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A phosphorylation site in the Toll-like receptor 5 TIR domain is required for inflammatory signalling in response to flagellin

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Abstract

Flagellin, the major structural subunit of bacterial flagella, potently induces inflammatory responses in mammalian cells by activating Toll-like receptor (TLR) 5. Like other TLRs, TLR5 recruits signalling molecules to its intracellular TIR domain, leading to inflammatory responses. Phosphatidylinositol 3-kinase (PI3K) has been reported to play a role in early TLR signalling. We identified a putative binding site for PI3K at tyrosine 798 in the TLR5 TIR domain, at a site analogous to the PI3K recruitment domain in the interleukin-1 receptor. Mutation of this residue did not affect homodimerization, but prevented inflammatory responses to flagellin. While we did not detect direct interaction of PI3K with TLR5, we demonstrated by mass spectrometry that Y798 is phosphorylated in flagellin-treated HEK 293T cells. Together, these results suggest that phosphorylation of Y798 in TLR5 is required for signalling, but not for TLR5 dimerization.

Keywords: Innate immunity; Signal transduction; Inflammation; Kinases; Chemokines; Mass spectrometry; Epithelial cells

Bacterial flagellin is an ancient and potent trigger of host innate immune responses in eukaryotes. Flagellin is the only known activator of Toll-like receptor (TLR) 5. Activation of TLR5 by flagellin signals inflammatory responses via a MyD88-dependent pathway, leading to IRAK-1 phosphorylation, and ultimately NF-κB and p38-MAPK activation, which are required for inflammatory phenotypes such as interleukin-8 release [1–3]. This signalling depends on the association of TLR5 with MyD88 via their homologous TIR domains. Moreover, like other TLRs, TLR5 can homodimerize, and constitutive dimerization activates NF-κB [4]. In some cell types, flagellin also activates inducible nitric oxide synthase via a mechanism requiring TLR5/TLR4 heterodimers [5].

While the formation of TLR5 homo- and heterodimers in different cell types can explain some of the tissue-specific differences in flagellin responses, additional layers of complexity are likely to exist. We and others have found that flagellin activates phosphatidylinositol 3-kinase (PI3K) in Caco-2 cells (Ivison et al., currently under review); [6,7]. PI3K is also recruited to TLR3 following stimulation with double-stranded RNA [8]. In response to IL-1 stimulation, PI3K is recruited to the IL-1 receptor (IL-1R1) at a specific phosphotyrosine in the TIR domain, contained within a Y–X–X–M SH2-binding motif [9]. This observation led us to identify a Y–X–X–M motif in TLR5 in the same location as the PI3K-binding site in IL-1R1. Based on this, we hypothesized that flagellin-induced inflammatory signalling in epithelial cells would require tyrosine phosphorylation of TLR5 at this site.

Methods

Cell culture. Culture media and supplements were from Sigma (St. Louis) except where otherwise noted. HeLa cells were from B. Brett Finlay (University of British Columbia) and HEK 293T cells were from American

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Type Culture Collection. Both were grown in high-glucose Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum (Hyclone; Logan, UT), 1× nonessential amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml), and 1 mM sodium pyruvate. Caco-2 cells from ATCC were grown in the same media, although non-heat-inactivated serum (Gibco) was used. Recombinant EAEC flagellin was prepared as previously described [10]. LPS was removed from flagellin preparations by polymyxin B chromatography (Detoxi-Gel, Pierce) and absence of endotoxin was confirmed by Limulus assay.

Transient transfection of HeLa and HEK cells. pEF6-V5-His-TLR5 was a gift from A. Aderem (University of Washington, Seattle, WA). pEGFP-N1 (Clontech, Palo Alto, CA) expressing green flourescent protein (GFP) was used as a transfection control. TLR5-Y798L was generated by circular mutagenesis with the following primers: 5′-ggtccttgtcccaattgcagttgatgaaa and 5′-tttcatcaactacaattgggacaaggacc. The FLAG-TLR5 constructs were generated by cloning the above constructs into pCMV-TAG 3 (Sigma).

For electroporation of HeLa cells, DNA (pEGFP, pTLR5, and sheared salmon sperm DNA to a total of 25 μg) was added to 400 μl of HeLa cell suspension (10^7/ml) in Opti-MEM. Cells were pulsed in 2-mm cuvettes in an ECM 630 electroporator (BTX, San Diego, CA) using capacitance 425 μF, resistance 25 Ω, and potential 280 V. Cells were rested 5 min at room temperature, transferred into growth media, and seeded in culture plates or dishes. HEK 293T cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, with 1.25 μl of reagent and 500 μg of DNA per well in 24-well plates. Cells were used 48–72 h after transfection.

IL-8 promoter/reporter assays. Caco-2 cells were trypsinized and suspended at 10^7/ml in Opti-MEM. Cells (4 million per condition) were transfected with 1 μg of pEGFP, 7.5 μg of IL-8pLuc (gift of B. Sahl, University of British Columbia), TLR5 constructs, and sheared salmon-sperm DNA to 30 μg total DNA by electroporation at 1000 μF, 50 Ω, 250 V. After 5 min at room temperature, cells were diluted into 1.2 ml of Caco-2 growth medium and seeded at 100 μl/well in 96-well plates. Medium was changed the following day and fluorescence verified by microscopy. Five days later, cells were stimulated 6 h and lysed with Bright-Glo reagent (Promega; Madison, WI). Luminescence was read in a microplate luminometer and GFP fluorescence measured in a Fluoroskan Ascent FL (Thermo Electron; Waltham, MA). The ratio of luminescence to fluorescence in arbitrary units was determined for each sample, and the fold increase in this value compared to controls within the same experiment was calculated.

IL-8 release assays. HeLa cells were transiently transfected with 1-2.5 μg of pEGFP and 5–10 μg of TLR5 construct per 4 million cells. Media was changed the following day and cells incubated for a further 24–48 h prior to stimulation. Flagellin was added in fresh growth media for 3 h and culture supernatants tested for IL-8 concentration by ELISA (Opt-EIA, BD Biosciences). Cells were then trypsinized and suspended in equal volumes of PBS with 2% fetal calf serum, and green fluorescence and cell number were measured by flow cytometry (FACSCalibur, BD Biosciences) The IL-8 value for each well was divided by the number of GFP expressing cells in the same well.

HEK 293T cells were transfected with pEF6-hTLR5 or its various mutant forms and used after 48 h. Stimulants were added and supernatants removed after 24 h for IL-8 EIA. Because the transfection efficiency was consistently >90% in these cells, results were not corrected for variation in GFP expression.

Cell fractionation and Western blotting. For p38 MAPK activation, HEK293T cells were transfected with 100 ng of TLR5 or TLR5-Y798L and treated with 0.5 μg/ml flagellin for 0, 5, 10, 20 or 30 min. Cells were lysed in 20 mM MOPS, pH 7.2, 0.5% NP-40, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 10 mM sodium pyrophosphate, 30 mM NaF, 2 mM Na_3VO_4 and 1× protease inhibitor for mammalian cells (Sigma). As a positive control, cells were treated with 1 μg/ml anisomycin for 10 min. 75 μg of cell lysate was analyzed by Western blot using anti-phospho-p38MAPK (Thr180/Tyr182, Cell Signaling). Anti-GAPDH (Fitzgerald; Concord, MA) was used as a loading control.

To measure tyrosine phosphorylation, HeLa cells were transfected with 1 μg of pEGFP and 5 μg of either TLR5 or TLR5-Y798 L, rested 48 h, and then treated with media or flagellin (1 μg/ml) for 10 min. Cells were washed in cold PBS and lysed in 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM β-glycero-phosphate, 1 mM Na_3VO_4, and 1× protease inhibitor cocktail. For immunoprecipitation (IP) of TLR5, 10 μg/mg total protein anti-V5 (anti-PK; Serotec, Oxford, UK) was added to cleared cell lysates. After overnight incubation at 4 °C, 50 μl of protein G agarose (Sigma) was added and lysates incubated for a further 2 h. Immune complexes were pelleted by brief centrifugation, washed three times in lysis buffer, and boiled in 2× SDS–PAGE buffer. Supernatants were analyzed by Western blot with 4G10 anti-phosphotyrosine (Upstate), followed by anti-V5.

For electrophoretic mobility shift assay (EMSA), HeLa cells transiently transfected with TLR5 contructs were stimulated for 20 min, lysed, fractionated and analyzed as in [3].

TLR5 dimerization experiments were performed in HEK 293T cells co-expressing V5-TLR5 and FLAG-TLR5 at amounts optimized to produce equivalent expression levels. After treatment with flagellin or media control, cells were lysed as described above and immunoprecipitated using anti-FLAG™ M2-affinity gel (Sigma). Immune complexes were eluted in 300 μM FLAG peptide (Sigma) and subjected to Western analysis with anti-V5. Blots were then stripped and probed with anti-FLAG M2 (Sigma) to control for efficiency of the IP.

For cell fractionation experiments, HeLa cells electroporated as described above were seeded in 15-cm dishes, incubated overnight, and surface biotinylated with sulfo-NHS-LC-biotin (Pierce) at 0.5 mg/ml for 30 min. Cells were lysed by two freeze–thaw cycles, scraped into 10 mM HEPES with 1× protease inhibitor cocktail, and sonicated. Lysates were spun at 750*g* for 10 min to pellet nuclei and insoluble debris (insoluble fraction). Supernatants were transferred to thick-walled tubes and spun at 100,000*g* for 1 h at 4 °C to pellet membranes (membrane fraction). The supernatant from this spin was saved as the cytosolic fraction. Fractions were suspended in 1% SDS in 10 mM HEPES and assayed for protein concentration. The entire insoluble fraction and 40 μg each of the other fractions were analyzed by Western blotting with anti-V5; membranes were then stripped and probed with streptavidin–peroxidase polymer (Sigma) at 1:10,000 in TBST. Equal protein loading was confirmed by staining with colloidal gold.

LC–MS analysis of TLR5 phosphorylation. HEK cells transfected in 15-cm dishes were treated with flagellin (1 μg/ml) for 10 min, and fractionated as above. Membrane pellets were solubilized in 1% SDS, diluted 10-fold in IP buffer, and incubated overnight with anti-V5 (10 μg/mg protein). Immune complexes were precipitated with protein G agarose and separated by SDS–PAGE. Gels were stained with Sypro Ruby (Invitrogen) and the band corresponding to TLR5 (verified by its absence in identically treated cells not expressing TLR5) was excised, trypsinized in situ, and analyzed by liquid chromatography–single mass spectrometry at the NAPS core MS facility at the University of British Columbia. Predicted tryptic peptides of TLR5 with a mass shift of 80 Da (the size of a phosphate group) were specifically sought.

Results

Tyr 798 of TLR5 is required for flagellin-induced TLR5 responses

As shown in Fig. 1A, TLR5 has a Y–X–X–M motif at an analogous location to the PI3K-binding site in the IL-1R1 and a putative TLR3 phosphotyrosine required for responses to double-stranded RNA. In order to test the importance of the Y–X–X–M motif in the TLR5 Tir domain, we expressed TLR5-Y798L in HeLa and HEK cells. As shown in Fig. 1B and C, IL-8 release from cells expressing TLR5-Y798L was significantly less than from