Modulation of Double-stranded RNA Recognition by the N-terminal Histidine-rich Region of the Human Toll-like Receptor 3*§

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Toll-like receptors (TLRs) are an essential component of the innate immune response to microbial pathogens. TLR3 is localized in intracellular compartments, such as endosomes, and initiates signals in response to virus-derived double-stranded RNA (dsRNA). The TLR3 ectodomain (ECD), which is implicated in dsRNA recognition, is a horseshoe-shaped solenoid composed of 23 leucine-rich repeats (LRRs). Recent mutagenesis studies on the TLR3 ECD revealed that TLR3 activation depends on a single binding site on the nonglycosylated surface in the C-terminal region, comprising H539 and several asparagines within LRR17 to -20. TLR3 localization within endosomes is required for ligand recognition, suggesting that acidic pH is the driving force for TLR3 ligand binding. To elucidate the pH-dependent binding mechanism of TLR3 at the structural level, we focused on three highly conserved histidine residues clustered at the N-terminal region of the TLR3 ECD: His39 in the N-cap region, His60 in LRR1, and His108 in LRR3. Mutagenesis of these residues showed that His39, His60, and His108 were essential for ligand-dependent TLR3 activation in a cell-based assay. Furthermore, dsRNA binding to recombinant TLR3 ECD depended strongly on pH and dsRNA length and was reduced by mutation of His39, His60, and His108, demonstrating that TLR3 signaling is initiated from the endosome through a pH-dependent binding mechanism, and that a second dsRNA binding site exists in the N-terminal region of the TLR3 ECD characteristic solenoid. We propose a novel model for the formation of TLR3 ECD dimers complexed with dsRNA, which incorporates this second binding site.

Mammalian Toll-like receptors (TLRs)² play an essential role in the innate immune response to molecular patterns associated with microbial pathogens. To date, more than 10 functional TLRs have been reported in humans and in mice (1). All TLRs are type I integral membrane glycoproteins composed of an ectodomain (ECD) containing varying numbers of leucine-rich repeats (LRRs) linked by a transmembrane domain to a cytoplasmic signaling Toll/IL-1 receptor (TIR) domain. TLR ECDs are responsible for ligand binding: specific ligands derived from viral and bacterial constituents and their respective TLRs have been identified, including lipoteichoic acid (TLR2), lipopolysaccharide (TLR4), flagellin (TLR5), single-stranded RNA (TLR7 and TLR8), and unmethylated CpG DNA motifs (TLR9) (1). The binding of a ligand to a TLR initiates a series of signaling processes that activate and mediate innate and adaptive immune responses (2). The basic mechanism of TLR signaling is thought to involve ligand-induced dimerization (3).

TLR3 is activated by polyinosinic-polycytidylic acid (poly(I:C)), an analog of double-stranded RNA (dsRNA), as well as by viral infection-associated dsRNAs (4). TLR3 is quite distinct from other TLRs in that it is not dependent on myeloid differentiation factor 88 for signaling. Upon ligand binding, the TLR3 TIR domain recruits the intracellular adaptor molecule TICAM-1, also known as TRIF (TIR-containing adaptor inducing IFN-β) (5, 6). The recruitment of this adaptor leads to the production of antiviral cytokines, such as IFN-β. TLR3 is specifically expressed in immune cells, such as conventional dendritic cells and natural killer cells, as well as in fibroblasts and intestinal epithelial cells (1, 7–9).

The crystal structure of the human TLR3 ECD was recently elucidated by two groups (10, 11). The TLR3 ECD is a horseshoe-shaped solenoid composed of 23 LRRs, which is capped on both ends by characteristic N- and C-terminal structures, and its surface is extensively modified with N-linked glycans. However, one surface of the LRR solenoid is free from glycosylation, and the charge properties and two loops protruding from LRR12 and LRR20 on this surface are predicted to be involved in TLR3 function. Recent mutagenesis studies on the TLR3 ECD revealed that a single binding site is present on the nonglycosylated surface near the C terminus. This binding site includes His539 and several asparagines in LRR17 to -20 and is essential for TLR3 activation, suggesting a model for TLR3 recognition of dsRNA and the formation of a signaling complex (12–14).
Because the inhibition of endosomal acidification abrogates poly(I:C)-driven TLR3 activation, TLR3 is thought to be localized in intracellular compartments, such as endosomes (8). Indeed, a recent report confirmed that TLR3 associates with c-Src tyrosine kinase on endosomes to initiate antiviral signaling (15). It is therefore likely that TLR3, as well as the nucleic acid-recognizing TLRs, TLR7, TLR8 (16), and TLR9 (17), resides in the endosomal membrane and that binding of each TLR to its ligand occurs in the endosomal compartment. The intracellular localization of nucleic acid-sensing TLRs seems to discriminate between self and nonself nucleic acids (18). In addition to this function, several studies have also shown that an acidic pH within endosomes is required for TLR3 recognition of dsRNA and subsequent downstream receptor signaling (13, 19).

To understand the mechanisms underlying TLR3 function and intracellular localization at the structural level, we focused our attention on conformational changes in the receptor ectodomain of TLR3, especially the ionization of histidine side chains. Because one pHₐ of histidine is 6.0, protonation of this group within endosomes can generate an ionic attraction to the negatively charged phosphate backbone of dsRNA. If this hypothesis is correct, then the highly conserved histidine residues clustered in the N-terminal region of the TLR3 ECD, namely His₃⁹ in the N-cap region, His₆₀ in LRR₁, and His₁₀₈ in LRR₃, may play a key role in TLR3 function in acidified endosomes.

Here, we demonstrate that His₃⁹, His₆₀, and His₁₀₈ are indeed critical for human TLR3 activation and direct binding to dsRNA. This indicates that, in addition to the C-terminal binding site, there is a second dsRNA binding site in the N-terminal region of the TLR3 ECD characteristic solenoid. Based on these data, we propose a novel model for the formation of TLR3 ECD dimers complexed with dsRNA.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—For the expression of plasmids encoding human TLR3 and its mutants, HEK293 (human embryonic kidney) cells were used and cultured in DMEM cell culture medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Biosource) and antibiotics (penicillin/streptomycin). Sf₂₁ cells used for baculovirus generation and protein expression were grown in suspension culture in Sf-900 II SFM (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Biosource) and antibiotics (penicillin/streptomycin and gentamicin).

**Plasmids Carrying Wild-type TLR3 and TLR3 ECD—pEFBOS/TLR3, which consists of a human TLR3 cDNA cloned into the mammalian expression vector pEFBOS, was previously described (20). To obtain the recombinant TLR3 ECD protein with the Bac-to-Bac baculovirus expression system (Invitrogen), a construct for the human TLR3 extracellular domain (residues 28–703) was generated by PCR using pEFBOS/TLR3 as a template and a 5’ primer encoding a HindIII site and a 3’ primer encoding a His₉ tag, STOP codon, and HindIII site. The PCR product was cloned into the HindIII site of pFastBac1/rTTLR5S (21), which contains a pFastBac1 donor plasmid backbone, resulting in the construction of pFastBac/TLR3-ECD. The protein expressed from pFastBac/TLR3-ECD contains the preprotrypsin signal sequence and a FLAG tag at the N-terminus.

**Site-directed Mutagenesis**—Mutations were introduced by PCR-mediated, site-directed mutagenesis using essentially the same procedure as described previously (22). Human TLR3 cDNA in pEFBOS/TLR3 was mutated to produce eight mutant plasmids for a reporter gene assay: H₃⁹A, H₃⁹E, H₆₀A, H₆₀E, H₁₀₈A, H₁₀₈E, H₅₃⁹A, and H₅₃⁹E. To generate the four recombinant mutant proteins rH₃⁹A, rH₆₀A, rH₁₀₈A, and rH₁₀₈E, a cDNA of the human TLR3 ECD in pFastBac/TLR3-ECD was mutated. The mutagenic primers used to mutate human TLR3 are listed in the supplemental materials (Table S1). The mutated DNA sequences were confirmed by sequencing with an Applied Biosystems model 3100A automatic sequencer (Applied Biosystems).

**Expression and Purification of Recombinant TLR3 ECD and Its Mutants**—Recombinant human TLR3 ECD protein (TLR3-ECD) was obtained using the Bac-to-Bac baculovirus expression system (Invitrogen). To generate the bacmid DNA encoding the TLR3 ECD, Escherichia coli DH10Bac cells were transformed with the recombinant donor plasmid pFastBac/TLR3-ECD. The isolated recombinant bacmid DNA was then transfected into Sf₂₁ cells with UniFECTOR (B-Bridge International, San Jose, CA) to generate the recombinant baculoviruses. To express TLR3-ECD, a suspension culture of Sf₂₁ cells in SF-900 II SFM (Gibco) was infected by the recombinant baculoviruses, and the cell culture supernatant was harvested 5 days after infection. Subsequently, TLR3-ECD was purified using a HiTrap chelating column (5 ml size; GE Healthcare) at 4 °C and analyzed by 8% SDS-PAGE. Protein-containing fractions were concentrated using YM-50 (Millipore Corp., Bedford, MA) to generate the recombinant baculoviruses.

**RNA and DNA Preparations**—For the production of siRNA and dsRNA₄₀, two transcription templates were generated by PCR by using two respective sets of primers (Table S2), which contained T₇ RNA promoter sequences on each end of the template. Subsequently, siRNA and dsRNA₄₀ were transcribed using a T₇ AmpliScribe kit (Epicient Technologies) with [α-³²P]CTP. Following in vitro transcription, siRNA was prepared according to the instructions for the Silencer siRNA Construction Kit (Ambion). After in vitro transcription, annealed dsRNA₄₀ was purified in an 8% native polyacrylamide gel. Corresponding, respective sense and antisense strands were used as follows: siRNA, 5’-CCUGUUCCAUUGGCGACACUU-3’ and 5’-GGUGUUGCCAUUGGAAACGGUU-3’; dsRNA₄₀, 5’-GGGAGACAGGCCUUGUCCAGGGCAACACGUUUUGCUCCC-3’ and 5’-GGGAGACAAACGUUGUGGGCGCGGACG-3’. The respective sense and antisense strands of dsRNA₄₀, 5’-UCGAGACAGUCAUGUAUCCUCGACGCUUACAGCGCGAAGACGAGAGCU-3’ and 5’-AGGCUCUAUACUUGCCUGGUAGCCUGAAGUGUGU-
TABLE 1
Analysis of the interaction between TLR3 ECD and nucleic acids under different pH conditions

| pH | siRNA | dsRNA<sub>40</sub> | dsRNA<sub>48</sub> | rC<sub>30</sub> | dsDNA<sub>40</sub> | dC<sub>30</sub> |
|----|-------|----------------|----------------|---------|----------------|---------|
| 4.2 | 13 ± 0.3 | 23 ± 0.8 | 24 ± 2.4 | 9.4 ± 0.3 | 9.2 ± 0.1 | 3.2 ± 0.8 |
| 5.0 | 18 ± 0.7 | 22 ± 2.8 | 23 ± 2.0 | 10 ± 0.6 | 8.4 ± 0.2 | 4.8 ± 1.0 |
| 6.0 | 21 ± 1.0 | 19 ± 2.0 | 21 ± 0.4 | 11 ± 1.5 | 6.9 ± 0.9 | 5.9 ± 0.3 |
| 6.3 | 24 ± 0.6 | 18 ± 0.9 | 20 ± 1.0 | 2.9 ± 0.7 | 3.8 ± 0.3 | 2.6 ± 1.0 |
| 7.6 | 5.7 ± 0.2 | 19 ± 2.2 | 22 ± 1.3 | 5.3 ± 0.1 | 4.3 ± 0.2 | 2.3 ± 0.6 |
| 8.0 | 8.8 ± 0.3 | 21 ± 3.3 | 24 ± 0.9 | 7.1 ± 1.2 | 5.2 ± 0.1 | 3.1 ± 0.6 |

To investigate the effect of dsRNA length on TLR3 ECD binding at pH 5.0, QCM analysis was performed as described under "Experimental Procedures." TLR3-ECD was injected stepwise into the incubation chamber containing the dsRNA<sub>34</sub> or dsRNA<sub>48</sub>-immobilized sensor tip to achieve various concentrations of TLR3-ECD (dsRNA<sub>34</sub> or dsRNA<sub>48</sub>-immobilized QCM, 0–121 nM; dsRNA<sub>34</sub>-immobilized QCM, 0–525 nM). The frequency change (Δf/Hz) of the dsRNA<sub>34</sub>-immobilized QCM was analyzed, and two binding curves, TLR3-ECD to dsRNA<sub>34</sub> (closed squares) and dsRNA<sub>48</sub> (closed circles), were plotted. Each value is the mean ± S.D. of three independent experiments. The apparent dissociation constant (K<sub>d</sub>) calculated for dsRNA<sub>34</sub> was 19 ± 0.9 nM, but the K<sub>d</sub> for dsRNA<sub>48</sub> could not be determined.

For the binding of TLR3-ECD (20, 50, or 100 nM) to siRNA, dsRNA<sub>40</sub>, dsRNA<sub>48</sub>, rC<sub>30</sub>, dsDNA<sub>40</sub>, and dC<sub>30</sub>, was purchased using an automated DNA/RNA synthesizer (model 394; ABI). The dsDNA<sub>40</sub>, with a sequence identical to the single-stranded RNA of rC<sub>30</sub>, was amplified by overlapped-PCR with primers 5'-GGGAGACAGGCCTGTTCCATGGCCA-3' and 5'-GGGAGACAAACGTGTTGGCCATGGA-3'. The dsDNA<sub>40</sub> was labeled at the 5'-end with [γ<sup>32</sup>P]ATP by using T4 polynucleotide kinase.

For the crystal microbalance (QCM) analysis, the respective sense and antisense strands of dsRNA<sub>24</sub> (5'-end-biotinylated), 5'-CGUAGACGACAGCCGTTCACATGGCCA-3' and 5'-GGGAGACAAACGTGTTGGCCATGGA-3', and 5'-end-biotinylated dsRNA<sub>48</sub> were purchased as duplex RNAs from Sigma-Aldrich Japan K.K.

Reported Gene Assay—HEK293 cells were seeded in 24-well plates (5 × 10<sup>4</sup> cells/well). Twenty-four hours later, Lipofectamine 2000 (Invitrogen) was used to transiently transfect the cells with pEFSB/TLR3 or TLR3 mutant expression vectors (0.1 μg) together with a p125-luc reporter (0.1 μg) and a Renilla luciferase reporter (0.25 μg). The total amount of transfected plasmid (0.8 μg) was held constant by supplementing with empty vector as needed. Twenty-four hours after transfection, the medium was replaced with fresh medium containing poly(I:C) (10 μg/ml), and the cells were incubated for an additional 6 h. Cells were collected and washed twice with 1 ml of phosphate-buffered saline. The collected cells were lysed using passive lysis buffer (Promega), and the cell lysates were assayed for dual luciferase activities (Promega). Data are expressed as mean relative stimulation ± S.D. for a representative experiment from three independent experiments, performed in triplicate.

Western Blots—HEK293 cells were transiently transfected with target expression vector (0.8 μg) as described above. After 24 h, the cells were collected, washed three times with 1 ml of phosphate-buffered saline, and suspended with lysis buffer (20 mm Tris-HCl (pH 7.4), 150 mm NaCl, 10 mm EDTA (pH 7.4), 1% Nonidet P-40, 25 mm iodoacetamide, 2 mm phenylmethylsulfonyl fluoride) supplemented with Complete Protease Inhibitor (Roche Applied Science). After incubation on ice for 30 min, the lysates were centrifuged, and the supernatants were obtained. The concentration of the supernatants was determined using a protein assay kit (Bio-Rad), and equal amounts of protein were subjected to 7.5% SDS-PAGE. Separated samples were blotted onto polyvinylidene difluoride membrane (Millipore) and probed with a mouse anti-TLR3 antibody (IMG315A; Imagene Inc.). Horseradish peroxidase-conjugated goat anti-mouse antibody (Biosource) was used as a secondary antibody. Detection of horseradish peroxidase was carried out using a chemiluminescent horseradish peroxidase substrate (Millipore).

Filter Binding Analysis—To analyze the ability of TLR3 to bind siRNA, dsRNA<sub>40</sub>, dsRNA<sub>48</sub>, rC<sub>30</sub>, dsDNA<sub>40</sub>, and dC<sub>30</sub>,
each $^{32}$P-labeled RNA and DNA (10 nM) was incubated with TLR3-ECD (20, 50, or 100 nM) in 50 μl of binding buffer (2 mM HEPES-NaOH (pH 7.6) and 5 mM MES-NaOH (pH 4.2, 5.0, or 6.0) with 100 mM NaCl and 3 mM MgCl₂) at 37°C for 1 h. To evaluate the binding of TLR3-ECD mutants, dsRNA$_{40}$ (10 nM) was incubated with mutants (20 nM) in 50 μl of binding buffer
N-terminal Binding Site in the TLR3 Ectodomain

**RESULTS**

Importance of Low pH and dsRNA Length for TLR3 ECD Binding—To study the molecular recognition events and biochemical interactions between TLR3 and dsRNA, we first examined the effect of pH on the binding of recombinant TLR3 ECD (TLR3-ECD) to dsRNA. A filter binding assay for TLR3-ECD (20, 50, or 100 nM) was carried out with siRNA, dsRNA<sub>40</sub>, and dsRNA<sub>48</sub> (10 nM) at pH 4.2–7.6 (Table 1). Binding to TLR3-ECD was then injected stepwise into the incubation chamber containing the immobilized dsRNA-coated sensor tip. The binding affinity was indicated by QCM frequency decrease of the emitted frequency, which occurs with increasing mass on the QCM sensor tip. When the dsRNA<sub>48</sub>-coated sensor tip was used, a positive response was observed at all tested concentrations of TLR3-ECD (0–52.5 nM), with an apparent dissociation constant (K<sub>D</sub>) of 19 nM (Fig. 1). Strikingly, when the sensor tip was coated with dsRNA<sub>32</sub> and higher concentrations of TLR3-ECD were used (0–121 nM), the positive response was dramatically reduced, and the K<sub>D</sub> value could no longer be determined (Fig. 1). These results suggest that the interaction of TLR3 ECD with dsRNA highly depends on acidic pH and dsRNA length.

His<sup>39</sup>, His<sup>60</sup>, and His<sup>108</sup> Are Essential for the Ligand-dependent Activation of TLR3—To assess how TLR3 senses acidic pH for binding dsRNA, we focused on three highly conserved histidine residues clustered in the N-terminal region of the TLR3 ECD: His<sup>39</sup> in the N-cap region, His<sup>60</sup> in LRR1, and His<sup>108</sup> in LRR3 (Fig. 2A). These histidine residues are located between the concave and nonglycosylated lateral surfaces of the TLR3 ECD structure, and all of their imidazole side chains are exposed on the outside of the protein (Fig. 2B). Because one pK<sub>a</sub> of histidine is 6.0, a pH change within the endosome from neutral to acidic protonates the imidazole group. This thought is to generate an ionic attraction between the histidine and the negatively charged phosphate backbone of the dsRNA.

To test our hypothesis that the N-terminal histidines are crucial for pH-dependent binding of TLR3 ECD to dsRNA, we constructed the site-specific substitution mutants H39A, H39E, H60A, H60E, H108A, and H108E and carried out a reporter gene assay to analyze how mutations in TLR3 influence TLR3 activation. TLR3-negative HEK293 cells were transiently transfected with wild-type (pEFBOS/TLR3) or mutant
TLR3 expression plasmids together with a reporter plasmid containing a luciferase gene under the control of the human IFN-β promoter; the cells were then stimulated with poly(I:C).

Although H108A failed to substantially abrogate the TLR3-mediated activation of luciferase activity, the H39A, H39E, H60A, H60E, and H108E mutants showed a nearly complete loss of function (Fig. 2C). The replacement of the histidine residues with glutamic acid rather than alanine had a stronger effect on TLR3 activation by poly(I:C), especially for His108. This may be due to the ionic repulsion between the glutamic acid introduced at His39, His60, and His108 and the negative charge on the phosphate backbone of the dsRNA. The mutants did not regain activity, even when 5-fold higher concentrations of the mutant TLR3 expression plasmids were employed (data not shown). This loss of activity was not due to low expression, since Western blot analysis showed that the expression level of each mutant protein was equal to or greater than that of the wild-type (Fig. 2D). Thus, His39, His60, and His108 in the N-terminal region of the TLR3 ECD are essential for ligand-dependent activation of TLR3.

Direct Binding between His39, His60, and His108 and dsRNA—To elucidate whether these essential histidine residues directly contacted dsRNA, we analyzed the binding between recombinant mutant TLR3 proteins and dsRNA40 at acidic pH. Purified TLR3-ECD mutant proteins, rH39A, rH60A, rH108A, and rH108E, were verified by performing 8% SDS-PAGE stained with Coomassie Blue R-250 (Fig. 3A). rH108A showed partial loss of binding (about 60% of the wild type) to dsRNA40 at pH 5.0 (Fig. 3C). In contrast, rH39A, rH60A, and rH108E exhibited remarkably diminished binding to dsRNA40 (Fig. 3, B and C). These observations clearly correlated with the results obtained in the reporter gene assay (Fig. 2C). It can therefore be concluded that the loss of function observed in the TLR3 mutants is due to their inability to recognize dsRNA and that His39, His60, and His108 are essential for the direct binding of TLR3 to dsRNA at acidic pH.

**DISCUSSION**

Mutational analyses have identified several important residues in the TLR3 ECD that are essential for ligand recognition and signal transduction. An impressive report by Bell et al. (12) showed that His539 and Asn541 in LRR20 are crucial for the activation of TLR3. Following this report, Ranjith-Kumar et al. (13) identified asparagines in LRR17 to LRR20 that also contribute to TLR3 activation. In this study, we identified three histidine residues, His39 in the N-cap region, His60 in LRR1, and His108 in LRR3, that are essential for human TLR3 activation and ligand binding. In contrast with our results, Bell et al. (12) reported that H39A and H60A had no effect on TLR3 activation. This discrepancy may be due to sensitivity differences in the cell-based assay systems used, as observed for the His539 substitution mutants. According to their report, the H539E mutation, but not the H539A mutation, resulted in almost complete loss of function. However, in our assay system, both H539E and H539A showed significant effects on TLR3 activation (Fig. 2C).

Because dsRNA40 was bound to TLR3-ECD more efficiently than to the same sequence of dsDNA40, we posit that there exists a difference between forms of dsRNA (A-DNA-like form) and dsDNA (B-form) with respect to nucleic acid backbone helical structures and/or recognition of the 2′-OH group in the ribose of dsRNA (Table 1). Indeed, it has been reported that the presence of the ribose 2′-OH group in poly(I:C) is essential for its recognition by TLR3 (20). Furthermore, the efficiency of TLR3-ECD binding to dsRNA was strictly modulated by the acidic pH conditions, as shown in Table 1. These results indicate that His39 and the histidine cluster at the N-terminal region might act as pH sensors to recruit dsRNA. Once the imidazole side chains are protonated in an acidic compartment, such as an endosome, the resulting positive electrostatic potential would presumably lead to the interaction of TLR3 with dsRNA. This suggests that TLR3 signaling is initiated from within the endosome, based on a pH-dependent binding mechanism that is regulated by these functional histidine residues. Consistent with this, Ranjith-Kumar et al. (13) previously reported observations from a UV cross-linking assay showing that the TLR3 ECD specifically interacts with dsRNA at acidic pH. Furthermore, Bouteiller et al. (19) have shown using a chimeric TLR3-CD32 receptor that recognition of dsRNA by TLR3 and subsequent signal transduction require an acidic pH. It is therefore likely that TLR3-dsRNA interactions occur in acidic compartments to activate signal transduction. Histidine residues on other receptors have also been implicated in acid-regulated mechanisms (23, 24). Other nucleic acid-recognizing TLRs, TLR7, TLR8, and TLR9, are localized in acidic compart-

**FIGURE 3. Binding of TLR3-ECD mutants to dsRNA40.** A, purified TLR3-ECD mutant proteins: Approximately 0.5 µg of wild type and each of the mutant proteins, rH39A, rH60A, rH108A, and rH108E, were loaded on an 8% SDS-PAGE. B and C, to analyze the binding of TLR3-ECD mutants, 32P-labeled dsRNA40 (10 nM) was incubated with TLR3-ECD (wild type), rH39A, rH60A, rH108A, or rH108E (20 nM) in 50 µl of binding buffer (5 mM MES-NaOH (pH 5.0), 100 mM NaCl, and 3 mM MgCl2) at 37 °C for 1 h. A filter binding assay was carried out as described under “Experimental Procedures.” To normalize the data, wild-type binding activity was set to 100%. The values ± S.D. are derived from three independent experiments.
ments, such as endosomes, indicating that the ligand-binding and signaling of these receptors are also likely to require acidic pH. Indeed, it has been reported that TLR9 interacts with its ligand, nonmethylated CpG DNA, at acidic pH (25).

The basic mechanism of TLR signaling is thought to involve ligand-induced dimerization. In a model proposed by Bell et al. (12), the TLR3 ECD contains a single ligand-binding site in LRR20 close to the C terminus, and two TLR3 monomers sandwich a dsRNA in a precisely symmetrical arrangement. Ranjith-Kumar et al. (13) proposed a similar architecture for TLR3 ECD complexed with dsRNA, although they suggested a model different from that of Bell et al. (12), in which ligand binding to the TLR3 ECD induces dimerization and subsequent TIR domain activation. In this study, we revealed a second histidine-enriched binding site in the N-terminal region of the TLR3 ECD, in addition to the limited C-terminal binding site. We also found that the binding constant of dsRNA24 was much higher than that of dsRNA48 (Fig. 1). This indicates that the stability of the TLR3-dsRNA complex is affected by the dsRNA length. Although the 24-bp length of dsRNA would be sufficient for TLR3 binding if the ECD contained only the limited C-terminal binding site, the binding constants indicate that dsRNA24 affinity for TLR3-ECD is quite low compared with that of dsRNA48. Given the approximate distance between the N- and C-terminal binding sites (~30 bp of dsRNA; gray surface in Fig. 4A), dsRNA24, but not dsRNA48, is sufficiently long to bridge the distance between the regions. These results, together with the recent report that TLR3 ECD monomers bind cooperatively to dsRNA to form stable dimeric complexes and require a dsRNA length of at least 40–50 bp to bind a single dimer (26), strongly suggest that there exist two binding sites in the TLR3 ECD, which are both essential for the dsRNA recognition by the TLR3 ECD.

Taking into account all of these data, we determined that a 29-bp length of dsRNA (Fig. 4A, gray surface) can lie across the two binding sites on the TLR3 solenoid, based on configuration adaptability predicted by a manual docking analysis (Fig. 4A). From this analysis, we propose a new model for the formation of symmetric TLR3 dimers (Fig. 4B). Given that the N- and C-terminal binding sites appear on the same glycosylation-free face, it would be possible to assemble at least four ternary architectural units, each composed of two TLR3s and a single dsRNA (Fig. 4B). A commonly observed feature is that two TLR3 monomers contact a dsRNA via two N- and C-terminal binding sites, so that a dsRNA is fixed at four different positions in a single unit. Although the capability to form dimers in the TIR domain is vital for subsequent signaling, the symmetric arrangement of the two TLR3 molecules can vary along the

FIGURE 4. Model of TLR3 ECD recognition of dsRNA and formation of symmetric TLR3 dimers. A, the N-cap region, C-cap region, and LRR domain are represented by blue, magenta, and green surfaces, respectively (Protein Data Bank code 2A0Z). His39, His60, and His108 in the N-terminal region (N-R) and His39 and Asn541 in the C-terminal region (C-R) are shown in red and are involved in the interaction with a dsRNA, 29 bp in length (gray surface; Protein Data Bank code 1QC0). Using manual docking performed with PyMOL (version 0.99, DeLano Scientific LLC), we determined that a 29-bp dsRNA can lie across both the N-terminal region and C-terminal region in the TLR3 ECD solenoid and that the minor groove provides the interaction surface for His39, His60, and His108. The interval between the two binding sites is estimated to be two helical turns of dsRNA, B, a dsRNA cross-links two molecules of the TLR3 ECD, related by a 180° rotation, resulting in a precisely symmetrical dimer of the TLR3 ECD. Two TLR3 ECD molecules sandwich a dsRNA at two sites in both the N-terminal region and C-terminal region, fixing the dsRNA at four sites. Because the symmetrical arrangement of two TLR3 ECD molecules can be changed by sliding along a dsRNA, it is difficult to determine a single, accurate signaling complex. Two TLR3 ECD molecules can sandwich a dsRNA in both the N-terminal region and C-terminal region (a), only the C-terminal region (b), or only the N-terminal region (c), d, the dsRNA is not sandwiched between the N-terminal region or the C-terminal region. The color scheme is the same as in A. The yellow boxes represent the TIR domains.

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length of a dsRNA (see the legend to Fig. 4B). Communication between the two nonglycosylated faces of the TLR3 ECD separated by the dsRNA (e.g. the loop structure protruding from LRR12), might determine the exact structure of the dimer. Further biochemical analyses or determination of the crystal structure of TLR3 ECD in complex with a dsRNA will be required to determine the precise mechanism by which TLR3 recognizes dsRNA.

Our study with single amino acid mutations in TLR3 demonstrates that the histidine cluster in the N-terminal region functions to recognize dsRNA based on acid-regulated mechanisms, indicating the presence of a second dsRNA binding site in the characteristic solenoid of the TLR3 ECD. This finding has led us to propose a novel dimer structure, which provides insight into the ligand recognition mechanism of TLR3.

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