Biochemical and Biophysical Research Communications 430 (2013) 1234–1239

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Recognition of CpG oligodeoxynucleotides by human Toll-like receptor 9 and subsequent cytokine induction

Suwarti Suwarti [a], Tomohiko Yamazaki [a,b], Chechetka Svetlana [c], Nobutaka Hanagata [a,c,*]

[a] Graduate School of Life Science, Hokkaido University, N10W8, Kita-ku, Sapporo 060-0810, Japan
[b] World Premier International (WPI) Research Center for International Center for Materials Nanoarchitectonics (MANA), National Institute for Materials Science (NIMS), 1-2-1 Sengen, Tsukuba, Ibaraki 305-0047, Japan
[c] Nanotechnology Innovation Station, NIMS, Ibaraki 305-0047, Japan

ARTICLE INFO

Article history:
Received 13 December 2012
Available online 22 December 2012

Keywords:
Toll-like receptor 9
Oligodeoxynucleotide
Immunostimulatory
Ligand-binding sites
Extracellular domain

ABSTRACT

Toll-like receptor 9 (TLR9) recognizes a synthetic ligand, oligodeoxynucleotide (ODN) containing cytosine–phosphate–guanine (CpG). Activation of TLR9 by CpG ODN induces a signal transduction cascade that plays a pivotal role in first-line immune defense in the human body. The three-dimensional structure of TLR9 has not yet been reported, and the ligand-binding mechanism of TLR9 is still poorly understood; therefore, the mechanism of human TLR9 (hTLR9) ligand binding needs to be elucidated. In this study, we constructed several hTLR9 mutants, including truncated mutants and single mutants in the predicted CpG ODN-binding site. We used these mutants to analyze the role of potential important regions of hTLR9 in receptor signaling induced by phosphorothioate (PTO)-modified CpG ODN and CpG ODNs only consist entirely of a phosphodiester (PD) backbone, CpG ODN2006x3-PD that we developed. We found truncated mutants of hTLR9 lost the signaling activity, indicating that both the C- and N-termini of the extracellular domain (ECD) are necessary for the function of hTLR9. We identified residues, His505, Gln510, His530, and Tyr554, in the C-terminal of hTLR9-ECD that are essential for hTLR9 activation. These residues might form positive charged clusters with which negatively charged CpG ODN could interact. Furthermore, we observed ODN–PD induced interleukin-6 (IL-6) through TLR9 in a CpG-sequence-dependent manner in human peripheral blood mononuclear cells and B cells, whereas ODN–PTO induced IL-6 in a CpG-sequence-independent manner. These finding are relevant for the mechanism of hTLR9 activation by CpG ODNs.

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1. Introduction

The Toll-like receptors (TLRs) are a class of pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs), as well as play a critical role in the innate immune response to invading pathogens [1–4]. In human, 10 kinds of TLRs, TLR1-10, have already been identified and classified into subfamily based on PAMPs type. TLR1, 2, and 6 are grouped into lipoprotein or lipopeptides recognizing-PAMPs, TLR4 and 5 are classified into flagellin and lipopolysacharide-PAMPs subfamily, while TLR3, 7, 8, and 9 are crucial for nucleic acid PAMPs recognition. TLR9 is activated by DNA from invasive bacteria and by synthetic oligodeoxynucleotides (ODNs) containing unmethylated cytosine–phosphate–guanosine (CpG) motifs [5]. TLR9 is localized in the early endosome/endolysosome of mainly B-cells and plasmacytoid dendritic cells (pDCs) in humans [6]. CpG ODNs stimulate the innate immune system and therefore have potential application in various immune therapies to treat infectious diseases, asthma, allergy, and cancer [7–9].

TLR9 harbors a leucine-rich repeat (LRR) motif at its extracellular domain (ECD) that is necessary for ligand recognition [10], as well as a transmembrane domain for localization [11,12]. The three-dimensional structure of TLR9 has not been reported; therefore, the structural details of the ligand–receptor interaction and any associated conformational changes remain unclear. Several analyses of the functional structure of human TLR9 (hTLR9) have been reported [13–15], but more detailed information regarding the ligand–protein structure is necessary to pursue the use of hTLR9 in adjuvant development.

Abbreviations: TLR, Toll-like receptor; hTLR9, human toll-like receptor 9; mTLR9, mouse toll-like receptor 9; CpG, cytosine–phosphate–guanine; ODN, oligodeoxynucleotide; PTO, phosphorothioate; PD, phosphodiester; ECD, extracellular domain; LRR, leucine rich repeat; PAMPs, pathogen-associated molecular patterns; PBMCs, peripheral blood mononuclear cells; IL-6, interleukin-6; IRS, inhibitory sequence; NF-κB, nuclear factor-kappa B.

* Corresponding author. Address: Nanotechnology Innovation Station, NIMS, 1-2-1, Sengen, Tsukuba, Ibaraki 305-0047, Japan. Fax: +81 29 859 2475.
E-mail address: HANAGATA.Nobutaka@nims.go.jp (N. Hanagata).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.12.068

Studies of TLR9-mediated immune-stimulation have been performed mostly with CpG ODNs consisting entirely or partially of a phosphorothioate (PTO) backbone, because this backbone is more stable and renders higher cellular uptake compared to the CpG ODN–PD backbone [16]. However, the ODN–PTO is associated with problems such as non-specific binding that may lead to bias when exploring the hTLR9 recognition mechanism. Roberts et al. reported that the binding affinity of CpG ODN–PD to TLR9 did not correlate with the established species-specific responses to CpG ODN–PTO [17]. Li et al. reported that CpG ODN–PTO cause TLR9 aggregation, but CpG ODN–PD induces TLR9 dimerization [18]. These results suggest that CpG ODN–PTO is in effect to TLR9 activation with the different mechanism from CpG ODN–PD. Wagner hypothesized that CpG ODN–PTO lead to the TLR9 clustering that might generate TLR9 hyperactivation hence CpG ODN–PTO do not faithfully recapitulate natural DNA-mediated TLR9 activation [19]. Therefore, study of TLR9-ligand recognition using ODN–PD gain importance. However, ODN–PD is rapidly degraded by nuclease and much less is known about ODN–PD-induced TLR9 activation. Recently, we reported a stable CpG ODN2006x3-PD [20]. This ODN–PD contains 9 CpG motifs and is nuclease resistant. These properties contributed to higher signaling activity of CpG ODN–PD stimulation in TLR9-expressing HEK293-xl cells than did CpG ODN–PTO.

In this study, we evaluated a functionally essential region in hTLR9 by using CpG ODN–PTO and CpG ODN–PD, which we developed. First, based on sequence prediction, we constructed a truncated form of hTLR9 and analyzed its signaling activity to investigate that whether truncated mutants of hTLR9 activate by CpG ODNs. Truncated mutants of hTLR9 lost the signaling activity. Second, to determine whether any of the proposed ligand-binding sites were responsible for TLR9 ligand recognition, we mutated residues that are highly conserved between different species according to homology modeling study [21] and identified an important positive cluster for CpG recognition. Taken together, these data provide structurally important information to clarify the mechanism of hTLR9 activation. In addition, we found that CpG ODN–PD induces interleukin-6 (IL-6) secretion via TLR9 activation in both human peripheral blood mononuclear cells (PBMCs) and B-cells in a CpG-sequence-dependent manner, although PTO–ODN induce IL-6 in a sequence-independent manner. By using CpG ODN–PD on behalf of CpG ODN–PTO, we were able to resolve the ligand recognition of hTLR9 and hTLR9-mediated pro-inflammatory cytokine induction mechanism to accurately mimic the immune reaction in mammalian cells.

2. Materials and methods

2.1. Cells and reagents

HEK293-xl-null cells (InvivoGen, San Diego, CA, USA) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Nichirei Biosciences, Tokyo, Japan), 50 units/mL penicillin, 50 mg/mL streptomycin, and 10 μg/mL blasticidin. Frozen PBMCs were purchased from Cellular Technology Limited (Shaker Heights, OH, USA) and thawed according to the manufacturer's instructions. B-cells were isolated from PBMCs by positive selection with CD20 cell isolation kits according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). ODNs were synthesized by Fasmac (Kanagawa, Japan), and are enlisted in Supplementary material Table 1. Anti-HA, anti-Calnexin, and anti-LAMP-1 antibodies were purchased from AbCam (Cambridge, UK); anti IgG-HRP was from Dakocytomation (Glostrup, Denmark); and anti-rabbit IgG-HRP, Alexa-488 anti-mouse, and Alexa-555 anti-rabbit antibodies were from Invitrogen (Carlsbad, CA, USA).

2.2. Plasmid and TLR9 mutant construction

The plasmid containing the TLR9-encoding sequence, pUNO-hTLR9-HA (here referred as hTLR9-WT) was purchased from InvivoGen. Site-directed mutagenesis using the QuikChange Kit (Stratagene, La Jolla, CA, USA) was performed. We also constructed truncated mutants as described in Fig. 1A by inverse PCR methods.

2.3. Reporter gene experiments

For reporter gene experiments, a firefly luciferase reporter construct with a nuclear factor-kappa B (NF-κB)-encoding gene was generated. HEK293-xl-null cells (3×10^4 cells/well) were transfected in 48-well format in a volume of 300 μL. hTLR9-WT (500 ng) or the indicated mutant plasmids were transfected using LyoVec (InvivoGen) with 500 ng of pNifty-luc (Invivogen), encoding five repeats of NF-κB-binding sites with a firefly luciferase reporter gene, and 100 ng of pGL4.74 (Promega, Madison, WI, USA), encoding *Renilla* luciferase. After 24 h, the cells were stimulated with 0.5 μM ODNs or unstimulated, and luciferase activities were determined after an additional 24 h using the Dual Luciferase Reporter Assay (Promega). The stimulation activity was expressed as the relative NF-κB activity by ODNs relative to that unstimulated. The data shown are the mean values of triplicates from one of at least two independent experiments. Control is un-transfected with TLR9 encoding vector in HEK293-xl-null cells.

2.4. Immunoblotting

HEK293-xl-null (1.0×10^7 cells) were seeded in 10-cm petri dishes and transfected with 3 μg of the indicated vector. After 48 h, cells were collected and rinsed twice with PBS and then lysed with RIPA buffer (Thermo Scientific, Rockford, IL, USA) on ice for 30 min. Lysates were cleared by centrifugation at 12,000*g* for 5 min. Equal amounts of lysates were fractionated by 4%–12% SDS–PAGE (NuPAGE, Invitrogen) and then electrotransferred to PVDF membranes (Invitrogen). The membranes were blocked with PBS containing 0.5% (w/v) skim milk (Millipore) and 1% (v/v) Tween-20. Cross-reactive bands were visualized using chemiluminescence (Millipore) on X-ray film.

2.5. Immunoprecipitation and pull-down assay

Cell lysates were purified using anti-HA antibodies immobilized on Protein A Mag Sepharose (GE Healthcare, Uppsala, Sweden) according to manufacturer's instructions. Eluates from the immunoprecipitation were used for pull-down analysis after pH adjustment to an endolysosomal environment using 1 M Tris–HCl (pH 5.0) buffer. A final concentration of 10 μM biotinylated CpG ODN was added to 20 μL of eluates, and the mixture was then incubated for 2 h at 4 °C. Subsequently, 20 μL of streptavidin-agarose Dynabeads (Invitrogen) was added, and the mixture was incubated for an additional 2 h at 4 °C. Beads were washed three times with RIPA buffer and eluted with lysis buffer for immunoblot analysis.

2.6. ELISA

Human PBMCs and $CD20^+$ B cells were seeded in 96-well plates at 5×10^6 and 5×10^5 cells, respectively. CpG ODNs were added at a final concentration of 0.5 μM to the cell culture medium. The cells were then incubated at 37 °C for 48 h, and the supernatants were collected and stored at −20 °C until further analysis. The IL-6 secretion level was measured with the human IL-6 Ready-Set-

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