Role of Receptor-interacting Protein in Tumor Necrosis Factor-α-dependent MEKK1 Activation

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Receptor-interacting protein (RIP), a death domain serine/threonine kinase, has been shown to play a critical role in tumor necrosis factor-α (TNF-α)-induced activation of the nuclear factor-κB (NF-κB) signaling pathway. We demonstrate here that ectopically expressed RIP induces I-κB kinase-β (IKKβ) activation in intact cells and that RIP-induced IKKβ activation can be blocked by a kinase-inactive form of MEKK1, MEKK1(K1253M). Interestingly, RIP physically associates with MEKK1 both in vitro and in vivo. RIP phosphorylated MEKK1 at Ser-857 and Ser-994. Our data also indicate that RIP induced the stimulation of MEKK1 but not MEKK1(S857A/S894A) in transfected cells. Furthermore, overexpressed MEKK1(S857A/S894A) inhibited the RIP-induced activation of both IKKβ and nuclear factor-κB. We also demonstrated that the TNF-α-induced MEKK1 activation was defective in RIP-deficient Jurkat cells. Taken together, our results suggest that RIP phosphorylates and activates MEKK1 and that RIP is involved in TNF-α-induced MEKK1 activation.

Proinflammatory cytokine tumor necrosis factor-α (TNF-α) stimulates various signaling pathways leading to proliferation, differentiation, or apoptosis of cells through its receptors TNFR1 and TNFR2 (1). TNF-α, by binding to TNFR1, can activate the apoptotic pathway in which TNFR1 associates with death domain proteins such as TNF-associated death domain (2) and Fas-associated death domain (3). The receptor complexes then form a death-inducing signaling complex with caspase-8, which subsequently activates the executors caspases, causing apoptotic cell death (4). TNF-α can also induce the association of TNFR with TNF receptor-associated factors (TRAFs). The TNFR/TRAF complexes activate the nuclear factor-κB (NF-κB) pathway, which functions as a cell survival signal (5–7).

TNFR-bound TRAFs have been shown to induce NF-κB activation through down-regulation of I-κB, an inhibitor of NF-κB (8). Down-regulation of I-κB results from I-κB phosphorylation by I-κB kinases (IKKs) and ubiquitin-dependent degradation of the phosphorylated I-κB (9). Three isoforms of IKK have been identified: α, β, and γ. Studies using knockout mice have shown that IKKβ is responsible for the TNF-α-induced NF-κB activation (10), whereas IKKα is dispensable for the TNF-α signaling (11). Nonenzymatic IKKγ/NEMO induces the formation of an IKKα and IKKβ complex (12). In addition to the activation of the IKKs by IKKγ-mediated complex formation, IKKα and IKKβ can be activated by phosphorylations catalyzed by upstream kinases such as the NF-κB inducing kinase (NIK) (13), MEKK1 (14) and Akt (15). NIK and MEKK1 have been reported to directly interact with TRAF2. Thus, TRAF2 has been thought to be an important component of the TNFR signaling complexes in the NF-κB activation (16, 17). However, a recent study using TRAF2-null mice has demonstrated that the TNF-α-induced NF-κB activation is intact while the TNF-α-induced c-Jun N-terminal kinase activation is defective in the TRAF2-deficient cells (18). Therefore, the major physiological function of TRAF2 may be the activation of the c-Jun N-terminal kinase pathway through physical interaction with mitogen-activated protein (MAP) kinase kinases such as MEKK1 (17) and apoptosis-stimulating kinase 1 (19) rather than the activation of the NF-κB pathway.

Receptor-interacting protein (RIP) has been shown to interact with cell surface receptors containing death domains such as TNFR1, Fas, and TNF-related apoptosis-inducing ligand (TRAIL), and with cytoplasmic adaptor proteins containing death domains such as Fas-associated death domain and TNFR-associated death domain (20–24). RIP can also associate with proteins involved in the NF-κB signaling such as TRAF2, Epstein-Barr virus-latent membrane protein 1, p62, and IKKγ (25–28). It has been reported previously that RIP-deficient thymocyte cells were defective in TNF-α-induced NF-κB activation but not c-Jun N-terminal kinase activation (29). Although RIP possesses serine/threonine kinase activity, the biological significance of the kinase activity is not yet clearly understood, and the physiological substrates of RIP have not yet been identified. In the present study, we investigated the mechanism underlying the RIP-mediated IKK activation and understood the role of RIP in the NF-κB signaling pathway. We demonstrate here that MEKK1 is a substrate of RIP. The RIP-induced phosphorylation of MEKK1 enhances enzymatic activity of MEKK1, and it then contributes to the TNF-α-induced activation of NF-κB.
EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Mouse monoclonal anti-hemagglutinin (HA) antibody was purchased from Roche Molecular Biochemicals. Mouse monoclonal anti-FLAG antibody was purchased from Stratagene, and mouse monoclonal anti-Myc antibody was from Santa Cruz, Inc. Human recombinant TNF-α was bought from R&D Systems. Mouse monoclonal anti-RIP and anti-MEKK1 antibodies were purchased from Pharmingen. 32PO4, [γ-32P]ATP, and [35S]methionine were purchased from Amersham Pharmacia Biotech.

Vector Constructs and Transfection—pRK7-FLAG-IKKα, pRK5-FLAG-IKKβ, and pRK5-FLAG-IKKβ(K44A) were kindly provided by Dr. David Goeddel (Tularik Inc.). Fragments of the human RIP gene (20) were isolated by polymerase chain reaction and cloned into the BamHI/XhoI sites of pcDNA3-HA or pGEX-4T-1. Derivatives of MEKK1 were produced by polymerase chain reaction from pcDNA3-HA-MEKK1 (30) and cloned into the BamHI/EcoRI sites of the pCMV2-FLAG vector (Kodak) or the BamHI/SalI sites of pGEX-4T-1 (Amersham Pharmacia Biotech). Point mutations of MEKK1 were produced using the QuikChange site-directed mutagenesis kit (Stratagene). Vector constructs were transfected into human embryonic kidney 293 (HEK293) cells using LipofectAMINE (Life Technologies, Inc.) and RIP(-) Jurkat cells by electroporation at 0.4 kV and 960 microfarads using an electroporator (Bio-Rad).

Cell Culture and Metabolic Labeling using 32PO4—HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. Wild type and RIP(-) Jurkat cells (31) were grown in RPMI 1640 with 10% fetal bovine serum. Phosphorylation of MEKK1 in cells was examined as described previously (32). Briefly, wild type or RIP(-) Jurkat cells were incubated in phosphate-free Dulbecco’s modified Eagle’s medium containing 10% phosphate-free fetal bovine serum for 1 h and labeled with 100 μCi/ml carrier-free 32PO4. Cells were then treated with 20 ng/ml TNF-α or 80 J/m2 UV and incubated further for 10 or 30 min, respectively. Cells were lysed and subjected to immunoprecipitation using anti-MEKK1 anti-
body. The immunopellets were analyzed by 8% SDS-PAGE and autoradiography.

Luciferase Reporter Assay—To assess the transcription stimulating activity of NF-κB, pcDNA3-3X-NF-κB-Luc, and pcDNA3-β-galactosidase were cotransfected with various vector constructs into the cells, and luciferase activities in the transfected cells were measured using a luciferase assay kit (Promega). Luciferase activity was normalized relative to the coexpressed β-galactosidase activity.

Immunocomplex Kinase Assays—IKK activity was measured by the method of Regnier et al. (30). Briefly, cells were transfected with FLAG-IKKb, FLAG-IKKa, or other indicated constructs. Where indicated, the cells were treated for 20 min with 20 ng/ml recombinant human TNF-α at 48 h after the transfection. The cell lysates were subjected to immunoprecipitation using anti-FLAG antibody. IKK activity in the immunocomplexes was determined by incubating them at 30 °C for 30 min in a reaction buffer containing 20 mM HEPES (pH 7.5), 20 mM β-glycerophosphate, 10 mM MgCl₂, 100 mM NaVO₃, 2 mM dithiothreitol, 20 mM ATP, 100 μCi/ml [γ-³²P]ATP, and 2 μg GST-I-κBβ. Phosphorylation of GST-I-κBβ was analyzed by 10% SDS-PAGE followed by autoradiography. c-Jun N-terminal kinase 1 and MEKK1 activity was measured by a procedure described previously (34). c-Jun N-terminal kinase 1 and MEKK1 activity was monitored by following the phosphorylation of I-κB through IKK activation and the subsequent IKK-mediated ubiquitin-mediated degradation (36). To understand better the role of RIP in the regulation of the NF-κB pathway, we investigated the mechanism by which RIP mediates the TNF-α-induced activation of NF-κB (29). TNF-α induces NF-κB activation through IKK activation and the subsequent IKK-mediated phosphorylation of I-κB. Phosphorylated I-κB undergoes ubiquitin-mediated degradation (36). To understand better the role of RIP in the regulation of the NF-κB pathway, we investigated the mechanism by which RIP mediates the TNF-α-induced IKK activation. We transfected HEK293 cells with expression vectors producing HA-tagged RIP variants and FLAG-tagged IKKα or IKKβ. We then examined IKKα or IKKβ activity in the transfected cells (Fig. 1). Because overexpression of RIP alone can induce apoptotic cell death (22), a caspase inhibitor crmA was coexpressed to prevent RIP-induced apoptosis. Ectopic expression of RIP or its mutants did not affect IKKα activity (Fig. 1B). In contrast, the ectopic RIP induced an increase in the IKKβ activity in the transfected cells.

RESULTS

RIP Induces IKKβ Activation—The death domain serine/threonine kinase RIP plays a key role in the TNF-α-induced activation of NF-κB (29). TNF-α induces NF-κB activation through IKK activation and the subsequent IKK-mediated phosphorylation of I-κB. Phosphorylated I-κB undergoes ubiquitin-mediated degradation (36). To understand better the role of RIP in the regulation of the NF-κB pathway, we investigated the mechanism by which RIP mediates the TNF-α-induced IKK activation. We transfected HEK293 cells with expression vectors producing HA-tagged RIP variants and FLAG-tagged IKKα or IKKβ. We then examined IKKα or IKKβ activity in the transfected cells (Fig. 1). Because overexpression of RIP alone can induce apoptotic cell death (22), a caspase inhibitor crmA was coexpressed to prevent RIP-induced apoptosis. Ectopic expression of RIP or its mutants did not affect IKKα activity (Fig. 1B). In contrast, the ectopic RIP induced an increase in the IKKβ activity in the transfected cells.

MEKK1(K1253M) Blocks the RIP-induced Activation of IKKβ and NF-κB—IKKβ can be phosphorylated and activated by

Fig. 3. Physical association between RIP and MEKK1. Panel A, GST-fused MEKK1 fragments were incubated with in vitro translated ³²S-labeled RIP. The association of the ³²S-labeled RIP with GST-fused MEKK1 fragments was examined by GST pulldown assay followed by 10% SDS-PAGE and autoradiography. Panel B, in vitro translated ³²S-labeled MEKK1 was incubated with GST-fused RIP fragments. The binding between the ³²S-labeled MEKK1 and GST fusion proteins was examined by GST pulldown assay. Panels C and D, physical association between ectopic RIP and MEKK1 in cotransfected HEK293 cells. Cells were transfected with pCMV2-FLAG vector expressing MEKK1, MEKK1-M, or ΔMEKK1 and pcDNA3-HA vector expressing RIP, ID, or RIP-3ID along with pCMV-crmA as indicated. After 48 h of transfection, the cell lysates were subjected to immunoprecipitation (IP) using anti-HA (panel C) or anti-FLAG antibody (panel D). The resultant immunopellets were analyzed by immunoblot (IB) probed with anti-FLAG (panel C) or anti-HA antibody (panel D), respectively. Panel E, effect of TNF-α on the physical interaction between ectopic RIP and MEKK1 in cotransfected cells. A fixed amount of pcDNA3-HA-MEKK1, pRK5-FLAG-RIP, or pCMV-crmA was transfected into HEK293 cells with various amount of pcDNA3-HA-3ID. Cell lysates were immunoprecipitated with anti-FLAG antibody after 48 h of transfection. The immunopellets were analyzed by immunoblot probed with anti-HA antibody. Panel F, physical interaction between the two endogenous MEKK1 and RIP proteins in intact cells. HEK293 cells were left untreated or treated with 20 ng/ml TNF-α for 10 min. Cell lysates were subjected to immunoprecipitation using mouse monoclonal anti-RIP or anti-MEKK1 antibody, respectively. The immunopellets were subjected to SDS-PAGE and analyzed by immunoblot probed with anti-MEKK1 or anti-RIP antibody, respectively.
MEKK1(922–951); M2 Ser-994. In phosphorylates MEKK1 at Ser-957 and nopellets, which were obtained from TNF-
M2 M3 1021); M2, MEKK1(982–1021); M3, MEKK1(1022–1172); M3-1, MEKK1(922–951); M2-2, MEKK1(952–981); M2-3, MEKK1(982–1021).

The TNF-
RIP-induced activation of IKK
The RIP-induced stimulation of NF-
k phosphorylation was examined by 8% SDS-PAGE autoradiography. Panel B, RIP phosphorylates MEKK1. Panels C and D, RIP phosphorylates MEKK1 at Ser-957 and Ser-994. In panels B–D, HA-RIP immunoprecipitated, which were obtained from TNF-
Panels C–D. These data suggest that MEKK1 may mediate the RIP-induced activation of IKK\(\beta\). The involvement of MEKK1 in the RIP-induced activation of IKK\(\beta\) was supported further by data showing that the RIP-induced stimulation of NF-\(\kappa\)B activity was repressed by coexpression of MEKK1(K1253 M) at (Fig. 2C).

MEKK1 Associates Directly with RIP—To test whether RIP could physically associate with MEKK1, we carried out in vitro binding experiments using in vitro translated \(^{35}\)S-labeled RIP and recombinant GST-MEKK1 variants that were immobilized on glutathione-agarose beads (Fig. 3A). \(^{35}\)S-Laabeled RIP physically associated with GST-MEKK1-M (amino acids 821-1172), which contains the conserved Cdc42/Rac interactive binding motif (38). We also carried out in vitro binding analyses using \(^{35}\)S-labeled MEKK1 and recombinant GST-RIP fragments (Fig. 3B). The in vitro binding data indicate that the binding of RIP to MEKK1 occurred in the intermediate domain (ID) of RIP (Fig. 3B). Next, we examined whether RIP and MEKK1 would bind to each other in intact cells. HEK293 cells were cotransfected with full-length MEKK1-FLAG and HA-tagged RIP variants (Fig. 3C) or full-length HA-RIP and FLAG-tagged MEKK1 variants (Fig. 3D). The communoprecipitation results indicated that MEKK1 physically interacted with RIP or RIP-ID but not with RIP lacking ID (RIP-\(\Delta\)ID) (Fig. 3C). RIP bound to MEKK1 or MEKK1-M in intact cells (Fig. 3D). Furthermore, TNF-\(\alpha\) treatment increased the interaction between RIP and MEKK1 in the cotransfected cells (Fig. 3E). The TNF-\(\alpha\)-dependent increase in the interaction between RIP and MEKK1 was reduced by overexpressed RIP-\(\Delta\)ID. RIP-\(\Delta\)ID contains a C-terminal death domain, which is involved in the interaction with TNFR-associated death domain during TNF-\(\alpha\) signaling (20, 21, 27). We also examined the physical interaction between the two endogenous RIP and MEKK1 proteins in HEK293 cells. Immunoblot analysis of protein complexes immunoprecipitated with anti-MEKK1 antibody showed that endogenous RIP protein physically interacted with endogenous MEKK1 in intact cells and that this interaction was enhanced when the cells had been exposed to TNF-\(\alpha\) (Fig. 3F).

RIP Phosphorylates MEKK1 at Ser-957 and Ser-994—Our
data in Figs. 2 and 3 suggested that MEKK1 functioned as a downstream signal of RIP and that MEKK1 was physically associated with RIP in intact cells. We therefore examined whether RIP could phosphorylate MEKK1. We used MEKK1(K1253M) to avoid the basal autophosphorylation of MEKK1. RIP and MEKK1(K1253M) were immunoprecipitated from TNF-α-treated HEK293 cells, and in vitro phosphorylation of the immunopellets was tested (Fig. 4A). Interestingly, RIP enhanced the phosphorylation of MEKK1(K1253M), suggesting that RIP could phosphorylate MEKK1. The phosphorylation of MEKK1 by RIP was examined further using GST fusion proteins as substrates. RIP phosphorylated MEKK1(821–1172) but not NIK, IKKα, or TRAF2 (Fig. 4B). Next, we constructed various MEKK1 fragments to map the in vitro phosphorylation sites of MEKK1(821–1172) (Fig. 4C). The in vitro phosphorylation study showed that MEKK1 phosphorylation by RIP occurred at a site(s) between amino acids 922 and 1021. MEKK1(922–1021) was then divided further into three fragments to identify the phosphorylation site(s). RIP-dependent in vitro phosphorylation was observed in two fragments: amino acids 953–981 and 982–1021 (Fig. 4C). Next, we replaced the serine or threonine residues in the two fragments with alanine and tested whether the mutations would prevent any phosphorylation by RIP (Fig. 4D). Our data demonstrate that RIP-dependent phosphorylation was abolished by the mutations of MEKK1 at Ser-957 and Ser-994. These findings strongly suggest that RIP phosphorylates MEKK1 at Ser-957 and Ser-994.

MEKK1(957A/994A) Blocks the RIP-induced Activation of IKKβ and NF-κB—We next examined the effect of the RIP-mediated MEKK1 phosphorylation on the activation of IKKβ and NF-κB (Fig. 5, A and B). Overexpression of RIP, MEKK1, or both induced IKKβ activation in the transfected HEK293 cells. The forced expression of MEKK1(S957A/S994A) resulted in a complete inhibition of the RIP-induced IKKβ activation (Fig. 5A). Overexpressed MEKK1(S957A/S994A) also abolished RIP-induced NF-κB activation (Fig. 5B). MEKK1(S957A/S994A) expression also blocked RIP-induced c-Jun N-terminal kinase 1 activation in the transfected cells (data not shown).

Phosphorylation of MEKK1 at Ser-957 and Ser-994 Is Critical for RIP-induced MEKK1 Activation—We examined further the effect of the RIP-induced MEKK1 phosphorylation on the MEKK1 activity (Fig. 6). Ectopically expressed RIP induced a stimulation of the MEKK1 activity phosphorylating its substrates GST-IKKα(1–200) and GST-SEK1(1–200), whereas RIP(K45R) did not affect the MEKK1 activity (Fig. 6A). MEKK1(S957A/S994A) failed to phosphorylate the substrates regardless of the coexpression of RIP or RIP(K45R). The involvement of MEKK1 phosphorylation in RIP-induced MEKK1 activation is supported further by our results showing that TNF-α induced the stimulation of MEKK1, but not that of MEKK1(S957A/S994A) (Fig. 6B). These results suggest that the RIP-catalyzed MEKK1 phosphorylation at Ser-957 and Ser-994 is important for TNF-α-induced MEKK1 activation.

TFN-α-induced MEKK1 Activation Is Defective in RIP-deficient Jurkat Cells.—To understand better the mechanism of the RIP-induced MEKK1 activation, we examined the phosphorylation of MEKK1 and the TNF-α-stimulated MEKK1 activity in RPM− Jurkat cells (31) (Fig. 7). Our metabolic labeling study using 32P-O4 demonstrated that TNF-α induced an increase in MEKK1 phosphorylation in wild type Jurkat cells but not in RPM− Jurkat cells (Fig. 7A). In comparison, UV irradiation resulted in an increase in MEKK1 phosphorylation both in wild type and RIP− Jurkat cells. These results suggest that RIP mediates the TNF-α-induced, but not UV-induced, phosphorylation of endogenous MEKK1 in Jurkat cells. Furthermore, the phosphorylation of MEKK1 was recovered in RIP(−) Jurkat cells when the cells were transfected with a RIP construct (Fig. 7B). However, RIP did not increase the phosphorylation of MEKK1(S957A/S994A) in RIP(−) Jurkat cells. We also examined the effect of TNF-α on the endogenous MEKK1 activity in phosphorylating IKKα or SEK1 in wild type and RIP(−) Jurkat cells (Fig. 7C). The TNF-α treatment resulted in an increase in MEKK1 activity in wild type Jurkat cells but not in RIP(−) cells. Collectively, these data suggest that RIP is a major mediator of the TNF-α-induced activation of MEKK1.

DISCUSSION

The death domain Ser/Thr kinase RIP has been shown to be a factor required for TNF-α-induced NF-κB activation (20, 22). A study using thymocytes from RIP(−)−deficient mice has shown complete absence of TNF-α-induced NF-κB activation (29). RIP can activate IKKs through interaction with IKKγ/ NEMO, a regulatory subunit of the IKK complex (27, 28), suggesting that RIP activates the NF-κB signal pathway through the direct interaction with this IKK complex. However, the role of kinase activity of RIP in the TNF-α-induced signaling cascade has not yet been clarified. In the present study, we show that RIP can phosphorylate MEKK1 and that the RIP-mediated MEKK1 phosphorylation results in both IKKβ and NF-κB activation.

We show in this study that RIP induces the activation of IKKβ but not IKKα in transfected cells. The intermediate domain of RIP alone can also induce the IKKβ activation. The IKKβ activity in cells overexpressing wild type RIP appears to be higher than that of cells overexpressing the intermediate...
Phosphorylation of MEKK1 by RIP

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Fig. 7. TNF-α-induced phosphorylation and activation of MEKK1 is defective in RIP(−) Jurkat cells. Panel A, TNF-α-induced MEKK1 phosphorylation is defective in RIP(−) Jurkat cells. Either wild type (WT) or RIP(−) Jurkat cells were incubated in phosphate-free medium in the presence of [32P]Pi (150 μCi/ml, 1 h) and then treated with 20 ng/ml TNF-α for 10 min or with 80 J/m2 UV. The cells were subjected to immunoprecipitation using anti-MEKK1 antibody. The immunopellets were analyzed by SDS-PAGE and autoradiography. Panel B, ectopically expressed RIP increases MEKK1 phosphorylation at Ser-957 and Ser-994 in RIP(−) Jurkat cells. RIP(−) Jurkat cells were transfected with vector constructs expressing HA-MEKK1(K1253M, S957/994A) (MEKK1(KMSA)), HA-MEKK1(K1253M, S957/994A) (MEKK1(KMSA)), and Flag-RIP, as indicated. pCMV-crmA was included in all transfections. After 48 h of transfection, the cells were labeled metabolically with [32P]Pi and were treated with 20 ng/ml TNF-α for 10 min. Cell lysates were immunoprecipitated with anti-HA antibody. Phosphorylation of HA-MEKK1 was visualized by SDS-PAGE and autoradiography. Panel C, TNF-α fails to activate MEKK1 in RIP(−) Jurkat cells. Either wild type or RIP(−) Jurkat cells were treated with 20 ng/ml TNF-α for 10 min. Cell lysates were immunoprecipitated with anti-HA antibody. Phosphorylation of HA-MEKK1 was visualized by SDS-PAGE and autoradiography. Panel C, TNF-α fails to activate MEKK1 in RIP(−) Jurkat cells. Either wild type or RIP(−) Jurkat cells were treated with 20 ng/ml TNF-α for 10 min. Cell lysates were subjected to immunoprecipitation using anti-MEKK1 antibody. The resultant immunoprecipitates were measured for MEKK1 activity by immunocomplex kinase assay using GST-SEK1(K129R) as substrate.

domain only. It may be proposed, therefore, that the noncatalytic intermediate domain of RIP is involved in the RIP-induced IKKβ activation, but the kinase activity of RIP is required for maximal activation of IKKβ by RIP. Because the intermediate domain of RIP can associate with IKKy (27), the activation of IKKβ by the intermediate domain may result from the interaction of the intermediate domain with IKKy. In addition, RIP has been shown to oligomerize itself through the C-terminal death domain in the absence of TNF-α (20, 21, 39). The oligomerization of RIP might induce a partial activation of the downstream signaling components, whereas the kinase activity might be required for maximal activation. This may reconcile our results with previous reports stating that the kinase activity of RIP is not essential for RIP-induced NF-κB activation (20, 31).

We show here that MEKK1 mediates RIP-induced IKKβ activation. IKKβ can be phosphorylated and activated by NIK (16), MEKK1 (14), and Akt (40). MEKK1 preferentially activates IKKβ, whereas NIK activates IKKα more effectively (37). We demonstrated here that the kinase activity of RIP is required for the stimulation of MEKK1 but not for the stimulation of NIK. Involvement of MEKK1 in RIP-induced IKKβ activation is supported further by the data showing that the kinase-inactive mutant, MEKK1(K1253M) suppressed the RIP-induced activation of IKKβ.

Our data suggest that RIP induces stimulation of the MEKK1-mediated NF-κB signaling pathway via a direct interaction of RIP with MEKK1. It has been reported previously that both RIP and MEKK1 can interact with TRAF2 (17), suggesting a complex formation between MEKK1 and RIP through TRAF2. Our in vitro binding data, however, indicate that RIP can bind to MEKK1 without the participation of TRAF2. The intermediate domain of RIP is important for the interaction between RIP and MEKK1. As reported previously, the intermediate domain also seems to be involved in physical interactions with other proteins such as TRAF2, p62, Epstein-Barr virus-latent membrane protein 1, and IKKy (21, 25–27). It may be proposed, therefore, that the intermediate domain may be a docking site for the interactions of RIP with various signaling components and that the downstream signaling may be determined by which signaling component(s) is bound to the intermediate domain of RIP.

RIP can phosphorylate MEKK1 at Ser-957 and Ser-994, resulting in MEKK1 stimulation. Interestingly, a replacement of one serine residue with alanine diminishes the phosphorylation of the other serine residue in MEKK1 by RIP. These findings imply that there might be a cross-regulatory interaction between the Ser-957 and Ser-994 residues in MEKK1, although the detailed mechanism underlying the MEKK1 phosphorylation by RIP needs to be investigated further. Both of the phosphorylation sites are followed by a proline residue, suggesting S/T-P as a putative RIP phosphorylation site. This motif has been also observed in sites phosphorylated by cyclin-dependent kinase (41) and MAP kinase (42). It suggests that the properties of the kinase activity of RIP might resemble those of cyclin-dependent kinase or MAP kinase. Interestingly, it has been reported recently that RIP2, one of the RIP kinase family members, possesses MAP kinase kinase activity (43), although RIP2 failed to phosphorylate MEKK1 in our experiments (data not shown).

RIP has been shown to mediate TNF-α-induced signaling for apoptosis (22, 44) as well as for cell survival via NF-κB activation (29). RIP can function as a proapoptotic factor when it is cleaved by caspase-8 during TNF-α-induced apoptosis (45, 46). The proapoptotic C-terminal cleavage product of RIP in turn stimulates caspase-8 and caspace-3, although it attenuates the TNF-α-induced IKKβ activation (46). In addition, the caspase-8-cleaved RIP does not stimulate MEKK1 (data not shown). Recent studies suggest that MEKK1 is a negative regulator of apoptosis (47, 48), whereas the caspase-3-cleaved MEKK1 fragment becomes proapoptotic (49, 50). The cleavage of MEKK1 by caspase-3 separates the RIP-catalyzed phosphorylation sites (Ser-957 and Ser-994) from the C-terminal catalytic domain of MEKK1, which is a constitutively active form. Thus, the caspase-3-cleaved active form of MEKK1 cannot be phosphorylated by RIP. Therefore, the caspase-3-induced MEKK1 cleavage and the caspase-8-induced RIP cleavage may prevent RIP from stimulating MEKK1 activity for activation of the IKKβ-NF-κB pathway. Furthermore, overexpression of...
MEKK1(S857A/S994A) enhances TNF-induced apoptotic cell death in HEK293 cells (data not shown). Collectively, our findings and those of others suggest that the RIP-mediated MEKK1 phosphorylation may be important for the cell survival mechanism of the IKK-β-NF-κB pathway, whereas the caspase signaling cascades may terminate the IKK-β-NF-κB signaling by cleaving both RIP and MEKK1.

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