TRIF Modulates TLR5-dependent Responses by Inducing Proteolytic Degradation of TLR5*1

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Proteolytic modification of pattern recognition receptors and their signaling adaptor molecules has recently emerged as an essential cellular event to regulate immune and inflammatory responses. Here we show that the TIR domain-containing adaptor-inducing interferon-β (TRIF), an adaptor molecule mediating TLR3 signaling and MyD88-independent signaling of TLR4, plays an inhibitory role in TLR5-elicited responses by inducing proteolytic degradation of TLR5. TRIF overexpression in human embryonic kidney (HEK293) and human colonic epithelial (NCM460) cells abolishes the cellular protein level of TLR5, whereas it does not alter TLR5 mRNA level. Thus, TRIF overexpression dramatically suppresses flagellin/TLR5-deriven NFκB activation in NCM460 cells. TRIF-induced TLR5 protein degradation is completely inhibited in the presence of pan-caspase inhibitor (benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone), whereas several specific inhibitors against cathepsin B, reactive oxygen species, or ubiquitin-mediated proteasome activity fail to suppress this degradation. These results indicate that TRIF-induced caspase activity causes TLR5 protein degradation. In addition, we identify that the C terminus of TRIF and extracellular domain of TLR5 are required for TRIF-induced TLR5 degradation. Furthermore, TRIF-induced proteolytic degradation is extended to TLR3, TLR6, TLR7, TLR8, TLR9, and TLR10, whereas the cellular level of TLR1, TLR2, and TLR4 is not affected by TRIF overexpression. These results suggest that, in addition to mediating TLR3- or TLR4-induced signaling as an adaptor molecule, TRIF can participate in proteolytic modification of certain members of TLRs to modulate the functionality of TLRs at post-translational level. Collectively, our findings propose a potential inhibitory role of TRIF at least in regulating host-microbial communication via TLR5 in colonic epithelial cells.

PRRs 2 mediate recognition of microbes in multicellular organisms, leading to the activation of innate and adaptive immune and inflammatory responses. Toll-like receptors (TLRs) are the most well-characterized members of PRRs and detect microbe-associated molecular patterns present in a wide range of microorganisms (1). The cellular and molecular mechanism by which PRRs mediate microbial recognition-generated intracellular signaling has been intensively studied. Those studies identified several adaptor molecules such as myeloid differentiation factor 88 (MyD88) and TRIF in TLR-dependent signaling (2–5). These adaptors are the key regulator in mediating TLR-dependent intracellular signaling pathways.

Along with PRR-mediated intracellular signaling, proteolytic modification of PRRs and PRR-associated adaptor molecules makes a significant impact on both the receptor functionality and the receptor-mediated immune and inflammatory signaling. Thus, proteolytic modification of these molecules has recently emerged as one of the critical cellular events regulating immune and inflammatory responses of PRRs. Indeed, cathepsin-cleaved fragments of TLR9 exhibit enhanced binding activity to CpG DNA compared with full-length TLR9, suggesting that the proteolytic cleavage of TLR9 is required for a full activation of TLR9 signaling (6, 7). Moreover, adaptor molecules such as TRIF are known to be cleaved by caspase activity, resulting in attenuated antiviral immunity (8). In addition to cathepsin and caspase activities, serine protease activity of hepatitis C virus (HCV) NS3-4A cleaves TRIF (9, 10), thereby allowing HCV to evade antiviral defense. Therefore, a proteolytic modification of PRRs and their key signaling adaptor molecules turn out to be critical to control immune and inflammatory responses against microbial factors.

TRIF and MyD88 adaptor molecules mediate MyD88-independent and MyD88-dependent signaling pathways, respectively, of various TLR family members. TRIF is a single adaptor molecule mediating TLR3-induced signaling and the MyD88-independent signaling pathway of TLR4, whereas MyD88 is responsible for mediating the MyD88-dependent pathway of TLR4. Whereas the involvement of TRIF is confined to TLR3 and TLR4, MyD88 is associated with mediating intracellular signaling of virtually most of the TLRs (except TLR3). Consequently, MyD88-mediated signaling leads to inhibitory κB kinases (IKKs) and MAPK activation and subsequently results in NFκB and AP-1 transcription factor activation to induce pleiotropic gene expression involved in immune and inflamma-

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tritory responses (4, 5, 11). In contrast, the involvement of TRIF in TLR3- or TLR4-induced responses primarily provokes interferon-regulatory factor-3 (IRF-3) activation to elicit interferon-β expression. Concomitantly, TRIF is known to induce caspase activation via the RIP (receptor interacting protein)/FADD (Fas-associated death domain)/caspase-8/caspase-3 cascade, which is uncoupled to NFκB or MAPK activation pathways (8, 12, 13). Therefore, TRIF-dependent caspase activation results in apoptosis (14).

TLR5, specifically recognizing flagellin, is abundantly expressed in virtually most types of epithelial cells from various organs including gastrointestinal tract (15), lung (16), or uterus (17), whereas a subset of lamina propria dendritic cells, CD11c⁺CD11b⁺, in the small intestine were suggested to respond to flagellin (18). TLR5 is known to exclusively utilize MyD88 adaptor molecule to mediate intracellular signaling pathways from flagellin/TLR5 engagement (5, 19, 20).

In this study we discovered that TRIF overexpression results in proteolytic degradation of TLR5 in a caspase-dependent manner and thereby negatively regulates TLR5-induced responses in intestinal epithelial cells. TRIF-induced TLR5 protein degradation is extended to TLR3, TLR6, TLR7, TLR8, TLR9, and TLR10, whereas the cellular levels of TLR1, TLR2, and TLR4 were not affected by TRIF overexpression. Our data suggest that, other than playing a role of signaling adaptor molecule for TLR3 and TLR4, TRIF may participate in proteolytic modification of certain members of TLRs to modulate the functionality of TLRs at the post-translational level.

**EXPERIMENTAL PROCEDURES**

**Materials and Mice**—Human colonic epithelial cells (NCM460) and its culture medium M3D were obtained from INCCELL Corp. (San Antonio, TX). NCM460 cells and human embryonic kidney 293 (HEK293) cells were cultivated as previously described (15, 20). Antibodies against human TRIF, phospho-ERK1/2, phospho-p38, phospho-c-Jun N-terminal kinase, phospho-p105 (NFκB), phospho-p65 (NFκB), cleaved caspase-3 or -8, cleaved poly(ADP-ribose) polymerase (PARP), ERK1/2, Akt, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2 (MEK1/2), Rab5, cathepsin D, and STAT3 were purchased from Cell Signaling Technology (Danvers, MA). HA or Myc antibody was from Roche Applied Science, and FLAG (M2) antibody was from Sigma. Flagellin purified from *Salmonella typhimurium* (< 0.1 endotoxin units/μg of purified protein, determined by the limulus amoebocyte lysate test) was purchased from Enzo Life Sciences (Farmingdale, NY) and dissolved in endotoxin-free water. Cathepsin B inhibitor (CA-074-Me), N-acetylcysteine, NH₄Cl, MG132, and benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone were purchased from EMD Chemicals, Inc. (Gibbstown, NJ) and dissolved in double distilled H₂O or DMSO. All other materials were purchased from Sigma unless specified.

**Quantitative Real-time PCR**—Total RNA was isolated from cultured cells using the RNeasy Plus mini kit (Qiagen, Valencia, CA), and an equal amount of RNA (2 μg) was transcribed into cDNA using the High Capacity Reverse Transcription kit obtained from Applied Biosystems (Carlabad, CA) by following the manufacturer’s instructions. To evaluate the cytokine gene expression, quantitative real-time PCR was performed on the Applied Biosystems 7500 Fast Real-time PCR System with the TaqMan Universal Master Mix using the standard conditions from Applied Biosystems. After the sample was incubated for 2 min at 50 °C followed by AmpliTaq Gold activation for 10 min at 95 °C, 40 cycles were run with a denaturing temperature of 95 °C (15 s) and an annealing/extension temperature 60 °C (1 min). The primer pairs and FAM™ dye-labeled TaqMan® MGB (minor groove binding) probes for human TRIF, MyD88, TLR4, and TLR5 and the internal control GAPDH gene were purchased from Applied Biosystems. The level of expression was calculated based upon the PCR cycle number (Cₜ) at which the exponential growth in fluorescence from the probe passes a certain threshold value (Cₜ). Relative gene expression was determined by the difference in the Cₜ values of the target genes after normalization to RNA input levels using the Cₜ value of GAPDH. Relative quantification was represented by standard 2⁻ΔCₜ calculations. ΔCₜ = (Cₜ-target gene − Cₜ-GAPDH). Each reaction was performed in triplicate.

**Expression Constructs**—Constructs of TLR5-HA and TLR5(ΔTIR)-HA were described previously (15, 20). Constructs of pcDNA3.1/zeo-HA-TLR4, pcDNA3-FLAG-MyD88, and NFκB-luciferase were previously described (15, 20, 21). TLR1-HA, TLR2-HA, TLR3-HA, TLR6-HA, TLR7-HA, TLR8-HA, TLR9-HA, and TLR10-HA expression constructs and pUNO2-human TRIF were obtained from Invivogen (San Diego, CA). The Myc-TRIF(AC) 1–541-amino acid construct was generously provided by Dr. Shizuo Akira (Osaka University, Japan). pcDNA3.1-myc-TRIF (22) or pLINX retroviral vector (23) was a gift from Dr. Rongtuan Lin (McGill University) or Dr. Fred H. Gage (The Salk Institute), respectively.

To generate TLR5 (ΔECD)-HA (640–858 amino acids), we performed PCR using pUNO-TLR5-HA as a template and primers A and B. The PCR product containing the transmembrane and TIR domain of TLR5 was inserted into NotI-EcoRV sites in the pFLAG-CMV3 (Sigma) vector for which the Met-preprotrypsin leader sequence precedes the FLAG sequence. Primer A was 5’-AAT ATA GCG GCC GGC TTC TCC CTT TCC ATT GTA TGA ACT-3’; Primer B was 5’-CTG AAA CAT AAA ATG ATT GCA ATT GTT-3’.

To generate FLAG-TRIF, we performed PCR using pUNO2-hTRIF as a template and primers C and D. The PCR product (180 bp) was subcloned into NotI-XbaI sites in pFLAG-CMV7.1 (Sigma) in which the XbaI-Smal fragment from pUNO2-hTRIF was subsequently inserted into XbaI-Hpal sites to generate pCMV-FLAG-TRIF. Primer C was 5’-TTATTATTAGCCGCCCAGTGCACCACAAGGCCCTACATCTT-C3’; Primer D was 5’-GGATTTTGCTTGAGGATTGGGTGGAGTGGAG-3’.

To generate the N-terminal-deleted TRIF expression construct (FLAG-TRIF(ΔN)), 380–712 amino acids), we performed PCR using pCMV-FLAG-TRIF as a template and primers E and F. NotI-BglII sites in full-length TRIF (pCMV-FLAG-TRIF) were replaced with the PCR product (500 bp) to give rise to FLAG-TRIF(ΔN). Primer E was 5’-AAT ATA GCG GCC GGC TTC CTT CCT CTT CCT CTT GCG GTG-3’; primer F was 5’-CAG GGC GGA GTC CTG TTC CTT-3’.
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To generate FLAG-TRIF(ΔN/ΔC), 387–566 amino acids, we performed the PCR using pDeNy-hTRIF (Invivogen) as a template and primers G and H. The PCR product containing only the TIR domain was inserted into NolI-SmaI sites in the pFLAG-CMV7.1. Primer G was 5’-TAT AAA GGG GCC GCC ATG GAA TCA TCA TCG GAA CAG-3’; primer H was 5’-TAT AAA CCC GGG TTA CAG TGC CGC CGC CTG CAT-3’.

To generate pLINX-FLAG-TRIF, we performed PCR using pUNO2-hTRIF as a template and primers I containing FLAG sequences and J. Then, the PCR product (550 bp) was inserted into KpnI-BamHI sites in the pcDNA3.1/zeo(+). Then, in BamHI-EcoRV sites of this construct, the DNA fragment (BamHI-HpaI) from pUNO2-hTRIF was inserted to obtain pcDNA3.1/zeo(+)-FLAG-TRIF. The cDNA for FLAG-TRIF was isolated by restriction enzyme digestion of Pmel and sub-cloned into HpaI site of pLINX, giving rise to pLINX-FLAG-TRIF. Primer I was 5’-ACTAA GGT ACC CGC GCC ATG GAT TAT AAA GAT GAT GATGAT AAA ATG GCC TGC ACA GCC CCA TCA CTT CC-3’; primer J was 5’-GCC TCC AGT CCG AAA CAC CGT CAA TGG-3’. The integrity of the whole sequence was confirmed by sequencing all DNA constructs.

Doxycycline-inducible Expression of TRIF—The packaging cell line for retroviral vectors, PT67, from Clontech (Mountain View, CA), was stably transfected with pLINX-FLAG-TRIF construct. The culture supernatant containing viral particles for pLINX-FLAG-TRIF was harvested and cleared by centrifugation. NCM460-TLR5-HA cells, stably expressing TLR5-HA, were treated with the supernatant mixed with Polybrene (8 µg/ml) to suppress the expression of TRIF cDNA inserted into pLINX vector. Each clone was cultivated with or without doxycycline for 2 days, and cell lysates were prepared in lysis buffer followed by immunoblot assay.

Cell Transfection—NCM460 or HEK293 cells were plated in 60-mm dishes (1.5 × 10⁶ cells) and stabilized overnight. Using SuperFect transfect reagent from Qiagen, cells were transfected with the appropriate plasmid DNA. The total DNA was kept consistent by adding the empty vector to each transfection. About 15 h after transfection, cell lysates were prepared in lysis buffer. To generate stably transfected cells, cells were plated in the proper medium containing selecting antibiotics.

Immunofluorescence Staining—NCM460 cells transfected with TLR5-HA construct were plated on a chamber slide (Nalgene Nunc, Rochester, NY). After overnight stabilization, cells were stimulated with flagellin (100 ng/ml) or vehicle for 30 min. Cells were then washed twice with phosphate-buffered saline, fixed in 10% formalin for 15 min, and permeabilized with ice-cold 100% methanol at −20°C for 10 min. Samples were washed with phosphate-buffered saline and blocked with 1% normal donkey serum and 0.1% Triton X-100 in phosphate-buffered saline for 1 h at room temperature. Samples were then incubated overnight at 4°C with primary antibodies HA-fluorescein (Clone 3F10, 1:10 dilution) and Rab5 (1:50 dilution) in blocking buffer. Samples were washed with phosphate-buffered saline and incubated with Texas Red-conjugated donkey anti-rabbit secondary antibody (1:100 dilution, Jackson ImmunoResearch Laboratories) for 1 h at room temperature. Samples were then rinsed and mounted with 4,6-diamidino-2-phenylindole mounting solution (Vector Laboratories, Burlingame, CA).

Luciferase Reporter Assays—Cells were plated in 6-well plates (0.5 × 10⁶ 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). One day after transfection cells were stimulated with flagellin (100 ng/ml) for 6 h, and the relative luciferase activity was determined by normalization with β-galactosidase activity as we previously described (15, 20). The total amount of plasmid DNA was kept consistent by adding the empty vector for each transfection.

Immunoblot Assay—Equal amounts of protein from cell lysates were subjected to SDS-PAGE analysis and immunoblot assay using the appropriate antibodies, performed as we previously described (15, 20).

RESULTS

TNFα Stimulation Enhances TRIF Expression in Human Colonic Epithelial Cells—Enhanced cytokine expression is well characterized in inflammatory diseases including inflammatory bowel diseases. Among those, TNFα plays an essential role in regulating the pathophysiology of inflammatory bowel disease, evidenced by several clinical studies using TNFα monoclonal antibodies to improve inflammatory bowel disease symptoms and pathology (24). We and others previously demonstrated that host-microbial recognition by TLR5 is associated with the development and progress of intestinal inflammation (25, 26). Moreover, aberrant activation of TLRs (e.g., TLR4) is responsible for intestinal inflammation (27–29). Accordingly, we tested whether TNFα affects the cellular levels of TLR4, TLR5, and TLR-associated adaptor molecules (MyD88 and TRIF) in human colonic epithelial cells (NCM460). Intriguingly, TNFα stimulation substantially enhanced the mRNA expression of TRIF in these cells, whereas the expression level of MyD88, TLR4, and TLR5 was not changed by TNFα stimulation compared with vehicle-treated cells (Fig. 1A). Similarly, TNFα stimulation up-regulated the protein production of TRIF in NCM460 cells in a time-dependent manner (Fig. 1B). These results demonstrate that the inflammatory stimulation with TNFα up-regulates TRIF expression in human colonic epithelial cells. This result may imply that increased TRIF expression is subsequently able to alter intracellular signaling triggered by microbial pattern recognition via TLRs.

TRIF Overexpression Causes Proteolytic Degradation of TLR5—Although TLR4 utilizes MyD88 and TRIF adaptor molecules to mediate MyD88-dependent and MyD88-independent signaling pathways, respectively, TLR5 is known to use only MyD88 to mediate flagellin-induced signaling pathways. In addition, many colonic epithelial cell lines (e.g., Caco-2 and T84) are hypo-responsive to TLR2 (Pam3Cys) and TLR4 (LPS) stimulation (19, 20, 30, 31). NCM460 cells in which the expression level of TRIF adaptor molecule is up-regulated by TNFα (Fig. 1) do
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FIGURE 1. TNFα stimulation up-regulates TRIF expression in human colonic epithelial cells. A, total RNA was prepared from human colonic epithelial cells (NCM460) stimulated with TNFα (100 ng/ml) for 0, 4, and 8 h. Quantitative real-time PCR was performed to evaluate the mRNA expression level of TRIF, MyD88, TLR4, and TLR5. Error bars indicate S.D. B, NCM460 cells were stimulated with TNFα (100 ng/ml) for the indicated time periods. Lysates were immunoblotted using antibodies recognizing human TRIF or STAT3. C, the validity of human TRIF antibody used in this study was confirmed by immunoblot (IB) assay. Human TRIF transfected into HEK293 cells is evidently recognized by TRIF antibody. HEK293 cells were transfected with Myc-TRIF or empty vector. Lysates were immunoblotted using antibodies recognizing human TRIF, Myc, or STAT3. All data are representative of at least three independent experiments.

FIGURE 2. TRIF induces TLR5 protein degradation. A and B, HEK293 cells were co-transfected with TLR5-HA or Myc-TRIF. Lysates were immunoblotted using antibodies to HA, Myc, or ERK1/2 (A). TLR5 or TRIF gene expression was detected by PCR using primers: TLR5, 5′-TATAAAGTCGACGCCCACCTCGTACGAC-3′ and 5′-CTGCATCAGTTCGACAGTAC-3′; TRIF, 5′-TATTTTGCCGCGGGAGTG-3′ and 5′-GGAGGCTTGCTGCTGAGG-3′ (B). C, NCM460-TLR5-HA cells were transduced with retroviral particles harboring the doxycycline-inducible pLINX-FLAG-TRIF or the empty pLINX vector. The presence of doxycycline suppresses FLAG-TRIF expression. All data are representative of at least three independent experiments.

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not respond to LPS or Pam3Cys, but rather strongly respond to TLR5 ligand (flagellin) (15, 20). Thus, to investigate a cellular effect of elevated TRIF on TLR5-dependent responses, we transiently transfected TLR5 and TRIF expression constructs into HEK293 cells. We found that TRIF overexpression dramatically reduced the expression level of TLR5 protein (Fig. 2A), whereas TRIF overexpression did not alter the mRNA expression of transfected TLR5 (Fig. 2B). In agreement with the study demonstrating that TRIF induces caspase activation leading to apoptosis in HEK293 cells (14), we could observe that TRIF transfection induced apoptosis in HEK293 cells. However, the diminished TLR5 expression is not a nonspecific outcome of a cell death process, because ERK1/2 expression as a loading control was intact in the cells transfected with TRIF or TLR5 or the empty vector (Fig. 2A), and the mRNA expression of transfected TLR5 and TRIF is intact in these cells (Fig. 2B).

As a next line of the evidence in human colonic epithelial cells, we induced doxycycline-regulated TRIF expression in NCM460 cells (Fig. 2C). Doxycycline-inducible TRIF expression resulted in substantially reduced TLR5 expression in NCM460 cells, whereas suppressing TRIF expression maintained the evident TLR5 expression (Fig. 2C). Together with the similar findings in HEK293 cells, this result demonstrates that elevated TRIF expression results in diminished cellular level of TLR5 protein in NCM460 cells as well as in HEK29 cells.

TRIF Inhibits TLR5-induced Responses in Human Colonic Epithelial Cells—Having found that TRIF expression results in reduced cellular levels of TLR5 protein, we next hypothesized that overexpression of TRIF would suppress TLR5-induced responsiveness to subsequent flagellin stimulation. To test this, we transiently transfected the TRIF expression construct into NCM460 cells together with NFκB reporter construct followed by flagellin stimulation. Interestingly, we found that TRIF expression completely blocked NFκB reporter activity in NCM460 cells stimulated with flagellin (Fig. 3A), whereas the control NCM460 cells transfected with the empty plasmid vector exhibited markedly induced NFκB reporter activity in response to flagellin. In contrast to an inhibitory role of TRIF in TLR5-induced responses, MyD88 overexpression did not show a negative effect in TLR5-induced NFκB reporter activity (Fig. 3B). These data indicated that TRIF and MyD88 may play a different role at least in TLR5-dependent responses, although they are immediate adaptor molecules associating with a member of TLR family. Despite that TRIF mediates MyD88-independent signaling of TLR3 or TLR4, our findings suggest that TRIF can also induce TLR5 protein degradation, contributing to attenuating TLR5-induced responses.

4 Y. J. Choi, E. Im, C. Pothoulakis, and S. H. Rhee, unpublished data.
TRIF Induces Degradation of Other TLRs, Except TLR1, TLR2, and TLR4—As shown above, overexpressing TRIF induces TLR5 protein degradation, which limits flagellin-induced TLR5 responses. This prompted us to examine whether the TRIF-induced TLR protein degradation is confined to TLR5. We further tested whether TRIF can also induce the degradation of other TRIF-associated TLRs, such as TLR3 and TLR4. In contrast to TLR5, TRIF did not alter TLR4 protein expression, whereas TLR3 expression was dramatically reduced by TRIF overexpression (Fig. 4, A and B). Moreover, TRIF overexpression did not alter the expression level of TLR1 and TLR2 (Fig. 4, D and E), whereas it dramatically diminished TLR6, TLR7, TLR8, TLR9, and TLR10 expression (Fig. 4, F–J). In contrast to TRIF, MyD88 overexpression did not change the expression level of these TLRs (Fig. 4, C–J).

Intriguingly, similar to TRIF, MyD88 overexpression reduced TLR5 protein expression in HEK293 cells, whereas it did not alter TLR5 mRNA expression (Fig. 5, A and B). However, although transfecting small amounts of TRIF expression construct (0.25 μg) almost completely abolished TLR5 protein expression (Fig. 5C), transfecting the same amount (0.25 μg) of MyD88 expression construct did not reduce the TLR5 expression level compared with the TLR5 expression in TLR5-alone-transfected cells. Moreover, higher amounts of MyD88 plasmid (4 μg) were required to induce the similar effect of the small amount (0.25 μg) of TRIF (Fig. 5D). Thus, we speculate that overexpressing MyD88 likely leads to indirect activation of an inherent TRIF-associated signaling in HEK293 cells, which in turn causes TLR5 protein degradation. This explanation appears to be reasonable, because MyD88 overexpression in NCM460 cells did not suppress flagellin-induced NFκB reporter activity (Fig. 3B), which was completely blocked by TRIF overexpression (Fig. 3A). These results indicate that MyD88 overexpression did not alter the cellular level of TLR5 in NCM460 cells, and TLR5 protein degradation by MyD88 transfection in HEK293 cells (Fig. 5, A and C) was not a direct outcome of MyD88 overexpression. Based on these observations, TLR5 protein seems to be more susceptible to TRIF-induced degradation than other TLRs. Collectively, TRIF rather than MyD88 has a capacity to down-regulate the cellular abundance of various TLRs including TLR5 but not TLR1, TLR2, and TLR4.
Extracellular Domain of TLR5 and C-terminal Domain of TRIF Are Essential for TRIF-induced TLR5 Degradation—TRIF adaptor protein consists of an N-terminal proline-rich region, TIR Domain, and C-terminal region. The TIR domain of TRIF is responsible for binding to the TIR domain of TLR3 or TLR4 adaptor TRAM. The N-terminal region of TRIF mediates IRF-3 activation (3) responsible for interferon-β expression, and the C-terminal region of TRIF is involved in caspase-induced apoptosis (13, 14, 32), whereas both the N- and C-terminal domains are able to induce NFκB activation through distinct mechanisms (13). To determine which region of TRIF molecule is involved in TLR5 degradation, we transfected HEK293 cells with the C-terminal-deleted TRIF (TRIF(C)) construct together with the TLR5 construct. Although full-length TRIF induces protein degradation of TLR5, TRIF(C) failed to induce TLR5 degradation (Fig. 6, A and B). In agreement with this result, both N- and C-terminal deleted TRIF (TRIF(NΔC)) lost its ability to induce TLR5 degradation (Fig. 6, A and C). In contrast, the N-terminal deleted TRIF (TRIF(NΔN)) was still able to induce TLR5 protein degradation (Fig. 6, A and D). These data indicate that the C-terminal region of TRIF is required for TRIF-induced TLR5 protein degradation.

Because the extracellular leucine-rich repeat domain and cytosolic TIR domain of TLR participate in ligand recognition and intracellular signaling, respectively, we next investigated the importance of these two domains in TRIF-induced TLR5 protein degradation. To assess this, we generated TIR domain-deleted TLR5 (TLR5(ΔTIR)) and extracellular domain-deleted TLR5 (TLR5(ΔECD)) constructs (Fig. 7A). We found that over-expressing TRIF did not reduce the expression level of TLR5(ΔECD) (Fig. 7B), whereas TLR5(ΔTIR), like full-length TLR5, was abolished by TRIF expression (Fig. 7C). These data indicate that the extracellular domain of TLR5, rather than its cytoplasmic TIR domain, plays a critical role in its degradation induced by TRIF. Together, our data demonstrate that the C-terminal domain of TRIF and the extracellular domain of TLR5 are essential for TRIF-induced TLR5 protein degradation.

TRIF-activated Caspases Causes TLR5 Protein Degradation—To gain specific insight into the mechanism of TRIF-induced TLR5 protein degradation, we tested various protease inhibitors known to block post-translational modification of PRRs. The lysosomal cysteine protease family (e.g. cathepsin B) is important in degradation of endocytosed and intracellular proteins (33). TLR9 proteolytic cleavage by cathepsin B is required for the activation of TLR9 signaling, suggesting that lysosomal proteolysis contributes to specific TLR cleavage (6, 7). Based on these considerations, we tested whether cathepsin B is responsible for TRIF-induced TLR5 protein degradation. However, we found that TRIF still diminishes TLR5 expression even in the presence of cathepsin B inhibitor (Fig. 8A), excluding the possibility that cathepsin B is involved in TRIF-induced TLR5 protein degradation. Given the fact that the activation of certain TLRs induces reactive oxygen species (ROS) (34) and ROS is able to elicit a proteolysis (35), we tested whether blocking ROS with an anti-oxidant such as N-acetylcysteine suppresses TRIF-induced TLR5 degradation. We found that N-acetylcysteine at various concentrations failed to block TRIF-induced TLR5 protein degradation (Fig. 8B). Furthermore, chemicals that block lysosomal acidification including endosomal acidification inhibitor NH4Cl was not able to alter TRIF-induced TLR5 degradation (Fig. 8C). This suggests that endosomal maturation is not related to TLR5 degradation. In addition, a ubiquitination-mediated proteolysis was known to promote the degradation of certain TLRs (36). However, we found that the cell-permeable
and selective proteasome inhibitor MG132 did not affect TLR5 protein degradation induced by TRIF, whereas at the same concentration, MG132 blocked LPS-induced IкΒα degradation (Fig. 8D).

More recently Rebsamen et al. (8) suggested that TRIF-induced caspase activity causes the degradation of both TRIF itself and Cardif (adapter protein of antiviral RNA helicase family member RIG-I or MDA5), leading to inhibition of IRF-3 and NFкB activation. Moreover, our data showed that the C-terminal domain of TRIF, which participates in caspase activation to elicit apoptosis, is required for TRIF-induced TLR5 protein degradation, whereas the N-terminal domain of TRIF is dispensable for TRIF-induced TLR5 protein degradation. In this context we hypothesized that caspase activation by TRIF overexpression would be involved in TLR5 protein degradation. Therefore, we tested whether the caspase inhibitor can suppress TRIF-induced TLR5 degradation. We found that blocking the caspase activity with pan-caspase inhibitor (benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) completely inhibited TRIF-induced TLR5 protein degradation (Fig. 8E, right two lanes).

Notably, TRIF overexpression elicited the cleavage of caspase-3 and -8 and PARP in HEK293 cells (Fig. 8E, second lane from the left), indicating that TRIF expression is sufficient to induce caspase-8 activation, which subsequently cleaves caspase-3 and PARP. Moreover, our data showed that co-expression of TLR5 and TRIF enhanced caspase-3 cleavage more than TRIF expression alone (Fig. 8E, fourth lane from the left). This result suggests that the expression of both TLR5 and TRIF is able to synergistically augment caspase-8 activation, leading to enhanced caspase-3 cleavage. In agreement with these results, the caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone blocked both caspase-3 and PARP cleavages in TLR5 and TRIF co-transfected cells. These findings indicate that TRIF overexpression induces caspase activity, and co-expression of TRIF and TLR5 synergistically enhances caspase activation, which subsequently causes TLR5 protein degradation.

**TLR5 Is Relocalized to Endolysosomes by Flagellin Stimulation**—Despite that TLR5 is believed to exist in the plasma membrane where its signaling is mediated by MyD88(37), in Arabidopsis the flagellin receptor FLS2 was shown to be endocytosed by ligand stimulation (38). Thus, we investigated whether flagellin stimulation in intestinal epithelial cells induces TLR5 relocalization to intracellular vesicles. To this end we used HA-tagged TLR5 expressing NCM460 cell (NCM460-TLR5-HA). Immunofluorescence staining against the HA epitope tag showed that TLR5 primarily resides in the plasma membrane in vehicle-treated control cells (supplemental Fig. 1). In contrast, in the flagellin-stimulated cells, TLR5 was observed in intracellular vesicles that were co-stained with the early endosomal marker Rab5 (Fig. 9A). This result suggested that flagellin stimulation induces intracellular trafficking of TLR5 into intracellular endosomes. Given the fact that early endosome is sorted to late endosome, which is then processed to be endolysosomes by subsequent fusions with
lysosomes (39), we hypothesized that TLR5 could also be observed at the subcellular vesicles stained with a lysosomal marker, cathepsin D. Immunofluorescence data showed that TLR5 was also co-localized with cathepsin D in flagellin-treated NCM460 cells (Fig. 9B). Collectively, our findings indicate that TLR5 residing in the plasma membrane follows the intracellular trafficking pathway from the plasma membrane to intracellular vesicles in response to flagellin stimulation.

**DISCUSSION**

Proteolytic modification of PRRs and their signaling adaptor molecules emerged as an important cellular event to regulate innate immune and inflammatory responses triggered by recognizing microbe-associated molecular patterns (6–10). These proteolytic modifications result in either enhanced or attenuated PRRs-induced responses. Cleaved fragments of TLR9 by caspase activity interact with CpG DNA more strongly than full-length TLR9, suggesting that the proteolytic cleavage of TLR enhances its responses (6, 7). On the other hand, proteolytic cleavage of PRR-associated adaptor molecules is targeted to suppress innate immune responses to evade antiviral defense against viral pathogens (8–10). In addition, to circumvent host immunity, proteolytic cleavage of PRRs could be one of inevitable measures that the host can exploit to limit uncontrolled activation of inflammatory responses initiated by host-microbial recognition. For instance, Triad3A, an E3 ubiquitin-protein ligase, enhances ubiquitination of TLR4 and TLR9 and subsequently induces proteolytic degradation of these receptors, leading to decreased proinflammatory responses (36).

Caspase enzymatic activity is one of the essential tools to execute proteolytic cleavage of PRRs and their adaptor molecules and thereby modulates inflammatory responses. Indeed, caspase-8 deficiency in mouse skin and liver was known to cause dermatitis and hepatocytome-associated hepatitis, respectively (40, 41). Interestingly, in line with causing skin inflammation, caspase-8 deficiency in mouse skin revealed enhanced IRF-3 phosphorylation (40). This finding may indicate that caspase-8 deficiency might allow prolonged activation of TRIF-dependent signaling such as enhanced IRF-3 activation and thereby result in enhanced TRIF-associated proinflammatory responses. These studies collectively imply that in addition to inducing apoptosis, caspase can also participate in restraining inflammatory responses. Similarly, our data showed that certain members of TLRs succumb to proteolytic degradation mediated by TRIF-induced caspase activity. These findings may suggest that TRIF is capable of keeping the cells from prolonged activation of TLRs by lowering its cellular abundance. Thus, TRIF-induced TLR degradation probably takes place for the purpose of preventing cells from sustained TLR activation, which may cause uncontrolled inflammatory responses. Given that continuous activation of TLRs by luminal microbes could be a critical event aggravating intestinal inflammatory diseases (26, 27), limiting TLR signaling is one of the essential modulatory events to ameliorate inflammatory responses (42).

Intestinal epithelial cells are in continuous contact with enteric microbes at the front line of host-microbial interaction of the gut. Given the facts that TLR2 and TLR4 responses are negligible in many intestinal epithelial cell lines (19, 20) and virtually most of epithelial cells are strongly responsive to flagellin via TLR5 (15–17), TLR5 appears to play an important role in mediating host-microbial communication between intestinal epithelium and enteric microbes. TLR5 contributes to preserving the crypt stem cell proliferation in the intestine and thereby protecting the gastrointestinal tract from radiation-induced damages (43). In contrast, aberrant activation of TLR5 by flagellin is associated with the development and progress of intestinal inflammation (25, 26). Regardless a protective or proinflammatory role of TLR5 engagement in the gastrointestinal tract, TLR5-dependent signaling in the intestinal epithelium should be properly controlled by a regulatory factor to maintain the intestinal homeostasis. In this context, several inhibitory molecules of TLR signaling (e.g. Tollip, IRAK-M, SIGIRR, MyD88s) have been determined to play an important role in maintaining the intestinal homeostasis by regulating TLR-induced signaling (44). In addition to those inhibitory molecules, our data show that TRIF can also suppress TLR5-mediated proinflammatory responses in intestinal epithelial cells, although it has never been suggested that TRIF is an adaptor molecule directly involved in TLR5-dependent responses.

Having found that extracellular domain-deleted TLR5 was protected from the TRIF-induced degradation, the extracellular domain of TLR5 should be critical for its proteolytic degradation. Intriguingly, although TRIF overexpression induces proteolytic degradation of TLR3, TLR5, TLR6, TLR7, TLR8, TLR9, and TLR10, it does not alter the cellular abundance of TLR1, TLR2, and TLR4. Although the mechanism by which TRIF differentially causes TLR protein degradation is not clear, we speculate that it may be associated with the complexity of TLR extracellular domains with which several accessory molecules of TLRs interact. Some members of TLRs directly interact with co-receptors or accessory molecules for a microbial recognition (45). TLR4 makes a receptor complex with its co-receptor CD14 and MD2 to recognize LPS. TLR2 forms a heterodimer with TLR1 to recognize triacylated lipopeptides (e.g. Pam3CysSKs), whereas TLR2/TLR6 heterodimer is stimulated by diacylated lipopeptides (e.g. MALP-2). Moreover, transmembrane receptor CD36 associates with TLR2/TLR6 complex upon lipid recognition (46). RP105 together with MD1 also interacts with TLR2 and TLR4 (45). Due to these accessory molecules collaborating with TLR at the cell surface, TLR1, TLR2, and TLR4 presumably form a large heteromorphic complex around cells compared with other TLRs. Thus, by analogy, it is reasonable to hypothesize that the presence of TLR accessory proteins at the cell surface might deter proteases from accessing the extracellular domain of TLR1, TLR2, and TLR4. Due to such structural hindrance, TLR1, TLR2, and TLR4 can probably circumvent the proteolytic degradation.

In addition, we also hypothesize that the requirement of the extracellular domain of TLR5 for TRIF-induced TLR5 degradation would be related to TLR5 internalization upon flagellin stimulation. Robatzek et al. (38) showed that the flagellin receptor FLS2 in Arabidopsis residing in the plasma membrane is transferred to intracellular vesicles upon its ligand stimulation, followed by FLS2 protein degradation. These observations indicated that both ligand recognition and receptor endocytosis should be essential for the protein degradation in cytosol. Thus,
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because extracellular domain deleted TLR5 is not able to recognize its ligand followed by impaired TLR5 endocytosis, TLR5 without its extracellular region may not be subjected to the caspase-associated protein degradation that occurs in cytosol. In summary, our data demonstrate that TRIF induces TLR5 protein degradation in a caspase-dependent manner and thereby suppresses flagellin/TLR5-induced responses in the intestinal epithelial cells.

Acknowledgments—We thank S. Akira (Osaka University) and R. Lin (McGill University) for pLINX retroviral vector.

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