

The Kinase Activity of Rip1 Is Not Required for Tumor Necrosis Factor- α -induced I κ B Kinase or p38 MAP Kinase Activation or for the Ubiquitination of Rip1 by Traf2*

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The death domain kinase Rip1 is recruited to the tumor necrosis factor receptor type 1 and mediates the I κ B kinase and p38 MAP kinase pathways. In response to tumor necrosis factor- α (TNF- α), we find Rip1 phosphorylated and ubiquitinated, suggesting that Rip1 phosphorylation may stimulate its ubiquitination. To address the contribution of the kinase activity of Rip1 to its ubiquitination and to TNF- α signaling, we introduced wild type Rip1 and a kinase-inactive form of Rip1, Rip1D138N, into *rip1*^{-/-} murine embryonic fibroblast cells by retroviral infection. TNF- α -induced ubiquitination of Rip1 is observed in Rip1D138N cells, supporting the argument that Rip1 autophosphorylation is not required for Rip1 ubiquitination. TNF- α -induced I κ B and p38 MAP kinase activation is normal, and the Rip1D138N cells are resistant to TNF- α -induced cell death, indicating that the kinase activity of Rip1 is not required to mediate its antiapoptotic functions. In the absence of Traf2, TNF- α -induced ubiquitination of Rip1 is impaired, suggesting that Traf2 may be the E3 ubiquitin ligase responsible for the TNF- α -dependent, ubiquitination of Rip1. Finally, recruitment of the ubiquitinated Tak1 complex is dependent on the presence of Rip1, suggesting that Rip1 ubiquitination rather than its phosphorylation is critical in signaling.

The proinflammatory cytokine tumor necrosis factor- α (TNF- α)¹ is a major mediator of apoptosis as well as inflammation and immunity. It has also been implicated in the pathogenesis of several human diseases including inflammatory bowel disease, arthritis, sepsis, diabetes, and cancer (1). TNF- α activates the transcription factors NF κ B and activator protein-1 and exerts its effects by binding to two receptors, the tumor necrosis factor receptor type 1 (Tnfr1 or p55) and the Tnfr type 2 (Tnfr2 or p75) (2). Signaling from the activated Tnfr1 is mediated by the adapter proteins Traf2 and the death domain serine/threonine kinase Rip1 (3–5); yet, how Rip1 and/or Traf2 mediates

activation of the downstream kinases remains unclear.

Ubiquitination has been implicated in the regulation of the NF κ B pathway as well as in many other biological processes. Ubiquitin is a 76-amino acid protein that is highly conserved and, like phosphorylation, is essential for the degradation of proteins that respond to changes or stresses in the microenvironment (6). Phosphorylation can stimulate or inhibit ubiquitination by affecting either the target protein or the ubiquitin enzymes. Typically, ubiquitin forms a linkage with proteins via one or more lysine residues. *In vivo*, Lys-48 polyubiquitin chains lead to the recognition and degradation of proteins by a proteasome. In contrast, Lys-63 linkages are not associated with the proteasomal degradation but have been implicated in other biological processes, including activation of the I κ B kinase by the E3 ubiquitin ligase Traf6 (7, 8).

On TNF- α treatment, we found that Rip1 undergoes autophosphorylation and ubiquitination, suggesting that one or both of these posttranslational modifications may be important in I κ B or Mkk-6 activation. Studies in transfected cells suggest that the kinase activity of Rip1 is not required for TNF- α -induced I κ B or p38 MAP kinase activation (9, 10); however, transfection of kinase-inactive Rip1 results in its oligomerization with Traf proteins and the subsequent activation of these pathways (5, 10, 11).

To determine whether Rip1 autophosphorylation triggers its ubiquitination and to test whether Rip1 ubiquitination contributes to MAP kinase activation, we introduced wild type *rip1* and a kinase-inactive *rip1* into *rip1*-deficient murine embryonic fibroblasts (MEFs) and examined TNF- α -induced ubiquitination of Rip1 as well as activation of I κ B and p38 MAP kinase. In response to TNF- α , both Rip1 and the kinase-inactive form of Rip1 are ubiquitinated, although phosphorylated Rip1 may be a preferred E3 substrate. In addition, TNF- α -induced activation of I κ B and p38 MAP is not affected in cells expressing an inactive Rip1 kinase, demonstrating that Rip1 autophosphorylation is not required for signaling. Although it is recruited to the Tnfr1, Rip1 ubiquitination is not observed when Traf-deficient cells are stimulated with TNF- α . Taken together, these studies suggest that TNF- α -induced I κ B and Mkk-6 activation is mediated by the ubiquitination of Rip1 and the subsequent recruitment of a ubiquitinated Tak1 complex.

EXPERIMENTAL PROCEDURES

Preparation of Murine Embryonic Fibroblasts—Targeted disruption of the *rip1* loci has been described previously (12). Embryonic day-14 wild type *rip1*^{+/+} and *rip1*^{-/-} embryos were equilibrated overnight in trypsin at 4° C. Trypsin was activated by incubating at 37° C for 15 min. The embryos were then dissociated in Dulbecco's modified Eagle's medium (BioWhittaker) containing 10% fetal calf serum. MEFs were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone), 1% penicillin/streptomycin, and 1% L-glutamine (Invitrogen). Experiments were performed on cells between passage 2 and passage 5. Immortalized wild type and *rip1*^{-/-} 3T3 cell

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¹ The abbreviations used are: TNF, tumor necrosis factor; Tnfr1, TNF receptor type 1; mTNF, murine TNF; MAP, mitogen-activated protein; MEFs, murine embryonic fibroblasts; IL, interleukin; GFP, green fluorescent protein; Ab, antibody; IKK, I κ B kinase; Mkk, MAP kinase; E3, ubiquitin-protein isopeptide ligase; HA, hemagglutinin; IRES, internal ribosome entry site; MSCV, murine stem cell virus.

A.

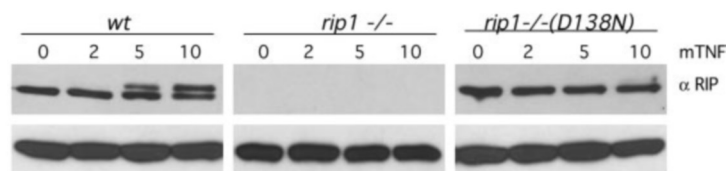
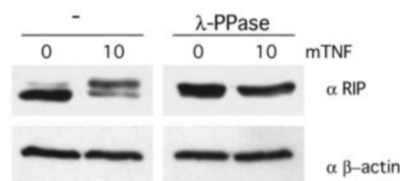
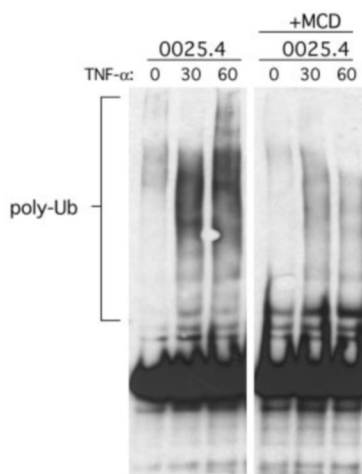


FIG. 1. Rip1 is phosphorylated and ubiquitinated in response to TNF- α . *A*, wild type (*wt*), *rip1*^{-/-}, or *rip1*^{-/(D138N)} cells were left untreated or stimulated with mTNF- α (10 ng/ml) for the indicated times. Cells were lysed, and 40 μ g of total protein was immunoblotted with α -RIP Ab (BD Biosciences). *B*, Rip1 is phosphorylated in response to TNF- α . Wild type cells were left untreated or stimulated with mTNF- α (10 ng/ml) (*left panel*) for 10 min. Cells were then lysed, and 40 μ g of total protein was treated with 10 units of λ -phosphatase (λ -PPase) (New England Biolabs) for 40 min at 30 °C. The lysates were then immunoblotted with α -RIP Ab. *C*, Rip1 is also ubiquitinated in response to TNF- α , and Rip1 ubiquitination is disrupted by pretreatment of cells with methyl- β -cyclodextrin (*MCD*), an agent that disrupts lipid rafts. Wild type MEFs were left untreated or treated with TNF- α for the time periods indicated. The cells were lysed, immunoprecipitated with α -RIP Ab, and immunoblotted with α -ubiquitin Ab. Cultures were also pretreated with methyl- β -cyclodextrin and either left untreated or stimulated with TNF- α and analyzed for the presence of ubiquitinated Rip1 proteins. *poly-Ub*, poly-ubiquitin.

B.



C.



lines were prepared according to established protocols. *traf2*-deficient and *traf2/traf5*^{-/-} MEFs were generously provided by Dr. Wen-chen Yeh (13) (Toronto University, Toronto, Ontario, Canada) and Dr. H. Nakano (Tokyo, Japan), respectively.

Plasmids and Constructs—The full-length RIP-Myc, Δ KIN-Myc, and Δ DD-Myc constructs and RIP-*K_D* have been described previously (9, 11). The HA-ubiquitin construct was provided by Dr. Zhijian James Chen (University of Texas, Southwestern, Dallas).

In Vitro Kinase Assay—Wild type or *rip1*^{-/-} cells infected with *rip1* or *rip1D138N* retrovirus were plated at 1×10^6 cells/10-cm plate. The cells were left untreated or treated with 10 ng/ml TNF- α for the time periods indicated. Cells were washed once with cold phosphate-buffered saline, and an *in vitro* kinase assay was performed as described previously (14).

Interleukin-6 Production Assays—Wild type, *rip1*^{-/-}, or *rip1*^{-/(D138N)} cells infected with *rip1* or *rip1D138N* retrovirus were plated at 3×10^4 cells/well on 24-well plates. The cells were left untreated or treated with 10 ng/ml TNF- α for 20 h. The supernatants were collected, and the levels of IL-6 produced from each cell line were assayed by an OptEIA mouse IL-6 enzyme-linked immunosorbent assay kit (BD Biosciences).

Co-immunoprecipitation and Western Blot Analysis—For all transfection studies, 293T cells were plated at 3×10^5 cells/plate on 6-well plates. The transfected cells were lysed in 0.2 ml E1A buffer (50 mM HEPES (pH 7.6), 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA), immunoprecipitated with α -RIP Ab (BD Biosciences), and immunoblotted with α -HA Ab. Expression of transfected constructs was confirmed by immunoblotting with an α -HA Ab. Rip1 proteins were detected by immunoblotting with an α -RIP1 antibody. To determine whether Rip1 ubiquitination occurs at the Tnfr1, wild type cells or cells expressing kinase-inactive Rip1 were left untreated or treated with mTNF- α for the time periods indicated. Cells were lysed in 0.5 ml of endogenous lysis buffer (0.5 M Tris (pH 7.6), 0.5 M NaCl, 0.1 M EDTA, 1% Triton X-100, 0.5 M NaF, and 0.5 M sodium pyrophosphate) supple-

mented with a protease mixture inhibitor (Roche Applied Science), immunoprecipitated with α -TNFR1 antibody, and then immunoblotted with α -RIP1, α -TAK1, or α -TRAF2 antibody (Santa Cruz Biotechnology). In other experiments, cells were lysed in radioimmune precipitation assay buffer and immunoprecipitated with α -RIP Ab, and ubiquitinated Rip1 proteins were detected by immunoblotting with an anti-ubiquitin antibody (Boston Biochem). To determine p38- α -MAP kinase activity, 40 μ g of total protein was immunoblotted with an α -phospho-p38 antibody (Cell Signaling Technology). To determine the relative expression levels of p38- α MAP kinase or Ikk, wild type *rip1*^{-/-} or *rip1*^{-/(D138N)} infected with *rip1* or *rip1D138N* retroviruses were lysed, and 30 μ g of total protein was immunoblotted with α -p38 α Ab (Santa Cruz Biotechnology) or with α -IKK- α antibody (Santa Cruz Biotechnology).

Cell Viability Assays—Briefly, 5×10^5 cells were plated overnight on 60-mm tissue culture dishes. Cells were then treated with 10 ng/ml mTNF and 250 ng/ml cycloheximide for 20 h. Cells were then collected and assayed either for cell viability by trypan blue staining or for cell death by annexin V/phosphatidylinositol staining on triplicate cultures.

RESULTS

Death Domain Kinase Rip1 Is Phosphorylated and Ubiquitinated on Tnfr1 Activation—Although biochemical and genetic studies have implicated the Rip1 kinase in TNF- α signaling (4, 9, 10, 12, 14), the mechanisms by which Rip1 mediates the NF κ B or p38 MAP kinase responses to TNF remain unclear. Both phosphorylation and, more recently, ubiquitination have been implicated in the activation of the IKK complex. To elucidate the role of Rip1 in the initiation of these pathways, wild type and *rip1*-deficient MEFs were left unstimulated or treated with TNF- α for 2, 5, and 10 min, and Rip1 protein was detected by immunoblotting. In the wild type MEF cells, we observed

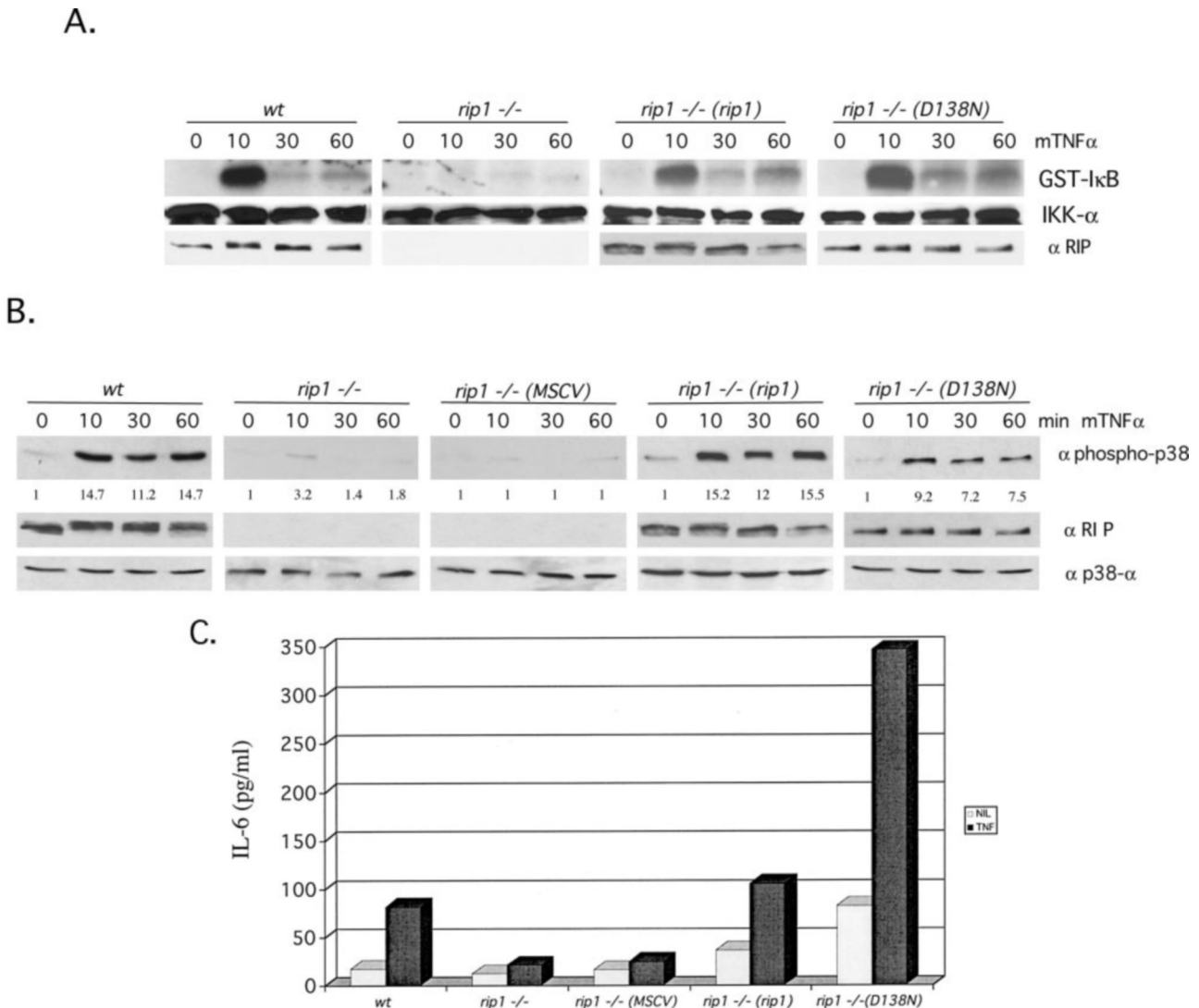


FIG. 2. The kinase activity of Rip1 is not essential for TNF-induced Ikk or p38 MAP kinase activation or for TNF- α -induced IL-6 production. A, TNF- α -induced Ikk activation is observed in cells expressing a kinase-inactive Rip1D138N. *rip1*^{-/-} MEFs were infected with vector alone (MSCV) or with retroviruses containing wild type Rip1 or a kinase-inactive Rip1 (*rip1*^{-/-}(D138N)). Wild type (*wt*), *rip1*^{-/-}, *rip1*^{-/-}(*rip1*), and *rip1*^{-/-}(D138N) were left untreated or stimulated with TNF- α for the time periods indicated. Cells were subsequently lysed and immunoprecipitated with α -IKK- α antibody, and the kinase activity of Ikk was measured by an *in vitro* kinase assay using glutathione S-transferase-IkB (*GST-IkB*) as a substrate. The amount of Ikk- α and Rip in each cell type was determined by immunoblotting with α -IKK- α Ab (Santa Cruz Biotechnology) and α -RIP Ab, respectively. B, normal p38 MAP kinase response to TNF in *rip1*^{-/-}(D138N) cells. Wild type, *rip1*^{-/-}, *rip1*^{-/-} (MSCV), *rip1*^{-/-} (*rip1*), and *rip1*^{-/-} (D138N) cells were left untreated or stimulated with TNF- α for the time periods indicated, and p38 MAP kinase activity was measured by immunoblotting with an α -phospho-p38 MAP kinase antibody (catalog no. 9211S, Cell Signaling Technology). The Rip1 and p38- α expression was determined by immunoblotting with anti-RIP and anti-p38- α antibodies. C, TNF- α -induced IL-6 production is restored in *rip1*^{-/-} cells infected with *rip1* or kinase-inactive *rip1* retrovirus. Wild type, *rip1*^{-/-} MEFs, *rip1*^{-/-} MEFs infected with vector (MSCV), *rip1* retrovirus (*rip1*^{-/-}(*rip1*)) or a kinase-inactive *rip1* retrovirus (*rip1*^{-/-}(D138N)) were plated at 3×10^4 cells/well on 24-well plates and left untreated or treated with 10 ng/ml TNF- α for 24 h. The supernatants were then analyzed for IL-6 levels using the OptEIA Mouse IL-6 enzyme-linked immunosorbent assay kit (BD Biosciences, catalog no. 2653KI). The amount of IL-6 is presented as the mean \pm S.D. of triplicate observations.

the presence of two forms of Rip1. On TNF- α stimulation, we observed induction of a slower, migrating form of Rip1. The presence of this form was abolished when the cell lysates were treated with λ -phosphatase, consistent with the idea that Tnfr1 activation stimulates Rip1 phosphorylation (Fig. 1, A and B). The kinetics of Rip1 phosphorylation with induction at 5 min after TNF- α stimulation suggest this is an early event in the TNF- α response and may function to initiate signaling.

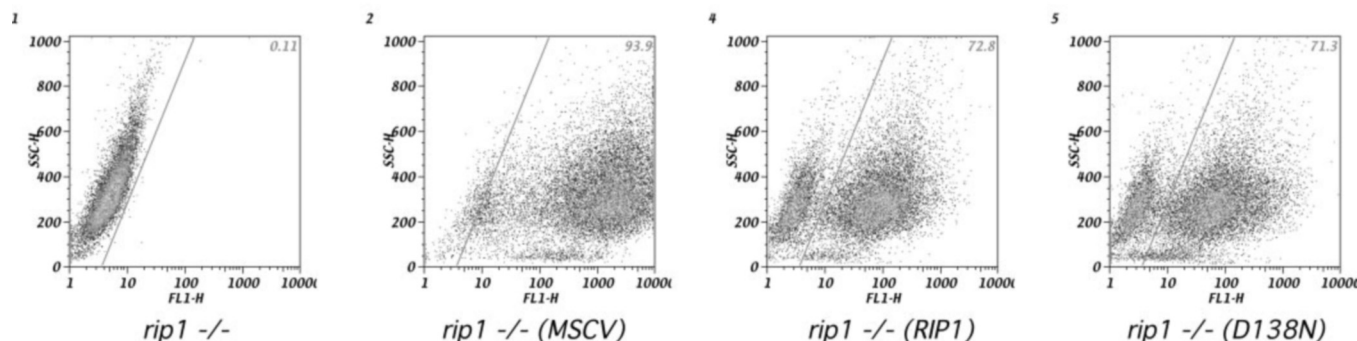
To examine whether Rip1 undergoes autophosphorylation or is phosphorylated by another kinase, we infected *rip1*^{-/-} MEFs with a kinase-inactive version of Rip1 (Rip1D138N) or with MSCV2.2-IRES-GFP retroviral vector alone (not shown). Cells expressing kinase-inactive Rip1 failed to undergo TNF- α -induced phosphorylation (Fig. 1A), revealing that Tnfr1 ac-

tivation stimulates Rip1 autophosphorylation.

In addition to phosphorylation and similar to published studies (15–17), the Rip1 kinase is polyubiquitinated when cells are stimulated with TNF- α (Fig. 1C). Moreover, Rip1 ubiquitination in response to TNF- α is inhibited by agents such as methyl- β -cyclodextrin that disrupt lipid rafts (Fig. 1C), suggesting that Rip1 ubiquitination occurs once the activated Tnfr1 is recruited into lipid rafts.

Kinase Activity of Rip1 Does Not Contribute to TNF- α -induced Ikk or p38 MAP Kinase Activation or TNF- α -induced IL-6 Production—The death domain kinase Rip1 undergoes two types of posttranslational modifications in response to TNF- α , but the precise functions of phosphorylated or ubiquitinated Rip1 in TNF signaling are unclear. We have previously

A.



B.

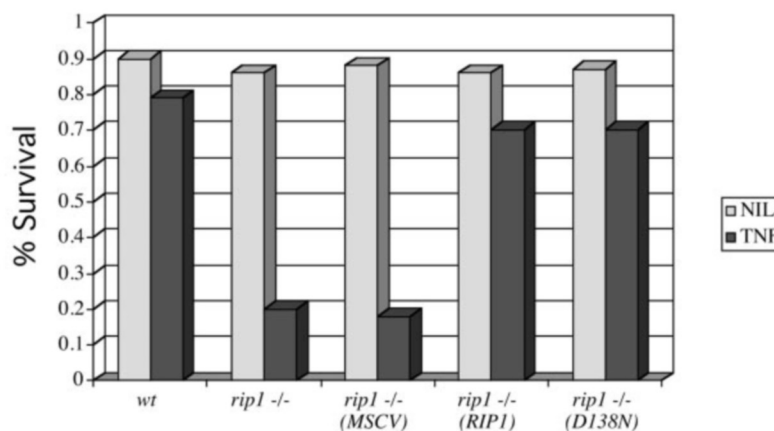


FIG. 3. The kinase activity of Rip1 is not required for the antiapoptotic response to TNF- α . A, *rip1*^{-/-} MEFs were left uninfected or were infected with vector only (*rip1*^{-/-}(MSCV)) or with a *rip1* (*rip1*^{-/-}(RIP1)) or kinase-inactive *rip1* (*rip1*^{-/-}(D138N)) retrovirus, and the percentage of GFP-positive cells was determined by flow cytometry. B, protection from TNF- α -induced cell death is observed in *rip1*^{-/-} MEFs infected with *rip1* or kinase-inactive *rip1* retroviruses. Wild type (wt), *rip1*^{-/-}, *rip1*^{-/-}(MSCV), *rip1*^{-/-}(rip1), and *rip1*^{-/-}(D138N) were left untreated (gray bars) or treated with mTNF- α (10 ng/ml) and cycloheximide (250 ng/ml) (filled bars) for 20 h. Cell viability was determined by trypan blue staining or by annexin V/phosphatidylinositol staining on triplicate cultures. The results are presented as the mean \pm S.D. of triplicate observations.

demonstrated that TNF- α -induced Ikk and p38 MAP kinase activation are both impaired in *rip1*-deficient MEFs (12, 14). To further examine the role of the kinase activity of Rip1 in TNF- α -induced Ikk and p38 MAP kinase activation, we infected *rip1*^{-/-} MEFs with MSCV2.2-IRES-GFP retroviruses that express wild type *rip1*, a kinase-inactive *rip1*D138N, or with the MSCV2.2-IRES-GFP retroviral vector alone. Cell lysates were prepared from wild type MEFs and from the infected *rip1*^{-/-} MEFs, and the Rip1 protein expression levels were compared. Importantly, *rip1*^{-/-} MEFs reconstituted with wild type *rip1* or kinase-inactive *rip1* expressed levels of Rip1 protein similar to those of wild type MEFs (Fig. 2), demonstrating that Rip1 overexpression and oligomerization are not induced in the infected cells.

Wild type, *rip1*^{-/-}, and *rip1*^{-/-} MEFs infected with vector alone, wild type *rip1*, or with the *rip1*D138N retrovirus were left untreated or were treated with mTNF- α for 10, 30, and 60 min. To measure TNF- α -induced Ikk activation, cell lysates were immunoprecipitated with anti-IKK α antibody, and an *in vitro* kinase assay was performed using glutathione S-transferase-IkB as the substrate. TNF- α -induced Ikk activation was not observed in cells lacking Rip1 (Fig. 2 and see Ref. 12), and TNF-induced NF κ B activity was restored in *rip1*^{-/-} cells reconstituted with wild type Rip1 or with a kinase-inactive version of Rip1 (Fig. 2A).

To measure TNF- α -induced p38 MAP kinase activity, cell

lysates were probed with a phospho-specific p38 MAP kinase antibody. TNF- α -induced p38 MAP kinase activation was not observed in *rip1*^{-/-} MEFs (as described in Ref. 14); however, TNF-induced p38 MAP kinase activity was observed in TNF- α -treated wild type MEFs and in *rip1*^{-/-} MEFs infected with the *rip1* retrovirus (Fig. 2B, *rip1*^{-/-}(*rip1*)) as well as the MEFs expressing the kinase-inactive version of Rip1.

The NF κ B and p38 MAP kinase pathways have been implicated in stress-induced cytokine production (18, 19), and TNF- α -induced IL-6 production is impaired in *Rip1*-deficient cells (14). To test whether the kinase activity of Rip1 contributes to TNF- α -induced cytokine production, we examined IL-6 production in response to TNF- α in the *rip1*^{-/-}(D138N) cells. Treatment of wild type cells with TNF- α resulted in increased production of IL-6, whereas little or no TNF- α -induced IL-6 production was observed in *rip1*^{-/-} cells (Fig. 2C and see Ref. 14). TNF- α -induced IL-6 production was restored in the *rip1*^{-/-} MEFs infected with wild type *rip1* or *rip1*D138N retroviruses (Fig. 2C). Although the *rip1*^{-/-}(D138N) cells exhibit a higher background of IL-6 production, a 4–5-fold increase in IL-6 production was observed when wild type MEFs or the *rip1*D138N-infected MEFs were treated with TNF- α . Consistent with the p38 MAP kinase and NF κ B responses to TNF observed in *rip1*D138N cells, these experiments suggest that the Rip1 kinase activity does not contribute to TNF- α -induced p38 MAP kinase or Ikk activation.

Kinase Activity of Rip1 Does Not Contribute to the Antiapoptotic Response to TNF- α —Previous transfection studies implicated the kinase activity of Rip1 in apoptosis and FasL-induced necrosis (11, 20). To examine whether the Rip1 kinase regulates programmed cell death in mammalian cells, we treated wild type and *rip1*^{-/-} MEFs with TNF- α and cycloheximide and quantitated the apoptotic cells. As expected, a *rip1* deficiency sensitizes cells to TNF- α -induced cell death, consistent with the impaired NF κ B response (Fig. 3B and see Ref. 12). The reintroduction of *rip1* into these cells is nearly completely protective, which provides survival to 70% of the cells and correlates with the fact that 72.8% of the cells are GFP-positive and express Rip1 (Fig. 3A). Cells expressing a kinase-inactive Rip1 remain resistant to TNF- α -induced cell death, presumably because of activation of NF κ B, indicating that kinase activity of Rip1 does not contribute to the antiapoptotic response to TNF- α (Fig. 2A).

Rip1 Phosphorylation Is Not Required for Its Ubiquitination—In the TNF- α -induced ubiquitination of the inhibitor I κ B α , phosphorylation stimulates recognition by the β -transducin repeat-containing protein/Skp1-Cul-F-box complex (21). To test whether Rip1 phosphorylation targets the protein for ubiquitination, cells were left untreated or stimulated with TNF- α for 30 or 60 min and immunoprecipitated with an α -RIP antibody, and then ubiquitinated Rip1 proteins were detected by immunoblotting with an anti-ubiquitin antibody (Fig. 4). Ubiquitinated Rip1 was induced by TNF- α stimulation, and polyubiquitinated Rip1 was also detected in cells expressing a kinase-inactive Rip1D138N only (Fig. 4). TNF- α -induced ubiquitination of Rip1 and Rip1D138N was inhibited when the cells were pretreated with methyl- β -cyclodextrin, indicating that Rip1 ubiquitination may occur within lipid rafts (Fig. 4). Thus, consistent with the signaling data (Fig. 2), kinase-inactive Rip1 is polyubiquitinated in response to TNF- α , revealing that Rip1 phosphorylation is not required for TNF- α -induced ubiquitination.

The previous experiments suggested that the ubiquitination of kinase-inactive Rip1D138N may be delayed in response to TNF- α , indicating that kinase-inactive Rip1D138N may be impaired in its ability to be recruited to the Tnfr1. To test this possibility, wild type and kinase-inactive Rip1D138N cells were treated with TNF- α , immunoprecipitated with an anti-TNFR1 Ab and then immunoblotted with an anti-RIP1 antibody. We observed a transient polyubiquitination of Rip at 10 min, co-incident with the activation of TNF- α -induced signaling (Fig. 5); however, Rip1 ubiquitination is transient, because polyubiquitinated Rip1 was not detected at the Tnfr1 at longer time points. Consistent with the signaling data (Fig. 2), Rip1 ubiquitination was also observed in TNF- α -treated cells expressing a kinase-inactive Rip1D138N (Fig. 5), indicating that the kinase-inactive Rip1 is recruited to the Tnfr1 and undergoes ubiquitination in response to TNF- α . Like I κ B proteins, Rip1 is phosphorylated in response to TNF- α , but Rip1 phosphorylation is not required for its ubiquitination at the Tnfr1.

Traf2 Ubiquitinates Rip1 at the Tnfr1—Although Rip1 ubiquitination has been observed by others (15–17), the role of Rip1 phosphorylation in its ubiquitination has not been addressed, nor has the E3 ubiquitin ligase for Rip1 been identified. Both Traf2 and Traf6 have been shown to act as E3 ubiquitin ligases and to undergo autoubiquitination through Lys-63-ubiquitin conjugations (7, 8). Moreover, TNF- α has been shown to stimulate Traf2 polyubiquitination and oligomerization (22).

To obtain genetic evidence that Traf proteins ubiquitinate Rip1 in response to TNF- α , we examined Rip1 ubiquitination in *traf2*^{-/-} and *traf2*^{-/-}/*traf5*^{-/-} MEFs. Three independent *traf2*-deficient cell lines were left untreated or stimulated with

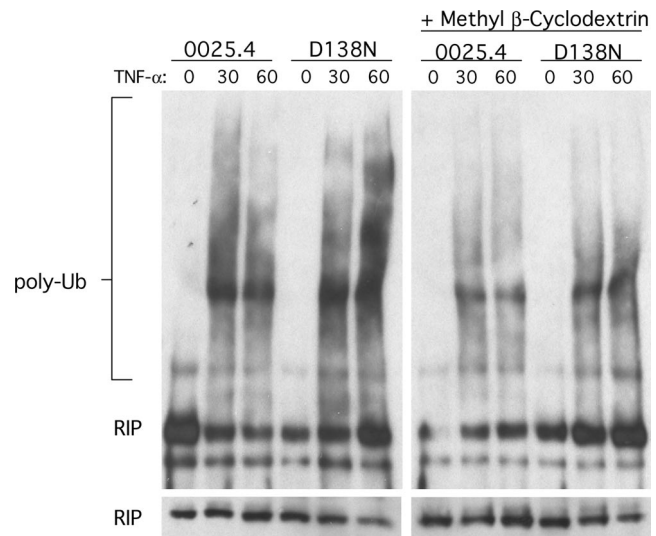


FIG. 4. Rip1 and kinase-inactive *rip1* are ubiquitinated in a TNF-dependent manner, and Rip1 and Rip1D138N ubiquitination is sensitive to agents that destroy lipid rafts. Wild type and *rip1*^{-/-}(D138N) cells were untreated or stimulated with mTNF- α (10 ng/ml) for the time periods indicated. The cells were lysed, immunoprecipitated with α -RIP Ab, and immunoblotted with α -ubiquitin Ab (poly-Ub). Separate cultures of wild type and *rip1*^{-/-}(D138N) cells were pretreated with methyl- β -cyclodextrin and then stimulated with TNF- α for various periods. The cells were lysed and analyzed for the presence of ubiquitinated forms of Rip1.

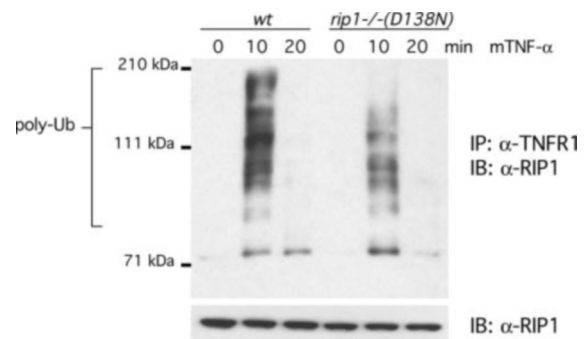


FIG. 5. Kinase-inactive Rip1 is recruited to the Tnfr1 and is ubiquitinated in response to TNF- α . Wild type and *rip1*^{-/-}(D138N) cells were left untreated or treated with TNF- α (10 ng/ml) for the indicated times. Cells were lysed and immunoprecipitated with α -TNFR1 Ab and immunoblotted with α -RIP antibody.

mTNF for 10 or 20 min, and the Tnfr1-associated signaling proteins were immunoprecipitated with an α -TNFR1 antibody. Immunoblotting with α -RIP antibody detected polyubiquitinated Rip1 in wild type MEFs treated with TNF- α for 10 min. By contrast, polyubiquitinated Rip1 was not detected in *traf2*^{-/-} or in *traf2*^{-/-}/*traf5*^{-/-} cells treated with TNF- α (Fig. 6A). To demonstrate more directly that polyubiquitinated Rip1 is not detected in Traf2-deficient cells, we stimulated wild type and *traf2*^{-/-} or *traf2*^{-/-}/*traf5*^{-/-} cells with TNF- α and immunoprecipitated the samples with the α -RIP antibody followed by immunoblotting with an anti-ubiquitin antibody. Polyubiquitinated Rip1 protein was readily detected in TNF- α -stimulated wild type cells but not in TNF- α -treated *traf2*^{-/-} or *traf2*^{-/-}/*traf5*^{-/-} cells (Fig. 6, B and C). Taken together, these data suggest that Traf2 may be the E3 ubiquitin ligase that initiates TNF- α signaling, potentially by ubiquitinating Rip1.

In the Absence of Rip1 a Ubiquitinated Tak1 Complex Is Not Recruited to the Tnfr1—These studies failed to reveal a role for

FIG. 6. TNF- α -induced ubiquitination of Rip1 is not observed in *traf2*^{-/-} or *traf2*^{-/-}/*traf5*^{-/-} cells. A, Wild type (*wt*), *traf2*^{-/-}, and *traf2*^{-/-}/*traf5*^{-/-} MEFs were left untreated or treated with TNF- α (10 ng/ml) for the indicated times. Cells were lysed, immunoprecipitated (IP) with α -TNFR1 Ab (BD Biosciences), and immunoblotted (IB) with an α -RIP antibody. B and C, wild type (*wt*), *traf2*^{-/-}, and *traf2*^{-/-}/*traf5*^{-/-} MEFs were stimulated with TNF- α for the time periods indicated, and ubiquitinated Rip1 was detected by immunoprecipitation with α -RIP antibody followed by immunoblotting with anti-ubiquitin antibody. Traf2 and Rip1 expression levels in *wt*, *traf2*^{-/-}, and *traf2*^{-/-}/*traf5*^{-/-} cells were examined by immunoblotting with α -TRAF2 and α -RIP antibodies.

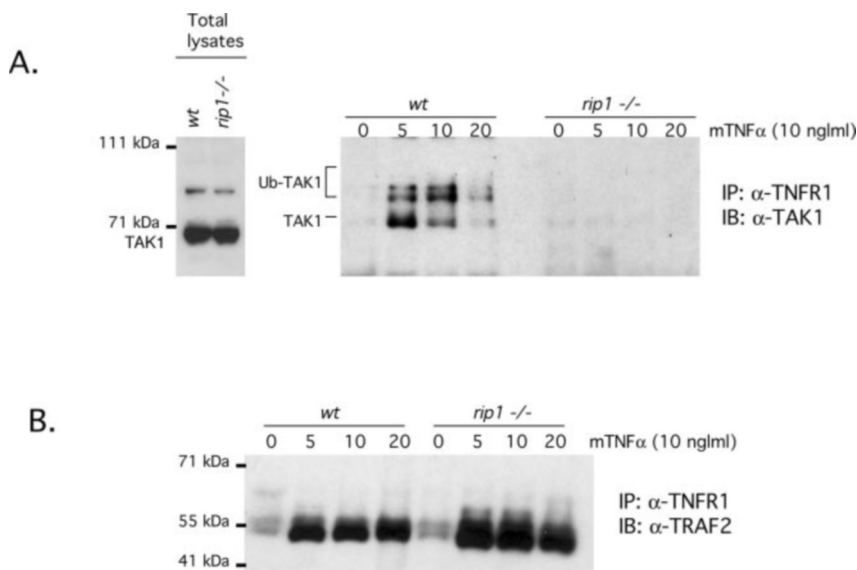
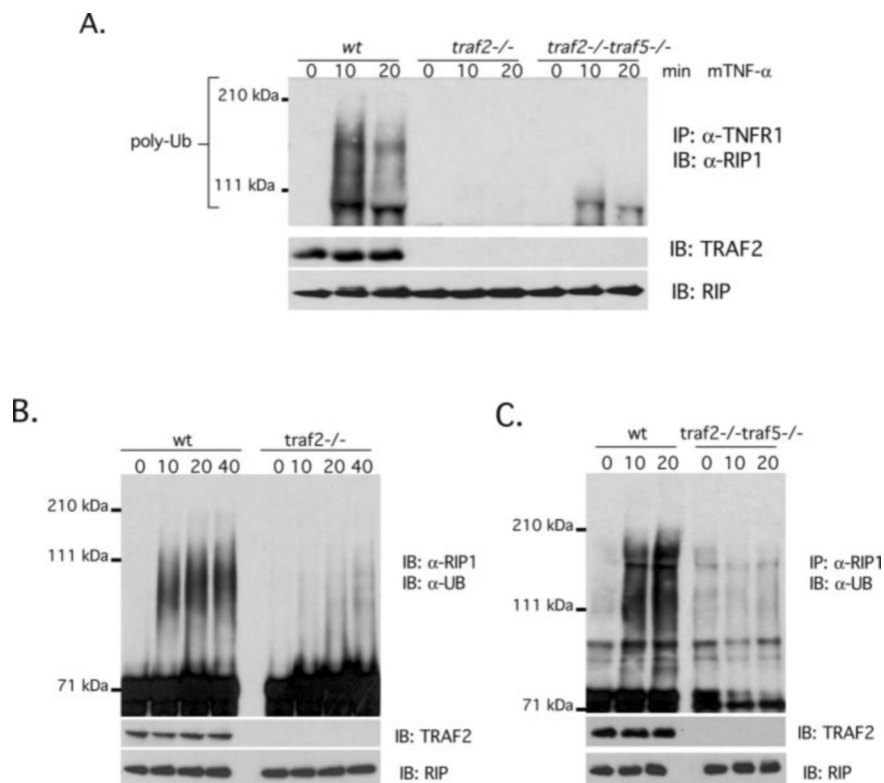


FIG. 7. The ability to recruit an ubiquitinated Tak1 complex in response to TNF- α is impaired in *rip1*^{-/-} cells. A, Rip1 mediates the recruitment of a ubiquitinated Tak1 (*Ub-TAK1*) complex to the activated Tnfr1. Wild type (*wt*) and *rip1*^{-/-} MEFs were left untreated or stimulated with TNF- α for the time periods indicated. Cells were then lysed and immunoprecipitated with an α -TNFR1 antibody, and associated proteins were detected by immunoblotting with an anti-Tak1 antibody. B, Rip1 is not required for the recruitment of a ubiquitinated Traf2 to the activated Tnfr1. Wild type and *rip1*^{-/-} MEFs were left untreated or stimulated with TNF- α for the time periods indicated. Cells were then lysed and immunoprecipitated with an α -TNFR1 antibody, and associated proteins were detected by immunoblotting with an α -TRAF2 antibody.

the Rip1 kinase in TNF- α signaling, but they suggest that ubiquitinated Rip1 may recruit other ubiquitinated proteins (kinases) capable of activating the Ikk complex or Mkk-6. To test whether ubiquitinated Rip1 recruits the Tak1 kinase complex, known to be activated by ubiquitination, to the Tnfr1 (8), we treated wild type cells and *rip1*^{-/-} cells with TNF- α for 5, 10, and 20 min and immunoprecipitated the Tnfr1-associated proteins. We observed the recruitment of a ubiquitinated Tak1 complex in wild type cells stimulated with TNF- α for 5 min (Fig. 7A). In contrast, in the absence of Rip1, TNF- α stimulation failed to induce recruitment of the Tak1 complex (Fig. 7A), suggesting that Rip1 and potentially ubiquitinated Rip1 are required for Tak1 recruitment to the Tnfr1. Traf2 recruitment, however, is unaffected by an absence of Rip1 and is readily recruited to the Tnfr1 when *rip1*^{-/-} MEFs are stimulated with TNF- α (Fig. 7B).

DISCUSSION

We demonstrate that the death domain kinase Rip1 undergoes autophosphorylation and ubiquitination as early as 2–5 min after TNF- α stimulation. Rip1 autophosphorylation occurs within the cytoplasm and appears to precede Rip1 ubiquitination at the Tnfr1. These inducible, posttranslational modifications are reminiscent of those observed for I κ B α and suggest that Rip1 autophosphorylation may trigger its ubiquitination; however, cells that express a kinase-inactive Rip1 undergo TNF- α -induced ubiquitination and respond normally to TNF- α . These data indicate that Rip1 autophosphorylation is not required for its recruitment to the activated Tnfr1 or for its ubiquitination in lipid rafts. Rip1 is required for the recruitment of a ubiquitinated Tak1 complex to the activated Tnfr1. Taken together, these studies suggest that Rip1 ubiquitination

rather than its phosphorylation leads to Ikk and Mkk-6 activation.

We provide genetic evidence that the E3 ubiquitin ligase Traf2 may be responsible for the ubiquitination of Rip1. Rip1 ubiquitination in response to TNF- α is not observed in cells lacking Traf2 or Traf 5 proteins. Although both proteins contain RING fingers, E3 ubiquitin ligase activity has been demonstrated only for Traf2 and Traf6 (8, 22). These findings suggest that Traf2 may regulate TNF- α signaling by ubiquitinating Rip1 and potentially other members of the Tnfr1 pathway. TNF- α -induced ubiquitination of Rip1 is transient, suggesting that Rip1 ubiquitination may be regulated by de-ubiquitinating enzymes such as Cyld (23, 24). Cyld overexpression can block NF κ B activation induced by Rip1 or Traf2 overexpression (24), suggesting that polyubiquitinated Rip1 may be a Cyld substrate *in vivo*.

Although Rip1 autophosphorylation is induced on TNF stimulation and the Rip1 serine/threonine kinase is recruited to the Tnfr1 on ligand binding, the kinase activity of Rip1 does not appear to participate in TNF- α signaling, nor is it required for down-regulation or termination of the TNF- α responses. Cells expressing a kinase-inactive Rip1 respond normally to TNF- α and terminate the NF κ B and p38 MAP kinase responses with kinetics similar to TNF- α -stimulated wild type cells. These data indicate that the kinase activity of Rip1 does not regulate the TNF- α pathway but may mediate signals from other receptor pathways. Recently, Rip1 has been implicated in the Trif-dependent, Toll-like receptor-3 antiviral response (25). Thus, the kinase activity of Rip1 may mediate Toll-like receptor-3 signals.

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