Role of MyD88 in Phosphatidylinositol 3-Kinase Activation by Flagellin/Toll-like Receptor 5 Engagement in Colonic Epithelial Cells*

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Bacterial flagellin, recognized by Toll-like receptor (TLR) 5, is suggested to be involved in colonic inflammation. However, the detailed signaling mechanisms mediated by flagellin/TLR5 engagement are not clear. Here we dissected the biochemical mechanism by which TLR5 engagement mediates phosphatidylinositol 3-kinase (PI3K) activation in colonic epithelial cells. We demonstrate that silencing TLR5 expression in nontransformed human colonic epithelial cells blocks flagellin-induced PI3K activation, indicating specific activation of PI3K by flagellin/TLR5 engagement. Moreover, we determine that TLR5 recruits the p85 regulatory subunit of PI3K to its cytoplasmic TIR domain in response to flagellin. However, the Src homology binding “YXXM” motif in the cytoplasmic TIR domain of TLR5 is not involved in p85 recruitment, implying that TLR5 indirectly recruits p85. Indeed, we demonstrate that the adaptor molecule MyD88 associates with TLR5 and silencing MyD88 expression blocks PI3K activation by disrupting the association between TLR5 and p85. Furthermore, we show that MyD88 associates with p85 in response to flagellin. Additionally, we determine that blocking PI3K activation reduces interleukin-8 production induced by flagellin in human colonic epithelial cells. Together, MyD88 bridges TLR5 engagement to PI3K activation in response to flagellin.

Toll-like receptors (TLRs) represent a type 1 transmembrane receptor protein family, recognizing a wide array of pathogen-associated molecular patterns, such as lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acid, bacterial CpG DNA, and bacterial flagellin, present in various microorganisms (1, 2). TLRs are composed of an extracellular leucine-rich repeat domain recognizing its specific agonists and a cytoplasmic Toll/IL-1R (TIR) domain responsible for mediating downstream signaling that plays a critical role in TLRs-associated innate immunity and proinflammatory responses (3). Among at least 11 members of the TLR family characterized to date, TLR5 is a specific receptor for flagellin from both commensal and pathogenic bacteria in the gut and highly expressed in colonic epithelial cells (4). Previously, we showed that TLR5 engagement by bacterial flagellin mediates MyD88/IRAK/TRAF6-dependent signaling resulting in the activation of transcription factor NFκB and AP-1 and leading to up-regulation of proinflammatory cytokine gene expression such as IL-8 and MIP3α in nontransformed human colonic epithelial cells, NCM460 (4). More recently, we also characterized the pathophysiological role of flagellin in an experimental colitis model using dextran sulfate sodium (DSS) (5). According to our recent results, only injured, not intact colonic mucosa is highly vulnerable to intracolonic flagellin, causing proinflammatory gene expression and histologically evident colonic inflammation. These data strongly indicate that bacterial flagellin in the gut participates in the development and progress of colonic inflammation.

Phosphatidylinositol 3-kinase (PI3K) is a lipid kinase that phosphorylates phosphoinositides at position 3 of the inositol ring, leading to activation of the well characterized serine/threonine kinase Akt/protein kinase B (6). Class I PI3Ks are composed of a p85 regulatory subunit interacting with an upstream signaling molecule, generally a membrane receptor, and a p110 catalytic subunit responsible for their enzymatic activity (6). This class of PI3Ks can be activated by various receptor-mediated extracellular stimuli, including TLR2, TLR3, TLR4, IL-1R, and platelet-derived growth factor receptor (7–12).

The human gut harbors a large collection of commensal microbes, releasing various pathogen-associated molecular patterns (2, 13). These microbial ligands are able to initiate TLR engagement and trigger PI3K activation. Sheng et al. (14) recently showed that PI3K activation is essential for intestinal epithelial cell proliferation in vitro and in vivo by inducing cyclin D1 expression. Moreover, the up-regulation of PI3K has been suggested to cause a hyperproliferative epithelium (15). Indeed, increased PI3K activity was observed in a majority of colon cancers (16). Therefore, inappropriate TLR engagement probably results in an aberrant PI3K activation leading to a hyperproliferative epithelium. Nevertheless, the molecular mechanisms by which TLR5 activates PI3K have not been studied.

In this study, we demonstrate that in nontransformed human colonic epithelial cells, the adaptor molecule MyD88 binds to TLR5 in response to flagellin, and MyD88 recruits the p85 regulatory subunit of PI3K, implying that MyD88 plays a bridging role between TLR5 and the p85 regulatory subunit. These results provide a molecular mechanism whereby TLR5 engagement by bacterial flagellin leads to activation of PI3K in human colonic epithelial cells.

EXPERIMENTAL PROCEDURES

Reagents—Flagellin from Salmonella typhimurium (Apotech Corp., Switzerland) was dissolved in LPS-free water (Sigma). Antibodies against phospho-Akt and inactive Akt were from Cell Signaling Tech-

* The abbreviations used are: TLR, Toll-like receptor; DSS, dextran sulfate sodium; FOXO, Forkhead transcription factors; LTA, lipoteichoic acid; MyD88, myeloid differentiation factor 88; PI3K, phosphatidylinositol 3-kinase; TIR, Toll/IL-1 receptor; TRIF, Toll/IL-1R domain-containing adaptor inducing IFN-β; HA, hemagglutinin; WT, wild type; PBS, phosphate-buffered saline; DAPI, 4,6-diamidino-2-phenylindole; siRNA, small interfering RNA; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; HEx, human embryonic kidney; SH, Src homology; LPS, lipopolysaccharide; DN, dominant negative.
nology (Beverly, MA), and antibodies against human TLR5 were purchased from Zymed Laboratories Inc. Plasmid constructs expressing human IL-8 luciferase reporter, NFκB-luciferase reporter, TLR5, and TLR5 (∆TIR) were described previously (4). The dominant negative plasmid of the PI3K regulatory subunit (p85-DN, lacks the binding site for the catalytic subunit p110) and wild type p85 expression plasmid were kindly provided by Dr. Julian Downward (Imperial Cancer Research Fund, London, UK) (17). p85ΔC/FLAG, a generous gift from Dr. David Fruman (University of California, Irvine), was subcloned into pcDNA3.1/zeo (−). Akt wild type and dominant negative constructs (Akt-DN) were from Upstate (Charlottesville, VA). All plasmids were prepared using the EndoFree plasmid purification kit (Qiagen, Valencia, CA) as recommended by the manufacturer. Monoclonal antibodies against FLAG and HA were from Sigma and Roche Applied Science, respectively.

The nontransformed human colonic epithelial cells, NCM460, and the culture medium M3D were obtained from INCELL Corp. (San Antonio, TX) (4). NCM460 cells and human embryonic kidney (HEK) 293 cells were cultivated as described in our previous studies (4, 18).

8-Week-old male mice (C57/16) were from The Jackson Laboratories (Bar Harbor, ME) and housed in a pathogen-free facility. To induce colitis, mice were fed dextran sulfate sodium (DSS, 2.5%) (MP Biomedicals, Irvine, CA) dissolved in regular tap water during the entire experimental period, as described previously (5). The Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center approved all animal procedures.

Generating the Various Mutant Constructs of TLR5 and MyD88—TLR5 (Y798F)-HA, TLR5 (Y815F)-HA, TLR5 (Y798F/Y815F)-HA, and TLR5 (Y691F/Y693F/Y696F)-HA were generated by PCR-based mutagenesis using Pfu enzyme (Stratagene, La Jolla, CA) and appropriate primers. To generate TLR5 (Y798F)-HA, we performed the first PCR using TLR5 WT-HA as template and primers F-1 harboring Y798F substitution and R-2. Then, by using the first PCR product as reverse primer and F-2 primer, we performed the second PCR with TLR5 WT as a template. The second PCR product was inserted into Xbal and BamHI sites in the pUNO-TLR5-HA construct, resulting in the TLR5 (Y798F)-HA construct. To generate TLR5 (Y815F)-HA and TLR5 (Y798F/Y815F)-HA, the first PCR was done with primers F-3 containing the Y815F mutation and R-1 and TLR5 WT-HA as a template. The second PCR was performed with the first PCR product and F-2 as primers, and TLR5 WT-HA or TLR5 (Y798F)-HA was used as a template. The PCR product from the second PCR was inserted into Xbal and BamHI sites in the pUNO-TLR5-HA construct, giving rise to TLR5 (Y815F)-HA and TLR5 (Y798F/Y815F)-HA constructs, respectively. To generate TLR5 (Y691F/Y693F/Y696F)-HA, the primers R-1 and F-4 harboring the Y691F mutation were used for the first PCR with a template TLR5 WT-HA. The primer F-2 and the first PCR product were used for the second PCR with TLR5 WT-HA to generate TLR5 (Y691F)-HA. PCR was again performed with primers R-1 and F-5 possessing Y693F/Y696F mutations and a template TLR5 (Y691F)-HA. The PCR product was used as primers along with F-2 and TLR5 (Y691F)-HA as a template. The PCR product was used to generate TLR5 (Y691F/Y693F/Y696F)-HA construct.

To generate human MyD88 (Y257F)-HA expressing construct, the first PCR was performed with an F-6 primer containing the Y257F mutation and an R-2 primer along with MyD88 WT-HA as a template. The MyD88 (Y257F)-HA fragment was amplified through PCR using the first PCR product and F-7 as primers and the pUNO-MyD88 WT-HA construct as a template. The integrity of the whole sequence was confirmed by sequencing all DNA constructs. Primers used for PCR were as described below, and substitution of tyrosine with phenylalanine is marked in boldface with an underline. The primers used are as follows: F-1, 5′-GGT GGT GGT GTG TCC TGT CCC CAG TTC CAG TGG ATG AAA CAT CAA TCC-3′; F-2, 5′-TTA CCG AAC CTC ATC CAC TTA TCAG-3′; F-3, 5′-GGC TTT GAT GCC CAA TAT GAT GCC TAT TTG TCC-3′; F-4, 5′-CCT GAG ATG TTC AAA TAT GAT GCC CAA TAT TTG TCG-3′; F-5, 5′-CCT GAT ATG TTC AAA TAT GAT GCC TTT TTG TGG TCC AGC ACG-3′; F-6, 5′-CCC CTT ACC AAC TTC TTT TTT ATG TCC-3′; F-7, 5′-CCC TGC TTC CTC ACC AAC TCT AGC TC-3′; R-1, 5′-GGG GTA GTC TGG CAC ATC ATA GGG GTA GGC-3′; and R-2, 5′-GGC GTA GTC TGG CAC ATC ATA AGG GTA GGA TTC-3′.

**Immunofluorescence Staining**—Cells grown on a chamber slide system (Nalge Nunc, Rochester, NY) were fixed in 10% formalin and permeabilized using 0.5% Triton X-100. Slides were washed with PBS and blocked with normal goat serum (1%) plus 1% free bovine serum albumin (1%) in PBS for 20 min at room temperature. Slides were then incubated for 1 h with primary antibodies (1:50 dilution) against FOXO1 (Santa Cruz Biotechnology, Santa Cruz, CA) in bovine serum albumin (1%), PBS. Samples were then washed with PBS and incubated with Cy3-conjugated anti-rabbit secondary antibody (1:200) for 1 h. The slides were then rinsed and mounted with DAPI mounting solution (Vector Laboratories, Burlingame, CA). Images were analyzed with a Zeiss Axioskop–2 microscope.

**Generating TLR5 or MyD88 Silenced Cells**—The silencing vector expressing siRNA targeting the human TLR5 or MyD88 were obtained from InvivoGen (San Diego) (19). NCM460 or HEK293 cells were transfected with the silencing vector and an empty control vector. Stably transfected cells were isolated in selection media containing Zeocin. Silencing of the target molecule was confirmed in several isolated colonies.

**Immunoblotting Analysis**—Cell lysates were prepared in lysis buffer (150 mM NaCl, 50 mM Tris·Cl (pH 8.0), 5 mM EDTA, 1% Nonidet P-40) containing a protease inhibitor mixture (Roche Applied Science) and a phosphatase inhibitor mixture (Sigma). The equal amount of total protein was subjected to SDS-PAGE analysis, and immunoblotting with the appropriate antibodies was performed as described previously (4).

**Immunoprecipitation Assay**—Total cell lysates were prepared in lysis buffer as described above. Equal amounts of total protein were mixed with a primary antibody or control IgG and incubated at 4 °C for 4–5 h. The protein-A or -G beads (Pierce) were then added and incubated overnight. The precipitants were washed three times with lysis buffer and fractionated on SDS-PAGE, followed by the immunoblotting procedure described above.

**Luciferase Reporter Assays**—Cells were plated in 6-well plates (0.5 × 10⁴ cells/well) and transfected with the appropriate plasmid DNA, including a β-galactosidase expression plasmid (HSP70-β-gal) as an internal control, using SuperFect transfect reagent (Qiagen, Valencia, CA) according to the manufacturer’s instructions. One day after transfection, cells were stimulated with flagellin (100 ng/ml), LPS (100 ng/ml), IL-1 (50 ng/ml), or tumor necrosis factor-α (50 ng/ml) for 6 h, and the relative luciferase activity was determined by normalization with β-galactosidase activity as described previously (4). The total amount of plasmid DNA was kept consistent by adding the empty vector for each sample. All assays were performed in triplicate, and a single representative experiment is shown. Data are expressed as mean values ± S.E.

**IL-8 and MIP3α Measurement**—An enzyme-linked immunosorbent assay (ELISA) was performed to measure the level of human IL-8 and MIP3α using the appropriate kits from BIOSOURCE and R & D Sys.
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RESULTS

Bacterial Flagellin Stimulation Activates PI3K in Human Nontransformed Colonocytes—We showed recently (5) that the disrupted colonic mucosa is sensitive to the proinflammatory responses of bacterial flagellin. We also showed in compromised colonic mucosa that bacterial flagellin is able to induce in vivo activation of MEK1/2, an important modulator of flagellin-induced proinflammatory cytokine expression (4, 5). In this context, we used the experimental DSS-induced colitis model as described in our recent publication (5) to investigate if intracolonic exposure of bacterial flagellin in injured colonic mucosa is able to induce PI3K activation. To test this, LPS-resistant C3H/HeJ mice were given water alone or DSS (2.5% in water) through the entire experimental period. Four days after starting DSS, mice were intracolonically exposed to flagellin (0.8–1.0 μg/mouse) for 7 days, as described previously (5). Total protein was prepared from colonic tissues taken separately from each mouse, and an equal amount of total protein was subjected to immunoblotting to evaluate Akt phosphorylation as a readout of PI3K activation. OD values obtained from the x-ray films were expressed as a relative ratio of phosphorylated to inactive Akt. Data are expressed as mean ± S.E. of five mice per group. B, NCM460 cells were stimulated with flagellin (100 ng/ml) as indicated. Equal amounts of total cell lysates were used for immunoblotting with an antibody against phosphorylated Akt. Membranes were then stripped and re-probed using an antibody against inactive Akt as control for equal loading. C, NCM460 cells were preincubated with LY294002 for 1 h. Cells were then stimulated with flagellin for 20 min with or without LY294002 at the indicated concentration. Akt phosphorylation and inactive Akt were measured by immunoblotting. D, NCM460 cells were stimulated with flagellin or vehicle (water) for 2 h followed by immunofluorescence staining of FOXO1 (red) expected to be localized into the nucleus in inactive cells. The slides were counterstained with DAPI to localize cell nuclei (blue). Shown here are representative images from each experimental group.
contrast, in cells stimulated with flagellin FOXO1 is localized outside the nuclei (Fig. 1D). The merged image of FOXO1 and DAPI nuclei counterstaining confirmed that FOXO1 is re-localized to the cytoplasmic region following flagellin exposure (Fig. 1D). Together, these data demonstrate that flagellin/TLR5 engagement induces PI3K activation in nontransformed human colonocytes.

Flagellin-stimulated PI3K Activation Is TLR5-specific—Although flagellin is a specific ligand for TLR5 and our flagellin preparation had minimal LPS contamination (<1 pg/μg LPS content as determined by Limulus Amebocyte Lysate Assay), we performed additional experiments to confirm the “TLR5 specificity” of our flagellin preparation using a TLR5 silencing approach. TLR5-silenced NCM460 cells were generated by stably transfecting a TLR5 siRNA-expressing vector into NCM460 cells as described under “Experimental Procedures.” Immunoblotting analysis verified that TLR5 expression is substantially reduced in TLR5-silenced NCM460 cells compared with control cells (Fig. 2A). We next verified whether NFκB activation is indeed knocked down in TLR5-silenced cell in response to flagellin. To address this, because NFκB activation is a canonical outcome of TLR engagement, TLR5-silenced NCM460 cells were transiently transfected with an NFκB-luciferase reporter plasmid followed by measuring NFκB reporter activity in response to flagellin. As shown in Fig. 2B, TLR5-silenced NCM460 cells were not responsive to flagellin stimulation, but they responded to IL-1 that utilizes a conserved molecular signaling pathway downstream of the TLR5 and IL-1 receptor, implying the specific inhibition of TLR5-mediated signaling at the receptor level (1). In contrast, NCM460 cells transfected with control vector were responsive to flagellin, as measured by NFκB reporter activity (Fig. 2B). Furthermore, because flagellin stimulation of NCM460 cells leads to IL-8 production (4), knockdown of TLR5 expression should inhibit flagellin-induced IL-8 production in TLR5-silenced NCM460 cells. To test this, we treated TLR5-silenced and control NCM460 cells with flagellin overnight and measured the level of IL-8 protein production in cell supernatants by using ELISA. We found that flagellin-induced IL-8 production was blocked in TLR5-silenced cells, whereas control cells produced large amounts of IL-8 in response to flagellin (Fig. 2C). In contrast, TLR5-silenced NCM460 cells had increased IL-8 production in response to IL-1 (Fig. 2C), indicating that TLR5 expression is selectively silenced in NCM460 cells. Next, we stimulated TLR5-silenced NCM460 cells with flagellin and examined PI3K activation by immunoblotting analysis. As shown in Fig. 2D, flagellin exposure stimulated Akt phosphorylation only in control, but not in TLR5-silenced NCM460 cells. Flagellin stimulation also failed to change the localization of FOXO1 in TLR5-silenced NCM460 cells (data not shown). Together, these data clearly indicate that PI3K activation is a specific outcome achieved from TLR5 engagement in response to flagellin in nontransformed human colonocytes.

TLR5 Associates with p85 Regulatory Subunit of PI3K, as Mediated by Its Cytoplasmic TIR Domain—Because TLR2 and TLR3 have been associated with the p85 regulatory subunit of PI3K (7, 8), we next investigated whether engagement of TLR5 by flagellin recruits the p85 regulatory subunit of PI3K in NCM460 cells. To study this, total cell lysates from NCM460 cells stimulated with flagellin, as indicated in Fig. 3A, were co-immunoprecipitated with an antibody against p85. The immunoprecipitant was next fractionated in SDS-PAGE followed by immunoblotting with an antibody against TLR5. As shown in Fig. 3A, the co-immunoprecipitation assay demonstrated that TLR5 associates with p85 in response to flagellin in nontransformed colon epithelial cells. Moreover, HEK293 cells were transiently co-transfected with an HA-tagged TLR5 wild type (TLR5 WT-HA) or an HA-tagged TLR5 (ΔTIR) (cytoplasmic TIR domain deleted TLR5, TLR5 (ΔTIR)-HA) construct along with a FLAG-tagged p85 (p85-FLAG) construct as indicated in Fig. 3B. Two days after transfection, total cell lysates were prepared and subjected to co-immunoprecipitation with FLAG antibody followed by immunoblot analysis with HA antibody, as described under “Experimental Procedures.” As shown in Fig. 3B, TLR5 was able to co-immunoprecipitate with the p85 regulatory subunit of PI3K, whereas TLR5 without the cytoplasmic TIR domain failed to associate with p85, implying that the cytoplasmic TIR domain of TLR5 mediates the association of TLR5 with the p85 regulatory subunit of PI3K.

The SH2 Domain Binding Motif (YXXM) in the Cytoplasmic TIR Domain of TLR5 Is Not Involved in Recruiting the p85 Subunit of PI3K to
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TLR5—PI3K is a heterodimer composed of a p85 regulatory and a p110 catalytic subunit. There are two SH2 domains (N-SH2 and C-SH2) in the p85 regulatory subunit that allow direct binding to the "YXXM" motif present in membrane-bound proteins, such as TLR2, IL-1 receptor, or platelet-derived growth factor receptor (7, 10–12). Previous studies showed that TLR2 directly associates with the p85 regulatory subunit of PI3K via the YXXM motif in the cytoplasmic TIR domain of TLR2 (7). Along these lines, we analyzed the amino acid sequence of the TLR5 and identified the presence of a YXXM motif at amino acid residues 798–801 YXXM within the cytoplasmic TIR domain of TLR5. Thus, we hypothesized that the YXXM motif in the cytoplasmic region of TLR5 is responsible for recruiting p85 protein, thereby resulting in TLR5-induced PI3K activation. To verify this hypothesis, we replaced the tyrosine residue in the YXXM motif within the cytoplasmic TIR domain of TLR5 with phenylalanine in order to disrupt the YXXM motif (TLR5 (Y798F)). TLR5 (Y798F)-HA expression construct was prepared as described under "Experimental Procedures." The HEK293 cells were then co-transfected with TLR5 (Y798F)-HA or TLR5 WT-HA expression construct and a p85-FLAG construct in the indicated combinations shown in Fig. 4B. Total cell lysates were prepared and subjected to co-immunoprecipitation with a FLAG antibody followed by immunoblot analysis using HA antibody to examine whether the disruption of YXXM motif prevents TLR5 (Y798F) from associating with p85. As presented in Fig. 4B, like TLR5 WT, TLR5 (Y798F) was still able to interact with p85. In addition, an adjacent tyrosine residue (Tyr-815) to the YXXM motif was also mutated to generate TLR5 (Y815F)-HA and co-transfected with p85-FLAG followed by co-immunoprecipitation assays. As shown in Fig. 4C, a point mutation of Y815F in the TIR domain of TLR5 is still capable of recruiting p85 protein. Furthermore, a double mutation of Y798F in the YXXM motif and Y815F in the TIR domain of TLR5 still enables p85 regulatory protein to co-immunoprecipitate with TLR5 (Fig. 4D). We also substituted various tyrosine residues (tyrosine 691, 693, and 696) at the tyrosine-rich region within the TIR domain of TLR5, and co-immunoprecipitation assays together with p85 were performed as described above. As shown in Fig. 4E, the triple mutation of tyrosine at 691, 693, and 696 amino acid residues did not alter the association of p85 protein with TLR5. Together, these data implied that the SH2 binding YXXM motif in the cytoplasmic TIR domain of TLR5 is not involved in recruiting the p85 regulatory subunit of PI3K. Furthermore, our data obtained from the various tyrosine mutants of TLR5 suggested that tyrosine residues at the TIR domain of TLR5 do not appear to be involved in p85 recruitment.

Flagellin Stimulation in Nontransformed Human Colonocytes Results in Recruitment of the MyD88 Adaptor Molecule to TLR5—Aksoy et al. (9) recently showed that TIRF, a TLR3 specific adaptor protein, recruits the p85 regulatory subunit of PI3K following TLR3 engagement. Moreover, MyD88 is the only known immediate downstream adaptor molecule of TLR5. Based on these considerations, and because the regulatory subunit p85 does not appear to directly associate with TLR5 (Fig. 4), we speculated that MyD88 might mediate recruitment of the p85 subunit to TLR5 in response to flagellin. We first determined whether MyD88 interacts with TLR5 upon flagellin stimulation in nontransformed human colonocytes expressing

FIGURE 3. The cytoplasmic TIR domain of TLR5 is involved in the association between TLR5 and the p85 regulatory subunit of PI3K. A, an endogenous p85 regulatory subunit was pull down in NCM460 cells with an antibody against p85 or control IgG, as described under "Experimental Procedures." Co-immunoprecipitation was evaluated by immunoblot analysis with TLR5 antibody. B, HEK293 cells were transiently transfected with TLR5 (Y798F)-HA, and p85-FLAG were co-immunoprecipitated with TLR5 WT-HA or TLR5 (ATIR)-HA along with p85-FLAG in the indicated combinations. Two days after transfection, p85-FLAG was pull downed with FLAG antibody followed by immunoblot analysis with HA antibody. Membranes were stripped and re-probed with p85 antibody to confirm precipitation of p85 protein. Whole lysates were processed for immunoblotting with p85 and HA antibodies. IP, immunoprecipitation; IB, immunoblot.

FIGURE 4. The SH2 domain-binding YXXM motif in the cytoplasmic TIR domain of TLR5 is not involved in recruiting the p85 regulatory subunit of PI3K. A, a schematic illustration of TLR5 shows the presence of the tyrosine residues mutated in our study. The amino acid at position 798 is the tyrosine at the YXXM motif inside the TIR domain of TLR5. B, TLR5 (Y798F)-HA and panel-FLAG constructs were co-transfected at the indicated combination followed by co-immunoprecipitation with HA antibody or control IgG and immunoblot (IB) analysis with HA antibody as described above. Whole cell lysates were subjected to immunoblot analysis to confirm the expression of TLR5 (Y798F)-HA and p85-FLAG. Similarly, co-immunoprecipitation assay was performed with TLR5 (Y815F)-HA (C), TLR5 (Y798/815F)-HA (D), and TLR5 (Y691F/Y693F/Y696F)-HA (E), as described under "Experimental Procedures." IP, immunoprecipitation.
TLR5. To do this, we first exposed NCM460 cells stably transfected with TLR5 WT-HA construct to flagellin, and then immunoprecipitation assay was performed with an HA antibody followed by immunoblot analysis with an antibody recognizing endogenous MyD88 protein. As shown in Fig. 5, flagellin stimulation recruited the endogenous MyD88 adaptor molecule to TLR5 in a time-dependent manner, indicating a biochemical association between TLR5 and the immediate downstream adaptor molecule MyD88 in flagellin-exposed human colonocytes.

**MyD88 Is Necessary for the Association of TLR5 with the p85 Regulatory Subunit of PI3K**—We next investigated whether the MyD88 adaptor molecule is involved in TLR5-induced PI3K activation. To test this, by using HEK293 cells expressing endogenous TLR5, we generated MyD88-silenced HEK293 cells by stably transfecting HEK293 cells with MyD88 siRNA expressing construct. Silenced MyD88 protein expression was confirmed by immunoblot analysis as shown in Fig. 6A, Moreover, to prove whether silenced MyD88 expression blocks flagellin-stimulated intracellular signaling, we transiently transfected MyD88-silenced and control HEK293 cells with an NFκB reporter construct followed by flagellin stimulation. As shown in Fig. 6B, NFκB reporter activity was not induced in MyD88-silenced HEK293 cells in response to flagellin stimulation, whereas strong NFκB reporter activity was evident in flagellin-exposed control HEK293 cells. In addition, because HEK293 cells do not express TLR4 to which MyD88 is a critical adaptor molecule, we transiently co-transfected TLR4 and NFκB reporter constructs in these cells followed by LPS stimulation. As shown in Fig. 6B, silencing MyD88 completely blocked LPS/TLR4-induced NFκB reporter activity, whereas LPS strongly induced NFκB reporter activity in control HEK293 cells. These data confirmed that MyD88 is biochemically and functionally silenced in HEK293 cells.

To test whether the association between TLR5 and the p85 regulatory subunit of PI3K is MyD88-dependent, we next transiently co-transfected TLR5-HA and p85-FLAG constructs into both MyD88-silenced and control HEK293 cells. As described under “Experimental Procedures,” the membranes were stripped with FLAG antibody to verify immunoprecipitation of p85. Whole cell lysates were subjected to immunoblotting with HA and FLAG antibodies to confirm the expression of TLR5 WT-HA and p85-FLAG, respectively. RLA, relative luciferase activity.

**FIGURE 5.** MyD88 adapter molecule is recruited to TLR5 in a time-dependent manner in NCM460 cells. NCM460-TLR5-HA cells were generated by stably transfecting a TLR5 WT-HA-expressing construct into the nontransformed human colonocytes NCM460. NCM460-TLR5-HA cells were stimulated with flagellin at the indicated times; total cell lysates were prepared, and TLR5-HA was co-immunoprecipitated with HA antibody to confirm immunoprecipitation (IP) of TLR5-HA.

**FIGURE 6.** Silencing MyD88 expression blocks the association between TLR5 and p85. A, MyD88-silenced and control HEK293 cells were generated as described under “Experimental Procedures,” and MyD88 silencing was confirmed by immunoblot analysis with an antibody recognizing MyD88. B, MyD88-silenced and control HEK293 cells were transiently transfected with NFκB-luciferase reporter construct (1 μg) either alone or together with TLR4-expressing construct, followed by stimulation with flagellin, or LPS (100 ng/ml) for 6 h. Relative luciferase activity was measured as described under “Experimental Procedures.” C, TLR5 WT-HA and p85-FLAG expression constructs were transiently co-transfected into MyD88-silenced and control HEK293 cells at the indicated combination. Co-immunoprecipitation assays were performed with FLAG antibody followed by immunoblot (IB) analysis with HA antibody as described under “Experimental Procedures.” The membranes were stripped with FLAG antibody to verify immunoprecipitation (IP) of p85. Whole cell lysates were subjected to immunoblotting with HA and FLAG antibodies to confirm the expression of TLR5 WT-HA and p85-FLAG, respectively. RLA, relative luciferase activity.

**FIGURE 7.** Silencing MyD88 in the human colonocytes blocks PI3K activation induced by flagellin. NCM460 cells were stably transfected with MyD88 siRNA expressing construct as described under “Experimental Procedures.” A, the silenced MyD88 protein expression was confirmed by immunoblotting analysis with MyD88 antibody. B, MyD88-silenced and control NCM460 cells were transiently transfected with NFκB-luciferase reporter construct (1 μg) followed by flagellin or tumor necrosis factor-α (TNFα) stimulation, and relative luciferase activity was measured as described above. C, MyD88-silenced and control cells were stimulated with flagellin overnight, and IL-8 production from the supernatant was evaluated by ELISA. D, MyD88-silenced and control cells were stimulated with flagellin as indicated time points followed by immunoblot analysis with antibody recognizing phosphorylated Akt. The membrane blot was stripped and re-probed with Akt antibody for the loading control. Data are reported as mean values ± S.E. from triple experiments.

MyD88 expression by immunoblotting analysis (Fig. 7A). Moreover, we demonstrated that flagellin-induced NFκB reporter activity was blocked in MyD88-silenced cells, in contrast to increased NFκB activation in control cells. However, tumor necrosis factor–α that mediates NFκB activation via MyD88-independent pathways was able to induce NFκB activation in both MyD88-silenced and control NCM460 cells (Fig. 7B), indicating that MyD88-dependent signaling was specifically blocked. Furthermore, we also evaluated if silencing MyD88 is able to inhibit flagellin-induced IL-8 production. As shown in Fig. 7C, flagellin-stimulated IL-8 production was
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YXXM Motif in MyD88 Is Responsible for Associating with the p85 Regulatory Subunit of PI3K—Ojaniemi et al. (23) showed that MyD88 associates with the p85 regulatory subunit of PI3K following LPS/TLR4 engagement in murine macrophages. Moreover, Aksoy et al. (9) suggested that the adaptor molecule TRIF downstream of TLR3 and -4 mediates PI3K activation. Because our data implied that MyD88 mediates TLR5-induced PI3K activation (Fig. 6), we investigated if flagellin/TLR5 engagement enables MyD88 to associate with the p85 regulatory subunit in nontransformed human colonocytes. To test this, NCM460 cells were co-transfected with a dominant negative mutant of p85 (p85-DN) or a wild type p85 expressing construct along with an IL-8 promoter reporter construct, as described under “Experimental Procedures” and evaluated flagellin-stimulated IL-8 promoter activity. As shown in Fig. 9C, overexpression of the p85 dominant negative mutant inhibited IL-8 promoter activity in response to flagellin. In addition, inhibition of the p85 downstream signaling molecule Akt by overexpressing a dominant negative Akt mutant (Akt-DN) substantially reduced IL-8 promoter activity stimulated by flagellin in NCM460 cells. To evaluate if overexpressing p85-DN is able to inhibit flagellin-induced IL-8 production, NCM460 cells were infected with p85-DN expressing adenoviruses (Adeno-p85-DN) or control viruses (Adeno-lacZ), as described elsewhere (20). Cells were next stimulated with flagellin overnight, and IL-8 levels were measured by ELISA in the cell supernatants. As shown in Fig. 9D, overexpressing p85-DN reduced flagellin-induced IL-8 protein production in NCM460 cells. Together, these data demonstrate that TLR5-linked PI3K activation is at least in part involved in IL-8 protein production in human colonocytes.

DISCUSSION

Here we show that exposure of disrupted mouse colon to the specific TLR5 ligand flagellin stimulates phosphorylation of the signaling molecule PI3K in this tissue. Similarly, stimulation of flagellin to human colonic epithelial cells leads to PI3K activation in a time-dependent manner. Flagellin stimulation of human colonocytes also recruited the downstream TLR adaptor molecule MyD88 to the cytoplasmic TIR domain of TLR5. Importantly, we demonstrate that silencing MyD88 blocks flagellin-stimulated PI3K activation in colonocytes. We also...
determined that the p85 regulatory subunit of PI3K associates with MyD88 in response to flagellin, in which the YKAM motif is responsible for the association between MyD88 and p85. To our knowledge, these are the first results to indicate that MyD88 is essential to link the activated signaling by TLR5 engagement to PI3K.

In general, the p85 regulatory subunit of PI3K has been shown to directly bind to the YXXM motif present in the cytoplasmic region of several transmembrane receptors, whereby mediating receptor-associated PI3K activation (7–12). In the case of TLR5, the cytoplasmic TIR domain of TLR5 harbors a YXXM motif that could be directly associated to p85 regulatory protein of PI3K. As shown in Fig. 4, however, disrupting the YXXM motif, or any other tyrosine residue in the TIR domain of TLR5, did not interfere with the interaction between TLR5 and the p85 regulatory protein, suggesting an indirect association between these two molecules. In this context, our results with MyD88-silenced cells revealed that the absence of MyD88 disrupted the association between TLR5 and the p85 regulatory subunit of PI3K and diminished PI3K activation in response to flagellin (Figs. 6 and 7). In addition, MyD88, which also contains a YXXM motif at its C-terminal region, was able to form a complex with the p85 regulatory protein. We also demonstrated that MyD88 (Y257F) containing a point mutation at the tyrosine residue of the YXXM motif failed to interact with p85 (Fig. 8). These data are consistent with recent studies suggesting that MyD88 associates with the p85 regulatory subunit in TLR4-mediated signaling in mouse macrophages (23). Along these lines, Aksoy et al. (9) also suggested that the adaptor molecule TRIF mediates PI3K phosphorylation following TLR3 and TLR4 activation.

Meanwhile, Sarkar et al. (8) recently showed that tyrosine phosphorylation of TLR3 is involved in PI3K activation, indicating that tyrosine phosphorylation of TLR may be an important biochemical event in TLR-associated signaling. In the case of TLR5, however, mutating the various tyrosine residues with phenylalanine in the cytoplasmic TIR domain did not appear to disrupt TLR5-mediated NFκB activation in NCM460 cells (data not shown). Further studies are needed to determine the biological significance of the tyrosine residues in the cytoplasmic TLR domain of TLR5.

PI3K activation mediates several critical cellular responses, including cell differentiation, proliferation, survival, and shape (6, 25). Of greater interest, Fukao and Koyasu (24) showed that blocking PI3K activation resulted in enhanced IL-12 production by several TLR ligands in mouse dendritic cells. However, using nontransformed human colonic epithelial cells stimulated with flagellin, our cytokine micro-array data did not show any enhanced cytokine production by blocking PI3K activation in flagellin/TLR5-induced signaling (Fig. 9). In contrast, pharmacologic antagonism or overexpression of a dominant negative PI3K mutant substantially reduced IL-8 production, but not MIP3α production in response to flagellin in colonocytes (Fig. 9). These results indicate that in human colonocytes, the IL-8 gene expression in response to flagellin is at least in part PI3K-dependent. In general, NFκB activation is a common regulator, critical for proinflammatory cytokine expression such as IL-8 and MIP3α (4). Interestingly, we determined that blocking PI3K activation by its specific inhibitor LY294002 substantially reduces flagellin/TLR5-derived NFκB reporter activity in colon epithelial cells, although LY294002 significantly inhibits IL-8 but not MIP3α production (Fig. 8A). Although the mechanism of these two different PI3K

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FIGURE 9. Inhibition of PI3K activation reduces flagellin-stimulated IL-8 production in NCM460 cells. A human cytokine micro-array was performed with equal amounts of total cell lysates from flagellin-stimulated NCM460 cells, as described under “Experimental Procedures.” IL-8 is indicated by the red arrow and MIP3α by the blue arrow. OD values obtained from the x-ray films were expressed as a relative ratio of IL-8. Data is expressed as mean ± S.E., n = 2. 8. NCM460 cells were stimulated with flagellin (100 ng/ml) overnight in the presence or absence of the PI3K antagonist LY294002 as indicated, and IL-8 and MIP3α levels were measured in the culture supernatants by ELISA. In parallel, to confirm the inhibitory effect of LY294002 on PI3K activity, NCM460 cells were stimulated with flagellin with or without LY294002 at the indicated concentrations. Akt phosphorylation was evaluated by immunoblotting analysis with antibodies against phospho-Akt and inactive Akt. C, IL-8 reporter construct was transiently co-transfected into NCM460 cells with p85 WT or p85-DN and Akt WT or Akt-DN. One day after transfection, cells were stimulated with flagellin for 6 h, and the relative luciferase activity was measured as described elsewhere (4). Transfection was performed in triplicate, and a single representative experiment is shown. D, NCM460 cells were infected with Adeno-p85-DN and control Adeno-lacZ viruses as described under “Experimental Procedures.” One day after infection, cells were stimulated with flagellin followed by IL-8 measurements in the supernatant as described above. Expression of p85-DN was confirmed by immunoblotting analysis (inset) in cell lysates. Because of the lack of the binding site to the catalytic subunit p110 of PI3K (17) (20), ectopic p85-DN is shown at slightly lower size than endogenous p85 WT. Data are reported as mean values ± S.E. RLA, relative luciferase activity.
responses is not clear, we hypothesize that, other than the canonical NFκB (p65/p50) transcription factor activation (26), TLR5-derived PI3K activation might be able to mediate the activation of the noncanonical transcription factors of the Rel family, such as RelB/p52. As a result PI3K activation might differentially influence expression of IL-8 and MIP3α in flagellin-stimulated colonic epithelial cells.

In conclusion, our results provide important information relevant to the molecular mechanism by which PI3K is activated in response to bacterial flagellin. These results might be critical to our understanding of the mechanism(s) by which TLR5/PI3K interactions participate in the pathophysiology of intestinal inflammation in response to bacteria, including inflammatory bowel disease.

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