Participation of Rip2 in Lipopolysaccharide Signaling Is Independent of Its Kinase Activity*

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Rip2 (Rick, Cardiak, CCK2, and CARD3) is a serine/threonine kinase containing a caspase recruitment domain (CARD) at the C terminus. Previous reports have shown that Rip2 is involved in multiple receptor signaling pathways that are important for innate and adaptive immune responses. However, it is not known whether Rip2 kinase activity is required for its function. Here we confirm that Rip2 participates in lipopolysaccharide (LPS)/Toll-like receptor (TLR4) signaling and demonstrate that its kinase activity is not required. Upon LPS stimulation, Rip2 was transiently recruited to the TLR4 receptor complex and associated with key TLR signaling mediators IRAK1 and TRAF6. Furthermore, Rip2 kinase activity was induced by LPS treatment. These data indicate that Rip2 is directly involved in the LPS/TLR4 signaling. Whereas macrophages from Rip2-deficient mice showed impaired NF-κB and p38 mitogen-activated protein kinase activation and reduced cytokine production in response to LPS stimulation, LPS signaling was intact in macrophages from mice that express Rip2 kinase-dead mutant. These results demonstrate that Rip2-mediated LPS signaling is independent of its kinase activity. Our findings strongly suggest that Rip2 functions as an adaptor molecule in transducing signals from immune receptors.

The Toll-like receptor (TLR) family of molecules mediates innate immunity by recognizing specific pathogen-associated molecular patterns (1, 2). Ten mammalian TLRs have been discovered to date. TLR4 is the receptor for lipopolysaccharide (LPS), a major cell wall component of Gram-negative bacteria. Activation of TLR4 by LPS leads to the activation of NF-κB and MAP kinase signaling pathways and induction of inflammatory cytokines (2–4). The TLR signaling pathway shares components common to IL-1 receptor signaling. Upon ligand binding, IL-1 receptor-associated kinase (IRAK1) is recruited to the TLR complex through the adaptor protein MyD88/TRAP. IRAK1 then dissociates from the receptor complex and associates with another adaptor protein TRAF6 to further activate downstream IKK kinase complex and MAP kinases. The IKK complex induces phosphorylation and degradation of IκBα, an inhibitor of transcription factor NF-κB, thus allowing NF-κB to translocate into the nucleus and induce target gene expression (5, 6).

Rip2 (also called Rick, Cardiak, CCK2, and CARD3) is a serine/threonine kinase containing an N-terminal kinase domain, a caspase recruitment domain (CARD) at the C terminus, and an intermediate domain between the kinase domain and the CARD domain (7–11). The CARD domain mediates Rip2 association with other CARD-containing proteins (9, 12, 13). The intermediate domain has been reported to be required for Rip2 interaction with the IκKα subunit (14). Overexpression of Rip2 activates NF-κB and Jun N-terminal kinase and induces apoptosis in mammalian cell lines (7, 9). Recent studies using Rip2 knock-out mice have shown that Rip2 mediates TLR signaling (15, 16). Rip2-deficient mice are resistant to LPS-induced septic shock. Cytokine production and activation of NF-κB and MAP kinases are impaired in Rip2-deficient cells in response to LPS stimulation. In addition to defective TLR signaling, Rip2 deficiency results in impaired signaling through the T-cell receptor (TCR) and IL-18/IL-12 receptor (15–17).

However, the role of Rip2 kinase domain remains unclear. The finding that Rip2 kinaseinactive mutant is capable of activating NF-κB reporter in 293 cells suggests that its kinase activity is dispensable (7, 9). We generated mice that expressed Rip2 kinase-dead mutant to investigate the role of the kinase activity in its function. We also generated Rip2 knock-out mice for comparison. The data reported here demonstrate that Rip2-mediated LPS signaling is independent of its kinase activity. Thus, Rip2 functions as an adaptor molecule, rather than an active kinase, in LPS/TLR4 signaling.

EXPERIMENTAL PROCEDURES

Expression Vectors and Antibodies—TLR4 cDNA in pFLAG-CMV-1 vector was a generous gift from Dr. Elizabeth Brint (Trinity College, Ireland). CD14 expression vector was purchased from Invitrogen (GeneStorm expression-ready clone). Myc-tagged human Rip2 vector, TRAF6, and IRAK1 expression vectors were described previously (13, 18).

Rip2 N-terminal peptide antibody, C-terminal peptide antibody and monoclonal antibody were purchased from Cayman Chemical, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and Alexis Biochemicals, respectively. Antibodies to IκBα, phospho-IκBα, p38 and phospho-p38 were from Cell Signaling Technology. IRAK1 antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), and anti-TRAF6 was from Santa Cruz Biotechnology. Monoclonal antibodies to FLAG tag, Myc tag, and V5 tag were purchased from Sigma, Santa Cruz Biotechnology, and Invitrogen, respectively.

Generation of Rip2 Knock-out and Kinase-dead Knock-in Mice—Rip2 knock-out (KO) mice and kinase-dead knock-in (KI) mice were generated by homologous recombination. The targeting vector for Rip2 KO was designed to remove a 204-base pair genomic fragment containing exon 1 that codes for the N-terminal sequences of Rip2 protein, including the kinase active site Lys47. To generate mice that express Rip2

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1 The abbreviations used are: TLR, Toll-like receptor; LPS, lipopolysaccharide; MAP, mitogen-activated protein; IL, interleukin; IRAK, IL-1 receptor-associated kinase; CARD, caspase recruitment domain; TCR, T cell receptor; KO, knock-out; KI, knock-in; RT, reverse transcription; WT, wild-type; TNF, tumor necrosis factor; bia-Tris, 2-bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

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kinase-dead mutant, Lys\(^{\text{144}}\) was mutated to Ala (K47A mutation) in the wild-type Rip2 allele. The mutated Rip2 allele was under the control of the endogenous Rip2 promoter. The KO and kinase-dead KI targeted alleles were confirmed by Southern blot of tail genomic DNA. Vector was electroporated into 129S5/SvEv ES cells. Targeted ES clones were identified by Southern blot analysis of genomic DNA and microinjected into C57BL/6 blastocysts. Germ line transmission of the targeted allele was confirmed by Southern blot of total genomic DNA. The homozygous KO and kinase-dead KI mice were viable and fertile and showed no gross developmental abnormalities. Homozygous KO and kinase-dead KI mice and their respective wild-type littermates were used in experiments.

**RNA Isolation and Reverse Transcription (RT)-PCR**—Total RNA was isolated from 1 \(\times\) 10\(^5\) mouse spleenocytes using the RNeasy RNA isolation kit from Qiagen according to manufacturer's instructions. RT was carried out using Omniscript reverse transcribe kit (Qiagen). Vent DNA polymerase (New England BioLabs) was used for PCRs. N-terminal PCR primers were 5'-ATGACGCGGACCACATCT-3' and 5'-GGAGGATGCGGAATCTCA-3', and a 377-base pair PCR product corresponding to nucleotides 1–377 of Rip2 cDNA was amplified. The C-terminal primers were 5'-AGAGGATACCTGTTGAGTC-3' and 5'-CAAGCTTTAATCTTGAGGG-3', and PCR amplified a 595-base pair Rip2 C-terminal fragment corresponding to nucleotides 1022–1617. The PCR products were separated on agarose gels and visualized by EtBr staining. The amplicons were purified using QIAquick gel extraction kit (Qiagen) according to manufacturer's instructions. RT was performed using Omniscript reverse transcriptase (Qiagen). The mixtures were separated on NuPAGE 4–12% bis-Tris gel (Invitrogen) and transferred to nitrocellulose membrane. The membrane was exposed in a PhosphorImager (Amersham Biosciences). The same membrane was probed with Rip2 antibody to determine the level of Rip2 protein.

Culture of Mouse Bone Marrow-derived Macrophages and LPS Stimulation—Bone marrow was collected from 8–12-week-old mice by gentle flushing of whole femurs with phosphate-buffered saline containing 2% fetal bovine serum. The cell suspension was layered onto lymphohy-M (Cedariane) and centrifuged for 30 min at 500 \(\times\) g. After centrifugation, the interface was collected, and cells were spun down and washed once with phosphate-buffered saline plus 2% fetal bovine serum. The bone marrow cells were cultured overnight in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. The following day, nonadherent cells were collected and plated in 10-cm tissue culture plates at a density of 1 \(\times\) 10\(^5\) cells/plate in Dulbecco's modified Eagle's medium plus 15% fetal bovine serum supplemented with 20 ng/ml mouse macrophage colony-stimulating factor. The cells were fed with fresh medium containing mouse macrophage colony-stimulating factor twice a week. On day 10, the cells were stimulated with 50 ng/ml LPS from *S. abortus equi* (Sigma) for various time periods. Cell lysates were prepared and subjected to SDS-PAGE and Western blot as described above.

Measurement of Cytokine Production by Macrophages—Total bone marrow cells were obtained as described above and plated in a 96-well plate at a density of 1 \(\times\) 10\(^5\)cells/well. After culture for 10 days in the presence of mouse macrophage colony-stimulating factor, macrophages were stimulated with 50 ng/ml LPS for 6 h. Culture supernatants were harvested, and production of IL-6 and TNF\(\alpha\) was determined using mouse IL-6 and TNF\(\alpha\) enzyme-linked immunosorbent assay kit, respectively (R&D).

## Results

### Rip2 Transiently Associates with TLR4 after LPS Stimulation

Recent studies have shown that LPS signaling is impaired in Rip2-deficient cells and mice (15, 16). We investigated whether Rip2 was recruited to the TLR4 signaling complex upon LPS treatment. 293T cells were transfected with FLAG-tagged TLR4 and CD14 and treated with LPS. These transfectants responded to LPS in the absence of MD2, since LPS treatment induced NF-\(\kappa\)B reporter activation (data not shown). Association of endogenous Rip2 with TLR4 was examined by coimmunoprecipitation. As shown in Fig. 1A, Rip2 transiently associated with TLR4 upon LPS treatment. Association peaked at 5 min and decreased after 15 min of LPS treatment. These results indicate that Rip2 transiently recruited to the TLR4 signaling complex after LPS stimulation.

We further tested whether Rip2 interacted with key mediators of LPS-induced signaling to NF-\(\kappa\)B activation. We first co-expressed Myc-tagged Rip2 with IRAK1, TRAF6, or IKK\(\beta\) in 293T cells. Coimmunoprecipitation experiments showed that Rip2 coimmunoprecipitated with IRAK1 and TRAF6 (Fig. 1, B and C), but not with IKK\(\beta\) (data not shown), indicating specific interaction of Rip2 with IRAK1 and TRAF6. We next examined association of endogenous Rip2 with IRAK1 and TRAF6 in THP1 cells. THP1 cells were treated with LPS for various time periods, and association of Rip2 with IRAK1 and TRAF6 was determined by coimmunoprecipitation. Endogenous Rip2 associated with IRAK1 after 5 min of LPS treatment, and the association decreased after 10 min (Fig. 1D). Association of Rip2 with...
TRAF6 was detected after LPS treatment for 5 min, peaked at 10–15 min, and decreased at 30 min (Fig. 1E). Thus, LPS induces transient association of endogenous Rip2 with IRAK1 and TRAF6. These findings strongly suggest that Rip2 directly participates in TLR4 signaling.

Rip2 Kinase Activity Is Induced by LPS Treatment—We investigated whether LPS treatment induced Rip2 kinase activity. THP1 cells were treated with LPS for various time periods. Endogenous Rip2 protein was immunoprecipitated, and Rip2 kinase activity was determined by in vitro autophosphorylation. Basal kinase activity was low (Fig. 2, top). LPS treatment for 5 min greatly increased Rip2 kinase activity and maximal kinase activity was detected at 15 min. By 30 min of LPS treatment, the kinase activity decreased to basal level. The level of Rip2 protein was not changed during LPS treatment (Fig. 2, bottom). Thus, LPS transiently induces Rip2 kinase activity.

Targeted Inactivation of Rip2 Kinase Activity in Mouse—To investigate the role of the kinase activity in Rip2 function, we generated Rip2 kinase-dead KI mice in which the conserved ATP-binding residue Lys47 in the kinase domain was replaced by alanine (K47A mutation). We also generated Rip2 KO mice for comparison. Rip2 deficiency in KO mice was confirmed by

Fig. 1. Rip2 associates with TLR4 signaling components. A, LPS-induced association of Rip2 with TLR4. 293T cells were co-transfected with FLAG-tagged TLR4 and CD14, and treated with 1 μg/ml LPS for the indicated time periods. Cell lysates were immunoprecipitated (IP) with antibody to the FLAG tag and analyzed by Western blot (WB) using Rip2 antibody (top). Lysates were analyzed by Western blot using anti-FLAG (middle) and anti-Rip2 antibody (bottom). B and C, association of Rip2 with IRAK1 (B) and TRAF6 (C) in 293T cells. Myc-tagged Rip2 was co-expressed with IRAK1 or TRAF6 in 293T cells. Cell lysates were analyzed by immunoprecipitation with antibody to the Myc tag and Western blot with antibodies as indicated. D and E, transient association of endogenous Rip2 with IRAK1 (D) and TRAF6 (E) in response to LPS. THP1 cells were treated with 1 μg/ml LPS for the indicated time periods. Endogenous Rip2 protein was immunoprecipitated with the C-terminal peptide antibody to Rip2 and analyzed by Western blot using anti-IRAK1 (D, top) or anti-TRAF6 (E, top). The same membranes were probed with the monoclonal antibody to Rip2 (D and E, bottom).

Fig. 2. Rip2 kinase activity is induced by LPS treatment. THP1 cells were treated with 1 μg/ml LPS for the indicated time periods. Endogenous Rip2 protein was immunoprecipitated with the C-terminal peptide antibody to Rip2 and subjected to an in vitro autophosphorylation assay. The kinase reaction mixtures were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was exposed in a PhosphorImager (top). The same membrane was probed with Rip2 monoclonal antibody (bottom).
Rip2 Kinase Activity Is Dispensable

**Fig. 3. Expression of Rip2 mRNA and protein in cells from Rip2 kinase-dead KI and KO mice.** A. mRNA expression. Total RNA was isolated from splenocytes of Rip2 kinase-dead KI or KO mice or WT littermates. Expression of Rip2 mRNA was determined by RT-PCR using the C-terminal primer pair (CT) and the N-terminal primer pair (NT) as described under “Experimental Procedures.” B. Rip2 protein expression in splenocytes. Lysates were prepared from splenocytes of KI or KO mice or WT littermates and analyzed by Western blot using the N-terminal antibody (NT-Ab) or the monoclonal antibody (mAb) to Rip2. The level of p38 protein was used for sample loading control. p38 protein level was not affected by Rip2 kinase-dead knock-in or Rip2 knock-out. C. Rip2 protein in kinase-dead KI macrophages is inducible by LPS stimulation. Bone marrow-derived macrophages from kinase-dead KI mice or WT littermates were treated with or without LPS (50 ng/ml) for 6 h. Cell lysates were prepared and analyzed by Western blot using antibodies as indicated.

The absence of Rip2 mRNA and protein (Fig. 3, A and B). Sequencing of Rip2 cDNA isolated from kinase-dead KI cells confirmed the presence of the K47A mutation in KI mice. Although the Rip2 mRNA level from kinase-dead KI cells was comparable with that in cells from WT littermates (Fig. 3A), the level of Rip2 protein in KI cells was greatly reduced compared with that from WT cells (Fig. 3B). However, the K47A mutant protein in KI macrophages was up-regulated by LPS stimulation similar to the WT protein (Fig. 3C), indicating that regulation of the Rip2 gene expression is intact in kinase-dead KI mice.

To further investigate the expression of Rip2 K47A mutant protein, Rip2 cDNAs encoding the K47A mutant and WT protein were isolated from kinase-dead KI and WT splenocytes, respectively, and expressed in 293T cells under the control of a constitutive promoter. The expression of the WT and K47A mutant protein was determined by Western blot. Consistent with the observation in cells from kinase-dead KI mice, the level of the full-length K47A mutant protein was significantly lower than the full-length WT protein expressed in 293T cells (Fig. 4A). However, the K47A mutant protein with the truncation of the CARD domain and the intermediate domain (amino acids 1–347) was expressed at a similar level to the truncated WT protein (Fig. 4A). These results suggest that the K47A mutation may cause instability of the full-length Rip2 protein.

To test that the K47A mutation indeed abrogated Rip2 kinase activity, the truncated WT and K47A proteins were immunoprecipitated from transfected 293T cells, and the kinase activity was determined by in vitro autophosphorylation (Fig. 4B). As expected, the K47A mutant protein failed to autophosphorylate.

We further tested autophosphorylation of Rip2 in response to LPS in cells isolated from kinase-dead KI mice. Macrophages from KI mice or WT littermates were treated with LPS, endogenous Rip2 was immunoprecipitated, and in vitro autophosphorylation was assayed. Similar amounts of Rip2 protein from WT and kinase-dead KI macrophages were used for an autophosphorylation assay (Fig. 4C, bottom). Although autophosphorylation of Rip2 from WT macrophages was detected, no autophosphorylation of Rip2 from kinase-dead KI cells was observed (Fig. 4C, top).

**Fig. 4. Expression and kinase activity of Rip2 WT and K47A mutant protein.** A. Rip2 WT and K47A mutant cDNAs encoding the full-length protein (FL) or the kinase domain (amino acids 1–347) were isolated from WT and kinase-dead KI macrophages, respectively, and expressed with V5 tag at the C terminus in 293T cells under the control of the EF-1α promoter. The expression level of Rip2 WT and K47A mutant protein was determined by Western blot using antibody to the V5 tag. Same amounts of plasmid DNA were used for 293T transfection. Two independent clones of each construct were analyzed. B. K47A mutation abolishes Rip2 kinase activity. Rip2 WT and K47A mutant protein (amino acids 1–347) was expressed in 293T cells and immunoprecipitated with anti-V5 antibody. The immunoprecipitates were subjected to in vitro autophosphorylation, followed by SDS-PAGE and phosphorimaging (top). The amount of immunoprecipitated Rip2 protein was determined by Western blot with Rip2 antibody (bottom). C. Rip2 from kinase-dead KI cells is kinase-inactive. Bone marrow-derived macrophages from kinase-dead KI mice or WT littermates were treated with LPS for 6 h. Rip2 protein was immunoprecipitated with the C-terminal peptide antibody to Rip2 and subjected to in vitro autophosphorylation, followed by SDS-PAGE and phosphorimaging (top). The amount of immunoprecipitated Rip2 protein was determined by Western blot using the monoclonal antibody to Rip2 (bottom).

Rip2 Kinase Activity Is Not Required for LPS Signaling—Recent reports show that Rip2 deficiency in mice results in impaired LPS signaling (15, 16). To investigate whether Rip2 kinase activity is required for LPS-triggered signaling events, we examined LPS-induced activation of p38 MAP kinase and NF-κB in macrophages from Rip2 kinase-dead knock-in mice and compared it with that in Rip2 knock-out cells. Bone marrow-derived macrophages were treated with LPS for various time periods. Activation of p38 was determined using a phos-
phosphospecific antibody in Western blot. Activation of NF-κB was determined by the phosphorylation state and degradation of IκBα, an inhibitor of NF-κB (5, 6). Consistent with previous reports, Rip2 knock-out macrophages showed reduced levels of p38 and IκBα phosphorylation and reduced IκBα degradation in response to LPS stimulation (Fig. 5A). By contrast, in Rip2 kinase-dead knock-in macrophages, LPS-induced phosphorylation of p38 and IκBα, and degradation of IκBα was normal, as shown by comparison with cells from wild-type littermates (Fig. 5A). These results indicate that Rip2 kinase activity is not involved in LPS-induced p38 MAP kinase activation and NF-κB activation pathways.

We next examined cytokine production by Rip2 kinase-dead knock-in macrophages. Whereas the levels of TNFα and IL-6 produced by Rip2 knock-out macrophages in response to LPS were significantly decreased compared with cells from wild-type littermates (Fig. 6, C and D), LPS-stimulated production of TNFα and IL-6 by Rip2 kinase-dead knock-in macrophages was indistinguishable from wild-type cells (Fig. 6, A and B). Thus, Rip2 kinase activity is not required for LPS-induced cytokine production. Together, these results demonstrate that participation of Rip2 in LPS signaling is independent of its kinase activity.

**Discussion**

In this report, we show that Rip2-mediated LPS signaling is independent of its kinase activity. We demonstrate that upon LPS stimulation, Rip2 is transiently recruited to the TLR4 receptor complex and associates with IRAK1 and TRAF6, two key mediators of the TLR signaling pathway. In addition, we find that Rip2 kinase activity is transiently induced by LPS treatment. These results provide evidence for direct participation of Rip2 in the LPS/TLR4 signaling pathway.

We find that macrophages from Rip2 knock-out mice are defective for NF-κB and p38 MAP kinase activation and cytokine production in response to LPS stimulation. Our data are consistent with previous reports that Rip2 deficiency in mice results in impaired LPS signaling (15, 16). In contrast, we find that in macrophages from Rip2 kinase-dead knock-in mice, LPS-induced NF-κB and p38 activation is intact and that TNFα and IL-6 production by kinase-dead knock-in macrophages in response to LPS is comparable with cells from wild-type littermates. These findings demonstrate that Rip2 kinase activity is not required for Rip2-dependent LPS signaling.

Although the amount of Rip2 protein in cells from kinase-dead knock-in mice is lower than that in wild-type cells, it is sufficient to mediate LPS signaling. A reduced protein level was also observed when the full-length K47A mutant protein was expressed under control of a constitutive promoter in 293T cells. The lower protein level is probably due to instability of the full-length kinase-inactive protein, since the mRNA level in kinase-dead knock-in cells is comparable with that in wild-type cells. Furthermore, the wild-type and K47A mutant proteins with the deletion of the CARD and intermediate domains expressed at comparable levels in 293T cells.

Previous reports have shown that T cell signaling and IL-18/IL-12 signaling in Rip2-deficient cells were impaired (15–17). However, these defects were not found in our Rip2-deficient mice. TCR-stimulated proliferation and IL-2 production by Rip2-deficient CD4+ T cells were similar to wild-type CD4+ T cells (data not shown). Comparable levels of interferon-γ production by Rip2-deficient NK cells and wild-type cells in response to IL-18 and IL-12 were observed (data not shown). Possible explanations for the discrepancy between our results and the published data include different strategies used for gene disruption, different mouse genetic backgrounds used to generate the knock-out mice, and the cleanness of environment for housing the animals. Similarly, we did not observe defects in TCR signaling and IL-18/IL-12 signaling in cells from Rip2 kinase-dead knock-in mice (data not shown). However, results from a recent report suggest that Rip2 participates in TCR signaling by phosphorylating Bcl10 (17). It is possible that Rip2 kinase activity is required for other Rip2-mediated functions.
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