Transcriptional Regulation of the Novel Toll-like Receptor Tlr13*

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Little has been known about Tlr13 (Toll-like receptor 13), a novel member of the Toll-like receptor family. To elucidate the molecular basis of murine Tlr13 gene expression, the activity of the Tlr13 gene promoter was characterized. Reporter gene analysis and electrophoretic mobility shift assays demonstrated that Tlr13 gene transcription was regulated through three cis-acting elements that interacted with the Ets2, Sp1, and PU.1 transcription factors. Furthermore, our work suggests that these transcription factors may cooperate, culminating in maximal transcription of the Tlr13 gene. In contrast, NF-κB appeared to act as an inhibitor of Tlr13 transcription. Overexpression of Ets2 caused a strong increase in the transcriptional activity of the Tlr13 promoter; however, overexpression of NF-κB p65 dramatically inhibited it. Additionally, interferon-β is capable of acting Tlr13 transcription, but the activated signaling of lipopolysaccharide/TLR4 and peptidoglycan/TLR2 strongly inhibited the Tlr13 gene promoter. Thus, these findings reveal the mechanism of Tlr13 gene regulation, thereby providing insight into the function of Tlr13 in the immune response to pathogen.

Upon infection, microorganisms are first recognized by cells of the host innate immune system, such as macrophages and dendritic cells, as well as mucosal epithelial cells (1–6). Recognition of pathogens is primarily mediated by a set of germ line-encoded molecules on innate immune cells that are referred to as pattern recognition receptors (7, 8). These pattern recognition receptors are expressed as either membrane-bound or soluble proteins that recognize invariant molecular structures from the pathogen called pathogen-associated molecular patterns (7, 8).

Recent studies on the recognition of microbial pathogen-associated molecular patterns have highlighted the vital role of one group of pattern recognition receptors, the Toll-like receptors (TLRs)2 (9, 10). It is already clear that TLRs play a crucial role in the recognition of “molecular signatures” produced by infecting microbes to engage differential signaling pathways (11, 12). Signaling through TLRs activates various transcription factors, such as nuclear factor-κB (NF-κB), activating protein-1 (AP-1), and interferon regulatory factors to induce an immunological response (3, 11).

Tlr13 is a novel and poorly characterized member of the Toll-like receptor family (3, 13). Although the elucidation of the function of Tlr13 depends mainly on the identification of its natural ligand, its transcriptional regulation also provide some clues. For example, which type of cells expresses Tlr13? Which transcription factors control Tlr13 expression? How do different pathogen-associated molecular patterns from different pathogens regulate Tlr13 expression? This information will perhaps help us understand not only how this novel TLR responds to different infections but also which pathogens might be recognized by Tlr13 to activate the innate immune response. Recently, Aderem et al. (14) reported that Tlr13 belongs to the Tlr11 subfamily based on phylogenetic analysis. We previously demonstrated that Tlr11 primarily expresses on epithelial cells and recognizes urinary pathogenic Escherichia coli (15) and profilin-like protein from parasite (16). We therefore studied transcriptional regulation of Tlr13 upon stimulation mainly with bacterial components, the results of which can be used as the starting point for characterization of this novel TLR.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—RAW 264.7, NIH 3T3, and HEK 293 cells were purchased from ATCC. These cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) and supplemented with 10% (v/v) heat-inactivated fetal bovine serum (HyClone), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO2 incubator. All of the TLR ligands were purchased from Invivogen. Antibodies for supershift analyses were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Bacteria used in this study, including the Staphylococcus aureus K2 strain and the urinary pathogenic E. coli 8NU strain (15), were frozen at −80 °C in 1-ml aliquots in 10% glycerol at 2 × 108 colony-forming units/ml. The frozen aliquots were thawed and heat-killed before each use. Recombinant mouse IFN-β was purchased from R&D Systems.

Isolation of Total RNA and RT-PCR—Total RNA was isolated with TRIzol reagent (Invitrogen). cDNA was prepared by oligo(dT)12–18 and reverse transcriptase SuperScript II from Invitrogen with 2 μg of DNase I-treated total RNA. One μl of cDNA was amplified using the primers shown in Table 1. The parameters of the PCR were as follows: denaturation at 94 °C
for 3 min followed by 25–35 cycles of 94 °C for 20 s, 57 °C for 20 s, and 72 °C for 30 s. The PCR products were subjected to electrophoresis in 1% agarose gels, visualized under UV light after ethidium bromide staining, and then imaged.

### TABLE 1

| Oligonucleotide | Sequence (5’→3’) | Purpose |
|----------------|-----------------|---------|
| TLR13R369      | GCCCAAGAAAGAACATTATGACACTGAATTCTG-3’ | 5’-RACE-PCR |
| TLR13R299      | AACTGCTTTGAACGATCACCTGACATTTTAC | 5’-RACE-PCR |
| NF-κB wild type | ATCTGGCGTGGAGGCTATTGACCATGAGCCTAGCCTCTG | EMSA/mutagenesis |
| NF-κB mutant   | GAGGAAAGAAAGAACATTATGACACTGAATTCTG-3’ | EMSA/mutagenesis |
| Ets2 wild type | GAGAAGAAAGAACATTATGACACTGAATTCTG-3’ | EMSA/mutagenesis |
| Ets2 mutant    | GAGAAGAAAGAACATTATGACACTGAATTCTG-3’ | EMSA/mutagenesis |
| Sp1mF          | GCCGCTTGTTAAGATAGATGAGCCTGTTTGG | Mutagenesis |
| Sp1mR          | GCCGCTTGTTAAGATAGATGAGCCTGTTTGG | Mutagenesis |
| PU.1mF         | TCTTTTGGAACTTCATAAGGAAATGTCTCA-5’ | Mutagenesis |
| PU.1mR         | TCTTTTGGAACTTCATAAGGAAATGTCTCA-5’ | Mutagenesis |
| TLR13F         | TGTCTGCTCTGGTGGACTTG | RT-PCR |
| TLR13R         | GGAAGCTTATGAGCGACCAAGATCACTC-3’ | RT-PCR |
| β-actin-F      | AAATCCTGTTGCCCCAGTAAAGAC | RT-PCR |
| β-actin-R      | AAGGCAATGCTGATACGACACTCCG | RT-PCR |
| Ets2-B-F       | GCAAGATCTTGCTTTCTTCTTGTGGTACCA-5’ | Cloning |
| Ets2-E-R       | GCAAGATCTTGCTTTCTTCTTGTGGTACCA-5’ | Cloning |
| PU.1-B-F       | CAGCTGCTCGTTTGTGGGCACTGCTG | Cloning |
| PU.1-E-F       | CAGCTGCTCGTTTGTGGGCACTGCTG | Cloning |
| Sp1-H-F        | GGAATTCAGATTTGAAAGCTAAATCCAC | Cloning |
| Sp1-R-F        | GGAATTCAGATTTGAAAGCTAAATCCAC | Cloning |
| Raf1-B-F       | AGTAAGCTGGTCCGACAGGAGGAGG | Cloning |
| Raf1-R-F       | AGTAAGCTGGTCCGACAGGAGGAGG | Cloning |

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—Mouse cDNA encoding full-length Tlr13 was first treated with calf intestinal phosphatase to remove free 5′-phosphates from all RNA molecules. The RNA was then treated with tobacco acid pyrophosphatase to remove the cap structure, leaving a 5′-monophosphate. A 45-base RNA adapter oligonucleotide was ligated to 10 μg of the treated RNA using T4 RNA ligase. A random-primed reverse transcription reaction was performed using Moloney murine leukemia virus reverse transcriptase and then followed by nested PCR to amplify the 5′-end of murine TLR13. The gene-specific primers are listed in Table 1. The PCR product was cloned into pJET1.2/blunt Cloning Vector (Fermentas) and sequenced.

**Plasmid Constructions**—Mouse cDNA encoding full-length TLR13 was first treated with calf intestinal phosphatase to remove free 5′-phosphates from all RNA molecules. The RNA was then treated with tobacco acid pyrophosphatase to remove the cap structure, leaving a 5′-monophosphate. A 45-base RNA adapter oligonucleotide was ligated to 10 μg of the treated RNA using T4 RNA ligase. A random-primed reverse transcription reaction was performed using Moloney murine leukemia virus reverse transcriptase and then followed by nested PCR to amplify the 5′-end of murine TLR13. The gene-specific primers are listed in Table 1. The PCR product was cloned into pJET1.2/blunt Cloning Vector (Fermentas) and sequenced.

**Cloning and Sequencing of the Tlr13 Gene**—Murine genomic DNA (C57/B6 strain) was amplified with the Expand High Fidelity PCR system (Roche Applied Science) according to the manufacturer’s instructions. PCR conditions were as follows: 92 °C for 2 min, one cycle; 92 °C for 15 s, 57 °C for 20 s, and 68 °C for 2 min for 30 cycles; and one cycle of incubation at 68 °C for 5 min. Murine TLR13-specific amplification was achieved using the sense primer 5′-GTGGTACCCAGTGCCTAAGATGACCTG-3′, containing a KpnI restriction enzyme site, and the antisense primer 5′-GCAGAATCTGCTAACAATGACATCGTCTG-3′, containing a BglII restriction enzyme site. An approximately 1.9-kb KpnI/BglII fragment was subcloned into the pGL3 basic vector (Promega). The complete sequence was determined with autosequencing by the Protein and Nucleic Acid Chemistry Facility at Baylor College of Medicine in Houston. Truncated mutants of the 5′-flanking region were also cloned into the pGL3 with the same restriction enzyme sites. Mutations and deletions of putative transcription factor binding sites were carried out by two-step PCR mutagenesis with the primers listed in Table 1.

**Transient Transfections and Luciferase Assay**—All transfections were performed in triplicate in 24-well plates. Approximately 2 × 10^5 cells/well were seeded 24 h before transfection. Following the manufacturer’s instructions, plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen). Briefly, 0.8 μg of reporter plasmids together with 0.02 μg of Renilla pRL-TK vector (Promega) were diluted with Opti-MEM and then mixed with diluted Lipofectamine 2000. After a 20-min incubation at room temperature, the mixtures were added to each well. At 24 h post-transfection, cells were either analyzed for luciferase activity or further challenged with different agonists of TLRs for the treatment times indicated. Luciferase assays were performed using the Dual Luciferase Assay System (Promega), which contains an internal control that is detectable simultaneously with the luciferase reporter gene. Each experiment was conducted a minimum of three times.

**Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)**—Nuclear extracts of RAW 264.7 cells were prepared as described previously (17). Briefly, RAW 264.7 cells were harvested by scraping in phosphate-buffered saline, pelleted, and then lysed in 500 μl of lysis buffer containing 0.5% Nonidet P-40, 10 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, and protease inhibitor mixture (Roche Applied Science). Intact nuclei were pelleted by centrifugation at 12,000 × g at 4 °C for 5 min and lysed in 150 μl of nuclear lysis buffer containing 20 mM HEPES (pH 7.5), 25% glycerol, 0.42 M NaCl, 0.2 mM EDTA, and protease inhibitor mixture. The protein concentration was determined using the BCA assay (Pierce). The double-stranded DNA probes used in the gel mobility shift assays are shown in Table 1. The EMSA was performed using Gel Shift Assay Systems (Promega). Briefly, 2.5 μg of nuclear extract was incubated with 10 ng of each labeled probe in binding buffer containing 0.5 mM EDTA, 0.5 mM dithiothreitol, 4% glycerol, 1 mM MgCl₂, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.05 mg/ml poly(dI–dC)-poly(dI–dC) for 20 min at room temperature. To demonstrate sequence-specific binding, some of the reactions contained a 100-fold excess of the same unlabeled probe and other unlabeled probes to determine specific and nonspecific binding. Furthermore, specific antibodies against p65 and p50 for supershift assays were included in other reactions. The reaction mixtures were then separated in a 6% non-denaturing polyacrylamide gel at room temperature in 0.5× TBE buffer at 100 V for 3 h. The gel was transferred to Whatman 3MM paper, dried, and exposed to x-ray film overnight at −70 °C with an intensifying screen. The
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**Figure 1. Suppression of Tlr13 mRNA expression by LPS, PGN, and bacterial lysates in macrophage RAW 264.7 cells.** A, regular RT-PCR was performed to analyze Tlr13 and β-actin mRNA expression in various murine cell lines, including RAW 264.7 cells, mouse embryonic fibroblasts, and NIH 3T3. B, real-time PCR analysis was performed to quantify Tlr13 expression in RAW 264.7 cells treated with 40 μl/ml bacterial lysates, 5 μg/ml PGN, 100 ng/ml LPS, or 25 μg/ml poly(I-C) for 1 or 3 h, as indicated. Staphylococcus aureus K2 strain; PGN, peptidoglycan; poly(I-C), mimic of viral double-stranded RNA. The graph shows the mean ± S.D. of three independent experiments; *, p < 0.05; **, p < 0.001. Statistical analysis was performed by Student’s t test.

Quantitative RT-PCR—Total RNA was isolated from cells using the RNAeasy kit (Qiagen). For each sample, 1 μg of total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen). The reverse transcription reaction was diluted 1:10, and 2 μl of the diluted sample was added to an 18-μl PCR assay mixture containing a 0.5 μM concentration of each primer and 1× SYBR Green JumpStart Taq ReadyMix (Sigma). PCR was conducted with the MyiQ single-color real time PCR detection system (Bio-Rad) using the following conditions: hot start activation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 61 °C for 30 s, and 72 °C for 30 s. Two sets of PCR assays were performed for each sample using the primers listed in Table 1. The threshold cycle number for each PCR product was determined after sequencing. Exon I in Raw264.7 was normalized to that of β-actin, and the resulting value was converted to a linear scale. All assays were performed at least three times from independent RNA preparations.

**RESULTS**

Characterization of Tlr13 Gene Expression in Macrophage RAW 264.7 Cells—Tlr13 is a novel member of the mammalian Toll-like receptor family, and little is known about its expression and function (3, 13). We therefore started by analyzing Tlr13 gene expression in various murine cell lines, including RAW 264.7 macrophages, mouse embryonic fibroblasts, and NIH 3T3 fibroblasts by semiquantitative RT-PCR. As shown in Fig. 1A, RAW 264.7 cells constitutively expressed the highest level of mRNA for Tlr13 among the cell types tested. We then pursued the transcriptional responses of Tlr13 upon stimulation in RAW 264.7 cells. Tlr13 mRNA levels were monitored by real time RT-PCR after incubation of RAW 264.7 cells with various TLR agonists, including 5 μg/ml peptidoglycan (PGN), 100 ng/ml LPS, and 25 μg/ml poly(I-C) (mimic of viral double-stranded RNA), as well as Gram-positive (S. aureus K2 strain) and Gram-negative (urinary pathogenic E. coli 8N strain) bacterial lysates. Surprisingly, as shown in Fig. 1B, we found that the expression of Tlr13 with various treatments was significantly reduced 1 h after treatment; levels subsequently declined over longer time periods (Fig. 1B). Specifically, at 3 h post-treatment, the original level of Tlr13 mRNA was reduced by more than 80% by heat-killed bacteria and reduced by about 60–90% by PGN and LPS, respectively. In contrast, poly(I-C) treatment did not overly alter Tlr13 expression levels (Fig. 1B).

**Determination of the Transcription Start Site of the Murine Tlr13 Gene**—To facilitate the cloning of the Tlr13 promoter constructs, the transcription initiation site of the mouse Tlr13 gene was determined by RNA ligase-mediated rapid amplification of cDNA 5′-ends (RLM-RACE) PCR using mRNA isolated from murine macrophage RAW 264.7 cells, which strongly express Tlr13 mRNA (Fig. 1A). The reverse primer was oligonucleotides that were complementary to the nucleotide position 299 bp downstream of the reported Tlr13 mRNA sequence (GenBankTM number NM_205820). The gene structure and the strategy designed for the 5′-RACE PCR are shown in Fig. 2A.

**Figure 2. Structure of murine Tlr13 gene and determination of the Tlr13 transcription start site.** A, physical map of the murine Tlr13 gene and the strategy of 5′-RACE PCR amplification are shown. Tlr13 has three exons; the transcriptional start site was mapped by 5′-RACE PCR. The oligonucleotide location used for 5′-RACE PCR was indicated by an arrow, and the size of the 211-bp PCR product was determined after sequencing. Exon I in Raw264.7 was obtained (Fig. 2B). B, 5′-RACE PCR product was resolved on a 2% agarose gel with only one specific band. The determined transcription start site is based on the specific PCR product after sequencing.
Identification of cis-Acting Elements within the Tlr13 Gene Promoter—Progressive 5′ deletions of Tlr13 gene promoter constructs were generated to determine DNA transcription regulatory elements. Mouse macrophage cell line RAW 264.7 cells, mouse embryonic fibroblasts, and HEK 293 cells were transfected with the Tlr13 plasmid DNA constructs as well as the pRL–TK vector as an internal control for normalizing transfection efficiency. Serial 5′-deletion mutations of the full-length promoter revealed a pattern of functional activity in transfected cells (Fig. 4A and supplemental Fig. 1). The highest level of luciferase activity was associated with the −341 fragments. Fragments larger than −1380 bp resulted in less luciferase activity, suggesting that the region from −1380 to −1000 bp contained negative regulatory elements. In contrast, deletions from −341 to −258 bp led to a remarkable reduction of the activity in RAW 264.7 cells (Fig. 3A) as well as in 293 cells and mouse embryonic fibroblasts (supplemental Fig. 1), indicating that this 83-bp region contained functional and essential transcription elements that drive maximal promoter activity. The region within −341 bp of the Tlr13 promoter contains multiple possible transcription factor binding sites, including NF-κB, Sp1, PU.1, and Est2 sites (Figs. 3 and 4A).

To pinpoint the functional significance of the NF-κB, Sp1, PU.1, and Est2 binding sites detected within the Tlr13 promoter, we used site-directed mutagenesis to mutate each of these sites and then assayed their effects on luciferase activity in RAW 264.7 cells (Fig. 4B). Disruption of the Sp1 site dramatically impaired the p−341 promoter’s activity by ∼70%. Furthermore, deletion of either Ets2 or PU.1 binding sites completely abolished its activity. In contrast, mutation of the NF-κB binding site, which plays an important role in the transcriptional regulation of most other TLRs, did not affect Tlr13 promoter activity (Fig. 4B). We observed that p65 overexpression can also inhibit the NF-κB–mutated Tlr13 promoter (Fig. 4B), indicating that the inhibition of Tlr13 expression by p65 is independent of the NF-κB binding site within the Tlr13 promoter. Thus, Sp1, PU.1, and Ets2 elements act as essential cis-acting elements within the TLR13 promoter, because they are necessary to reach maximal transcriptional activity.

Suppression of the Murine Tlr13 Gene Promoter by LPS and PGN but Not by Poly(I-C)—To determine the molecular mechanisms underlying LPS-, PGN-, Gram-positive bacterial lysate-, and Gram-negative bacterial lysate-mediated decrease in Tlr13 mRNA (Fig. 1B), the effects of LPS, PGN, and other compounds on Tlr13 gene promoter activity were examined using macrophage RAW 264.7 cells transfected with the Tlr13 promoter construct p−341. The p−341 construct was chosen for these studies, because it showed the highest activity. After the cells were treated with LPS or PGN, luciferase activity levels were significantly decreased. In contrast, poly(I-C) treatment did not dramatically alter the promoter’s activity, whereas the treatment with IFN-β significantly increased it (Fig. 5A). Furthermore, PS1145 (an IKK inhibitor) did abolish the capacity of LPS-mediated Tlr13 down-regulation (Fig. 5, B and C), indicating that NF-κB was involved in this down-regulation.

Identification of Transcription Factors That Interact with the Essential cis-Acting Elements—To elucidate potential transcription factors that interact directly with the identified cis-acting elements of Tlr13, a gel EMSA was performed. Oligonucleotides corresponding to the binding sites from NF-κB, PU.1, Ets2, and Sp1 in the Tlr13 promoter were designed for these experiments. The mobility of each labeled DNA probe was altered in the presence of nuclear protein prepared from RAW 264.7 cells (Fig. 6); a weak, but positive binding signal was detected in the case of NF-κB and Sp1 (data not shown). The binding specificity of each probe was verified using anti-Ets2 antibody, in the case of Ets2, or the addition of excessive unlabeled oligonucleotide competitor, in the case of PU.1. Interestingly, NF-κB p65 overexpression is capable of inhibiting Ets2 binding in a dose-dependent manner (Fig. 6C).

Characterization of the trans-Activators of the Tlr13 Promoter—To further investigate the role of potential trans-activators (including Ets2, PU.1, and Sp1) in transcriptional regulation of the Tlr13 gene, we co-transfected the p−341 Tlr13 promoter with Ets2, PU.1, and Sp1 expression vectors of into

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FIGURE 3. Sequence of the 5′-flanking region of the murine Tlr13 gene. Shown is a 1.9-kb sequence of the 5′-flanking region of murine Tlr13. Underlined sequences are the potential transcription factor binding sites predicted by MatInspector software. The arrow indicates the transcription start site (TSS), which was determined by 5′-RACE.
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Raw 264.7 cells. As shown in Fig. 7A, overexpression of Ets2 increased the transcription activity of the Tlr13 promoter by 15–20-fold. In contrast, overexpression of Sp1 and PU.1 failed to activate it. Instead, overexpression of PU.1 inhibited Ets2-mediated Tlr13 promoter activity, perhaps because the overexpression of PU.1 might compete with endogenous PU.1. Since transcription factors are able to directly bind to cis-acting elements, we believe that the transcriptional factor Ets2 activates the Tlr13 promoter through its binding motif. Furthermore, we also confirmed that p65 directly interacts with Ets2 after LPS stimulation (Fig. 7B). We explored the Ets2 role in the Ets2 wild type compared with Ets2 mutated promoter activity. Indeed, Ets2 increased the activity only in Ets2-wild type promoter but not in the Ets2 mutated promoter in a dose-dependent pattern (Fig. 7C). Since Ets2 activation is controlled by the Raf/MEK/ERK pathway, and overexpression of Raf is able to activate Ets2 expression (20), we overexpressed Raf to check whether it can also activate mTLR13 promoter activity in RAW 264.7 cells. As expected, Raf overexpression stimulated the Tlr13 promoter activity and co-transfection with Ets2 and showed the apparent synergy (Fig. 7D).

DISCUSSION

In this study, the activity of the Tlr13 gene promoter was characterized to elucidate the molecular basis of murine Tlr13 gene expression. We demonstrate that Ets2, PU.1, and Sp1 sites within the Tlr13 promoter region act as cis-acting elements and have a critical role in the transcriptional regulation of the Tlr13 gene. In contrast, NF-κB acted as a suppressor. Overexpression of Ets2 and NF-κB p65 potently trans-activated and inhibited the Tlr13 gene promoter, respectively. The activated signaling of LPS/TLR4 and PGN/TLR2 strongly inhibited the Tlr13 gene promoter.

Ets2 is a member of the Ets transcription factor family that plays a

FIGURE 4. Identification of essential cis-acting elements within the Tlr13 promoter. A, deletion analysis of the Tlr13 gene promoter. The truncated promoter fragments with luciferase reporter gene constructs (Luc) were cotransfected with the Renilla-TK luciferase vector into RAW 264.7 cells. Firefly luciferase activity is relative to the Renilla-TK luciferase activity; values are the means ± S.D. obtained from three independent experiments. Deletions from -341 to -238 bp led to an extreme reduction of the activity. The region within -341 bp of the Tlr13 promoter contains multiple potential important transcription factor binding sites, including NF-κB, Sp1, and Est2. B, site-directed mutation analysis of the Tlr13 gene promoter. RAW 264.7 cells were transiently transfected with p65 expression vector or control vector plus the p-341 promoter plasmid or the constructs with different mutations of the Sp1, NF-κB, PU.1, or Ets2 site, respectively. Transfected cells were harvested after 24 h of transfection for the luciferase assay. Firefly luciferase activity was normalized to Renilla luciferase activity, and the values represent the means ± S.D. of three independent experiments.

FIGURE 5. Suppression of the Tlr13 gene promoter by LPS and PGN. A, Raw264.7 cells were transfected with Tlr13 promoter p-341 plus Renilla-TK luciferase vector by Lipofectamine 2000. Twenty-four hours after transfection, cells were treated with medium alone, 5 μg/ml PGN, 100 ng/ml LPS, 25 μg/ml poly(I-C), or 100 units/ml IFN-β. Firefly luciferase activity was assayed 6 h after treatment and normalized to Renilla luciferase activity. Data present the mean ± S.D. of three independent experiments. Statistical analysis was performed by Student’s t-test; *, p < 0.05; **, p < 0.005. B, all of the procedure was performed in the same way as in A except for the pretreatment with PS1145 (10 μM for 3 h) or PBS before LPS stimulation. C, Raw264.7 cells were pretreated with PS1145 (10 μM for 3 h) or left untreated (UN) and then stimulated with LPS (100 ng/ml) for 0, 1, or 3 h. The endogenous Tlr13 expression was analyzed by real-time PCR.
target gene in the regulation of the immune response; ETS2 can significantly increase murine Tlr13 promoter activity and strongly up-regulate endogenous Tlr13 expression. All of the ETS members can be directly phosphorylated by the ERK molecules through the Raf/MEK/ERK pathway (20). For example, Ets2 can be phosphorylated by ERKs on Thr72, which leads to Ets2 activation (23). The activated ETS2 binds to target promoters and triggers transcription of the regulated genes. ETS2 regulates the expression of several cytokines in the inflammatory reaction. In mice, Thr72 phosphorylation of Ets2 is required for the persistent activation of tumor necrosis factor-α in macrophages stimulated with LPS (24). Moreover, Ets2 can directly bind to and activate the promoters of IL-5 (25), IL-10 (26), and IL-12 (27, 28). More interestingly, Ets2 is involved in the development and differentiation of macrophages and T cells. This implies that Ets2 has a role in host defense. To exemplify, studies with Ets2-lacZ transgenic mouse have showed that these mice undergo abnormal macrophage development during the first 40 days after birth. Furthermore, peritoneal macrophages obtained from these transgenic animals did not exhibit the characteristic macrophage morphological features when cultivated in vitro with CSF-1 stimulation (29, 30).

PU.1 is also a member of the Ets family of transcription factors that is specific for macrophage and B cells (31). PU.1 regulates TLR expression; it up-regulates TLR2 and TLR4 (32) but down-regulates TLR9 (33). However, it is not clear how PU.1 distinctly regulates each TLR. For the Tlr13 promoter, we found that mutating the PU.1 binding site abolished the promoter activity. In fact, the PU.1 and Ets2 binding sites are overlapping. Comparing the results of our PU.1 and Ets2 overexpression studies, we concluded that Ets2 exerted the more significant activator for Tlr13 expression.

Tlr13 gene transcription is also regulated through another cis-acting element that interacts with the transcription factor Sp1. Sp1 is a ubiquitous factor that regulates the constitutive
expression of many genes and is frequently localized at the proximal promoter regions as an enhancer (34, 35). Sp1 is a transcription factor containing a zinc finger motif that binds directly to DNA and enhances gene transcription. It is generally believed that Sp1 is part of the basal transcription initiation machinery, particularly for the promoter without a typical TATA box. Because the Tlr13 promoter lacks a TATA box, Sp1 may function as a linkage with the transcriptional complex. Indeed, we demonstrated that mutation of the Sp1 site dramatically reduced the transcription of Tlr13 by 75% at the basal level. Sp1 has been reported to mediate the induction of several genes, including human and murine Tlr2 (36, 37). We show herein that Sp1 alone is necessary but not sufficient for maximal transcriptional activity of Tlr13.

To explore the function of Tlr13 in the innate immune response to infection, we stimulated cells with microbial agents that potentially have the capacity of inducing Tlr13 expression and activating its specific signaling pathway. However, our data showed that Tlr13 expression is down-regulated by different microbial stimuli.

The machinery that controls the activation of TLR signaling is complex (38). Known strategies for controlling TLRs signaling include receptor down-regulation, sequestration of Toll-IL-1 receptor adaptors, TRAF6 ubiquitination, and NF-κB degradation (39). However, a simple way in which the immune system could accomplish this regulation might be by tightly control the expression of the TLRs themselves. TLR overexpression is, in fact, detected in various inflammatory diseases. For example, in vivo expression of TLR2 and TLR4 has been shown to be modulated in patients with rheumatoid arthritis, chronic obstructive pulmonary disease, and sepsis (40–42).

NF-κB is known to regulate the expression of many genes (43), including TLRs. Previous work has demonstrated that NF-κB up-regulates the transcriptional expression of human and mouse TLR2 (44–46). In contrast, NF-κB down-regulates the transcriptional expression of TLR9 gene (33). How does NF-κB up-regulate or down-regulate different TLR expression? The mechanism underlying this distinct role of NF-κB is not understood. One possibility is that NF-κB might cooperate with other transcription factors, such as Ets2. Ets2 can up-regulate Tlr13 through direct binding; however, NF-κB p65 inhibits binding of Ets2 and its ability to activate TLR13 transcriptional activity.

Although the exact role of TLR13 is currently unknown, phylogenetic analysis indicates that Tlr13 is a member of the Tlr11 subfamily (14). We have previously demonstrated that TLR11 recognizes urinary pathogenic E. coli (15). Therefore, to generate more information concerning the possible role of Tlr13, we tested bacterial components, including LPS, PGN, and whole bacterial lysates, for their ability to influence Tlr13 promoter activity. Our work indicates that these components significantly inhibit Tlr13 promoter activity. In contrast, viral components, such as poly(I-C), do not severely alter Tlr13 promoter activity, whereas IFN-β slightly increased Tlr13 promoter activity in our tested fragment. Actually, one possible clue concerning the role of Tlr13 might be found in recent work generated by the Beutler laboratory (47) and the Ploegh laboratory (48). They claim that Tlr13, like TLR3 and TLR9, colocalizes and interacts with UNC93B1, a molecule located in the endoplasmic reticulum (47, 48), and strongly suggest that Tlr13 might be found inside cells. Our current knowledge about TLR biology indicates that all of the intracellular TLRs, including TLR3, -7, -8, and -9, are nucleic acid sensors and are mainly involved in the recognition of viral infections (3). Therefore, Tlr13 may also play a similar role in recognizing viral infections. Our multiple tissue Northern blot demonstrated that Tlr13 is mainly expressed in murine spleen; quantitative real time RT-PCR revealed that Tlr13 is highly expressed in plasmacytoid dendritic cells, indicating that Tlr13 might play a role in innate immune responses to virus to activate type I interferon. Thus, Tlr11 and Tlr13 seem quite different from each other. Tlr11 recognizes bacteria, whereas Tlr13 might recognize virus. However, we have not yet identified the responsible elements, such as interferon regulatory factors, to regulate Tlr13 promoter activity in response to virus. A further analysis of the upstream regions in the Tlr13 promoter may reveal elements that control Tlr13 transcriptional activity upon viral infection.

In summary, we identified three cis-acting elements, Ets2, PU.1, and Sp1 sites, which play a critical role culminating in the maximal transcriptional activity of Tlr13. NF-κB acted as a suppressor. Overexpression of Ets2 potently trans-activated the Tlr13 gene promoter. INF-β is capable of acting TLR13 tran-

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