Reciprocal Cross-talk between Nod2 and TAK1 Signaling Pathways*

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Mutations in the leucine-rich repeat (LRR) domain of Nod2 have been implicated in the pathogenesis of Crohn’s disease, yet the function of Nod2 and regulation of the Nod2 pathway remain unclear. In this study, we determined that mitogen-activated protein kinase kinase transforming growth factor (TGF)-β-activated kinase 1 (TAK1) interacts with Nod2 and is required for Nod2-mediated NF-κB activation. The dominant negative form of TAK1 abolished muramyl dipeptide-induced NF-κB activation in Nod2-expressing cells. Nod2, acting in a reciprocal manner, inhibited TAK1-induced NF-κB activation in RICK-deficient embryonic fibroblasts. Nod2 appears to interact with TAK1 through its LRR region to exert its inhibitory effect on TAK1-induced NF-κB activation. Further, wild-type LRR more effectively suppressed NF-κB activation induced by TAK1 than LRR with a 3020insC mutation. Considered together, these findings demonstrate a critical role for Nod2 in Nod2-mediated innate immune responses and reveal a novel function for Nod2 in the regulation of the TAK1 signaling pathway.

Crohn’s disease is a chronic inflammatory bowel disease with an estimated prevalence of 1 in 1000 in Western countries (1, 2). Despite its unknown etiology, research indicates a strong association between Crohn’s disease and mutation of the Nod2 (CARD15) gene (3–9). A member of the CED/APAF1 superfamily of apoptosis regulatory proteins (11), Nod2 contains two N-terminal CARD domains, a nucleotide-binding domain, and multiple C-terminal leucine-rich repeat (LRR)1 regions (10). Nod2 interacts with RICK via CARD-CARD interaction. A recent study shows that Nod2 activates NF-κB and that RICK is essential for this process (11, 12). Embryonic cells from RICK knockout mice (RICK−/−) are deficient in Nod2-mediated NF-κB activation, which suggests that RICK involvement in signaling occurs downstream of Nod2 (11, 12). Muramyl dipeptide (MDP) enhances Nod2-mediated NF-κB activation, which suggests that RICK involvement in signaling occurs downstream of Nod2 (11, 12). Muramyl dipeptide (MDP) enhances Nod2-mediated NF-κB activation, which suggests that RICK involvement in signaling occurs downstream of Nod2 (11, 12). The LRR region of Nod2 inhibited TAK1-induced NF-κB activation. Only the dominant negative form of TAK1 (TAK1DN) suppressed Nod2-induced NF-κB activation. TAK1DN also inhibited MDP-induced NF-κB activation in Nod2-expressing cells. In vitro and in vivo coimmunoprecipitation and confocal microscopic analysis confirmed a biochemical interaction between Nod2 and TAK1. Interestingly, Nod2 had a negative regulatory effect on TAK1-induced NF-κB activation. The LRR region of Nod2 inhibited TAK1-induced NF-κB activation. This is the first report of Nod2 and TAK1 interaction and evidence of reciprocal cross-talk between the Nod2 and inflammatory cytokine signaling pathways.

EXPERIMENTAL PROCEDURES

Cell Culture—HEK293T and COS cells were grown in Dulbecco’s modified Eagle’s medium, and LS174T cells were grown in minimum Eagle’s medium. Both media were supplemented with 10% fetal bovine serum and penicillin, and cultures were maintained in a humidified atmosphere of 95% air and 5% CO₂. Embryonic fibroblasts derived from RICK−/− and wild-type littermates were kindly provided by Dr. Richard Flavell (11).

Expression Constructs—TAK1, the dominant negative form of TAK1 (K63W), and TAB1 constructs were kindly provided by Dr. Kuni Matsumoto (25, 26). Nod2 and mutant Nod2 (3020insC) constructs were kindly provided by Dr. Gabriel Nunez (3). The expression constructs, FLAG-Nod2 and FLAG-LRR, which, respectively, encode full-length Nod2 and the LRR region of Nod2, were generated by PCR and cloned into LPCX vector, kindly provided by Dr. Derynick. TAK1 deletion constructs encoding aa 1–303, 1–403, and 286–632 were generated by restriction enzyme digestion or PCR and cloned into plasmid vector pGEM-2 (Promega). Glutathione S-transferase (GST), GST-CARD (aa 28–265), the CARD domain of Nod2; and GST-LRR (aa 744–1040), the LRR domain of Nod2 were generated by PCR and cloned into plasmid type Nod2 (13, 14). These results suggest that Nod2 mutation confers susceptibility to Crohn’s disease by dysregulation of NF-κB (5). The NF-κB family of transcription factors exerts pleiotropic effects on the regulated expression of many genes involved in inflammation (15–17). The importance of NF-κB in Crohn’s disease is manifested by dysregulation of NF-κB and enhanced production of proinflammatory cytokines (17).

TGF-β-activated kinase 1 (TAK1) is an essential component of the signaling pathways of many inflammatory cytokines (18–24). TAK1 is required for interleukin (IL)-1, tumor necrosis factor-α, IL-18, RANKL, and TGF-β-mediated NF-κB activation (18–24). Proinflammatory mediators, such as IL-1, activate TAK1. Activated TAK1 phosphorylates IkB-related kinase and MKK6, which leads to activation of the NF-κB and JNK-p38 kinase pathways (18). Proinflammatory cytokines mediate their effect primarily by reprogramming gene expression in inflamed tissues. Among the many genes induced are those of proinflammatory cytokines such as IL-8 (25).

Understanding of the regulation of Nod2-mediated NF-κB activation remains limited. Whether cross-talk exists between the Nod2 pathway and other signaling pathways of inflammatory cytokines is still not known. In this study, we examined the regulatory effect of TAK1, TBK1, Ubc13, Rip, and MEKK1, proteins implicated in NF-κB activation, on Nod2-mediated NF-κB activation. The dominant negative form of TAK1 (TAK1DN) suppressed Nod2-induced NF-κB activation. TAK1DN also inhibited MDP-induced NF-κB activation in Nod2-expressing cells. In vitro and in vivo coimmunoprecipitation and confocal microscopic analysis confirmed a biochemical interaction between Nod2 and TAK1. Interestingly, Nod2 had a negative regulatory effect on TAK1-induced NF-κB activation. This is the first report of Nod2 and TAK1 interaction and evidence of reciprocal cross-talk between the Nod2 and inflammatory cytokine signaling pathways.

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The abbreviations used are: LRR, leucine-rich repeat; TGF, transforming growth factor; TAK1, TGF-β-activated kinase 1; MDP, muramyl dipeptide; IL, interleukin; GST, glutathione S-transferase; aa, amino acids; HA, hemagglutinin; Luc, luciferase; JNK, c-Jun N-terminal kinase; siRNA, small interfering RNA.

This paper is available on line at http://www.jbc.org
vector pGEX (Amersham Biosciences). Construct authenticity was verified by sequencing.

**Immunoprecipitation and Immunoblotting—** HEK293T cells were co-transfected with pHA-TAK1 and pLPCX-FLAG-Nod2 using the transfection reagent FuGENE 6 (Roche Diagnostics). L5174T cells were treated with tumor necrosis factor-α (50 ng/ml) for 20 min to induce Nod2 expression. Cells were lysed on ice for 30 min in lysis buffer containing 20 mM Hepes (pH 7.2), 0.5% Triton X-100, 12.5 mM NaCl, 0.1% sodium deoxycholate, 2 mM dithiothreitol, 2 mM EDTA, 10 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor (Roche Diagnostics). Lysates were immunoprecipitated with the indicated antibodies for 2 h at 4 °C.

Complete proteinase inhibitor (Roche Diagnostics). Lysates were immunoprecipitated with the indicated antibodies for 2 h at 4 °C. The immune complexes were precipitated with protein G-agarose (Roche Diagnostics). All immunoblots were developed by an enhanced chemiluminescence imaging assay developed by Amersham Biosciences. Anti-HA and anti-FLAG antibodies were obtained from Santa Cruz Biotechnology and Stratogene.

**In Vitro GST Pull-down Assay—** GST, GSTCARD, and GST LRR fusion proteins were each expressed in the Escherichia coli BL21 strain and purified. TAK1 proteins were synthesized by in vitro transcription and translation using rabbit reticulocyte lysates (Promega) and [35S]-S-actamine (Invitrogen) according to the manufacturer’s instructions (Promega). Results were normalized for transfection efficiency with GST alone.

**NF-κB Activation Assays—** Cells were cotransfected with RSV-KB-Luc (100 ng), a reporter construct encoding the luciferase reporter gene using rabbit reticulocyte lysates (Promega) and [35S]-methionine (Amersham Biosciences), and incubated in lysis buffer with the indicated GST fusion proteins for 4 h at 4 °C. The beads were washed four times with lysis buffer, resolved in 4–20% gradient SDS-PAGE, and visualized by autoradiography.

**Immunofluorescence Microscopy—** For immunofluorescence studies, COS cells were transfected with the indicated expression constructs. To detect FLAG-Nod2 and HA-TAK1, cells were stained 24 h after transfection with anti-FLAG rabbit polyclonal antibody and anti-HA monoclonal antibody, respectively, and incubated with Alexa 488 goat anti-rabbit and Alexa 594 goat anti-mouse antibody (Molecular Probes). Images were captured by an Olympus FV300 confocal laser scanning microscope.

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MDP-induced NF-βB activation in HEK293T-expressing cells. HEK293T cells were transfected with pCDNA-Nod2 (5 ng) and reporter constructs plus the indicated amounts of TAK1DN or control vector. Cells were stimulated with MDP (100 ng/ml) 8 h after transfection. Luciferase activity was determined 16 h after stimulation and normalized on the basis of β-galactosidase activity. All experiments were performed in triplicate and repeated three times with equivalent results. *, p < 0.05.

Fig. 2. Dominant negative form of TAK1 (TAK1DN) inhibits MDP-induced NF-κB activation in NF-κB-expressing cells. HEK293T cells were transfected with pCDNA-Nod2 (5 ng) and reporter constructs plus the indicated amounts of TAK1DN or control vector. Luciferase activity was determined 16 h after stimulation and normalized on the basis of β-galactosidase activity. All experiments were performed in triplicate and repeated three times with equivalent results. *, p < 0.05.

To determine which region of TAK1 interacts with Nod2, GST-LRR fusion protein was incubated with different regions of TAK1 translated in vitro. Full-length TAK1 and two deletion mutants (aa 1–303 and aa 1–403) bound to GST-LRR, but not GST-CARD or GST protein. TAK1 bound to GST-LRR but not GST-CARD or GST protein (Fig. 3C). These results suggest that Nod2 binds to TAK1 via its LRR domain.

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Nod2 expression does not activate TAK1—Because Nod2 has a negative regulatory effect on TAK1-mediated NF-κB activation, we tested whether Nod2 induces activation of NF-κB by activating TAK1. TAB1 and IL-1 are known to activate TAK1 and to induce autophosphorylation of TAK1 (27). We therefore performed an in vitro phosphorylation assay to examine TAK1 activation, as described previously (27). HEK293T cells were transfected with TAK1 plus Nod2 or control vector. The positive control was HEK293T cells transfected with TAK1 and TAB1. TAK1 protein was immunoprecipitated with anti-TAK1 antibody and subjected to phosphorylation. Nod2 expression did not activate TAK1 (Fig. 5). These results indicate that Nod2 does not activate NF-κB via the TAK1 pathway.

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Coexpression of Nod2 and TAK1 does not have a synergistic effect on NF-κB activation—The requirement of TAK1 activation for Nod2-induced NF-κB activation led us to hypothesize that coexpression of Nod2 and TAK1 may exert a synergistic effect on NF-κB activation. To test this hypothesis, we transfected HEK293T cells with TAK1, TAB1, and Nod2. HEK293T cells transfected with TAK1, TAB1, and control vector were used as the control. NF-κB activation in HEK293T cells transfected with TAK1, TAB1, and Nod2 was comparable with that in the control cells. (Fig. 6). Further, the level of NF-κB activation in cells transfected with TAK1, TAB1, and Nod2 tended to be lower than the sum of NF-κB activation in cells transfected with only TAK1 and TAB1 and in cells transfected with only Nod2 (Fig. 6). Thus, Nod2 and TAK1 do not appear to exert a synergistic or additive effect on NF-κB activation. Rather, a negative regulatory interaction between TAK1 and Nod2 is implied.

Nod2 inhibits TAK1-induced NF-κB activation—Recent studies have shown that RICK is a downstream molecule in the Nod2-mediated NF-κB activation pathway (11, 12). Because Nod2 expression does not induce NF-κB activation in RICK−/− fibroblasts (11, 12), we used these cells to examine the effect of Nod2 on TAK1-induced NF-κB activation. We first showed that the level of TAK1-induced NF-κB activation was similar in wild-type and RICK−/− fibroblasts (Fig. 7, A and B), which suggests that TAK1-induced NF-κB activation is not RICK-dependent. To study the effect of Nod2 expression on TAK1-induced NF-κB activation, we transfected RICK−/− fibroblasts with TAK1, TAB1, and Nod2. RICK−/− fibroblasts transfected with TAK1, TAB1, and control vector were the control. The expression plasmids RSV-KB-Luc and RSV-β-galactosidase were included in each transfection. As shown in Fig. 7C, we confirmed that Nod2 expression did not activate NF-κB in RICK−/− fibroblasts. Further, Nod2 effectively suppressed TAK1-induced NF-κB activation in RICK−/− fibroblasts; the control vector exerted no inhibitory effect. As seen in HEK293T cells, NF-κB activation in RICK wild-type fibroblasts transfected with TAK1, TAB1, and Nod2 was comparable with that in RICK wild-type fibroblasts transfected with TAK1, TAB1, and control vector (data not shown). These results show that in the absence of RICK, Nod2 has a negative regulatory effect on TAK1-mediated activation of NF-κB. In the presence of RICK, Nod2 may also suppress TAK1-mediated NF-κB activation. However, the decreased NF-κB activity is less obvious because NF-κB activation induced by Nod2 compensates for the suppression of NF-κB activity induced by TAK1. To confirm the negative role of Nod2 in TAK1-dependent NF-κB activation, we reduced endogenous Nod2 expression through RNA-mediated interference. Fig. 7D shows a marked decrease in exogenous Nod2 induced by expression of Nod2-specific siRNA; evidence that Nod2-specific siRNA can efficiently reduce Nod2 expression. NF-κB activity was reduced in LS174T cells transfected with Nod2-specific siRNA as compared with cells transfected with control siRNA, which suggests that Nod2 siRNA efficiently suppresses NF-κB activation induced by endogenous Nod2 (Fig. 7E). Interestingly, TAK1 induced higher NF-κB activation in LS174T cells pretreated with Nod2-specific siRNA than in LS174T cells pretreated with control siRNA (Fig. 7F). These results show that Nod2 siRNA expression potentiates TAK1-induced NF-κB activation and further support the observation that Nod2 negatively regulates TAK1-induced NF-κB activation.

LRR domain of Nod2 suppresses TAK1-induced NF-κB activation—Previous studies have shown that Nod2 activates NF-κB activation through the CARD domain; the LRR domain of Nod2 does not activate NF-κB (10). Because the LRR region of Nod2 interacts with TAK1 (Fig. 3), we examined its effect on TAK1-induced activation of NF-κB. HEK293T cells were transfected with LRR, TAK1, and TAB1. As shown in Fig. 8A, LRR effectively suppresses TAK1-induced NF-κB activation; the inhibitory effect of the control vector is insignificant. Nod2 with deletion of LRR exerts no inhibitory effect (data not shown).

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These results suggest that Nod2 suppresses TAK1-induced NF-\(\kappa\)B activation through the LRR domain.

**Mutant LRR Less Effectively Suppresses TAK1-induced NF-\(\kappa\)B Activation**—Because the 3020\(\text{insC}\) mutation in the LRR region of Nod2 is the main mutation in patients with Crohn’s disease (3, 4), we compared the effects of wild-type LRR and mutant LRR (3020\(\text{insC}\)) on the suppression of TAK1-induced NF-\(\kappa\)B activation. HEK293T cells were transfected with TAK1...
and TAB1 plus wild-type LRR or mutant LRR. Mutant LRR was less effective than wild type at suppressing TAK1-induced NF-κB activation even at comparable levels of protein expression (Fig. 8B). These results suggest that mutation in the LRR region of Nod2 may affect the ability of Nod2 to regulate the TAK1 signaling pathway.

FIG. 4. Nod2 colocalizes with TAK1. COS cells were seeded in six-well plates. A cover slide was placed in each well. Cells attached to the slides after overnight culture were cotransfected with FLAG-Nod2 (50 ng) and HA-TAK (150 ng). The cells were subjected to immunofluorescence staining 24 h after transfection: anti-FLAG antibody (A) and anti-HA antibody (B). C, an overlay of Nod2 and TAK1 staining shows colocalization of the proteins in the cytoplasm. Images were captured by confocal microscopy, as described under “Experimental Procedures.” All experiments were performed in duplicate and repeated two times with similar results.

FIG. 5. Nod2 does not induce TAK1 activation. HEK293T cells were transfected with equal amounts of the indicated expression constructs: control vector and TAK1 (leftmost lane), Nod2 and HA-TAK1 (3 μg) (middle lane), TAB1 and TAK1 (rightmost lane). TAK1 protein was immunoprecipitated with anti-TAK1 antibody and subjected to phosphorylation as described under “Experimental Procedures.” All experiments were repeated two times with equivalent results.

FIG. 6. Coexpression of Nod2 and TAK1 does not exert a synergistic effect on NF-κB activation. HEK293T cells were transfected with Nod2 (5 ng) alone, TAK1 (25 ng), and TAB1 (2 ng) together, TAK1 (25 ng) and TAB1 (2 ng) plus Nod2 (5 ng), or TAK1 (25 ng) and TAB1 (2 ng) plus control vector (5 ng). Expression plasmids RSV-β-galactosidase were included in each transfection. Luciferase activity was determined 24 h after transfection and normalized on the basis of β-galactosidase activity. All experiments were performed in triplicate and were repeated three times with equivalent results.

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Discussion

Research has demonstrated that mutations in the Nod2 gene are associated with Crohn’s disease. However, regulation of Nod2 function remains unclear. In this study, we showed that activation of TAK1 is required for Nod2-associated NF-κB activation. TAK1 is known to be activated by multiple proinflammatory cytokines (18–24). Proinflammatory cytokines IL-1, tumor necrosis factor-α, and IL-18 have been shown to activate NF-κB via activation of TAK1. These results suggest that cross-talk exists between inflammatory cytokines and the Nod2 pathway through TAK1.

TAK1 may play a role in host defense against infection. Null mutations in the Drosophila dTAK1 gene reveal a conserved function for TAK1 in the control of rel/NF-κB-dependent innate immune responses (31). dTAK1 mutant flies do not produce antibacterial peptides and are highly susceptible to Gram-negative bacterial infection (31). The bacterial product lipopolysaccharide activates multiple protein kinases via TAK1 (32). The bacterium, nontypeable Haemophilus influenzae, uses the TAK1-NIK-IκB-related kinase-β-γ-IκBα pathway to mediate NF-κB-dependent transcription of MUC2 mucin, which is a primary innate defensive response for mammalian airways and intestines (33). In the current study, we showed that the dominant negative form of TAK1 abolished MDP-induced NF-κB activation in Nod2-expressing cells, which suggests that TAK1 plays an important role in regulating the host response to MDP. Further, these results elucidate a novel mechanism by which TAK1 regulates innate immune responses.

The underlying mechanism involving Nod2 mutation and the pathogenesis of Crohn’s disease is still unclear. Mutant Nod2 is found to elicit a weaker response in NF-κB activation (3). Further, MDP less effectively induces NF-κB activation in cells transfected with mutant Nod2 than in cells transfected with wild-type Nod2 (13, 14). Why a lower level of NF-κB activation leads to chronic inflammation in Crohn’s disease remains unclear. In this study, we have shown for the first time that Nod2 has a negative regulatory effect on TAK1-induced NF-κB activation. In RICK–/– cells, Nod2 markedly suppresses TAK1-mediated NF-κB activation, suggesting that Nod2 may act as a negative regulator of TAK1-mediated NF-κB activation.

Previous studies have shown that Nod2 expression, which mainly occurs in monocytes (10), can be readily detected without stimulation (10). In comparison, the expression of RICK in monocytes occurs only after treatment with lipopolysaccharide (11). These results suggest that Nod2 and RICK expression occur at different temporal points. Therefore, Nod2 may have a dual function: 1) in the absence of RICK, Nod2 may suppress TAK1-induced activation of NF-κB, and 2) in the presence of RICK, Nod2 may serve as an intracellular receptor for MDP to activate NF-κB. Inflammatory mediators such as lipopolysaccharide may regulate Nod2 function by modulating the expression of RICK.

Emerging research has shown that TAK1-mediated NF-κB activation plays an important role in IL-1 and tumor necrosis factor-α-induced secretion of proinflammatory cytokines (34). Our finding that Nod2 suppresses TAK1-mediated NF-κB activation suggests that Nod2 may exert a negative regulatory effect on TAK1-mediated inflammation. The discovery that the LRR region of Nod2 suppresses TAK1-induced NF-κB activation suggests that Nod2 may inhibit TAK1-mediated NF-κB activation.
via its LRR domain. Further, the LRR mutant was a less effective inhibitor of TAK1-mediated NF-κB activation. Considered together, these results raise the possibility that mutations in the LRR domain may reduce the capability of Nod2 to suppress TAK1-mediated NF-κB activation, which, in turn, may lead to a dysregulated TAK1-associated inflammation.

In summary, we have shown that TAK1 regulates Nod2-mediated NF-κB activation. TAK1 modulates MDP-induced NF-κB activation in Nod2-expressing cells. These results reveal a novel mechanism by which TAK1 regulates an innate immune response. These results also indicate that cross-talk exists between the Nod2 and proinflammatory cytokine pathways. Proinflammatory cytokines may regulate Nod2-mediated NF-κB by activating TAK1. Also, this is the first evidence that Nod2 exerts an inhibitory effect on TAK1-induced NF-κB activation. The LRR domain of Nod2 appears to be involved in Nod2 suppression. The ability of Nod2 to interact physically and functionally with TAK1 represents a novel interaction.
between a Nod protein and a mitogen-activated protein kinase kinase. Further understanding of Nod2 function and regulation may shed light on the pathogenesis of Crohn’s disease.

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