Nucleic Acids Exert a Sequence-independent Cooperative Effect on Sequence-dependent Activation of Toll-like Receptor 9*

Received for publication, August 23, 2006, and in revised form, February 23, 2007
Published, JBC Papers in Press, March 13, 2007, DOI 10.1074/jbc.M608089200

Jason Kindrachuk1†, Jean E. Potter1‡, Robert Brownlie§, Andrew D. Ficzycz9, Philip J. Griebel1, Neeloofer Mookherjee1, George K. Mutwiri1, Lorne A. Babiuk1, and Scott Napper1†

From the 1Vaccine and Infectious Disease Organization, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5E3 and the 9Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

Toll-like receptor 9 (TLR9) activates the innate immune system in response to microbial DNA or mimicking oligodeoxynucleotides. Although cell stimulation experiments demonstrate the preferential activation of TLR9 by CpG-containing nucleic acids, direct binding investigations have reached contradictory conclusions with respect to the ability of this receptor to bind nucleic acids in a sequence-specific manner. To address this apparent discrepancy, we report the purification of the soluble ectodomain of human TLR9 with characterization of its ligand binding properties. We observe that TLR9 has a high degree of specificity in its ability to bind nucleic acids that contain CpG dinucleotides as well as higher order motifs that mediate species-specific activation. However, TLR9 is also functionally influenced by nucleic acids in a sequence-independent fashion as both stimulatory and nonstimulatory nucleic acids sensitize TLR9 for in vitro ligand binding as well as in vivo activation. We propose a model in which receptor activation is achieved in a sequence-dependent manner, and sensitivity is modulated by the absolute concentration of nucleic acids in a sequence-independent fashion. This model bears resemblance to that recently proposed for Toll in that activation is a two-step process in which formation of a ligand-bound monomer precedes formation of the activated dimer. In each model receptor sensitivity is determined within the second step with the crucial distinction that Toll undergoes negative cooperativity, whereas TLR9 is sensitized through a positive cooperative effect.

The mammalian innate immune system initiates conserved responses against an array of microbial challenges by targeting biomolecules that are conserved within microbes but largely absent from the host. These pathogen-associated molecular patterns (PAMPs)2 are recognized by germ line-encoded, pattern recognition receptors that include the Toll-like receptors (TLRs) (1). TLRs are highly conserved type I integral membrane proteins that sample PAMPs in both the extracellular and intracellular compartments (2). The ligand-binding regions of the TLRs consist primarily of repeating elements of a leucine-rich repeat motif (3). This motif is present in a large number of eukaryotic proteins that often share the unifying characteristic of mediating biomolecular interactions (4). Indeed, the primary function of the leucine-rich repeat motif appears to be to provide an adaptable structural matrix for biomolecular interactions, the versatility of which is exemplified by the vast and structurally diverse ligands that are recognized by different TLRs as follows: microbial DNA (TLR9), lipopolysaccharides (TLR4), and bacterial flagella (TLR5) (5, 6).

There is considerable evidence that microbial DNA serves as the physiological ligand for TLR9. Most convincingly, TLR9 knock-out mice are unresponsive to bacterial DNA but can be made responsive through expression of TLR9 (7). Foreign and host DNA appear to be discriminated on the basis of unmethylated cytosine-phosphate-guanine (CpG) dinucleotide sequences, which are frequent within microbial DNA but largely absent from host genetic material (8). The ability for CpG motifs, in the context of bacterial DNA or synthetic oligodeoxynucleotides (ODN) analogs, to activate innate immune responses is well documented (9, 10).

Although the CpG dinucleotide represents the minimum recognition element, TLR9 undergoes preferential activation, in a species-dependent manner, by specific higher order hexameric sequences. For example, mice are most responsive to a GACGTT motif, whereas human and bovine cells are preferentially activated by GTCGTT sequences (11, 12). The ability for specific sequences of ODNs to mediate species-dependent responses is generally assumed to reflect the unique ligand preferences of TLR9s from various species, although there is little direct experimental evidence to support this hypothesis.

The low cost, ease of production, and stability of nucleic acids make TLR9 an attractive target for immunotherapeutic intervention. CpG-ODNs have the additional advantage of being one of the most selective stimulators of dendritic cells with minimal systemic toxicity (13). There have been numerous human trials examining the immunotherapeutic potential of

---

* This work was supported by funding from Qiagen Inc. and The Krembil Foundation and grants provided by the Natural Sciences and Engineering Research Council (to S. N. and L. A. B.), and Canadian Adaptation and Rural Development. Published with permission of the Director of the Vaccine and Infectious Disease Organization as journal series number 451. 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.†To whom correspondence should be addressed. Tel: 306-966-1546; Fax: 306-966-7478; E-mail: scott.napper@usask.ca.
§The abbreviations used are: PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptor; CMV, cytomegalovirus; ODN, oligodeoxynucleotide; PTO, phosphothioate; PD, phosphodiester; PBMC, peripheral blood mononuclear cell; PNGase F, peptide N-glycosidase F; LBD, ligand-binding domain.
Specificity and Cooperativity of Ligand Binding by TLR9

CpG-ODNs toward the applications of priming the innate immune system to mediate protection against infectious pathogens, as adjuvants, as anti-allergens, and as immunotherapeutics in the treatment of various malignancies (14, 15).

Despite the considerable therapeutic potential of CpG-ODNs, reasonable concerns have been raised with respect to their safety, in particular their potential to trigger autoimmune disorders. Studies have shown that high doses of bacterial DNA elicit the production of auto-antibodies against double-stranded DNA in normal mice (16) and accelerate the production of autoimmune antibodies in lupus-prone animals (17). CpG-ODNs may also facilitate the development of toxic shock by lowering the pathological threshold of lipopolysaccharide tolerance (18). Although the risks associated with these scenarios are low, they nevertheless emphasize the importance of optimization of TLR9 agonists.

Currently, the rational design of TLR9 agonists is limited by a lack of basic information on the receptor-ligand interaction. This is largely a consequence of difficulties associated with purification of sufficient quantities of the receptor for biochemical characterization. As such, TLR9 ligands are currently evaluated primarily on the basis of their ability to elicit responses in immune cells. An inherent danger to this approach is that the reduction of numerous potential points of regulation to a single measured output does not permit discrimination of specific events, such as ligand binding, from the overall process. This is particularly problematic given the emerging evidence that the current paradigm of CpG-mediated activation likely underestimates the complexity of this system in terms of inputs and functional outputs (19), as well as the observation that nucleic acids can elicit TLR9-independent cellular responses (20).

A wealth of cell stimulation experiments has demonstrated the preferential ability for CpG, rather than GpC-ODNs, to initiate innate immune responses (21, 22), and this is generally assumed to reflect sequence-specific binding by TLR9. However, ligand binding investigations have reached contradictory conclusions with respect to the ability of TLR9 to bind nucleic acids in a sequence-specific fashion. Although two in vitro studies have shown the preferential association of TLR9 with single-stranded CpG-ODNs (23, 24), both CpG- and non-CpG-ODNs were equally effective in mediating immunoprecipitation of TLR9, indicating that the receptor interacts with both activating and nonactivating ODNs (25). Furthermore, recent demonstrations of in vivo activation of TLR9 by phosphodiester ODNs in a CpG-independent fashion (26), as well as the suggestion that the discrimination of host and foreign DNA is dependent upon TLR9 localization rather than specificity of ligand binding (27), undermine the hypothesis of exclusive CpG-mediated activation of TLR9. Collectively these data highlight the current lack of consensus on the ligand-binding proclivities of TLR9 as well as the emerging distinction between TLR9 ligand binding and receptor activation.

The complexity of TLR activation is perhaps predicted by investigations of Toll, a receptor that bears considerable structural and functional similarity to the Toll-like receptors. Activation of Toll by the cytokine ligand Spätzle involves two nonequivalent binding events and is influenced by receptor-receptor and receptor-ligand interactions (28, 29). As full-length Toll ectodomains are able to form dimers in the absence of ligand, the resting state of Toll is suggested to involve ligand-free, inactive dimers (28). Ligand binding segregates these inactive dimers into ligand-free and ligand-bound subunits. Their re-association to the formation of the active ligand-bound dimer is influenced by the ability of Spätzle to induce structural alterations within the binding monomer that are translated to the incoming monomer. The functional consequence of these induced alterations is to decrease the affinity of the second subunit for Spätzle such that ligand binding by the second Toll monomer occurs with an ∼3-fold lower affinity than that of the first (28). This negative cooperative effect is proposed to represent a mechanism to increase the range of substrate concentrations to which Toll is responsive. Negative cooperativity in the activation of Toll is supported by kinetic investigations in which 400-fold increases in ligand concentration are required to achieve full activation of the receptor (30).

To investigate the specificity of ligand binding, as well as the occurrence of higher order regulatory events that may influence receptor function, we have expressed and purified the ectodomain of human TLR9 as a soluble protein for investigation of its ligand binding properties. Through this model we resolve a number of key issues with respect to the sequence and structural parameters that influence the ability of nucleic acids to function as TLR9 ligands. We also demonstrate that sequences that are unable to mediate direct activation of TLR9 nevertheless influence TLR9 function by sensitizing the receptor for in vitro ligand binding as well as in vivo activation.

Materials and Methods

Cloning—With some species-specific variations, TLR9 is a protein of ∼1030 amino acids. The ligand-binding ectodomain consists of residues 1–815; residues 815–825 represent the transmembrane region, and the final C-terminal residues form the intracellular TIR domain. As the ligand-binding region of TLR9 has not been well characterized, we selected the entire ectodomain, amino acids 1–815, to represent the ligand-binding domain TLR9(LBD).

A eukaryotic expression system, which employs the bovine hsp70A gene promoter to direct the heat-regulated synthesis of proteins in transfected bovine kidney epithelial cells, was used to express TLR9(LBD). This system has proven highly effective for the overexpression of other glycosylated proteins (31).

DNA encoding the ectodomain was PCR-amplified, with the addition of a 6-residue histidine tag at the C terminus, using a plasmid containing the human TLR9 open reading frame as a template (kindly provided by Grayson Lipford). The resulting PCR product was cloned downstream of a bovine hsp70A gene promoter, and the expression cassette was stably introduced into Madin-Darby bovine kidney cells by lentiviral delivery (32).

Purification—An advantage of this expression system is that the recombinant proteins are secreted into the culture media to greatly facilitate purification. Although the yields of the recombinant protein are relatively low, ∼50 µg of protein per liter of culture media, the nondestructive nature of this system permits large scale production by splitting the culture and ongoing heat-shock cycling. Sufficient quantities of the protein could be produced from 10 rounds of temperature shifting of 40 T150...
Deglycosylation of TLR9(LBD)—To confirm the mass of the unmodified polypeptide, purified TLR9(LBD) was subjected to deglycosylation. TLR9(LBD) (20 μg) was denatured in 5% SDS with 0.4 M dithiothreitol for 10 min at 100 °C. Following this procedure, reaction buffer (0.5 M sodium phosphate (pH 7.5)) and 10% Nonidet P-40 were added at 1:10 reaction volume with 2.5 μl of peptide-N-glycosidase F (PNGase F). The deglycosylation reaction was allowed to proceed at 37 °C for 1 h.

Agarose Electrophoretic Mobility Shift Assays—Unless otherwise specified, DNA binding assays were performed with the incubation of 8 μg of TLR9(LBD) (20 mM phosphate-buffered saline (pH 7.2), 100 mM NaCl, 0.5 mM β-mercaptoethanol) with 1.4 μg of DNA (pFLAG-CMV-2 expression vector (Sigma)) in the presence of 10 mM MgCl₂. Reactions were buffered with 100 mM sodium acetate (pH 5.2). The final volume of each reaction was 50 μl. Reactions were incubated at 37 °C for 10 min, stopped with the addition of 10 μl of agarose loading solution (30% glycerol), and immediately electrophoresed through a 0.8% agarose gel at 95 V for 1 h. DNA visualization was through ethidium bromide staining.

Agarose Electrophoretic Supershift Assays—Supershift assays were performed by the same methodology as described for the mobility shift assays with the exceptions that the TLR9(LBD) was dialyzed against (50 mM phosphate-buffered saline (pH 7.2), 100 mM NaCl), and the binding reactions were performed at pH 7.2 (50 mM Tris). For the supershift assay, 1 μg of a human TLR9 monoclonal antibody (Oncogene Research Products (EMD Biosciences)) was preincubated with 8 μg of TLR9(LBD) in reaction buffer for 1 h at room temperature. Plasmid DNA was added after this incubation period and electrophoresis performed as described earlier.

Western Blot Analysis—Proteins were resolved through a 10% SDS-PAGE gel. Following electrophoresis, the proteins were transblotted onto polyvinylidene difluoride membranes (PolyScreen, PerkinElmer Life Sciences). The membranes were blocked with 3% molecular grade fat-free skim milk powder in phosphate-buffered saline containing 0.02% (v/v) Tween 20. Primary and secondary washes were carried out using this sample buffer. The monoclonal antibody to human TLR9, α-hTLR9, was used at a dilution of at 4 μl/ml. The secondary antibody (Kirkegaard & Perry Laboratories, Inc.) (goat α-mouse IgG (H + L) at 0.5 μl/ml) was used to probe for TLR9-antibody complexes.

Acrylamide Electrophoretic Mobility Shift Assays—ODNs were end-labeled with [γ-32P]dCTP (Mandel) using the Klenow fragment of DNA polymerase I (Promega). Binding reactions containing 0.2 ng of double-stranded labeled probe, 8 μg of TLR9(LBD), 10 mM Tris (pH 7.8), 50 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, and 5% glycerol were incubated at 37 °C for 20 min. Nondenaturing polyacrylamide gels (5%) containing 7.2% acrylamide were run at 9 V/cm for 2 h at 10 V/cm. Gels were dried for 1 h at 80 °C and exposed to a Kodak Storage Phosphor Screen GP. Storage phosphor screens were scanned with a Bio-Rad Molecular Imager FX using Bio-Rad Quantity One software.

ELISAs for TLR9—The isolation and culture of bovine peripheral blood mononuclear cells (PBMC) were described previously (33). Briefly, PBMC were isolated from three mature (>1 year old) male and female cattle. Lymphocyte proliferative responses for each animal were assayed by stimulating triplicate cultures (2 × 10⁵ cells/well) in a U-bottom 96-well culture plate. Cells were cultured for 72 h at 37 °C in a humidified atmosphere of 5% CO₂, and 0.4 μCi of [³H]thymidine (Amersham Biosciences) was added to each well. Cells were harvested using standard liquid scintillation protocols, and the incorporation of [³H] was measured using a beta-counter (Topcount, Packard Instrument Co.). The lymphocyte proliferative response was calculated as the mean counts/min for the triplicate cultures.

Oligodeoxynucleotides—The sequences of the ODNs used in this investigation are presented in Table 1. The phosphothioate (PTO) versions of the 2007 ODNs consist exclusively of phosphothioate linkages. Phosphodiester (PD) ODNs were purchased from Invitrogen, and PTO ODNs were kindly provided by Qiagen.

Determination of the Relative Abundance of TLR9 and DNA in Nucleoprotein Complexes—The stoichiometry of the shifted nucleoprotein complexes was determined by comparing the relative abundances of both nucleic acid and TLR9(LBD) within uniquely migrating species hypothesized to represent monomeric and dimeric forms of the TLR9 nucleoprotein complex. To qualify the nucleic acid component, agarose gels were scanned with an Alphalmager gel dock system (Alpha Innotech Corp.), and the intensities of the bands were expressed as a percentage of the total intensity of the two bands. To determine the relative ratio, the protein component of the shifted bands was excised, and proteins were extracted from the agarose by overnight freezing at −80 °C followed by high speed centrifugation at 30,000 × g for 30 min. Supernatants from the spins were subjected to Western blot analysis utilizing the same protocol and TLR9-specific antibody as described earlier. The blots were scanned using the same gel dock system, and the relative intensities of each of the bands was expressed as a percentage of the total intensity of the two bands.
RESULTS

Purification of TLR9(LBD)—From our expression system, the recombinant TLR9(LBD) is secreted into the media as a soluble glycosylated protein. Following nickel-column purification, a single protein species, which is reactive with a monoclonal antibody to human TLR9, was observed. The mass of this reactive band is consistent with that reported by others for the glycosylated form of comparable fragments of the receptor (25). This protein was not present in media collected from cells that were not transfected with the TLR9(LBD) expression vector (Fig. 1A). Treatment of TLR9(LBD) with the deglycosylation enzyme PNGase F resulted in a protein species of ~100 kDa, which is consistent with the predicted mass of the non-glycosylated polypeptide, as well as the observations of others (25) (Fig. 1B).

The principal objective in the expression/purification of the recombinant receptor was to obtain sufficient quantities of the protein for biochemical characterization through mobility shift assays. Importantly, these assays are not dependent upon the absolute purification of the receptor as the presence of the protein in nucleoprotein complexes is confirmed through super-shift assays employing a specific monoclonal antibody.

TLR9(LBD) Binds Plasmids—The ability of TLR9(LBD) to bind nucleic acids was investigated through a modified agarose electrophoretic mobility shift assay. This assay is based upon differential patterns of DNA migration in the presence of DNA-binding proteins. This system permits high-throughput analysis in a manner that is not dependent upon immobilization of either the receptor or the ligand. This permits more flexible characterization of receptor/ligand dynamics with respect to the formation of higher order structures such as dimers (34). Within this system, patterns of nucleoprotein migration are dependent upon the charge/mass ratio of the complexes, as well as the formation of higher order structures that influence the overall size of the molecule. As such, there is not a linear relationship between the calculated molecular mass and the distance migrated within the gel. The shifts are qualitative in nature, permitting discrimination and visualization of nucleoprotein complexes of differing compositions. This is of particular value for discrimination of nucleic acid binding by monomeric or dimeric forms of proteins as these complexes migrate as distinct entities.

The ability of TLR9(LBD) to influence the migration pattern of a plasmid demonstrates the ability of TLR9 to bind double-stranded DNA, which has been a point of contention in the literature. The ligand employed in our assay system is a closed circular plasmid that is present in both supercoiled and relaxed forms. We observe that TLR9(LBD) undergoes preferential association with supercoiled forms of plasmids, which may account for the reported discrepancies with regard to the efficiency of plasmid binding by TLR9. Investigations that report minimal association with plasmid have employed linear, double-stranded nucleic acids, whereas investigations supportive of plasmid binding have employed circular plasmids (24, 25). The difference in the ability of these ligands to form supercoiled species would account for their unique abilities to serve as TLR9 ligands. The association with nucleic acids is dependent upon the maintenance of a correctly folded structure as deliberate disruption of the structure of TLR9(LBD) by thermal denaturation prior to its addition to the reaction mixture abolished DNA binding activity (Fig. 2A).

That the differential patterns of plasmid migration result specifically from TLR9(LBD) binding was verified by a super-shift assay employing a monoclonal antibody to the extracellular domain of human TLR9. The binding of the antibody to TLR9(LBD) resulted in a uniquely migrating species of the plasmid, confirming the presence of TLR9(LBD) in the nucleoprotein complex (Fig. 2B). Furthermore, proteins extracted from the shifted bands were shown to be reactive with a human TLR9 monoclonal antibody (data not shown).

TLR9 Forms Monomeric and Dimeric Nucleoprotein Complexes—TLR9(LBD) influences the migration of plasmid DNA as evidenced by the formation of two distinct migrating bands (Fig. 2A). These two nucleoprotein species likely represent nucleoprotein complexes involving monomeric and dimeric forms of the receptor. Ligand-induced dimerization is a common theme within the Toll and Toll-like family (35, 36), and the sequential binding of ligands, first by monomeric and then dimeric forms of the receptor, has been demonstrated for Toll (28, 29). As demonstrated later, the two distinctly migrating species are observed in a sequential fashion that is dependent upon the ligand concentration. The proposed monomeric nucleoprotein complex, which migrates farthest away from the unbound plasmid, is observed at lower ligand concentrations, whereas formation of the second and presumed dimeric nucleoprotein complex occurs at higher concentrations of nucleic acid. This is consistent with the model proposed for Toll.

To confirm that these shifted species corresponded to monomeric and dimeric nucleoprotein complexes, the relative quantities of DNA and TLR9 in each of the shifted species was determined. Each of the shifted complexes had an approximately equivalent amount of DNA, whereas the species corresponding to the proposed dimeric complex had approximately double (1.95:1) the quantity of TLR9(LBD) as observed in the monomeric complex (Fig. 2C).

pH Dependence of DNA Binding—Ligand binding by TLR9 occurs in the acidic environment of the endosome (37). TLR9 signaling requires acidification and maturation of the endo-
somes and is disrupted by agents that interfere with either of these processes (38). As such, it would be anticipated that the interaction between TLR9 and DNA would have evolved to be optimized under acidic conditions similar to those found in the late endosome. Surface plasmon resonance experiments with TLR9 suggest that the pH dependence of signaling is determined at the level of ligand binding as optimal interaction between the receptor and ODNs occurs at acidic pH values (23). Comparable pH dependence has been demonstrated for TLR3, another endosomal, nucleic acid-binding Toll-like receptor (39).

Titration of increasing concentrations of TLR9(LBD), at either pH 5.2 or 7.5, against a constant quantity of plasmid verified that the receptor was better able to bind plasmid at acidic pH (Fig. 3). This improvement in efficiency is demonstrated both by the disappearance of the unbound plasmid band as well as the increased formation of the dimeric nucleoprotein complex. This likely reflects the improved ability for TLR9 to bind nucleic acids and to form activated dimeric complexes, respectively. That the TLR9(LBD) recombinant protein maintains a consistent pH dependence as reported by others confirms the ability of our assay system to accurately describe the behaviors of the receptor.

Activating and Nonactivating ODNs Promote Plasmid Binding by TLR9—Unlike plasmid DNA, the limited net charge of an ODN is insufficient to cause migration of TLR9(LBD) into an agarose gel. As such, direct visualization of ODN binding is not possible through this system. However, it is possible to indirectly monitor the association of ODNs with TLR9(LBD) through their impact on TLR9(LBD)-plasmid complexes. Based on the expectation that plasmids and ODNs bind to a conserved site of TLR9, it was anticipated that these molecules would function as competitive inhibitors of each other such that binding of either molecule would preclude the binding of the other. Instead, ODNs were found to promote plasmid binding by TLR9(LBD). This is primarily demonstrated by the appearance of increased quantities of the dimeric nucleoprotein complex in the presence of increasing concentrations of the ODN (Fig. 4A). The ability of ODNs to exert this effect does not involve their incorporation into the complex as radiolabeled ODNs do not co-migrate with any of the distinctly migrating species in the agarose shift assays (data not shown). To a lesser extent, activation of ligand binding by TLR9 is also demonstrated by the decreased quantities of the unbound plasmid in the presence of increasing concentrations of ODN. Both of these events are independent of sequence as both activating and nonactivating PD-ODNs are equally efficient in promoting these effects (Fig. 4A).

**Phosphodiester ODNs More Effectively Sensitize TLR9**—To achieve greater stability through nucleic acid resistance, many
investigations of TLR9 utilize PTO-modified ODNs in which one of the nonbridging backbone oxygen atoms is replaced with sulfur. Clinical trials of TLR9 agonists, as well as much of the information that has been extrapolated to TLR9 ligand binding, are based upon investigations with these PTO-modified ODNs. There are reports however that this is not a neutral substitution and that this modification alters the specificity of interaction of ODNs with TLR9 (40–42).

To determine whether the PTO modification has any significance on the ability to influence TLR9-ligand binding, pairs of CpG- and GpC-containing ODNs of identical sequence, but consisting of either natural PD or modified PTO backbones, were examined for a cooperative effect on TLR9 ligand binding. Although both activating and nonactivating phosphodiester ODNs were equally effective in promoting plasmid binding, the PTO-ODNs were far less effective in this capacity (Fig. 4). That PTO-ODNs serve as analogs of bacterial DNA to initiate immune cell responses, but differ in their ability to mediate the same cooperative effect as their natural counterparts, emphasizes the functional distinction of this modification.

**Plasmids Enable TLR9 Ligand Binding**—The ability for nucleic acids to promote TLR9 ligand binding is not limited to ODNs as plasmids also exert a similar influence on the formation of dimeric nucleoprotein complexes. This was demonstrated in two ways. First, in the presence of two distinct plasmids, the first maintained at a constant concentration and the second titrated into the reaction mixture, TLR9 showed increased binding, and increased formation of the dimeric nucleoprotein complex involving the plasmid held at constant concentration, as the absolute concentration of plasmids increases (Fig. 5). This cooperative effect is independent of the identity of the plasmids, and titrating either of the plasmids against a constant quantity of the second plasmid results in an identical effect. Second, increasing the concentration of a single plasmid also results in the formation of the second distinctly migrating species over a range of concentrations consistent with those observed in the two plasmid system.

**TLR9 Specifically Binds CpG and Higher Order Motifs**—A wealth of cell stimulation experiments demonstrates the preferential activation of the innate immune system by CpG-, rather than GpC-, containing ODNs. This is suggestive that TLR9 binds nucleic acids in a sequence-specific manner. There have also been reports, perhaps most convincingly the immunoprecipitation of TLR9 by either CpG- or GpC-ODNs (25), to suggest TLR9 binds nucleic acids in a sequence-independent fashion. Our observations that ODNs, independent of their sequence, functionally influence TLR9 ligand binding also suggests that TLR9 has the ability to interact with, and be modulated by, nucleic acids in a sequence-independent fashion.

The interaction between TLR9(LBD) and ODNs can be directly visualized through acrylamide gel shift assays through which we observed that the recombinant receptor discriminates CpG from GpC ODNs with a high degree of efficiency (Fig. 6). This illustrates that although the protein can interact and be influenced by ODNs in a sequence-independent manner, stable complex formation is highly sequence-specific.

Different species demonstrate preferential responsiveness to particular sequences around the CpG dinucleotide as follows: murine cells to GACGTT and human and bovine cells to GTCGTT. The ability for these sequences to preferentially activate immune responses in different species has been hypothesized to arise as a consequence of their preferential recognition by the corresponding TLR9s. In particular, expression of TLR9 in a TLR9-deficient background confers maximal responsiveness to ODNs bearing the motif corresponding to maximal activation of the species from which the TLR9 originated (12).

Through direct binding assays we verify that the ability for higher order motifs to activate innate immune responses is a result of higher order sequence discrimination by TLR9. TLR9(LBD) is able to discriminate ODNs bearing CpG motifs in either the human or mouse higher order motifs with a high degree of efficiency. Our human TLR9(LBD) forms nucleoprotein complexes with ODNs bearing the human rather than mouse activating motif (Fig. 6). This specificity corresponds to the discrimination of ODNs that are identical at 31 of 33 positions.

**Plasmids Promote ODN Binding by TLR9**—The cooperative effect that was observed in ODNs promoting plasmid binding also occurs in a reciprocal fashion with plasmids promoting the
Formation of complexes between TLR9(LBD) and specific ODNs. At the ODN concentrations employed in this experiment, little association between TLR9(LBD) and ODNs was observed in the absence of plasmid (Fig. 6). This verifies the significance of the allosteric effect in sensitizing the receptor for ligand binding. The mutual cooperativity of plasmids promoting ODN binding, and ODNs promoting plasmid binding, would be anticipated to be occurring under all reaction conditions. The different techniques of agarose or acrylamide shift assays are able to visualize either plasmid or ODN-based nucleoprotein complexes, respectively. Therefore, the general conclusion is that nucleic acids, independent of structure or sequence, promote formation of TLR9 dimers with either single- or double-stranded ligands that contain activating sequences.

**Allosteric Activation of Endogenous TLR9**—That ODNs and plasmids exerted a mutually cooperative effect on ligand binding in vitro prompted consideration of whether a similar effect would be observed in cellular responses mediated by endogenous TLR9. The ability for stimulatory and nonstimulatory ODNs to enhance cellular responses to plasmid DNA was determined through cellular proliferation assays, which are standard assay for quantification of TLR9-mediated cellular activation.

For phosphothioate ODNs, the magnitude of the cellular responses to co-stimulation with plasmid and either CpG or GpC ODNs was higher than the sum of the individual responses to these stimuli given in isolation (Fig. 7). The data presented represent the average of experiments performed in triplicate for a single animal. Although all three animals, each tested in triplicate, gave the same trend of responses, the magnitude of these responses is unique for each animal. This is typical of the animal-to-animal variation in the magnitude of TLR9-mediated responses that has been observed by our group (33). Notably the fold induction of the responses to the co-stimulation by ODN and plasmid, as compared with the sum of the individual responses to plasmid and ODN administered separately, was quite consistent across animals. For CpG-PTO 2007 ODN, the magnitude of the allosteric effect was a 2.9 ± 0.9-fold (mean ± 1 S.D.) induction, whereas GpC-PTO 2007 ODN mediated a 1.6 ± 0.7-fold induction. The fold inductions are calculated as the ratio of the response to co-stimulation with both plasmid and ODN as compared with the sum of responses to plasmid and ODN alone. That a more potent induction of immune responses was observed with the CpG-PTO-ODNs reflects not only the ability for this ODN to promote a cooperative effect of plasmid-mediated activation but also plasmid-mediated cooperativity on ODN-mediated responses that, as would be anticipated, are highly sequence-specific.

A point of discrepancy between the in vitro and in vivo assays was that in ligand-binding experiments, the PTO-modified ODNs were considerably less efficient in mediating the cooperative effect of plasmid binding by TLR9. However, these modified ODNs elicited a much stronger cooperative effect in vivo. This distinction is likely a result of the concentrations of PD-ODN and PTO-ODNs that are presented to the endogenous TLR9. Although equal quantities of the natural and modified ODNs were added to the cell stimulation mixtures, the increased stability, as well as increased rates of uptake of PTO-ODNs, approximately an order of magnitude higher (43), would mean the functional concentration of PTO-ODNs in the endosome would be much higher than for PD-ODNs. A more
appropriate statement may be that in the endosomes phosphodiester ODNs exert a cooperative effect on TLR9 at much lower concentrations than phosphothioate ODNs, but it is exceedingly difficult to achieve these endosomal concentrations through direct administration. Bacterial DNA would normally be taken up into the endosome via phagocytosis of intact bacteria, offering protection from nuclease degradation until bacterial lysis releases microbial DNA in proximity to TLR9.

**Kinetics of TLR9 Activation Support Allosteric Activation**

Based on Michaelis-Menten kinetics, an ~80-fold increase in the substrate concentration is required to increase signaling from 10 to 90% in the absence of any cooperative effect. In the event of positive cooperativity, where the subsequent binding events are favored over the first, this magnitude of activation can be achieved with less than 5-fold increases in substrate concentration. With negative cooperativity, where the second binding event is less favored than the first, up to 400-fold increases in substrate concentration may be required to increase receptor signaling from 10 to 90% (44).

For Toll, negative cooperativity is apparent as 300–500-fold increases in the concentration of the ligand Spätzle are required to increase signaling activity from 10 to 90% (30). In contrast, activation of TLR9 occurs over a much narrower range of substrate concentrations. Using the example of the 2007 CpG-ODN, full activation of TLR9 occurs within a range of substrate concentrations of under an order of magnitude, consistent with positive cooperativity (Fig. 8). Other investigations of TLR9, employing different activating ODNs, have reported activation over a comparable range of substrate concentrations (45, 46).

**DISCUSSION**

TLR9 has attracted considerable attention for its role in the initiation of innate immune responses as well as for the ability to modulate its responses through nucleic acid-based therapeutics. Agonists of TLR9 are under active clinical trials for a variety of applications, including as immune adjuvants, cancer therapies, anti-allergens, and immunoprotective agents. Given the emerging therapeutic promise of this class of molecules, coupled with their potential to induce deleterious consequences such as autoimmune disorders, it is timely and appropriate to characterize the structural parameters that define TLR9 ligands to facilitate the design of more efficient agonists. To date, investigations of TLR9 ligand binding have been limited by difficulties associated with the purification of sufficient quantities of the receptor for biochemical characterization. As such, many of the assumptions of TLR9 ligand binding properties have been extrapolated from cell stimulation readouts. An inherent danger of this approach is that the reduction of complex physiological responses to a single measured output fails to consider the numerous potential points of regulation within the system, making it difficult to reach definitive conclusions on discrete events such as ligand binding.

Collectively, there is currently little consensus on the fundamental issues of TLR9 activation as follows: 1) the efficiency of the receptor in binding double-stranded DNA; 2) the absolute requirement for CpG motifs within nucleic acids to mediate ligand binding and cellular activation; 3) the ability of TLR9 to recognize higher order motifs in a species–specific fashion; 4) the influence of the commonly utilized phosphothioate modification on receptor binding; and 5) whether TLR9 ligand binding is influenced by higher order allosteric mechanisms similar to those that have recently been reported for Toll. Here we report novel methodologies for both the purification of the soluble ligand-binding domain of human TLR9 as well as a convenient high throughput assay that permits comparative analysis of nucleic acid binding by the receptor. By employing these systems, we are able to address all of these outstanding issues.

Although it has been demonstrated that innate immune responses can be initiated by plasmid DNA (9) and time course experiments verify that plasmids remain intact within the endosomes for a sufficient duration to serve as TLR9 ligands (47), previous investigations have reached opposing conclusions with respect to the ability of TLR9 to bind double-stranded DNA. Although a system established by Cornélié et al. (24) demonstrated effective binding of plasmids, Rutz et al. (23) reported a weak interaction between TLR9 and double-stranded ligands. Our observation that TLR9 undergoes preferential association with supercoiled species of plasmids likely accounts for the differing conclusions. In the investigation by Rutz(23), double-stranded ODNs, rather than plasmids, were employed as ligands. The inability of double-stranded ODNs to form supercoiled species would account for their reduced efficiency as ligands.

With respect to pH specificity, investigations of TLR3, another nucleic acid-binding TLR of the endosome, demonstrate that ligand binding and dimerization are dependent upon the acidic pH environment of the endosome (39). Similarly, surface plasmon resonance investigations of ligand binding by TLR9 demonstrate similar pH dependence (23). This would seem to indicate that the pH dependence of TLR9 signaling occurs at the level of ligand binding. Consistent with this hypothesis, we also observe that the efficiency of TLR9 ligand binding, in a system that does not require immobilization of either the receptor or ligand, is highly pH-dependent. At acidic pH values, comparable with the physiological environment of...
Specificity and Cooperativity of Ligand Binding by TLR9

the late endosomes where TLR9 encounters its ligands, the recombinant receptor had greater ability to form and maintain specific nucleoprotein complexes.

From investigations of immune cell responses the paradigm of CpG-mediated activation of TLR9 has been well established. The hypothesis of CpG-specific activation is consistent with the observation that these motifs are favored in microbial rather than host genetic material, providing a structural mechanism by which the innate immune system can differentiate PAMPs from a structurally similar endogenous molecule. For which CpG-specific activation of the innate immunity has generally been assumed to reflect sequence-specific binding by TLR9, recent in vitro binding assays have reached contradictory conclusions with respect to the ability of the receptor to associate with nucleic acids in a sequence-dependent manner (23–29). Our investigation may offer an explanation into this apparent discrepancy by demonstrating both the ability of TLR9 to form stable and highly sequence-specific nucleoprotein complexes but also to be functionally influenced by, and therefore presumably interact with, nucleic acids in a sequence-independent fashion.

Through our in vitro binding assays, we demonstrate the ability for TLR9 to discriminate the CpG motif, as well as higher order sequences of 6 bp in length. The 6-bp CpG motif preferentially bound by our recombinant human TLR9 protein corresponds with the sequence that elicits the greatest in vivo responses in humans. As such, formation of stable complexes is highly sequence-dependent, and this likely dictates the specificity of activation of the innate immune system.

In contrast to the high specificity of ODN complex formation, our results also demonstrate the ability for single- and double-stranded nucleic acids, in a sequence-independent fashion, to promote TLR9 ligand binding and subsequent activation both in vitro and in vivo. That activation of TLR9 is regulated by such high order regulatory mechanisms is consistent with the recent observation that Toll, a receptor with significant structural and functional similarity to the TLRs, is regulated through a complex mechanism involving both receptor-receptor and receptor-ligand interactions.

Activation of Toll is proposed through a two-step mechanism in which formation of a ligand-bound monomer precedes the formation of the activated dimer. Spätzle, the Toll ligand, functions to modulate Toll responsiveness by exerting a negative cooperative effect on the binding by the second subunit (Fig. 9). This has been verified through binding experiments as well as the kinetics of activation of Toll (Fig. 8). As Spätzle binds to the active site of Toll and exerts an inhibitory influence on receptor activation, it can be classified as a homotropic allosteric inhibitor. In the study by Gay et al. (28), this effect is reported as negative cooperativity and is proposed to increase the range of substrate concentrations to which Toll is responsive. These authors (28) hypothesize that a similar mechanism may influence the activation of the Toll-like receptors.

In contrast to the negative cooperativity reported for Toll, we have observed the ability for single- and double-stranded nucleic acids to exert a positive cooperative effect on TLR9 ligand binding. TLR9 is better able to form nucleoprotein complexes with plasmid molecules in the presence of ODNs and is also better able to bind ODNs in the presence of plasmid. This occurs in a sequence-independent fashion as CpG and GpC ODNs are equally effective in promoting this behavior. This positive cooperative effect is observed at the level of ligand binding as well as during activation of endogenous TLR9 both through cellular activation experiments as well as for the kinetics of TLR9-mediated responses.

This positive cooperativity of TLR9 is hypothesized to result from the requirement for the receptor to form dimers to discriminate activating from nonactivating nucleic acids. Although nonactivating nucleic acids are rapidly released, the dimer momentarily persists during which time it has a higher ligand binding affinity. The associations with nonactivating ODNs are sufficient to cause dimer formation and shift the monomer-dimer equilibrium of TLR9 to the sensitized form of the receptor. The schematic in Fig. 9 illustrates the proposed equilibrium between the different forms of TLR9 as follows: monomer, occupied dimer, and ligand-free dimer (Fig. 9). This model requires TLR9 to associate with nucleic acids in a sequence-independent fashion, the occurrence of which is becoming increasingly well established.

Notable for TLR9, we did not observe the presence of labeled ODNs in plasmid-TLR9 nucleoprotein complexes, which indicates that the ability to exert a cooperative effect does not involve the simultaneous binding of each type of nucleic acid. Although for TLR9 it has not been definitively demonstrated that activating and nonactivating sequences bind to the same site of the receptor, indeed the ligand-binding site of TLR9 has yet to be defined, our demonstration of the inability to simultaneously bind two types of nucleic acids suggests that binding of both
classes of molecules occurs at a conserved site. These modulatory interactions would therefore represent a form of homotropic allosteric activation. There is the possibility, however, that the ability for nucleic acids to influence TLR9 function may not require specific interactions with the receptor but rather influence the monomer/dimer equilibrium through a more general mass action mechanism.

Collectively, these results demonstrate that nucleic acids influence TLR9 in both sequence-dependent and sequence-independent fashions. Although the binding of ligands for activation of the system is highly sequence-specific, nonactivating nucleic acids are able to modulate TLR9 responsiveness through sensitizing the receptor for ligand binding. That ODNs are able to modulate TLR9 function through influencing sensitivity helps to reconcile the ability of these molecules to interact, but not directly activate, TLR9. Taken together with the observations of Toll, this suggests that both of these receptor families activate through a two-step process with the initial binding event determining specificity, and the second binding event dictating sensitivity in a positive or negative fashion.

The physiological significance of this cooperative effect may be in determining the set point for activation of TLR9. That increased quantities of nucleic acid are able to sensitize the system in a sequence-independent fashion suggests that release of nucleic acids following bacterial uptake within the phagosome primes the system for innate immune responsiveness if activating CpG motifs are present. This dual requirement for activation may function as a safeguard to prevent the induction of inappropriate innate immune responses.

REFERENCES
1. Hallman, M., Rämet, M., and Ezekowitz, R. A. (2001) Pediatr. Res. 50, 315–321
2. Ahmad-Nejad, P., Hacker, H., Rutz, M., Bauer, S., Vabulas, R. M., and Wagner, H. (2004) Eur. J. Immunol. 34, 2541–2550
3. Krieg, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Sumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000) Nat. Immun. 4, 594–600
4. Kowalski, J., Gilbert, S. A., van Drunen-Little-van der Hurk, S., van der Hurk, J., Babyak, L. A., and Zamb, T. J. (1993) Vaccine 11, 1070–1077
5. Rouas, R., Uch, R., Cleuter, Y., Jordier, F., Bagnis, C., Mannoni, P., Lewalle, H., Ray, K. P., Morse, M. A., Imler, J. L., and Gay, N. J. (2003) Nat. Immun. 4, 794–800
6. de Bouteiller, O., Merck, E., Hasan, U. A., Hubac, S., Benguigui, B., Trinchieri, G., Bates, E. E. M., and Caux, C. (2005) J. Biol. Chem. 280, 38133–38145
7. Bennett, R. M., Gabor, G. T., and Merritt, M. M. (1985) J. Clin. Investig. 76, 2182–2190