N-terminal Binding Site in The Human Toll-like Receptor 3 Ectodomain

Tomoya Watanabe¹, Takashi Tokisue¹, Tadayuki Tsujita², Misako Matsumoto², Tsukasa Seya², Satoshi Nishikawa³, Tsunemi Hasegawa¹ and Kotaro Fukuda¹

¹Department of Material and Biochemical Chemistry, Faculty of Science, Yamagata University, Yamagata 990-8560, Japan, ²Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Kita-ku, Sapporo 060-8638, Japan and ³Age Dimension Research Center, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan

ABSTRACT
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 signals in response to virus-derived 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 TLR3 ECD revealed that TLR3 activation depends on a single binding site on the nonglycosylated surface in the C-terminal region that includes H539 and several asparagines in LRRs 17 to 20. The localization of TLR3 within endosomes is required for ligand recognition, suggesting that acidic pH is the driving force for the ligand binding of TLR3. To clarify the pH-dependent binding mechanism of TLR3 at the structural level, we focused on some highly conserved histidine residues clustered at the N-terminal region of the TLR3 ECD. Mutagenesis approach showed that these residues were essential for the ligand-dependent activation of TLR3 in a cell-based assay. Furthermore, the binding of dsRNA to recombinant TLR3 ECD was strongly pH-dependent, and the binding was reduced by these mutations, demonstrating that TLR3 signaling is initiated from the endosome through a pH-dependent binding mechanism and that a second dsRNA binding site is present in the N-terminal region in the characteristic solenoid of the TLR3 ECD. We propose a novel model for the formation of TLR3 ECD dimers complexed with dsRNA that incorporates this second binding site.

INTRODUCTION
Mammalian Toll-like receptors (TLRs) have an essential role in the innate immune response to molecular patterns associated with microbial pathogens (1). More than 10 functional TLRs have been reported in human and mouse so far. TLR ectodomains (ECDs) are responsible for ligand binding, and specific ligands derived from bacterial and viral constituents and their respective TLRs have been identified. The binding of a ligand to a TLR initiates a series of signaling processes that activate and mediate innate and adaptive immune responses. TLR3 is activated by dsRNA associated with viral infection (2). Upon ligand binding, the TLR3 TIR domain recruits TIR-containing adaptor molecule (TICAM)-1 (3). The recruitment of the adaptor leads to the production of antiviral cytokines, such as IFN-β. The crystal structure of the human TLR3 ECD was recently elucidated (4). The TLR3 ECD is a horseshoe-shaped solenoid composed of 23 LRRs, and its surface is extensively modified with N-linked glycans. However, one surface of the LRR solenoid is free from glycosylation which surface is 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 H539 and several asparagines in LRRs 17 to 20 and is essential for the activation of TLR3, suggesting a model for TLR3 recognition of dsRNA and the formation of a signaling complex (5, 6).

Several studies have shown that the acidic pH within endosomes is required for the recognition of dsRNA by TLR3 and the subsequent downstream receptor signaling (7). To understand the mechanisms underlying TLR3 function and intracellular localization at the structural level, we focused our attention on a conformational change in the receptor ectodomain of TLR3, especially the ionization of histidine side chains. Because one pH₅₀ of histidine is 6.0, protonation of this group within endosomes is thought to generate an ionic attraction to the negative charge on the phosphate backbone of dsRNA. If this hypothesis is correct, then the highly conserved histidine residues clustered in the N-terminal region of TLR3 ECD must play a key role in TLR function in acidified endosomes. Here we report that some histidine residues in the N-terminal region are indeed critical for human TLR3 activation and direct binding to dsRNA.

RESULTS AND DISCUSSION
The importance of low pH for binding between TLR3 ECD and dsRNA — To study the molecular recognition events and biochemical interactions between TLR3 and dsRNA, we first examined the effect of pH
condition for 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 and dsRNA$_{40}$ (10 nM) at pH 4.2-7.6. Binding to TLR3-ECD was undetectable or slight at pH 7.6 and 6.0. However, both siRNA and dsRNA$_{40}$ bound strongly at lower pH, especially at pH 4.2. Furthermore, TLR3-ECD interacted more efficiently with the longer dsRNA$_{40}$ than with the siRNA. These results suggest that the interaction of TLR3-ECD with dsRNA depends on acidic pH.

Histidine residue essential for the ligand-dependent activation of TLR3 – To assess how TLR3 senses acidic pH for binding dsRNA, we focused on highly conserved histidine residues clustered in the N-terminal region of the TLR3 ECD. Because one pK$_a$ of histidine is 6.0, the pH change within endosome from neutral to acidic pH protonates the imidazole group. This is thought to generate an ionic attraction between the histidine and the negative charge on the phosphate backbone of the dsRNA. To test our hypothesis that the N-terminal histidines are crucial for the pH-dependent binding of the TLR3 ECD to dsRNA, we constructed the site-specific substitution mutants which histidine residues is replaced by alanine or glutamic acid, and carried out a reporter gene assay to analyze how the mutations in TLR3 would influence TLR3 activation. TLR3-negative HEK293 cells were transiently transfected with wild-type (pEFBOS/TLR3) and mutant TLR3 expression plasmids together with a reporter plasmid containing a luciferase gene under the control of the human IFN-$

con$-b promoter; the cells were then stimulated with poly(I:C). Some mutants showed a nearly complete loss of function. The loss of activity was not due to low expression, because a Western blot analysis showed that the expression levels of all mutant proteins were equal to or greater than that of the wild type. Thus, these histidine residues in the N-terminal region of the TLR3 ECD are essential for the ligand-dependent activation of TLR3.

Direct binding between TLR3 mutants and dsRNA – Next, to elucidate whether these essential histidine residues directly contact dsRNA, we conducted a binding analysis between recombinant mutant TLR3 proteins and dsRNA$_{40}$ at acidic pH (pH 5.0). The results concluded that the loss of function observed in TLR3 mutants is due to their inability to recognize dsRNA, and that these histidine residues are indispensable for the direct binding of TLR3 to dsRNA at acidic pH.

CONCLUSION

These results indicate that, in addition to the C-terminal binding site, there is a second dsRNA binding site in the N-terminal region of the characteristic solenoid of TLR3 ECD. Based on these data, we also propose a novel model for the formation of TLR3 ECD dimers complexed with dsRNA.

REFERENCES


*Corresponding author. E-mail: kotaro.f@sci.kj.yamagata-u.ac.jp