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*J Immunol* 2006; 177:1772-1779; doi: 10.4049/jimmunol.177.3.1772
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A Protein Associated with Toll-Like Receptor 4 (PRAT4A) Regulates Cell Surface Expression of TLR4

Yasutaka Wakabayashi,*2 Makiko Kobayashi,*2 Sachiko Akashi-Takamura,* Natsuko Tanimura,* Kazunori Konno,* Koichiro Takahashi,* Takashi Ishii,* Taketoshi Mizutani,† Hideo Iba,† Taku Kouro,‡ Satoshi Takaki,‡ Kiyoshi Takatsu,‡ Yoshiya Oda,§ Yasushi Ishihama,§ Shin-ichiroh Saijoh,* and Kensuke Miyake*†‡

TLRs recognize microbial products. Their subcellular distribution is optimized for microbial recognition. Little is known, however, about mechanisms regulating the subcellular distribution of TLRs. LPS is recognized by the receptor complex consisting of TLR4 and MD-2. Although MD-2, a coreceptor for TLR4, enhances cell surface expression of TLR4, an additional mechanism regulating TLR4 distribution has been suggested. We show here that PRAT4A, a novel protein associated with TLR4, regulates cell surface expression of TLR4. PRAT4A interacts with the immature form of TLR4 but not with MD-2 or TLR2. PRAT4A knockdown abolished LPS responsiveness in a cell line expressing TLR4/MD-2, probably due to the lack of cell surface TLR4. PRAT4A knockdown down-regulated cell surface TLR4/MD-2 on dendritic cells. These results demonstrate a novel mechanism regulating TLR4/MD-2 expression on the cell surface.

The Journal of Immunology, 2006, 177: 1772–1779.

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Received for publication February 15, 2006. Accepted for publication May 5, 2006.

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1 This study was supported by Special Coordination Funds of the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government, Uehara Memorial Foundation, the Takeda Foundation, and the Naito Foundation.

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4 Abbreviations used in this paper: DC, dendritic cell; ER, endoplasmic reticulum; FH, Flag/His, epitope; HPRT, hypoxanthine phosphoribosyltransferase; siRNA, small interfering RNA; shRNA, short hairpin RNA.

1 This work was supported by the Core Research for Engineering, Science, and Technology, Japan Science and Technology Corporation, Tokyo, Japan.

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Materials and Methods

Reagents, cells, and mice

Anti-Flag Ab and anti-Flag-agarose were purchased from Sigma-Aldrich. Rat anti-mouse TLR4 mAb (MT5S10 and Sa15-21) and rat anti-mouse...
CD14 (Sz-28) were established in our laboratory and described previously (19). Pre-made Northern blot for mouse normal tissues was purchased from Seegene. HEK293 cells were maintained in DMEM supplemented with 10% FCS and antibiotics. IL-3-dependent Ba/F3 cells were cultured in 10% FCS-RPMI 1640 supplemented with antibiotics, 100 μM 2-ME, and recombinant murine IL-3 (~70 U/ml) at 37°C in a humidified atmosphere of 5% CO2. A rat was immunized with the GST-PRAT4A (protein associated with TLR4) fusion protein and used for hybridoma production. The anti-PRAT4A mAb (Sz-28; rat IgG) can be used for immunoprecipitation. C57BL/6 mice were purchased from Japan SLC. TLR4+/- mice were kindly provided by Dr. S. Akira (Osaka University, Osaka, Japan), and MD-2+/- mice were established in our own laboratory (16). These mice were maintained in the animal facility at the Institute of Medical Science, the University of Tokyo, Tokyo, Japan. Bone marrow-derived DCs and macrophages were prepared as described previously (16). Briefly, bone marrow cells were plated at 1 × 10⁶ cells/ml in 24-well plates with 10% FCS-RPMI 1640 supplemented with 10 ng/ml recombinant murine GM-CSF (Genzyme/Techne) or 100 ng/ml recombinant murine macrophage CSF (M-CSF) (Genzyme/Techne). On day 7, cells were harvested and used for flow cytometry. For retroviral transduction, bone marrow cells during DC induction were transduced on days 1, 3, and 5 with retroviruses and 25 μl of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) transfection reagent (Roche) and centrifuged at 2000 rpm for 1 h at room temperature. After centrifugation, cells were cultured in 10% FCS-RPMI1640 supplemented with GM-CSF and cultured at 37°C in a humidified atmosphere of 5% CO2. On day 7, cells were harvested and used for flow cytometry. To detect DCs expressing short hairpin RNA (shRNA), we used a retroviral vector encoding GFP. In flow cytometry, the gate was set so that only DCs with GFP expression were analyzed (Fig. 7, A and B). To enrich DCs expressing shRNA, we used a retroviral vector with the purmorphycin resistance gene. Bone marrow was isolated from C57BL/6 mice that had been injected i.p. 4 days earlier with 5 mg of 5-fluorouracil. After 48 h of culture, cells were transduced with retroviral supernatant on two successive days. After the second transduction, cells were tagged with the FH epitope (TLR4FH, MD-2FH, and TLR4FH) or without epitope tags (see Fig. 3C). Retroviruses were transduced into Ba/F3 cells, and stable cells expressing mouse PRAT4A were established by enriching GFP-positive cells with a cell sorter. Human and mouse PRAT4A cDNAs were also cloned into the retroviral vector, pMX-IRES-neomycin, which encodes mouse TLR4, MD-2, and TLR2, all of which can be used for immunoprecipitation. Immunoprecipitation and immunopробing

For immunopробing, we used a retroviral vector expressing shRNA (26). The target sequences were 5'- gag tta gga ggt gtt gaf g-3'.

RNA extract and Northern hybridization

Premade Northern blot for mouse normal tissues was purchased from Seegene. The RNA blot was hybridized with a 32P-labeled cDNA probe encoding mouse PRAT4A (from aa 1 to aa 166). The probe was labeled using a random primed labeling system (Rediprime II; Amersham Biosciences). Hybridization was conducted at 65°C for 17 h in a buffer containing 10% dextran sulfate, 1% SDS, 50 mM Tris-HCl (pH 7.5), 1 M NaCl, and denatured salmon sperm DNA (100 μg/ml). Blots were washed three times in 2 × SSC/0.1% SDS at 65°C and three times in 0.2 × SSC/0.1% SDS for 10 min at 65°C before exposure to imaging plate (Fuji Film).

RT-PCR and semiquantitative PCR

Total pure RNA (1 μg) from Ba/F3 or HEK293 cells was reverse transcribed into cDNA using ReverTra Ace-a (Toyobo) according to the manufacturer’s instruction. cDNA was diluted at 1/1, 1/4, and 1/16, and each diluted cDNA was used as PCR template. RT-PCR was conducted with initial denaturation at 94°C for 3 min followed by 25 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min. The primer pairs used were as follows: 5'-ggcctgagattcagttgaggt-3' and 5'-ggaggtcttccaggccttgg-3' for human TLR4; 5'-cttcatctcagaggtgagaa-3' and 5'-acctgccacctgcctg-3' for human TLR4; and 5'-aggtcaggaacagaacg-3' and 5'-ctcaacaaactctqgtg-3' for human hpyoxan phosphoribosyltransferase (HPRT).

To determine the efficiency of gene silencing, semiquantitative PCR analyses were conducted using a 7300 fast real-time PCR System (Applied Biosystems) with TaqMan gene expression assays (Applied Biosystems) for mouse PRAT4A (Mm00511161). To normalize the amount of cDNAs, each sample was standardized by using internal TaqMan gene expression assays for mouse β-actin (Mm00607939).

Results

HEK293 cells expressing mouse TLR4 alone

Because HEK293 cells do not express endogenous MD-2, HEK293 cells expressing TLR4 responded to LPS/MD-2 but not to LPS alone (25), suggesting that TLR4 was expressed on the cell surface and bound to the LPS/MD-2 complex. We established HEK293 cells stably expressing mouse TLR4 fused with GFP. Established HEK293 cells, in which TLR4 expression was confirmed by GFP expression, were stained with two distinct mAbs for mouse TLR4, anti-mouse TLR4 mAb, anti-His6 mAb, and anti-mouse MD-2 polyclonal Abs. The second Abs were goat anti-mouse IgG-alkaline phosphatase conjugate (American Qualex) and goat anti-rat IgG-alkaline phosphatase conjugate (American Qualex).

Small interfering RNA

To investigate the biological function of PRAT4A, we purchased an RNA interference duplex (Invitrogen Life Technologies). The oligonucleotide sequences of target sequences for human and mouse PRAT4A were ggag gaacagcaggaauuucc (sense strand for human PRAT4A, no.9) and accggacauauuucuacuccgca (sense strand for mouse PRAT4A, no.11). Sequenced scrambled negative controls for no.9 and no.11 were gggaagucgcaauaaugccg and accggacauauuucuacuccgca, respectively. HEK293 transfectants were seeded onto 24- or 6-well plates in antibiotic-free medium on the day before RNA interference transfection. LipofectAMINE 2000 (Invitrogen Life Technologies) was used as a transfection reagent. Cells were collected 3–4 days after transfection and used for further analyses.

To inhibit mouse PRAT4A expression, we produced a retroviral vector expressing shRNA (26). The target sequences was 5'- gag tta gga ggt gtt gaf g-3'.

Microsequencing of N-terminal amino acids

HEK293/mLTLR4-GFP cells were collected up to ~5 × 10⁶ cells. Cell lystate extraction and purification with the anti-GFP Abs were conducted as described in the paragraph title Immunoprecipitation and immunopробing below in this section. Bound proteins were eluted and fractionated with the elution buffer containing 30 mM glycine/Tris (pH 2.5), 30 mM NaCl, and 0.1% Triton X-100. Each fraction was neutralized immediately with 1 M Tris-HCl (pH 8.0), and peak fractions were subjected to freeze drying, SDS-PAGE, electroblo требт, and staining with Coomassie brilliant blue R-250 (Bio-Rad). The coprecipitated protein was excised, and its N-terminal amino acid sequence was determined by APRO Science.

Expression constructs and transfectants

Mouse PRAT4A cDNA was cloned into a retroviral vector, pMX-IRES-GFP. PRAT4A was tagged with the Flag epitope followed by the His6 epitope (collectively designated hereafter as the FH epitope) at the C-terminus (PRAT4A-FH). PRAT4A-FH was expressed in Ba/F3 cells stably expressing TLR4/MD-2 and CD14. Retroviral vectors were transfected into the Plat-E cells using the FuGENE 6 transfection reagent (Roche). Retroviruses were transfected into Ba/F3 cells, and stable cells expressing mouse PRAT4A were established byenriching GFP-positive cells with a cell sorter. Human and mouse PRAT4A cDNAs were also cloned into the expression vector pEBSOS. We also established retroviral expression vector (pMX-purmorphycin) encoding TLR4, MD-2, and TLR2, all of which were either tagged with the FH epitope (TLR4/FH-MD-2/MD-2/FH and TLR4/FH) or without epitope tags (see Fig. 3C).

Immunoprecipitation and immunopробing

Cells were lysed on ice for 30 min with a lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.6), 2 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, and 1% Triton X-100. Lysates were separated from debris by centrifuging at 13,000 rpm for 10 min and then incubated with Ab-conjugated beads at 4°C for 2 h. Beads were washed three times, and bound proteins were boiled at 95°C for 5 min in the sample buffer. Samples were subjected to SDS-PAGE and electroblotting. Reagents used for immunoprobning were anti-Flag (M2), anti-mouse PRAT4A mAb, anti-mouse TLR4 mAb, anti-His6 mAb, and anti-mouse MD-2 polyclonal Abs. The second Abs were goat anti-mouse IgG-alkaline phosphatase conjugate (American Qualex) and goat anti-rat IgG-alkaline phosphatase conjugate (American Qualex).
Sa15-21 was able to detect TLR4 on DCs or macrophages from MD-2/H11002/H11002 mice (Fig. 1, B and C). Although a role for MD-2 in augmenting cell surface expression of TLR4 was confirmed, MD-2 was found to be dispensable for cell surface expression of TLR4 at least in certain types of cells such as HEK293 cells, bone marrow-derived DC, or macrophages.

We have previously demonstrated with Ba/F3 cells that the above two mAbs to TLR4, MTS510 and Sa15-21, reacted with TLR4/MD-2 but poorly with TLR4 alone as judged by immunoprecipitation (19, 27). Sa15-21 was not able to detect cell surface TLR4 alone on Ba/F3 cells by flow cytometry. Because Sa15-21 was found to react with TLR4 on HEK293 cells, MD-2-deficient macrophages, or DCs, Sa15-21 but not MTS510 was able to bind to TLR4 without MD-2. TLR4 without MD-2 expressed in Ba/F3 cells might be distinct in conformation from TLR4 on HEK293 cells, macrophages, or DCs.

**Molecular cloning of a molecule interacting with TLR4**

We hypothesized that cell surface expression of TLR4 is regulated not only by MD-2 but also by another unknown molecule. With an aim to identifying such a molecule, we sought for a molecule co-precipitated with TLR4-GFP in HEK293 cells. An ~40 kDa signal was consistently coprecipitated with TLR4/MD-2, and we therefore determined its N-terminal amino acid sequence. The determined amino acid sequence (EENDWVRLPS) was completely matched with aa 38–47 of a gene containing CAG trinucleotide repeats (28). A similar 40-kDa molecule was also found to be co-precipitated with TLR4 in Ba/F3 cells, and its N-terminal amino acid sequence was determined. The N-terminal amino acid sequence was precisely the same portion of the mouse homologue (Fig. 2A) that was previously described as a retinoic acid-induced

**FIGURE 1.** TLR4 alone is expressed on the cell surface. A, HEK293 cells were stained with two biotinylated mAbs to TLR4, MTS510, or Sa15-21, followed by streptavidin-PE. The stained cells were HEK293 cells (top row), HEK293 cells stably expressing mouse TLR4 (middle row), and HEK293 cells stably expressing mouse TLR4 and transiently expressing mouse MD-2 (bottom row). Open histograms depict staining with the second reagent alone. B and C, Bone marrow-derived DCs (B) and macrophages (C) were prepared from wild-type, TLR4/H11002/H11002, and MD-2/H11002/H11002 mice as indicated. Cells were stained with biotinylated mAbs to CD14 or TLR4 (MTS510 and Sa15-21), followed by streptavidin-PE. Open histograms depict staining with the second reagent alone.

**FIGURE 2.** The amino acid sequences of PRAT4A and its tissue distribution. A, The amino acid sequences of mouse and human PRAT4A were aligned. The signal sequence and the experimentally determined N-terminal sequence were boxed and underlined by a dashed line, respectively. Cysteine residues and an N-glycosylation site were marked by closed circles and an asterisk, respectively. B, Northern hybridization was conducted with a cDNA probe encoding mouse PRAT4A.
gene (29). We hereafter describe this molecule as PRAT4A, a protein associated with TLR4A. Data base analyses identified a molecule similar to PRAT4A. We named it PRAT4B, which was described elsewhere (30). The experimentally determined N-terminal amino acid sequence of PRAT4A clearly identified the first 37 amino acids as a signal sequence (Fig. 2A, boxed area). The mature peptide has a canonical N-glycosylation site and six cysteine residues. PRAT4A is highly conserved among a variety of species, including cow, chick, pig, catfish, sea squirt, nematode, zebra fish, and fruit fly (29). We examined tissue distribution of mRNA for mouse PRAT4A. Abundant expression of PRAT4A mRNA was detected in all of the organs tested, including lymphoid organs such as spleen and thymus (Fig. 2B).

PRAT4A is associated with the hypoglycosylated form of TLR4

We first confirmed the physical association between TLR4 and PRAT4A. HEK293 cells expressing mouse TLR4-GFP or TLR4-GFP/MD-2 were transiently transfected with human PRAT4A tagged with the Flag epitope followed by the His6 epitope (PRAT4A-FH). TLR4-GFP and PRAT4A-FH were precipitated, and coprecipitated RPA4-FH or TLR4-GFP was immunoprecipitated by the anti-Flag mAb or anti-GFP Ab, respectively (Fig. 3A). Mouse TLR4-GFP was coprecipitated with PRAT4A-FH (Fig. 3A, upper panel, lanes 4 and 6), and human PRAT4A-FH was coprecipitated with mouse TLR4-GFP (lower panel, lanes 4 and 6). The amount of PRAT4A coprecipitated with TLR4-GFP appeared to decrease when MD-2 was coexpressed (Fig. 3A, upper, lanes 4 and 6).

To confirm the physical association between TLR4 and PRAT4A with another cell line and to study the interaction between PRAT4A and other TLRs, Ba/F3 transfecants expressing TLR4-FH, TLR4-FH/MD-2, MD-2-FH, or TLR2-FH were used for immunoprecipitation studies. Coprecipitation of endogenously expressed PRAT4A was detected by a mAb to PRAT4A. PRAT4A was coprecipitated with TLR4-FH and TLR4-FH/MD-2, but not with MD-2-FH, TLR2-FH, or RP105/MD-1 (Fig. 3B, and data not shown), demonstrating specific association between TLR4 and PRAT4A. In contrast to the results with HEK293 cells, PRAT4A coprecipitated with TLR4 increased with MD-2 coexpression (Fig. 3B, lanes 2 and 3). The association of human PRAT4A with mouse TLR4 might be weaker than that of mouse PRAT4A and may be affected by MD-2 association. Next, epitope-tagged PRAT4A (PRAT4A-FH) was expressed in Ba/F3 cells expressing TLR4, MD-2, and CD14. PRAT4A-FH was precipitated by the anti-Flag mAb, and coprecipitated TLR4 and MD-2 were immunoprecipitated (Fig. 3C). Interestingly, the smaller species of TLR4 and MD-2 were coprecipitated with PRAT4A-FH (Fig. 3C, lane 3). Considering that PRAT4A-FH was not associated with MD-2 alone (Fig. 3B), PRAT4A-FH was likely to be associated with the hypoglycosylated form of TLR4 that was expressed either alone or as a complex with the hypoglycosylated form of MD-2. As published previously (19), coexpressed MD-2 facilitated TLR4 glycosylation as revealed by the larger form in SDS-PAGE (Fig. 3B, lane 2 compared with lane 3). The glycosylated mature form was preferentially precipitated by the MTS510 mAb (compare Fig. 3B, lane 2 with C, lane 1). Coprecipitation of PRAT4A-FH was hardly detected when the MTS510 mAb was used for immunoprecipitation of TLR4/MD-2 (Fig. 3C, middle, lane 1). The MTS510 mAb would bind to the mature TLR4/MD-2 complex from which PRAT4A has been already dissociated, or the mAb might disrupt the association between PRAT4A and TLR4/MD-2.

PRAT4A knockdown negatively regulates cell surface expression of TLR4

To address a role for PRAT4A in cell surface expression of TLR4, we used siRNA for inhibiting the expression of endogenous PRAT4A. The effect of siRNA was validated by detecting the transiently expressed human PRAT4A protein (Fig. 4A) or mRNA.
encoding endogenously expressed PRAT4A by RT-PCR (Fig. 4B), both of which were apparently down-regulated by two distinct siRNA, siRNA1 and siRNA2, but not by sequence-scrambled control RNA, ctrl1 and ctrl2 (Fig. 4, A and B). The PRAT4A siRNA did not down-regulate mRNA expression encoding human TLR4-FH, indicating specific silencing by the siRNA (Fig. 4B). HEK293 cells stably expressing human TLR4 were transiently transfected with the human PRAT4A siRNA. Cell surface TLR4 was detected by flow cytometry with an anti-human TLR4 mAb, HTA125 (Fig. 4C). The PRAT4A siRNA down-regulated cell surface human TLR4 without influencing the expression of mRNA encoding hTLR4-FH (Fig. 4B). Cell surface CD44 was not influenced by the siRNA, and the sequence-scrambled control siRNA had no effect on cell surface hTLR4-FH.

**FIGURE 4.** Cell surface murine TLR4 is down-regulated by the knockdown of human PRAT4A (hPRAT4A). A. HEK293 cells were transiently transfected with the expression vector encoding human PRAT4A-FH (hPRAT4A-FH; lanes 2–6) and 2 nM siRNA (PRAT4A siRNA1 and siRNA2 are in lanes 4 and 6, respectively, and control siRNAs for siRNA1 and siRNA2 are in lanes 3 and 5, respectively) 3 days before analyses. HEK293 cells were subjected to SDS-PAGE, Western blotting, and immunoprobing with the anti-Flag mAb and immunoprobing with the anti-His6 mAb, tagged with the FH epitope, these molecules were precipitated with the anti-Flag mAb and immunoprobed with the anti-His6 mAb, respectively. B, HEK293 cells stably expressing human TLR4-FH were transfected with the 20 nM PRAT4A siRNA (control 1 and control 2) or 2 nM siRNA (PRAT4A siRNA1 and siRNA2) or 20 nM sequence-scrambled control RNA (control 1 and control 2) as indicated. RT-PCR was conducted with template cDNAs without dilution or with 4-fold or 16-fold dilution. cDNAs encoding human PRAT4A (top panel), human TLR4 (middle panel), or human HPRT (HPRT; bottom panel) were amplified, electrophoresed on an agarose gel, and visualized. C. HEK293 cells stably expressing human TLR4 were transfected with the PRAT4A siRNA or control siRNA at the indicated concentration (2 or 10 nM). HEK293 cells were stained 4 days later with an anti-human TLR4 mAb HTA125 or anti-human CD44 as indicated.

**PRAT4A knockdown negatively regulates cell surface expression of TLR4/MD-2 and LPS responsiveness**

We next asked whether PRAT4A regulates cell surface expression of TLR4/MD-2. We established two Ba/F3 cell lines, PRAT4A8 and PRAT4A11, stably expressing TLR4/MD-2 and the mouse PRAT4A shRNA. The effect of the PRAT4A shRNA was confirmed by semiquantitative PCR (Fig. 5A). The PRAT4A mRNA was reduced to 15% (PRAT4A8) or 13% (PRAT4A11) by the shRNA when compared with the Ba/F3 transfectant without transduction or with the Ba/F3 transfectant expressing an empty vector (Fig. 5A). PRAT4A mRNA reduction by ~85% appeared to be sufficient for down-regulating cell surface TLR4/MD-2 on Ba/F3 cells to the undetectable level by flow cytometry (Fig. 5B). Such a drastic down-regulation was not observed in cell surface TLR2 or CD43 (Fig. 5B, and data not shown). We asked whether the lack of cell surface TLR4/MD-2 influenced LPS responses. Because the Ba/F3 transfectants had been transfected with a luciferase reporter construct driven by NF-κB activity, transfectants were stimulated with lipid A, LPS, another TLR4/MD-2 ligand (Taxol), or TNF-α. Luciferase activity in the cell lysate was determined (Fig. 5C). Whereas Ba/F3 transfectants without any treatment or with an empty control vector both responded to the TLR4 ligands, those with the PRAT4A shRNA did not respond to lipid A or LPS from *E. coli* even at a concentration as high as 10 μg/ml (Fig. 5C). Similar results were obtained with another TLR4/MD-2 ligand (Taxol) (data not shown). PRAT4A knockdown had no effect on NF-κB activation induced by TNF-α (data not shown).

**PRAT4A knockdown led to the lack of mature TLR4/MD-2 on the cell surface**

To ask how the PRAT4A shRNA led to the lack of cell surface TLR4/MD-2 and hyporesponsiveness to LPS, the expression of TLR4 and PRAT4A was studied. RT-PCR revealed that the amount of mRNA encoding TLR4 or MD-2 was not down-regulated by the PRAT4A shRNA (Fig. 6A). Because TLR4 and MD-2 had been tagged with the FH epitope, these molecules were precipitated with the anti-Flag mAb and immunoprobed with the anti-His6 mAb, respectively. The amount of TLR4 protein was decreased in one of the transfectants, PRAT4A11, but not in the other transfectant, PRAT4A8, when compared with Ba/F3 cells without transduction or with the Ba/F3 transfectant expressing PRAT4A shRNA, demonstrating its silencing effect (Fig. 6B). Despite the expression of TLR4 and MD-2, TLR4 was much less precipitated by the PRAT4A shRNA when compared with the Ba/F3 transfectant without transduction or with the Ba/F3 transfectant expressing an empty vector (Fig. 6A). PRAT4A mRNA reduction by ~85% appeared to be sufficient for down-regulating cell surface TLR4/MD-2 on Ba/F3 cells to the undetectable level by flow cytometry (Fig. 5B). Such a drastic down-regulation was not observed in cell surface TLR2 or CD43 (Fig. 5B, and data not shown). We asked whether the lack of cell surface TLR4/MD-2 influenced LPS responses. Because the Ba/F3 transfectants had been transfected with a luciferase reporter construct driven by NF-κB activity, transfectants were stimulated with lipid A, LPS, another TLR4/MD-2 ligand (Taxol), or TNF-α. Luciferase activity in the cell lysate was determined (Fig. 5C). Whereas Ba/F3 transfectants without any treatment or with an empty control vector both responded to the TLR4 ligands, those with the PRAT4A shRNA did not respond to lipid A or LPS from *E. coli* even at a concentration as high as 10 μg/ml (Fig. 5C). Similar results were obtained with another TLR4/MD-2 ligand (Taxol) (data not shown). PRAT4A knockdown had no effect on NF-κB activation induced by TNF-α (data not shown).
the transfectants with RPAT4A shRNA (Fig. 6B, lower panel), indicating the lack of mature TLR4. These results demonstrate an important role for PRAT4A in TLR4 maturation.

PRAT4A knockdown negatively regulates cell surface TLR4 and TLR4/MD-2 on DCs

To confirm that the role for PRAT4A on TLR4 was not restricted to in vitro cell lines such as Ba/F3 or HEK293 cells, bone marrow-derived DCs were transduced with retrovirus encoding the PRAT4A shRNA or an empty control vector. Cell surface expression of TLR4 alone, TLR4/MD-2, or TLR2 was determined by flow cytometry analyses. TLR4 alone was detected on MD-2-deficient DCs without any treatment or with an empty control vector but not on those with the PRAT4A shRNA (Fig. 7A). Similarly, cell surface TLR4/MD-2 on wild-type DCs was drastically downregulated with the PRAT4A shRNA (Fig. 7B). The control vector had no effect on cell surface expression of TLR4/MD-2. To make sure that the TLR4 protein is expressed in PRAT4A-silenced DCs, DCs expressing the PRAT4A shRNA were enriched and subject to immunoprecipitation with the anti-TLR4 mAb Sa15-21 and to immunoprobing with anti-TLR4 Ab (Fig. 7C). We were able to detect the TLR4 protein in DCs expressing the RPAT4A shRNA, but the amount of TLR4 protein in PRAT4A-silenced DCs was much less than the amount in control cells, DCs without any treatment, or DCs expressing an empty vector. When compared with TLR4/MD-2, cell surface TLR2 was much less influenced by the

FIGURE 5. Down-regulation of cell surface TLR4/MD-2 by the PRAT4A knockdown. A, Expression of PRAT4A mRNA was determined by semiquantitative PCR in TLR4/MD-2-expressing Ba/F3 transfectants without any treatment (no treat), with transduction with an empty vector (control), or with the PRAT4A shRNA (PRAT4A no.8 and PRAT4A no.11). RTPCR was conducted with template cDNAs without dilution or with 4-fold or 16-fold dilution for each sample. cDNAs encoding murine TLR4 (mTLR4; top panel), murine MD-2 (mMD-2; middle panel), or murine HPRT (mHPRT; bottom panel) were amplified, electrophoresed on an agarose gel, and visualized. B, Cell surface TLR4/MD-2 and TLR2 on Ba/F3 transfectants were stained with biotinylated mAbs to TLR4 (MTS510) or TLR2, followed by streptavidin-PE. Open histograms depict staining with the second reagent alone. The cell lines studied are indicated in the figure. C, Ba/F3 cells expressing TLR4/MD-2 and the NF-κB luciferase reporter construct were stimulated with lipid A or LPS from E. coli as indicated. Luciferase activity was determined and represented as fold increase from the activity without stimulation. The results were shown as the mean from triplicates.

FIGURE 6. PRAT4A knockdown leads to the lack of mature TLR4/MD-2. A, Expression of mRNAs encoding TLR4, MD-2, and HPRT was determined by RT-PCR in TLR4/MD-2-expressing Ba/F3 transfectants without any treatment (no treat), with transduction with an empty vector (control), or with the PRAT4A shRNA (PRAT4A no.8 and PRAT4A no.11). RTPCR was conducted with template cDNAs without dilution or with 4-fold or 16-fold dilution for each sample. cDNAs encoding murine TLR4 (mTLR4; top panel), murine MD-2 (mMD-2; middle panel), or murine HPRT (mHPRT; bottom panel) were amplified, electrophoresed on an agarose gel, and visualized. B, TLR4 and MD-2 with the FH epitope (TLR4-FH and MD-2-FH) were precipitated (IP) with anti-Flag mAb (top and middle panels) or with the anti-TLR4 mAb MTS510 (bottom panel) from TLR4/MD-2-expressing Ba/F3 transfectants as indicated. Precipitated TLR4-FH and MD-2-FH were probed with the anti-His mAb (top panel). Coprecipitated endogenous PRAT4A was probed with the anti-PRAT4A mAb (middle panel). TLR4-FH precipitated with MTS510 was probed with the anti-His mAb (bottom panel). WB, Western blotting.
mice, HEK293 cells, and Ba/F3 cells (Fig. 3). Second, association between TLR4 and PRAT4A was shown in human and This conclusion was based on the following results. First, physical associated with TLR4 and regulates its cell surface expression.

The present study identified RPAT4A as a novel molecule that is associated with TLR4 and regulates cell surface expression of TLR2. This issue is currently being studied further.

**Discussion**

The present study identified RPAT4A as a novel molecule that is associated with TLR4 and regulates its cell surface expression. This conclusion was based on the following results. First, physical association between TLR4 and PRAT4A was shown in human and mouse cell lines, HEK293 cells, and Ba/F3 cells (Fig. 3). Second, gene silencing of the PRAT4A mRNA down-regulated cell surface TLR4 alone on human HEK293 cells and mouse MD-2-deficient DCs (Figs. 4 and 7). Third, PRAT4A knockdown decreased cell surface TLR4/MD-2 on Ba/F3 transfectants and wild-type DCs. Fourth, mature TLR4/MD-2 precipitated by the MTS510 mAb was reduced by the PRAT4A knockdown (Fig. 6).

PRAT4A binds hypoglycosylated TLR4 with or without MD-2 (Fig. 3C), but not with mature TLR4/MD-2. PRAT4A knockdown decreased the amount of mature TLR4 precipitated with the MTS510 mAb, but not that of immature TLR4 (Fig. 6B). These results suggest a role for PRAT4A in TLR4 maturation/glycosylation. Although the MTS510 mAb was unable to precipitate TLR4/MD-2 in Ba/F3 transfectants with RPAT4A shRNA (Fig. 6B), it is possible that hypoglycosylated TLR4 is still associated with hypoglycosylated MD-2 even in the lack of PRAT4A. A role for PRAT4A in the assembly of the TLR4/MD-2 complex remains to be clarified.

An ER chaperon, gp96, was shown to have a similar role. Randow and Seed (22) described a pre-B cell line, deficient in gp96, in which TLR4 failed to associate with MD-2, get to the cell surface, and respond to LPS. Altered localization of gp96 from ER to the membrane led to the redistribution of TLR4 (20). gp96 regulates the subcellular distribution of not only TLR4 but also TLR2, TLR1, and some integrins (22). Although PRAT4A is not associated with TLR2, it remains to be determined whether PRAT4A is associated with other TLRs, including TLR1 or TLR6.

A majority of TLR4 was detected inside the cells in the Golgi apparatus (16) as a hypoglycosylated immature form as judged by SDS-PAGE analyses (Fig. 3B, lane 3). Overexpression of MD-2 increased the mature form of TLR4 on the cell surface, but most TLR4 still remained immature in the Golgi apparatus (Fig. 3B, lane 2) (16). There must be machinery regulating the subcellular distribution of TLR4 between Golgi/ER and the cell surface. The Golgi apparatus is reported to be another site allowing TLR4/MD-2 to recognize LPS. TLR4 in intestinal epithelial cells resides in the Golgi apparatus, and internalized LPS is recognized in the Golgi apparatus (20). The molecular mechanisms by which TLR4/MD-2 stays in Golgi/ER or traffics back from the cell surface are currently unknown. PRAT4A is likely to be a component of the machinery facilitating TLR4/MD-2 trafficking to the cell surface. Further study might reveal a difference in cytokine production between TLR4/MD-2 on the cell surface and in the Golgi apparatus.

PRAT4A knockdown led to the profound defect in LPS responsiveness that is probably due to the impaired maturation of TLR4, leading to the lack of mature TLR4/MD-2 on the cell surface (Fig. 5). PRAT4A was required for cell surface expression of TLR4/MD-2, not only in Ba/F3 cells but also in normal DCs (Fig. 7), suggesting a role for PRAT4A in DC response to LPS. We are currently studying a role for PRAT4A in LPS responses by DCs or macrophages by using shRNA. Up-regulation or down-regulation of PRAT4A may influence LPS responses. PRAT4A was previously shown to be induced by all-trans retinoic acid (29). It is important to study induction and reduction of PRAT4A mRNA upon a variety of stimulations.

In conclusion, the present study identified a novel molecule that is associated with TLR4 and regulates cell surface expression of TLR4. This molecule might reveal a novel mechanism regulating the subcellular distribution of TLR4.

**Acknowledgments**

We thank Dr. Shizuo Akira (Osaka University, Osaka, Japan) for providing us with TLR4-deficient mice.

**FIGURE 7.** Cell surface TLR4/MD-2 is down-regulated by the knockdown of murine PRAT4A DCs from MD-2−/− (A) or wild-type mice (B) were transduced with a retroviral vector encoding the PRAT4A shRNA (bottom) or with an empty control vector (middle). Because a retroviral vector encodes GFP, transduced cells can be detected as GFP-positive cells. Cell surface expression of TLR4/MD-2 (left column), TLR4 (center column), or TLR2 (right column) on GFP-positive, transduced DCs was shown by staining with biotinylated MTS510, Sa15-21, or anti-TLR2 mAb, respectively, followed by streptavidin-PE. DCs without transduction (no treat; top row) were also stained as a control. KO, knockout. C, DCs were subjected to immunoprecipitation with the anti-TLR4 mAb Sa15-21 and immunoprobed with the anti-TLR4 Ab (top panel). A part of cell lysate was used for immunopробing with an anti-actin Ab to show that the approximately equal amount of cell lysate was used (bottom panel). The cells used were DCs without any treatment (no treat; lane 1), DCs expressing an empty vector (lane 2), or DCs expressing the PRAT4A shRNA (lane 3).

PRAT4A shRNA (Fig. 7B). However, we cannot exclude a possibility that PRAT4A knockdown had a small but significant effect on cell surface expression of TLR2. This issue is currently being studied further.
Disclosures
The authors have no financial conflict of interest.

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