

Ubiquitination of RIP Is Required for Tumor Necrosis Factor α -induced NF- κ B Activation*

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Stimulation of cells with tumor necrosis factor (TNF α) triggers a recruitment of various signaling molecules, such as RIP, to the TNF α receptor 1 complex, leading to activation of NF- κ B. Previous studies indicate that RIP plays an essential role for TNF α -induced NF- κ B activation, but the molecular mechanism by which RIP mediates TNF α signals to activate NF- κ B is not fully defined. Earlier studies suggest that RIP undergoes a ligand-dependent ubiquitination. However, it remains to be determined whether the ubiquitination of RIP is required for TNF α -induced NF- κ B activation. In this study, we have identified Lys³⁷⁷ of RIP as the functional ubiquitination site, because mutating this residue to arginine completely abolished RIP-mediated NF- κ B activation. The K377R mutation of RIP cannot undergo ligand-dependent ubiquitination and fails to recruit its downstream signaling components into the TNF α receptor 1 complex. Together, our studies provide the first genetic evidence that the ubiquitination of RIP is required for TNF α -induced NF- κ B activation.

NF- κ B is a homo- or heterodimeric transcription factor that controls the expression of various genes involved in inflammatory, apoptotic, and immune responses. In resting cells, the activity of NF- κ B is controlled through its cytoplasmic sequestration by a family of inhibitors, I κ B (Inhibitor of NF- κ B) (1). In response to extracellular stimuli, I κ B proteins are phosphorylated by the I κ B kinase (IKK)² complex, then ubiquitinated, and rapidly degraded, which leads to the nuclear localization and activation of NF- κ B (2). One of the most potent NF- κ B activators is the proinflammatory cytokine, tumor necrosis factor (TNF) α . TNF α functions through the TNF receptors, mainly TNFR1, on the cell surface. The binding of TNF α to TNFR1 causes trimerization of the receptor and recruitment of the adaptor protein TRADD (TNF receptor-associated death domain) (3). TRADD further recruits TARF2 (TNF receptor-associated factor 2) (4), FADD (Fas-associated death domain) (4), and RIP (receptor-interacting protein) (5, 6).

RIP is a serine/threonine kinase that plays an essential role in TNF α -induced NF- κ B activation (6, 7). It contains an N-terminal kinase domain, an intermediate domain, and a C-terminal death domain. Earlier studies indicate that the RIP kinase domain is dispensable for the

activation of NF- κ B (6, 8), whereas its death domain is required for the association with the upstream signaling component TRADD (5). The intermediate domain is required for RIP-mediated NF- κ B activation (6) and plays an important role for interacting with its downstream signaling components such as NEMO, the regulatory subunit of the IKK complex, and other molecules (9). Therefore, RIP likely functions as an adaptor molecule to mediate the TNF α signaling cascade. Although genetic studies have demonstrated that TAK1 (tumor growth factor β -activated kinase 1) and MEKK3 are involved in mediating TNF α -induced NF- κ B activation downstream of RIP (10–15), the mechanism by which RIP activates these downstream kinases is not fully defined. Our recent studies show that RIP fusion proteins, which contain the death domain of RIP and MEKK3 or TAK1, can effectively rescue TNF α -induced NF- κ B activation in RIP-deficient cells (10, 16), suggesting that one of the roles of RIP is to recruit these downstream kinases to the TNFR1 complex.

Ubiquitination has been shown to play various roles in the regulation of NF- κ B signaling pathways (17) as well as many other biological processes. Ubiquitin is a 76-amino-acid protein that is highly conserved among different species and is covalently attached to lysine residues of target proteins (18). This process is catalyzed in three steps. First, ubiquitin is activated in an ATP-dependent reaction by an ubiquitin activation enzyme (E1). Then, the activated ubiquitin is transferred to an ubiquitin-conjugation enzyme (E2). Subsequently, the ubiquitin is attached to the target protein through an isopeptide bond between the C-terminal of ubiquitin and the [epsilon]-amino group of the lysine residue in the target protein in the presence of ubiquitin ligase (E3) (17). Ubiquitin itself contains several lysine residues, in which Lys⁴⁸ or Lys⁶³ could be linked to another ubiquitin to form polyubiquitin chain on target proteins. *In vivo*, Lys⁴⁸-linked polyubiquitin chains lead to the recognition and degradation of proteins by the proteasome complex. In contrast, Lys⁶³-linked polyubiquitination does not undergo the proteasome degradation but has been implicated in other biological processes (17).

Both forms of polyubiquitination play important roles in TNF α -induced NF- κ B activation. I κ B is ubiquitinated through Lys⁴⁸-linked polyubiquitination and degraded in a proteasome-dependent manner, whereas Lys⁶³-linked polyubiquitination has been suggested to be involved in the modification of NEMO (11, 19), which plays an important role in the activation of those proteins. It has been shown that RIP is also ubiquitinated following TNF α stimulation (8, 11, 20). The ubiquitination of RIP may occur in both Lys⁶³ and Lys⁴⁸ linkages (21). It has been suggested that RIP is ubiquitinated by TRAF2 through the Lys⁶³ linkage immediately following TNF α stimulation (21). This ubiquitination may provide a docking site on RIP for its association with TAB2, a TAK1-associated protein (11). In contrast, RIP may also be ubiquitinated by A20 through a Lys⁴⁸ linkage (21), which is suggested to play an important role for the down-regulation of TNF α -induced NF- κ B activation and degradation of RIP (22). However, the remaining key question is whether the ubiquitination of RIP is required for TNF α -induced

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² The abbreviations used are: IKK, I κ B kinase; TNF, tumor necrosis factor; TNFR, TNF receptor; TAK1, tumor growth factor β -activated kinase 1; MEKK3, mitogen-activated protein kinase kinase kinase 3; E1, ubiquitin-activating enzyme; E2, ubiquitin conjugation enzyme; E3, ubiquitin ligase; HEK, human embryonic kidney; WT, wild type; FITC, fluorescein isothiocyanate; CTxB, cholera toxin B.

NF- κ B activation. In this study, we have generated a series of RIP mutants and reconstituted RIP-deficient cells with these mutants. Our results indicate that Lys³⁷⁷ of RIP is the functional ubiquitination site and is required for the recruitment of downstream signaling components to the TNFR1 complex, leading to NF- κ B activation. Together, our results provide both biochemical and genetic evidence that the ubiquitination of RIP is required for TNF α -induced NF- κ B activation.

MATERIALS AND METHODS

Reagents and Plasmids—Antibodies specific for Myc (A14 and A7404), TAK1 (C-9), TRAF2 (C-20), IKK α/β (H-470), ubiquitin (P4D1), NEMO (FL-419), Bcl10 (H-197), and β -tubulin (D-10) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-I κ B α monoclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Recombinant human TNF α was obtained from Endogen (Woburn, MA). The plasmids encoding Myc-RIP and deletion mutants of RIP were constructed by inserting the BglII/HindIII fragments into corresponding sites of pRK6-Myc vector. Plasmids encoding Flag-RIP and Flag-RIP-K377R were constructed by inserting the HindIII/XbaI fragment into pcDNA3(–) vector. Point mutations of RIP were constructed using the QuikChangeTM site-directed mutagenesis kit (Stratagene). Lentivirus expression vectors were constructed by inserting RIP into the ClaI and SmaI sites in a lentiviral vector, pRV3, which contains a multiple cloning site of ClaI-Sall-BamHI-SmaI-EcoRI and a bicistronic internal ribosome entry site green fluorescent protein cassette downstream of the multiple cloning site.

Cell Cultures and Transfection—RIP-deficient Jurkat T cells (RIP[–]) were kindly provided by B. Seed (Massachusetts General Hospital, Boston, MA) (6). Jurkat T and RIP[–] cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Human embryonic kidney 293T (HEK293T) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and the antibiotics described above. Cells were grown in 5% CO₂ at 37 °C and passed every 3 days. Stable transfectants of RIP[–] cells were established by electroporation using a gene pulser (Bio-Rad) at 250 V, 950 mF. Plasmids (10 μ g) encoding RIP-WT or RIP-K377R were transfected into 1×10^7 RIP[–] cells. The cells were incubated at 37 °C for 2 days and then treated with G418 (1 mg/ml) for 2 weeks. The clones resistant to G418 were collected and examined for the presence of RIP proteins using anti-FLAG antibodies. HEK293 cells were transfected using the calcium phosphate precipitation method ($1\text{--}4 \mu$ g of DNA/ 7×10^5 cells).

Generation of Stable Cell Lines Using Lentiviral Infection—HEK293T cells (3×10^6) were seeded in 100-mm dishes 1 day before transfection. The cells cultured in Dulbecco's modified Eagle's medium were transfected with 10 μ g of pRV3 vector encoding different mutant forms of RIP together with 5 μ g of pHep (packaging vector) and 5 μ g of pEnv (Envelope vector) using the calcium phosphate precipitation method. Ten to sixteen hours later, 3–5 ml of warm RPMI 1640 medium was used to replace the Dulbecco's modified Eagle's medium. Viral supernatants were collected 48 h after transfection by spinning to remove cells. RIP[–] cells were resuspended in viral supernatant for infection at the concentration between 0.8 and 1.0×10^6 and incubated in 24-well plates for 30–48 h. The efficiency of the viral infection was determined by the flow cytometry or Western blot analysis of the expression of RIP proteins in the infected cells.

Western Blot and Immunoprecipitation—Cells were transfected with different vectors and lysed in a buffer containing 50 mM HEPES (pH 7.4), 250 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM Na₃VO₄, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and a prote-

ase inhibitor mixture (Roche Diagnostics). The cell lysates were subjected to SDS-PAGE and Western blotted or immunoprecipitated with anti-c-Myc-agarose affinity gel (Sigma-Aldrich). The immunoprecipitates were washed with lysis buffer four times and eluted with 2 \times SDS loading buffer. After boiling (4 min), the samples were fractionated on 10% SDS-PAGE and transferred to nitrocellulose membranes. Immunoblots were incubated with specific primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies and were developed by the enhanced chemiluminescence method according to the manufacturer's protocol (Pierce).

Luciferase Reporter Assay—HEK293T cells (3×10^5 in 12-well plates) were transfected with reporter plasmid encoding 5 \times NF- κ B-luc (60 ng) and pEF-Renilla-luc (10 ng) together with plasmids encoding the desired genes using the calcium phosphate precipitation method. Twenty hours later, cell lysates were prepared, and luciferase activities were measured with the Dual-Luciferase assay kits (Promega, Madison, WI). NF- κ B activities were determined by normalization of NF- κ B-dependent firefly luciferase to Renilla luciferase activity.

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared from Jurkat or its derivative cells following various stimulations. Cells (2×10^7) were resuspended in 400 μ l of lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.4% Nonidet P-40, and 1% protease inhibitor mixture) and incubated on ice for 15 min. The nuclei were pelleted, and the cytoplasmic proteins were carefully removed. The nuclear pellets were then resuspended in 100 μ l of extraction buffer (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 1% protease inhibitor mixture). After vortexing for 30 min at 4 °C, the samples were centrifuged ($13,000 \times g$, 10 min), and the nuclear proteins in the supernatant were collected. Protein concentrations of nuclear extracts were determined by the Bio-Rad protein assay (Bio-Rad Laboratories) using bovine serum albumin as the standard. The nuclear extract (10 μ g) was incubated with a ³²P-labeled, double-stranded, NF- κ B-specific oligonucleotide probe or Oct-1 probe as a control (Promega) for 15 min at room temperature. After incubation, samples were fractionated on a 5% polyacrylamide gel and visualized by autoradiography.

Cell Viability Assay—RIP[–] cells reconstituted with different forms of RIP were seeded into 6-well plate (0.4×10^6 /well) and treated with or without TNF α (20 ng/ml) for 22 h. Cells were then collected and assayed either for cell viability by flow cytometry or for cell death by annexin V staining on triplicate cultures.

Confocal Microscopy—Jurkat T cells or RIP[–] cell lines expressing FLAG-RIP-WT or FLAG-RIP-K377R were used to examine the capping of TNFR1 and recruitment of signaling components into the lipid raft. Briefly, 1 million cells were stained with FITC-conjugated cholera toxin B (CTxB) (8 μ g/ml (Sigma)) at 4 °C for 20 min. Cells were then washed and treated with 2 μ g/ml TNFR1 agonistic antibodies (Goat anti-TNFR1 antibodies, R&D systems, Minneapolis, MN) at 4 °C for 60 min. Unbound antibody was removed by washing twice. To show the capping of TNFR1, cells were incubated with Alexa 594-conjugated donkey anti-goat secondary antibodies at 1:100 dilution at 4 °C for 60 min. Capping was induced by warming cells to 37 °C in a water bath with mild agitation for 10 min. Cells were harvested and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Fixed cells were washed and mounted onto poly-L-lysine-coated glasses by cytospin. Unstimulated controls were sequentially exposed to TNFR1 antibodies and secondary antibody at 4 °C only. Thereafter, fixed cells were permeabilized and treated with primary antibodies as indicated followed by Fluor 594-conjugated secondary antibodies (Invitrogen Molecular Probes,

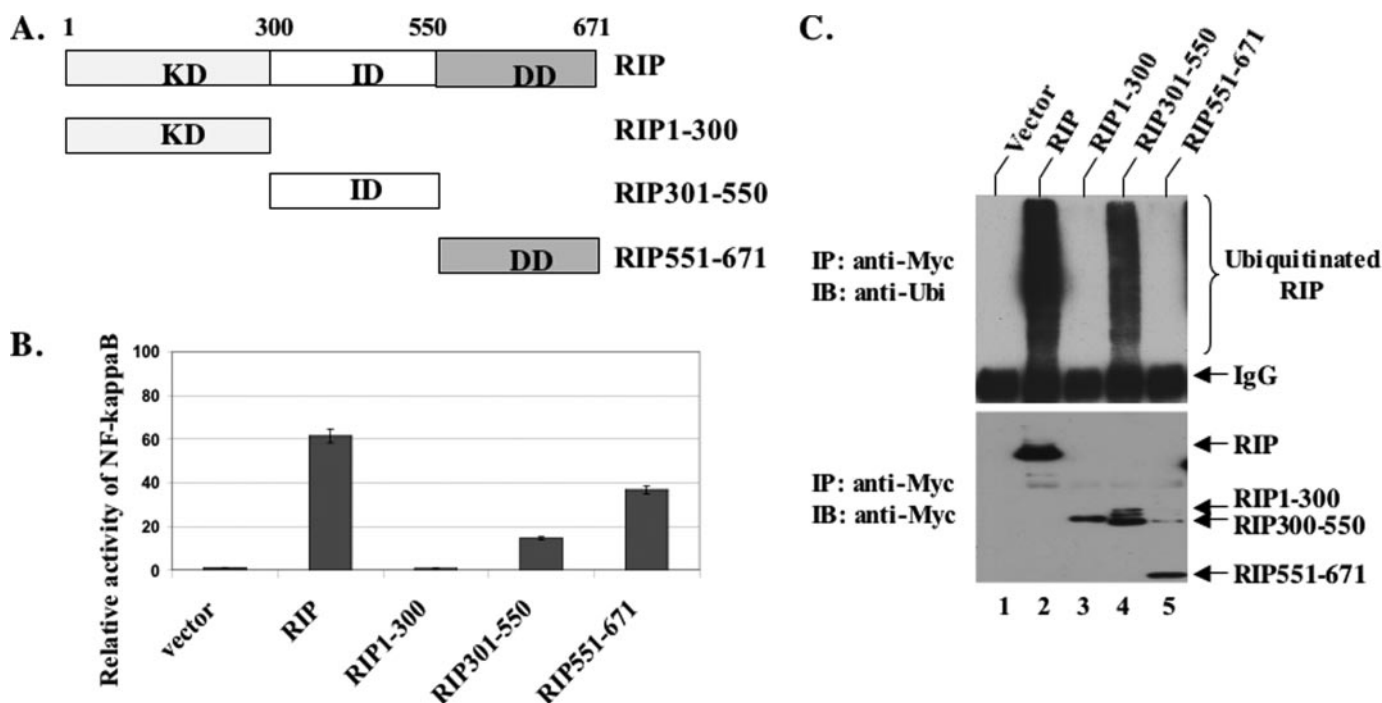


FIGURE 1. Intermediate domain of RIP is essential for the activation of NF- κ B and RIP ubiquitination. *A*, schematic diagram of RIP truncation mutants that contains different domains of RIP. *KD*, kinase domain; *ID*, intermediate domain; *DD*, death domain. *B*, HEK293T cells were transfected with plasmids encoding different truncation forms of RIP together with the plasmid encoding NF- κ B-dependent luciferase. Vectors encoding CrmA were also included in each transfection to inhibit the apoptotic effect induced by the overexpression of RIP. At 24 h post-transfection, the cells were lysed. The cell lysates were used to determine NF- κ B activation by the dual luciferase reporter assay. Data are the mean of triplicates from a representative experiment. *C*, the cell lysates were also immunoprecipitated (IP) with anti-c-Myc-agarose affinity gel. The resulted immunoprecipitates were subjected to SDS-PAGE and analyzed by Western blot (IB) using anti-ubiquitin (top panel) or anti-Myc antibodies (bottom panel), respectively.

Eugene, Oregon). Fluorescence was detected using a Olympus Fluoview FV300 confocal laser scanning biological microscope.

RESULTS

Previous studies indicate that RIP undergoes a TNF α -dependent ubiquitination (8, 11, 20). To investigate whether the ubiquitination of RIP is required for TNF α -dependent NF- κ B activation, we decided to map the ubiquitination site(s) of RIP. RIP is composed of three structural domains, the N-terminal kinase domain (*KD*), an intermediate domain (*ID*), and the C-terminal death domain (*DD*) (Fig. 1*A*). Thus, we constructed a series of RIP deletion mutants that encode different domains (Fig. 1*A*). The expression vectors encoding these mutants were transfected into HEK293T cells together with a vector encoding the NF- κ B-dependent luciferase to measure their ability to activate NF- κ B. To prevent transfection-associated cell death, a plasmid encoding CrmA was included in the transfection. Consistent with previous observations (5, 6), overexpression of full-length (residues 1–671), the intermediate domain (residues 301–550), and the death domain (residues 551–671) of RIP could induce NF- κ B activation (Fig. 1*B*). In contrast, overexpression of the kinase domain (residues 1–300) of RIP failed to activate NF- κ B (Fig. 1*B*). To determine which domain of RIP contains the ubiquitination site of RIP, the full-length and deletion mutants of RIP were immunoprecipitated from the transfected cells, subjected to SDS-PAGE, and blotted with anti-ubiquitin antibodies. As shown in Fig. 1*C*, both full-length and the intermediate domain of RIP were ubiquitinated, whereas the kinase domain and the death domain were not ubiquitinated. These results indicate that the intermediate domain of RIP contains the ubiquitination sites.

To identify which lysine residue(s) in the intermediate domain of RIP is the functional ubiquitination site, we aligned the sequence of the intermediate domain of human, rat, and mouse RIP. We found that Lys

residues 305, 306, 377, 396, and 530 are conserved among different species (Fig. 2*A*), any of which might be the ubiquitination site in the intermediate domain of RIP. Therefore, we first constructed a series of RIP intermediate domain mutants, in which Lys residues 377, 396, 530, or both 305 and 306, were mutated to Arg. Although overexpression of these mutants, as well as wild type RIP intermediate domain, can induce NF- κ B activation (Fig. 2*B*), the RIP intermediate domain mutant with Lys³⁷⁷ mutated to Arg (K377R) had the most significant defect for NF- κ B activation (Fig. 2*B*). Interestingly, the overexpression-associated ubiquitination was significantly reduced for the K377R mutant (Fig. 2*C*, lane 3). Similarly, the RIP full-length mutant with Lys³⁷⁷ mutated to Arg (RIP-K377R) had the most significant defect for NF- κ B activation (data not shown). Together, these results suggest that Lys³⁷⁷ may be the ubiquitination site in the intermediate domain of RIP and plays an important role for RIP-mediated NF- κ B activation.

To demonstrate that Lys³⁷⁷ of RIP plays a crucial role for TNF α -induced NF- κ B activation, we reconstituted RIP-deficient (RIP[−]) Jurkat T cells with wild type RIP and its mutants, in which Lys residues in the intermediate domain were mutated to Arg. As shown in Fig. 3*A*, all of these mutants of RIP are expressed at comparable levels. Next, we examined TNF α -induced NF- κ B activation by electrophoretic mobility shift assay (Fig. 3*B*) and determined the induction of phosphorylation of I κ B α (Fig. 3*C*). We found that expression of RIP-K396R, RIP-K530R, RIP-K305/306R (data not shown), as well as wild type version of RIP (RIP-WT), could effectively restore TNF α -induced NF- κ B activation in RIP[−] cells (Fig. 3*B*). In contrast, mutant forms of RIP, containing mutated lysine 377 (RIP-K377R and RIP-K377/396/530R), failed to restore TNF α -induced NF- κ B activation in RIP[−] cells (Fig. 3*B*). The defect observed in Lys³⁷⁷ mutants was specific for TNF α signaling, because phorbol 12-myristate 13-acetate (PMA) CD28 costimulation-induced NF- κ B activation was normal in these cell lines (Fig. 3*B*). Con-

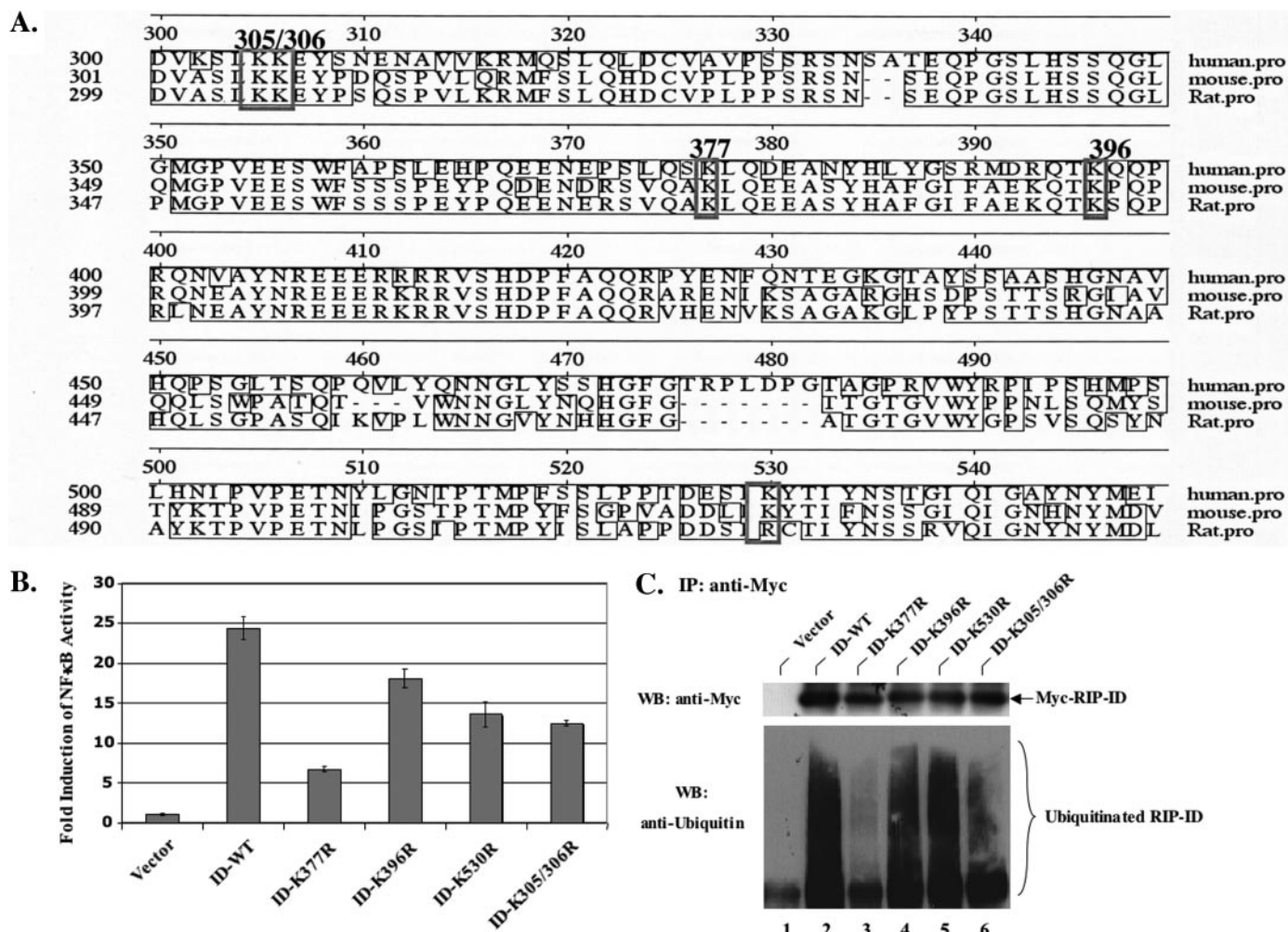


FIGURE 2. Lys³⁷⁷ in the intermediate domain of RIP is conserved among different species and is important for the activation of NF- κ B. A, the intermediate domain of RIP from human, mouse, and rat were used to perform an alignment analysis. Lysine residues 305, 306, 377, 396, and 530 are conserved in this domain. B, the RIP intermediate domain (ID) mutants, in which individual Lys residues were mutated to Arg, were transfected into HEK293T cells together with NF- κ B luciferase reporter. NF- κ B activation was determined by measuring luciferase activity as described in Fig. 1. Data are the mean of triplicates from a representative experiment. C, the transfected RIP proteins were immunoprecipitated (IP) using anti-Myc antibody-conjugated beads. The immunoprecipitated complexes were then subjected to SDS-PAGE and analyzed by Western blots (WB) using various antibodies.

sistent with these results, inducible I κ B α phosphorylation was detected in RIP-deficient cells expressing RIP-WT, RIP-K396R, or RIP-K530R but significantly defective in RIP-deficient cells expressing RIP-K377R or RIP-K377/396/530R following TNF α stimulation (Fig. 3C). Together, these results indicate that lysine 377 of RIP plays a crucial role for mediating TNF α -induced NF- κ B activation.

TNF α treatment activates both apoptotic and survival signals. The outcome of cell survival or death following TNF α treatment is dependent on the balance of these two signals. Earlier studies indicate that the TNF α -induced survival signal is dependent on NF- κ B activation, which induces the expression of several anti-apoptotic or survival genes. These anti-apoptotic or survival proteins protect cells from TNF α -induced apoptosis. Because TNF α stimulation cannot activate NF- κ B in RIP-deficient (RIP⁻) cells, these cells are sensitive to TNF α -induced apoptosis (Fig. 4) (6, 8). To examine whether the mutant form of RIP can rescue RIP⁻ cells from TNF α -induced apoptosis, stable cells with different mutants of RIP were stimulated with TNF α for 22 h before the cell viability was measured. Cells without TNF α treatment are normalized to 100% and used as the negative control. TNF α treatment induced more than 50% of apoptosis in RIP⁻ (Mock), RIP⁻ (RIP-K377R), and RIP⁻ (RIP-K377/396/530R) cells, whereas the expression of RIP-WT, RIP-K396R, or RIP-K530R protected RIP⁻ cells from TNF α -induced

apoptosis (Fig. 4). This protective effect is highly specific, because Fas-induced apoptosis was not affected in these cells (Fig. 4). Together, these data further support the conclusion that Lys³⁷⁷ of RIP is important for TNF α -induced NF- κ B activation.

To further explore whether the functional deficiency of the RIP-K377R mutant is because of the lack of ubiquitination following TNF α stimulation, RIP ubiquitination was measured in RIP-deficient (RIP⁻) cells expressing RIP-WT, RIP-K377R, or vector control. As shown in Fig. 5A, the ubiquitinated form of RIP can be detected in RIP⁻ (RIP-WT) cells treated with TNF α for 4 min, whereas RIP-K377R expressed in RIP⁻ cells cannot be ubiquitinated following TNF α stimulation, although RIP-WT and RIP-K377R are expressed at comparable levels (Fig. 5A). Consistent with these results, TNF α effectively induced phosphorylation of I κ B α in RIP⁻ (RIP-WT) but not in RIP⁻ (RIP-K377R) cells (Fig. 5B, lanes 4 and 6). These results demonstrate that Lys³⁷⁷ in the intermediate domain of RIP is the functional ubiquitination site for TNF α -induced NF- κ B activation.

Earlier studies suggest that RIP may provide docking sites to recruit downstream signaling components such as TAK1 and NEMO (11, 23), and our recent data also suggest that the role of RIP is to recruit TAK1 to the TNFR1 complex leading to NF- κ B activation (10). Therefore, we hypothesized that the defect of TNF α -induced NF- κ B activation in the

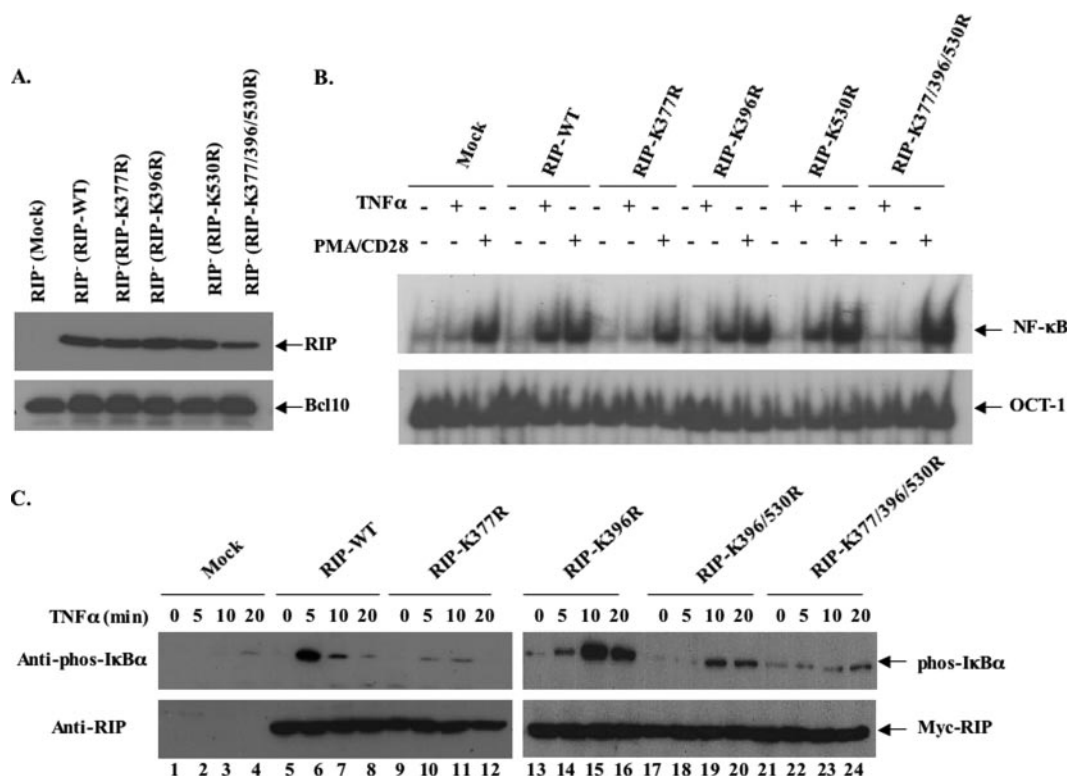


FIGURE 3. RIP mutants containing K377R fail to restore TNF α -induced NF- κ B activation in RIP-deficient cells. A, stable cell lines with Myc-tagged mutants of RIP were established by lenti-viral infection of RIP-deficient (RIP^{-/-}) Jurkat T cells. RIP^{-/-} cells infected with empty vector are used as mock control. Expression levels of mutant RIP in these cells were determined by Western blot using anti-Myc antibodies (upper panel), whereas endogenous Bcl10 levels in these cells were used as the loading control (lower panel). B, these RIP-reconstituted RIP^{-/-} cells were stimulated with TNF α . TNF α -induced NF- κ B activation in these cells was examined by electrophoretic mobility shift assay (top panel). The nuclear extracts from the cells stimulated with phorbol 12-myristate 13-acetate plus anti-CD28 antibodies were used as positive controls. An electrophoretic mobility shift assay with OCT-1 binding was used as loading controls (bottom panel). C, TNF α -induced I κ B α phosphorylation in these cells was examined by Western blot analysis using anti-phospho-I κ B α antibodies (top panel). The membrane was reprobed with anti-RIP antibodies as loading controls (bottom panel).

RIP-K377R cannot protect RIP-deficient cells from TNF α -induced apoptosis.

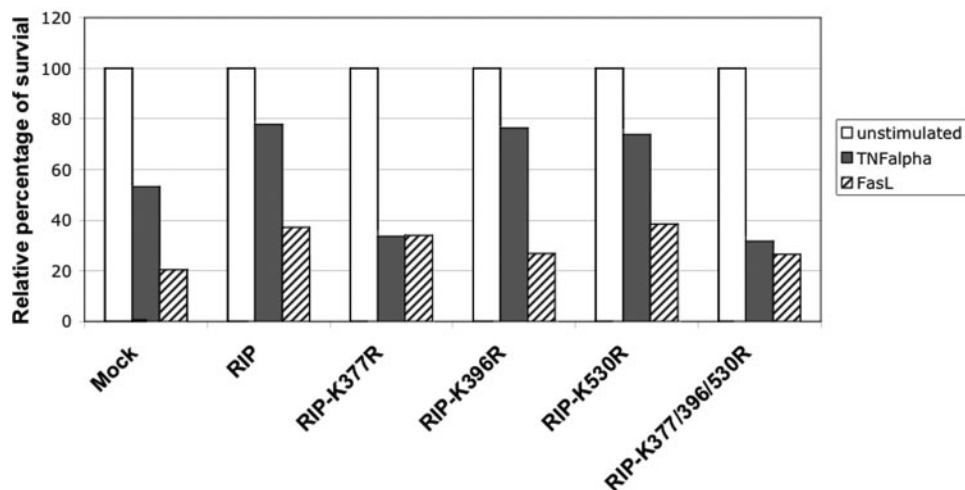


FIGURE 4. RIP-K377R cannot protect RIP-deficient cells from TNF α -induced apoptosis. RIP-deficient cells expressing mutants of RIP were left untreated (white bars), or treated with either TNF α (dark bars) or FasL (hatch bars) for 22 h. Cell apoptosis was determined by Annexin-V staining and flow cytometry. The survival rate of untreated cells was normalized to 100%.

RIP-deficient cells expressing RIP-K377R is because RIP-K377R cannot effectively associate with TAK1 or NEMO. To test this hypothesis, plasmids encoding Myc-RIP-WT, Myc-RIP-K377R, Myc-RIP-K396R, or Myc-RIP-K530R were transiently transfected into HEK293T cells (Fig. 6A). The Myc-tagged RIP proteins were immunoprecipitated from the transfected cells, subjected to SDS-PAGE gel, and blotted with anti-TAK1 antibodies. Compared with RIP-WT and other mutants of RIP, the interaction between RIP-K377R and endogenous TAK1 was significantly reduced (Fig. 6B). However, although it also slightly reduced, RIP-K377R could still associate with the endogenous NEMO (Fig. 6B).

These results suggest that the association of RIP with TAK1 is mainly dependent on the ubiquitination of Lys³⁷⁷, whereas the association of RIP with the IKK complex may be independent of Lys³⁷⁷ ubiquitination.

The lipid raft, the cholesterol- and sphingolipid-enriched membrane microdomains, has been suggested to involve in the activation of NF- κ B in the TNF α signaling pathway. It has been shown that TNFR1 and downstream signaling components are recruited to the lipid raft following TNF α stimulation, and this recruitment may be required for NF- κ B activation. To examine the recruitment of signaling components to TNFR1 after the stimulation of TNF receptor 1, we examined the local-

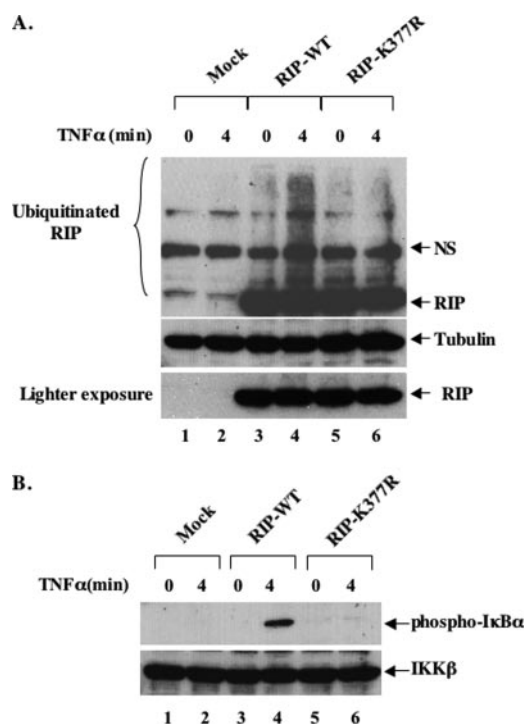


FIGURE 5. RIP-K377R cannot be ubiquitinated upon TNF α stimulation. RIP-deficient cells reconstituted with mock, WT, and RIP-K377R mutant were stimulated with TNF α for time indicated. *A*, whole cell lysates were subjected to SDS-PAGE and analyzed by Western blot analysis using anti-RIP antibodies to detect the ubiquitinated RIP. A Western blot with anti-tubulin antibodies is used as the loading control. NS, none specific. *B*, Western blot with anti-phospho-I κ B α antibodies is performed to show the activation of NF- κ B upon TNF α stimulation, and IKK α / β levels in these lysates are used as loading controls.

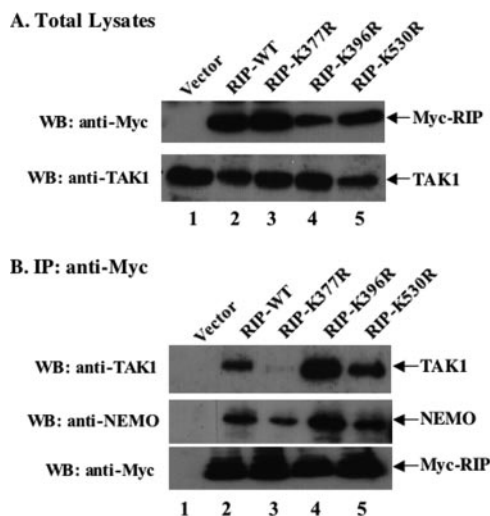


FIGURE 6. Lys³⁷⁷ of RIP plays an important role for the interaction between RIP and downstream signaling components. HEK293T cells (5×10^5) were plated in 6-well plates and then transfected with plasmids encoding Myc-RIP-WT, Myc-RIP-K377R, Myc-RIP-K396R, and Myc-RIP-K530R, as well as the empty vector. *A*, transfected cells were lysed 24 h after transfection, and the cell lysates were subjected to SDS-PAGE and immunoblotting analysis for detecting the expression of RIP and its mutants using anti-Myc antibodies. Endogenous TAK1 levels were used as loading controls. *B*, portion of cell lysates was immunoprecipitated (IP) with anti-Myc antibody-conjugated agarose. The immunocomplexes were subjected to SDS-PAGE and immunoblotting analysis. The interaction between overexpressed RIP and endogenous TAK1 or NEMO was examined using anti-TAK1 or anti-NEMO antibodies. The immunoblotting analysis using anti-Myc antibodies is used to show the level of different forms of RIP in the immunoprecipitates (bottom panel). WB, Western blot.

ization of signaling components using fluorescent confocal microscopy methods. Jurkat T cells were first labeled with FITC-conjugated CTxB (green), which specifically associates with the lipid raft. To show the

TNFR1 and Lipid Raft

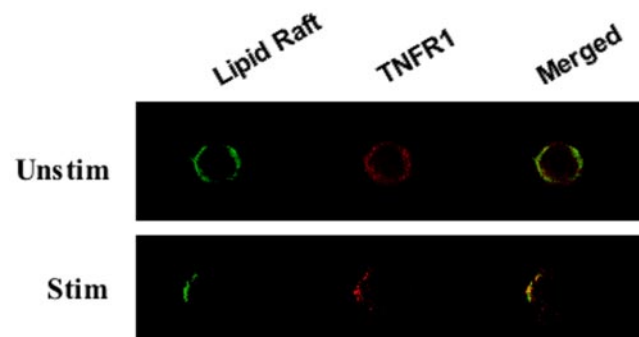


FIGURE 7. TNFR1 is colocalized with lipid rafts following the stimulation of TNFR1. Jurkat cells were stained with FITC-conjugated CTxB ($8 \mu\text{g/ml}$) to label lipid rafts (green). Cells were then washed and treated with $2 \mu\text{g/ml}$ goat anti-TNFR1 agonistic antibodies at 4°C for 60 min. To show the capping of TNFR1, cells were incubated with Alexa 594-conjugated donkey anti-goat secondary antibodies (red) at 4°C for 60 min. TNFR1 capping (red) was induced by warming cells to 37°C for 10 min. Unstimulated (Unstim) controls were sequentially exposed to TNFR1 antibodies and secondary antibody at 4°C only. The cells were mounted onto poly-L-lysine-coated glass slides by cytospin. Fluorescence was detected using a fluorescent confocal microscope.

localization of TNFR1, CTxB-labeled cells were stimulated with or without TNFR1 agonist antibodies followed by the staining with Alexa594-conjugated secondary antibody (red). In unstimulated cells, lipid raft and TNFR1 are evenly distributed on the cell surface (Fig. 7). After cross-linking TNFR1, TNFR1 was recruitment into aggregated lipid rafts (Fig. 7). Therefore, we can determine whether signaling components are recruited into the TNFR1 complex by examining their colocalization with lipid rafts following the stimulation of TNFR1.

To further determine whether Lys³⁷⁷ of RIP plays an essential role for the recruitment of downstream signaling components to TNFR1 complex, we reconstituted RIP-deficient (RIP⁻) cells with plasmids encoding RIP-WT or RIP-K377R. RIP-WT and RIP-K377R were expressed at comparable levels in the reconstituted RIP⁻ cells (data not shown). The resulting stable cells were also labeled with CTxB and cross-linked with or without TNFR1 agonist antibodies. As found in Jurkat T cells, TNFR1 was colocalized with lipid rafts following the stimulation of TNFR1 in both RIP-WT and RIP-K377R-reconstituted RIP⁻ cells (Fig. 8A, lower panels). To examine the localization of signaling components, these cells were immunolabeled with anti-RIP (Fig. 8B), anti-TAK1 (Fig. 8C), or anti-TRAF2 (Fig. 8D) antibodies. In the unstimulated cells, RIP, TAK1, and TRAF2 were uniformly distributed in cytoplasm. Following the stimulation, RIP (WT), TAK1, and TRAF2 were recruited to the region close to lipid raft on the cytoplasm in the RIP⁻ cells expressing RIP-WT. In contrast, the recruitment of TAK1 was defective in the RIP⁻ cells expressing RIP-K377R (Fig. 8C, lower right panels), although the recruitment of TNFR1, RIP (K377R), and TRAF2 was normal in these cells (Fig. 8, A, B, and D, lower right panels). Together, our results indicate that the ubiquitination of Lys³⁷⁷ in RIP is required for the recruitment of its downstream signaling components to the TNFR1 complex in lipid rafts.

DISCUSSION

RIP is an essential signaling component for the NF- κ B activation in TNF α signaling pathway (6, 7) and is ubiquitinated following TNF α stimulation (8, 11, 20), which has been suggested to play an important role in the NF- κ B activation. In this study, we have investigated the role of RIP ubiquitination by identifying the ubiquitination site and examining TNF α -induced NF- κ B activation in the RIP-deficient cells reconstituted with various RIP mutants. Our results indicate that Lys³⁷⁷ in the intermediate domain of RIP is the functional ubiquitination site,

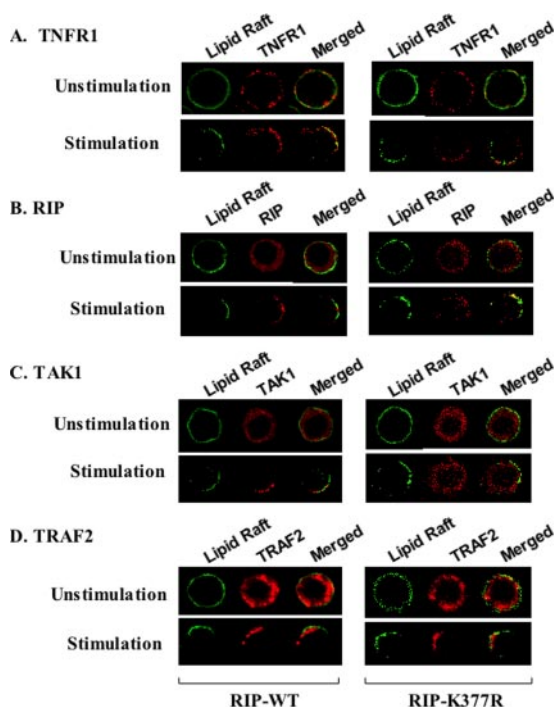


FIGURE 8. Lys³⁷⁷ of RIP is required for the recruitment of its downstream signaling components to the lipid raft. RIP-deficient cells expressing RIP-WT or RIP-K377R were labeled with FITC-conjugated CTxB for staining lipid rafts (green) and then were stimulated by cross-linking with (lower panels) or without (upper panels) agonist antibodies for TNFR1. The cells were stained with specific antibodies against TNFR1 (A), RIP (B), TAK1 (C), and TRAF2 (D), and followed by Alexa 594-conjugated secondary antibodies (red). The labeled cells were visualized using a fluorescent confocal microscope.

because expression of the ubiquitination-deficient mutant of RIP, in which Lys³⁷⁷ was replaced with an arginine residue, cannot restore the TNF α -induced NF- κ B activation in RIP-deficient cells. This defect is likely because this RIP mutant fails to recruit the downstream signaling components (TAK1-TAB complex) into the TNFR1 complex. Together, our studies demonstrate that ubiquitination of RIP is required for TNF α -induced NF- κ B activation.

RIP is composed of an N-terminal kinase domain, an intermediate domain, and a C-terminal death domain. Consistent with previous studies (5, 6), we found that the kinase domain of RIP cannot activate NF- κ B (Fig. 1B) and, in addition, it is not ubiquitinated (Fig. 1C). In contrast, the intermediate domain is ubiquitinated (Fig. 1C) and can also activate NF- κ B, supporting the hypothesis that ubiquitination of RIP is important for the NF- κ B activation. Of note, although the death domain, which is required for the recruitment of RIP to the upstream component TRADD, does not contain an ubiquitination site, it can still activate NF- κ B. One explanation for the death domain-induced NF- κ B activation is that the overexpressed death domain may lead to oligomerization-dependent NF- κ B activation (24) by oligomerizing the endogenous RIP. Consistent with this hypothesis, we have found that RIP indeed forms oligomers through its death domain (data not shown).

The intermediate domain of RIP contains several lysine residues, including lysine 305, 306, 377, 396, and 530, which are conserved among different species (Fig. 3A). However, only the mutation on Lys³⁷⁷ in the intermediate domain leads to the defect for ubiquitination (Fig. 3C), indicating that Lys³⁷⁷ may be the key ubiquitination site in the intermediate domain. Although the NF- κ B activation induced by overexpression of ID-K377R was significantly reduced in comparison with wild type intermediate domain or other mutants, it remained partial activity to activate NF- κ B (Fig. 2B). This remaining activity is likely because of the fact that other Lys residues in the intermediate domain may partially

compensate the function of Lys³⁷⁷ when ID-K377R is overexpressed in HEK293 cells. Nevertheless, RIP-deficient cells expressing RIP-K377R mutant are completely defective in TNF α -induced NF- κ B activation, whereas mutations on Lys³⁹⁶, Lys⁵³⁰ (Fig. 3), and Lys³⁰⁵/Lys³⁰⁶ (data not shown) have no significant defect for the NF- κ B activation. Consistent with the functional assay, TNF α -induced ubiquitination is also significantly reduced for RIP-K377R (Fig. 5).

It has been reported that TRAF2 may work as an E3 ligase for the Lys⁶³-linked polyubiquitination of RIP, and this Lys⁶³-linked polyubiquitination of RIP has been suggested to provide a docking site for the recruitment of TAK1-TAB2 complex (11). Our studies indicate that the mutation of Lys³⁷⁷ significantly disrupts the interaction between RIP and TAK1 (Fig. 6), suggesting that the Lys³⁷⁷-mediated ubiquitination is likely involved in this Lys⁶³-linked ubiquitination and is required for the association of TAK1. Consistent with this conclusion, RIP-K377R fails to recruit TAK1 to the TNF α receptor complex following the stimulation of TNFR1 (Fig. 8). However, we have not been able to demonstrate directly that Lys³⁷⁷ only undergoes the Lys⁶³-linked polyubiquitination *in vivo*, because the high level of endogenous ubiquitin interferes a direct assessment of Lys⁶³-linked polyubiquitination.

It has been suggested that A20 functions as a deubiquitination enzyme to remove the Lys⁶³-linked polyubiquitination of RIP and then functions as an E3 ligase to further ubiquitinate RIP through the Lys⁴⁸-linked polyubiquitination. This Lys⁴⁸-linked polyubiquitination may play a role in the down-regulation of TNF α -induced NF- κ B activation by targeting RIP for proteasome-mediated degradation (21). Because the mutation of Lys³⁷⁷ does not significantly increase the stability of RIP (data not shown), we believe that the Lys⁴⁸-linked polyubiquitination of RIP is through a Lys³⁷⁷-independent process. Of note, when full-length RIP-K377R was overexpressed in HEK293 cells, it could still be ubiquitinated (data not shown). It remains to be determined whether this Lys³⁷⁷-independent ubiquitination of RIP is Lys⁴⁸-linked polyubiquitination and what is the functional significance of Lys³⁷⁷-independent ubiquitination in RIP.

It has been shown that the NEMO-IKK complex can be recruited to the TNF α receptor complex through a RIP-independent but TRAF2-dependent mechanism (23). However, it has also been shown that NEMO can directly associate with the intermediate domain of RIP (9). Although the association of RIP-K377R with the IKK complex (NEMO subunit) is slightly decreased, RIP-K377R can still associate with NEMO (Fig. 6). Therefore, these results suggest that the association of RIP with IKK complex may not be completely dependent on the ubiquitination of Lys³⁷⁷, and suggest that RIP functions as an adaptor molecule to bring TAK1 and IKK together by the Lys³⁷⁷-dependent and Lys³⁷⁷-independent process following TNF α stimulation. In this complex, TAK1 induces the phosphorylation of IKK β directly or indirectly, leading to NF- κ B activation.

Recruitment of signaling components to the lipid raft is required for the activation of NF- κ B following the cross-linking of TNFR1 (20, 25). Consistent with these observations, we have found that TNFR1 and other signaling components are colocalized with lipid rafts (10). Although RIP-K377R and wild type RIP were effectively recruited to lipid rafts following the stimulation of TNFR1, the recruitment of TAK1 to lipid rafts was defective in RIP-deficient cells expressing RIP-K377R. Together, our results indicate that Lys³⁷⁷-dependent ubiquitination of RIP is essential for the recruitment of TAK1 to the TNFR1 complex in the lipid raft following the stimulation of TNFR1 (Fig. 8D).

In summary, our results provide the direct genetic and biochemical evidence that the ubiquitination of RIP is required for the TNF α -induced NF- κ B activation through recruiting TAK1 to the TNFR1 com-

plex. Importantly, we have found that Lys³⁷⁷ in the intermediate domain of RIP is the functional ubiquitination site.

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