Direct Evidence that Toll-like Receptor 9 (TLR9) Functionally Binds Plasmid DNA by Specific Cytosine-phosphate-guanine Motif Recognition*

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Cytosine-phosphate-guanine (CpG) motifs in bacterial DNA are known to activate the mammalian immune system, and this activation is thought to depend on the Toll-like receptor 9 (TLR9) signaling pathway. Previous studies strongly suggested that TLR9 is involved as the specific receptor for CpG motifs but did not provide direct evidence of their interaction. In this study, we demonstrate for the first time that murine TLR9 binds an unmethylated CpG-containing plasmid. This interaction is sequence-specific and is influenced by the methylation status of the plasmid. Furthermore, we demonstrate that this interaction leads to the activation of the NF-κB pathway in mTLR9-expressing cells. Our results provide a molecular basis for the interaction between CpG-DNA and TLR9.

The recognition of pathogens by the mammalian immune system is a key step in the initiation of the immune response, and the toll-like-receptor (TLR) family is an essential component of this process (1, 2). In humans, tenTRLs (TLR1–10) have been identified (1, 3). Among these, TLR4 is involved in the recognition of lipopolysaccharide (LPS) from Gram-negative bacteria, TLR2 detects a variety of cell wall components, TLR6 in association with TLR2 senses lipoproteins, and TLR5 recognizes flagellin (4–11). Members of the TLR family have some common features: they are transmembrane proteins with a large extracellular domain containing several leucine-rich repeats and a cytoplasmic Toll/IL-1-receptor domain. In general, activation through the Toll/IL-1-receptor domain involves the MyD88-dependent interleukin 1 receptor-TLR signaling pathway which leads to the activation of nuclear factor-κB (NF-κB) (12–14).

Bacterial DNA is a potent stimulus for immune cells. This stimulatory activity is due to a sequence motif containing unmethylated CpG deoxyribonucleotides, the methylation status being a crucial distinction between bacterial and mammalian DNA (15–18). Unmethylated oligodeoxyribonucleotides (ODN) including a CpG motif (CpG-ODN) can mimic the effects of bacterial DNA, inducing B-cell proliferation and activating cells of the myeloid lineage (dendritic cells; Refs. 19–26). A molecular understanding of the cellular recognition of CpG-DNA is only now beginning to emerge. Indeed, it was first demonstrated by Hemmi et al. (27) that TLR9 is involved in the signaling induced by synthetic CpG-ODN. Chuang et al. have established that the signaling cascade induced by CpG-ODN involves MyD88, IL-1 receptor-associated kinase (IRAK), and TRAF6 (28). Moreover, several investigators have demonstrated that CpG motifs present in plasmid DNA could induce the activation of the immune response (29, 30).

In this paper, we demonstrate for the first time that a plasmid containing unmethylated CpG motifs physically binds to TLR9. This interaction is sequence-specific and depends on the methylation status of the CpG motifs. Furthermore, this interaction is sufficient to activate the NF-κB transcription factor.

Experimental Procedures

Cloning mTLR9—The full-length mTLR9 was PCR-amplified from a mouse lung single-stranded cDNA library. Specific primers for amplification of mTLR9 were designed based on the GenBank™ accession number AF314224 (28). The forward primer was 5′-TCCTCCATCTC-CCACATggTTCCTC, and the reverse primer sequence was 5′-TGCTT-gCTggTAGGTCCggCggAgAg. PCR was performed with 20 μl of platinum Taq polymerase (Invitrogen). The amplified full-length cDNA was subcloned into pCR4 containing the V5 and polyhistidine tags (Invitrogen) and sequenced.

For expression in SF9 insect cells, mTLR9 was sub-cloned in the pI7Z expression vector containing the V5 and poly-histidine tags (Invitrogen). This vector expressed the green fluorescent protein constitutively as a positive control of transfection.

Cell Culture, NF-κB Reporter Assay, and Immunolocalization—Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco’s modified Eagle’s medium with Glutamax (Invitrogen, France) supplemented with 10% fetal bovine serum. The HEK293 cells were plated at 4 × 10⁴ cells in six-well plates and transfected by Exgen (Euromedex, France) with 475 ng of mTLR9 expression vector plus 1 μg of NF-κB luciferase reporter plasmid (Stratagene, France). Twenty-four hours later, the cells were treated with indicated agonists for 6 h. The cells were lysed, and luciferase activity was determined using reagents from Promega Corp. Results were normalized according to the protein content measured by the BCA reagent (Pierce-Perbio, France). Results are expressed as fold induction compared with unstimulated controls, and the data presented are the mean ± S.E. (n = 3). To demonstrate that the mTLR9 was expressed on the cell surface, 4 × 10⁵ HEK293 cells or SF9 cells were plated on six-well plates and transfected with Exgen (Euromedex, France) with 475 ng of mTLR9 expression vector plus 1 μg of NF-κB luciferase reporter plasmid (Stratagene, France). Twenty-four hours later, the cells were treated with indicated agonists for 6 h. The cells were lysed, and luciferase activity was determined using reagents from Promega Corp. Results were normalized according to the protein content measured by the BCA reagent (Pierce-Perbio, France). Results are expressed as fold induction compared with unstimulated controls, and the data presented are the mean ± S.E. (n = 3). To demonstrate that the mTLR9 was expressed on the cell surface, 4 × 10⁵ HEK293 cells or SF9 cells were plated on six-well plates and transfected with 1 μg of mTLR9 DNA per well. Forty-eight hours later, cells were washed twice in phosphate-buffered saline and fixed in 4% paraformaldehyde. HEK293 cells were stained with anti-V5 antibody-fluorescein isothio-cyanate antibody (Invitrogen) 1 h at room temperature, washed three times with phosphate-buffered saline, and incubated for a few minutes with phosphate-buffered saline plus 4′,6-diamidino-2-phenylindole for...
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DNA staining. SF9 (Spodoptera frugiperda) cells were stained with anti-V5 as a primary antibody and then with anti-mouse IgG phycoerythrin-conjugated antibody (Sigma).

The insect cell line SF9 was used to produce large amounts of mTLR9 and was cultured in Grace insect cell medium (Invitrogen, France) supplemented with 10% fetal bovine serum and 5% glutamine. SF9 cells were transfected with Cellfectin (Invitrogen) at 50% of confluence with 25 μg of DNA per 75 cm² flask. Forty-eight hours later, cells were centrifuged, and pellets were frozen for use in surface plasmon resonance experiments.

Surface Plasmon Resonance—The Biacore 3000 instrument (Uppsala, Sweden) was used with an NTA sensor chip (nitriloacetic acid bound to a solid support). SPR buffers and solutions were as follows: eluent buffer (10 mM HEPES, 0.15 M NaCl, 50 mM EDTA, 0.005% surfactant P20, pH 7.4); nickel solution (500 μM NiCl₂ in eluent buffer); and regeneration buffer (10 mM HEPES, 0.15 M NaCl, 350 mM EDTA, 0.005% surfactant P20, pH 8.3). Two flow cells were run in parallel: on the first one, irrelevant His-tagged chloramphenicol acetyl transferase (CAT) protein was immobilized to provide background corrections for the first one, irrelevant His-tagged chloramphenicol acetyl transferase (CAT) protein was immobilized for binding purposes.

Surfacing and background corrections were determined by using the limulus amebocyte lysate assay (BioWhittaker, Norway) with 1 μg of the appropriate expression plasmid. a, an anti-V5 as a primary antibody and then an anti-mouse phycoerythrin antibody were used to stain SF9 transfected cells.

HEK293 cells. We transiently cotransfected 4 × 10⁵ cells with the mammalian expression vector for mTLR9 together with a luciferase-reporter gene driven by an NF-κB promoter. As a first step, cells were activated 24 h later with 3 μg of bioactive CpG-ODN or 3 μg of control inverted GpC-ODN for 6 h. In HEK293, bioactive CpG-ODN markedly induced NF-κB activity (Fig. 2a), which was 13-fold higher than that observed with unstimulated cells. Activation of the cells by control inverted GpC-ODN induced only marginal relative NF-κB activity, comparable with that detected when mTLR9 was not expressed by the cells. Furthermore, this activity was not observed when the cells were incubated in the presence of other bacterial stimuli such as LPS. These results indicate that the NF-κB activation of the HEK293 transfected cells by the bioactive CpG-ODN is strongly dependent upon mTLR9 expression and is sequence-specific. These results are consistent with those obtained with human TLR9 by other authors (28, 31, 32).

Plasmid DNA Activates NF-κB in a CpG-dependent Manner—Induction of NF-κB activity via mTLR9 by a CpG-containing plasmid was investigated next. To address this question, we used two plasmids similar in their sequences which do not code for any eukaryotic protein. These two plasmids were either fully methylated or unmethylated on the CG sequences and were derived from the pACY184 replicon. Both methylated and unmethylated CpG-containing plasmids contained about 60 CpG motifs. The pAyt2 plasmid expresses the methylase SsI gene, allowing the de novo methylation specifically on the CG sequences in bacteria; we called this the methylated CpG-containing plasmid. By introducing a frameshift in the methylase gene, we generated the unmethylated CpG-containing plasmid which contains the same number of CpG motifs, but which is not methylated. The methylation status of plasmids was verified by using a functional test with restriction enzymes. Plasmids were treated with HpaII, which cut the unmethylated restriction site (C ↓ CGG), or by MspI, which recognizes the same restriction site independently of its methylation state. This treatment proved that no one CpG was found unmethylated in the plasmid coding the SsI methylase gene. We made use of these two plasmids to test whether they could induce NF-κB activity in vitro. HEK293 cells were transiently transfected with both mTLR9 and NF-κB-expressing plasmids as described above. Cells were then activated with 10 μg of plasmid DNA, corresponding to 80 nM of equivalent integrated CpG motifs. As shown in Fig. 2b, the unmethylated

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Fig. 1. Membrane localization of the mTLR9 in SF9 insect cells and HEK293 cells. 4 × 10⁵ cells were transfected with 1 μg of the appropriate expression plasmid. a, an anti-V5 as a primary antibody and then an anti-mouse phycoerythrin antibody were used to stain SF9 transfected cells. b, expression of the green fluorescent protein as a positive control for transfection in SF9 cells. c, images a and b merged, expression of mTLR9 on the plasma membrane. d, HEK293 cells were stained with a conjugated fluorescein isothiocyanate-anti-V5 antibody. e, DNA staining with 4′,6-diamidino-2-phenylindole. f, images of d and e merged. Magnification, ×40.
CpG-containing plasmid induced a 5-fold increase in NF-κB activity, whereas the same amount of methylated CpG plasmid provoked only a weak induction of 1.5-fold. Furthermore, only a 1-fold background activity was observed when the mTLR9 was not expressed by the cells. This result reinforced the hypothesis that unmethylated CpG-containing plasmid requires TLR9 for the activation of the NF-κB transcription factor. These results suggest that activation of the NF-κB transduction pathway is strongly dependent upon the methylation status of the CpG-containing plasmid.

NF-κB Activation by Plasmid DNA Requires Endosomal Internalization—To confirm these findings, a dose-response experiment with both plasmids was performed. The activation of the NF-κB transcription factor by unmethylated CpG-containing plasmid increased with the concentration of the plasmid (up to 20-fold for the 100 μM dose; 833 nM of equivalent integrated CpG motifs) (Fig. 3a). It seems, therefore, that the effect of unmethylated CpG-containing plasmid is dose-dependent. Furthermore, increasing the concentration of methylated plasmid had no influence on the relative NF-κB activity. This indicates that the weak activation observed with the methylated plasmid is nonspecific. These results strongly suggest that the stimulation by unmethylated CpG-containing plasmid is mediated by a receptor, probably the mTLR9. Bauer et al. (31) have already suggested that endosomal maturation is required for the NF-κB activity induced by CpG-ODN. Therefore, we tested whether unmethylated CpG-containing plasmid required endosomal maturation to mediate its effects. Pre-treatment of HEK293-transfected cells with chloroquine prevented the induction of NF-κB activity by either unmethylated CpG-containing plasmid or bioactive CpG-ODN (Fig. 3b). This result is consistent with the previously described inhibitory effect of chloroquine on CpG-ODN-induced activation in macrophages, dendritic cells, and B cells (33).

Plasmid DNA Binds mTLR9—BIAcore technology was used to study the physical interaction of CpG-containing plasmid with mTLR9 produced in SF9 cells. Expression of mTLR9 was tested by Western blotting for each batch of transfected cells (data not shown). After cell transfection and lysis, the supernatant containing His-tagged TLR9 was used to cover an Ni-NTA sensor chip. Several concentrations of the CpG-containing plasmid were tested in this experiment. To ensure that the observed interactions occurred with the TLR9, a correction for nonspecific binding was applied by subtraction of the control channel data where the chip was coated with an irrelevant His-tagged protein, the chloramphenicol acetyl transferase protein for each binding experiment, as described under "Experimental Procedures." As shown in Fig. 4a, when 250 nM (calculated as CpG motifs) of unmethylated CpG-containing plasmid was injected onto the sensorchip, a significant increase in the absorption of signal (37.1 resonance units (RU); corresponds to 1 pg/mm²) was observed. This difference was enhanced with
increased doses of unmethylated plasmid, demonstrating that the unmethylated CpG-containing plasmid binds to mTLR9. The kinetics of association are slow and saturable. On the other hand, injection of methylated CpG-containing plasmid showed flattened association curves and a much lesser increase in RU at the dissociation phase, suggesting nonspecific interactions. Therefore, we subtracted this nonspecific interaction from the sensorgrams of unmethylated CpG-containing plasmid and ODNs. 200 nM of unmethylated plasmid was injected alone (−), with 2 μM bioactive CpG-ODN (gray line), or with 2 μM control inverted GpC-ODN (−).

**DISCUSSION**

Recent research on pathogen-associated molecular patterns linked to immunity has highlighted the importance of the innate response (34). The patterns in question involve peptidoglycan, LPS, bacterial flagellin, lipoteichoic acid, double-stranded RNA, and bacterial DNA. These components are specific for bacteria and viruses and can signal the presence of potential infectious agents. The case of DNA is particularly specific for bacteria and viruses and can signal the presence of DNA, which is used by viruses as their genetic material. The discrimination between self and foreign DNA would depend upon certain sequence motifs and modifications such as methylation (35). Indeed, mammalian DNA is highly suppressed in CpG motifs, and most of them are methylated. In contrast, these CpG motifs are more frequent in bacteria and are not methylated in the cytosine residue (36). These features would allow the immune system to recognize specifically bacterial DNA and lead to the initiation of signal transduction within the cells (37). This would mark the starting point for the immune system to secrete cytokines and co-stimulatory molecules needed for the adaptive response (35).

Functional analyses in gene-deficient mice have identified TLR9 as a major component necessary for the responses mediated by synthetic CpG motifs, i.e. CpG-ODNs (27). The results suggest that TLR9 activation and signal transduction from the endosome to the cytoplasm activate MyD88, IRAK, and TRAF6 (38). These first mediators then induce transduction through mitogen-activated protein kinase and then the NF-κB transcription factor which initiates transcription of many immune response genes (39, 40).

In the present study, we demonstrate that murine TLR9
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selectively binds CpG-containing plasmid, and we provide evidence that this specific binding occurs only if CpG motifs are unmethylated. Biacore technology allowed us to determine the affinity constant ($K_a$) of this interaction, which is close to 200 nM (taking into account the molarity of CpG motifs in the plasmid). The affinity constant lies in the same range as the Speatzle-Drosophila Toll receptor interaction (90 nM) described recently (41). Moreover, our results show that the interaction between TLR9 and plasmid DNA is strictly sequence-specific. In competition experiments, CpG-ODN dramatically abolished the fixation of the CpG-containing plasmid to mTLR9, whereas the control inverted GpC-ODN was unable to interfere with this interaction. A recent paper of Verthelyi and Zeuner (42) suggested that other proteins could be involved in the signaling of the CpG DNA. This hypothesis was based on (i) no direct evidence of binding interaction between TLR9 and CpG-ODN, (ii) the localization of the TLR9 in the endosomes, and (iii) that a certain type of CpG-ODNs (D-ODNs), which are unable to activate HEK293 cells transfected with human TLR9. Here we demonstrate that a native form of bacterial DNA, i.e. plasmid DNA, binds directly to the TLR9 and is effective in activating the NF-$\kappa$B signaling pathway. The results presented here emphasize the fundamental role of TLR9 in the signaling of bacterial DNA but do not exclude the involvement of other intracellular or membrane molecules and/or the engagement of an adapter molecule different from MyD88, as suggested elsewhere. Indeed, Bandholtz et al. (43), using an electromobility shift assay, established that both unmethylated and methylated CpG-ODN could bind to the HSP90 chaperone protein but not ODN without the CpG motif. They hypothesized that the HSP90 protein could act as a transfer ligand for mTLR9. In our study, surface plasmon resonance experiments reveal that isolated mTLR9 does not require any co-receptor for the binding of CpG motifs. The mTLR9 saturation we observed was CpG dose-dependent, and this is consistent with a direct interaction between plasmid and mTLR9. However, HSP90 proteins are broadly distributed, and their involvement in the binding of CpG motifs to TLR9 in vivo or in vitro cell culture could not be excluded.

The interaction between TLR9 and CpG-containing plasmid is consistent with the results obtained in cell activation experiments. Indeed, our results show that the unmethylated CpG-containing plasmid induces the activation of NF-$\kappa$B transcription factor in an mTLR9-dependent manner. This activation is highly specific because methylated CpG-containing plasmid as well as methylated CpG-ODN (data not shown) is ineffective in inducing NF-$\kappa$B activation. We also showed that chloroquine blocked, at least in part, cellular activation by the unmethylated CpG-containing plasmid or by bioactive CpG-ODN. This effect suggests that endosomal maturation might be required for TLR9 signaling (44). This process probably allows the recruitment of the adapter MyD88 and the subsequent proteins contributing to the TLR9 transduction pathway (37, 45). Furthermore, it is interesting to note that the LPS transduction pathway via TLR4, which can use the same adapter proteins as TLR9, was not inhibited by chloroquine and derived compounds (46). Our results suggest that the activation of the transduction pathway induced by plasmid DNA requires its internalization and the acidification of the endosomal structures. However, it has been reported elsewhere that TLR9 is expressed in the endosomal compartment; therefore, this hypothesis strongly suggests the engagement of another molecule in allowing the entry of DNA into the cell. In this view, the scavenger receptor A, which is known to interact with large polyanionic structures, could be involved in the entry of DNA into the cells and might act as a co-receptor for the TLR9 (47).

The question of the binding site for the CpG motifs on the TLR9 is still open, and our data do not shed any light on this issue. Few data are available on the binding sites for the TLR. Mizel et al. (48) studied the fixation of bacterial flagellin on human TLR5. They generated several truncated forms of the extracellular domain of the hTLR5 and identified a region which is critical for the flagellin binding. This region contains a consensus sequence for a leucine-rich repeat. The known leucine-rich repeat-containing proteins share two fundamental characteristics, namely, the presence of repetitive leucine-rich sequences and their involvement in protein-protein interactions (49). However, this kind of interaction is not suitable for bacterial DNA and TLR9. Based on analogies with the methyl-CpG-binding proteins 1–4 (MDB 1–4) and DNA-methyl transferase (50, 51) which bind DNA by means of a CXXC motif (C = cysteine), such a motif has been identified in the sequence of both human and murine TLR9 (52). Bauer and Wagner (52) suggested that this domain is implicated in the recognition of bacterial DNA by the TLR9. However, it is possible that both leucine-rich repeat and CXXC domains contribute to the binding of bacterial DNA. Further experiments are needed to explore this issue, and we have initiated site-directed mutagenesis on the CXXC region.

In this study, we provide for the first time molecular evidences that DNA is more than the support of genetic material and constitutes a biochemical signature of species susceptible to infect mammals. Indeed, bacterial DNA can be directly recognized by the mammalian immune system by means of a receptor of the innate immune system, the TLR9. These data have a great importance for the rational use of DNA technology in both DNA vaccines and gene therapy. These results allow us a better understanding of how the mammalian immune system has evolved to selectively recognize pathogens. Unmethylated CpG DNA represents an original pathogen associated molecular pattern, which can be sensed by a specific receptor to engage the immune response to clear the infection and maintain the integrity of the organism.

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