RIP2 Is a Raf1-activated Mitogen-activated Protein Kinase Kinase*

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**RIP2 is a serine-threonine kinase associated with the tumor necrosis factor (TNF) receptor complex and is implicated in the activation of NF-kB and cell death in mammalian cells. However, the function of its kinase domain is still enigmatic as it is not required in engaging these responses. Here we show that RIP2 activates the extracellular signal-regulated kinase (ERK) pathway and that the kinase activity of RIP2 appears to be important in this process. RIP2 activates AP-1 and serum response element regulated expression by inducing the activation of the Elk1 transcription factor. RIP2 directly phosphorylates and activates ERK2 in vitro and in vivo. RIP2 in turn is activated through its interaction with Ras-activated Raf1. Kinase-defective point and deletion variants of RIP2 also significantly blocked the activation of ERK2 by TNFα but not epidermal growth factor. These results describe a novel pathway of ERK activation and the first catalytic function ascribed to any of the RIP-like kinases associated with the TNF receptor superfamily.

The ERK/MAPK signaling pathway is critical for a number of biological processes including proliferation and differentiation (1, 2). The ERKs can be activated by a variety of ligands including growth factors such as EGF and cytokines such as TNFα (3–6). In response to EGF, Ras becomes activated by a guanylate exchange reaction, and GTP-Ras recruits Raf1 to the membrane where Raf1 is activated by mechanisms that may involve phosphorylation and conformational changes (7–9). Active Raf1 phosphorylates MEK1 which in turn phosphorylates and activates the ERKs (10). However, the mechanism by which TNFα activates the ERKs is still less well understood.

TNFα binds two distinct cell surface receptors, TNFR1 (55 kDa) or TNFR2 (75 kDa) (11). Whereas TNFR2 binds TNFα more tightly, TNFR1 is thought to be the predominant receptor that signals both cytotoxic and inflammatory responses. The cytoplasmic domains of these receptors do not possess any catalytic activity although they do contain binding sites for other receptor-associated proteins that mediate the signaling responses triggered by TNFα (12, 13). The intracellular mediators of the TNFα response include proteins characterized by “death domains” such as TRADD (14), FADD/MORT1 (15), RIP (16, 17), and RAIDD (18). These proteins bind a conserved 80-amino acid region found in the cytoplasmic domain of TNFR receptor family members and serve as adaptors to assemble a signaling complex that leads to apoptosis (19). Another group of receptor-interacting molecules includes the TNF receptor-associated factors (TRAFs; reviewed in Ref. 20). Upon TNFR-1 trimerization, TNFR-1 binds TRADD that recruits FADD, RIP, and TRAF2 (16). FADD initiates apoptosis by mediating the activation of caspase-8 (21–24). RIP appears to mediate NF-κB activation (25, 26), whereas TRAF2 activates JNK (27–29). Although RIP is an active serine-threonine kinase, the functional significance of its kinase activity is still unknown since it is not essential for NF-κB activation and apoptosis (16).

In addition to the activation of JNK and induction of apoptosis, TNFα also activates the ERK pathway. However, Raf1 activation, which is a key step in ERK activation by receptor tyrosine kinases, has been observed in response to TNFα only in some (4, 30–32) but not all reports (6, 33, 34). Recently, the TNF receptor has been found to interact with the adaptor protein Grb2 and the exchange factor SOS in response to TNFα (35). In other circumstances, these interactions lead to Ras and Raf1 activation, but in response to TNFα, it may not be sufficient for ERK activation. An alternative derives from the observation that TNFα induces neutral and acidic sphingomyelinases that convert sphingomyelin to the secondary messenger, ceramide (reviewed in Ref. 36). It has been reported that ceramide produced by neutral sphingomyelinase activates the ERK pathway through the sequential activation of ceramide-activated protein kinase and Raf1 (4, 31). In apparent contrast to these results, Muller et al. (33) demonstrated that ceramide directly binds Raf1, but this interaction leads to the formation of an inactive complex with GTP-Ras. Thus, whereas ERK activation is known to be a direct consequence of TNF stimulation by TNFR1 and TNFR2, the mediation through Raf1 is still uncertain.

Recently, three groups reported the isolation of a novel serine-threonine kinase RIP2 (also known as RICK or CARDIAC) (37–39). RIP2 is homologous to RIP and interacts with members of the TNFR-1, CD40, and Fas signaling complexes, in particular with TRAF1, TRAF5, TRAF6 (38), and TRAF2 (39). RIP2 contains an N-terminal kinase domain and a C-terminal caspase-recruiting domain (CARD) domain that binds CLARP, caspase-1, and caspase-8. RIP2 can activate NF-κB and can induce apoptosis, although none of these activities was shown to be dependent on the kinase activity of RIP2.

We independently isolated RIP2 and show here that RIP2 kinase activity directly activates ERK1/2 and appears to mediate TNF-dependent ERK activation. These results describe a novel pathway of ERK activation and the first catalytic function ascribed to any of the RIP-like kinases associated with the TNF receptor superfamily.

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‡ The abbreviations used are: ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; TNF, tumor necrosis factor; TNFR, TNF receptor; EGF, epidermal growth factor; TRAFs, TNF-receptor-associated factors; CARD, caspase-recruiting domain; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; MBP, myelin basic protein; MOPS, 4-morpholinepropanesulfonic acid; JNK, c-Jun N-terminal kinase; MEK, MAPK/ERK kinase; MAP, mitogen-activated protein; GST, glutathione S-transferase; SRE, serum response element; GFP, green fluorescent protein.
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MATERIALS AND METHODS

Cloning of RIP2—RIP2 was cloned from a human fetal lung cDNA library in pRK vector using standard hybridization methods. The oligonucleotides used for screening were designed from an EST sequence homologous to Raf1 which was obtained from the Incyte data base. An oligonucleotide encoding the FLAG epitope (DYKDDDK) was cloned in frame to the N terminus to generate FLAG-RIP2. FLAG- RIP2 expression was generated by in vitro translation (Stratagene) and confirmed by DNA sequencing and Western analysis.

Cell Culture and Transfections—COS-7 and 293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM t-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. CHO cells were maintained in F-12/Dulbecco’s modified Eagle’s medium (provided by C. Crowley, Genentech, Inc.) containing 5% fetal bovine serum (Hyclone), 2 mM t-glutamine, and 100 μg/ml streptomycin. Transient transfections were all carried out using Superfect (Qiagen) according to manufacturer’s instructions. For Pathdetect and Luciferase reporter assays, 2 × 10^5 cells on six-well (35-mm) dishes were used. For co-immunoprecipitations and kinase assays, 2 × 10^5 cells were seeded on 100-mm dishes.

Plasmids and Reagents—Expression plasmids pUSE-Ras, pUSE-RasL61Q, pUSE-ERaS, pUSE-Raf1, and pUSE-ERK2 were purchased from Upstate Biotechnology, Inc. Human recombinant TNFα and EGF were from R & D Systems. RIP2 deletion plasmids (311–541) and (455–541) were provided by J. McCarthy and V. M. Dixit (38). Raf1(K375M) was generated by subcloning Raf1 into pRK5D and performing in vitro mutagenesis using the Gene Engine System (Promega). Mutagenesis was confirmed by DNA sequencing and Western analysis.

Expression in vitro c-Jun and Elk1 phosphorylation was determined using the Pathdetect c-Jun and Elk1 Trans-Reporting Systems (Stratagene). Reporter plasmids pFR-Luc, pAP1-Luc, and pSRE-Luc were from Stratagene, and pRL-TK was from Promega. Firefly and Renilla luciferase assays were performed using the Dual-Luciferase Reporter assay system (Promega). The fold induction of luciferase expression is calculated by obtaining the ratios of firefly luciferase to Renilla luciferase expression for each gene and dividing the result with that of the vector control.

Co-immunoprecipitations and Western Analysis—Transiently transfected cells (100-mm dishes) were lysed in Co-IP buffer (200 mM Heps, pH 7.4, 1% Triton X-100, 100 mM NaCl, 2 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, and 2 mM orthovanadate) containing complete protease inhibitors (Roche Molecular Biochemicals). Samples were immunoprecipitated overnight at 4 °C with either anti-Raf1 agarose-conjugated antibodies (Santa Cruz Biotechnology) or anti-FLAG M2 affinity beads (IBI, Eastman Kodak Co.). Immunoprecipitates were washed three times in lysis buffer, resolved by SDS-PAGE, transferred onto a nitrocellulose membrane (Novex), and immunoblotted using rabbit polyclonal anti-FLAG (IBI Kodak), anti-Raf1, anti-Ras (Santa Cruz Biotechnology), anti-phosphoprotein antibodies (Zymed Laboratories Inc.), or anti-phospho MAP kinase (T202/Y212) antibody (Promega or NEB) and then visualized using the ECL system (Pierce). Unless otherwise indicated all results were reproduced at least two independent experiments.

Protein Kinase Assays—Transiently transfected cells were lysed and immunoprecipitated in RIPA buffer (phosphate-buffered saline containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor mixture) with rabbit polyclonal agarose-conjugated anti-ERK2 (Santa Cruz Biotechnology), anti-FLAG, or anti-Raf1 antibodies. Immunoprecipitates were washed three times with lysis buffer and once with kinase wash buffer (50 mM Tris-HCl, 15 mM MgCl2). Kinase assays were performed at 30 °C for 30 min in 30 μl of Assay Dilution Buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 6.5 mM MgCl2, and 5 μM ATP) (Upstate Biotechnology, Inc.) containing 20 μM protein kinase C inhibitor peptide, 2 μM protein kinase A inhibitor peptide, and 20 μM calmodulin inhibitor (compound R24571) and supplemented with 100 mM γ-32P-ATP (Amer sham Pharmacia Biotech). ERK2 activity was assayed by using 2.0 μg of MBP substrate (Upstate Biotechnology, Inc.). To assay for RIP2 activity, 0.5 μg of purified uninduced ERK2 (Upstate Biotechnology, Inc.) was added with MBP. To assay for Raf1 activity, 0.5 μg of purified uninduced MEK1, 0.5 μg of ERK2, and 2 μg of MBP were added to the reaction. Alternative substrates used include 1.0 μg of GST-ERK1 (K71A) (Upstate Biotechnology, Inc.) and 2.0 μg of poly-His-MEK1 (Santa Cruz Biotechnology). Stress-activated protein kinase/Jun NH2-terminal kinase activity was assayed using materials and protocols provided by Bio-Rad. Unless otherwise indicated, all results were reproduced in at least two independent experiments.

RESULTS

Isolation of RIP2—In a search for additional kinases that could be implicated in the MAP kinase pathway, we searched EST data bases and identified an open reading frame that has significant homology to the kinase domain of Raf1. By using the EST sequence, we isolated a novel full-length human cDNA that contains a 1623-base pair open reading frame, encodes 540 amino acid residues, and has a predicted molecular mass of about 60 kDa. While this work was in preparation, the cloning and pro-apoptotic activity of this kinase was reported by three other groups (37–39). In accordance with the earlier published nomenclature and because of its extensive homology to both the kinase and intermediate domains of RIP (21% identity), we refer to the kinase we report here as RIP2.

In addition to having high homology with the RAF family of kinases (24% identity), the kinase domain of RIP2 also contains two TXY motifs (amino acid residues 95–97 and 111–113) which are conserved in the ERK family of kinases but are not found in RIP. All MAPKs contain signature TXY motifs within the activating phosphorylation site, and the X residue distinguishes the three subfamilies of MAPKs (ERK, JNK, and p38) (40). We therefore determined whether RIP2 can activate any of the MAP kinase pathways.

Activation of ERK but Not the JNK Pathway by RIP2—We examined whether expression of RIP2 in a heterologous system can stimulate the transcription of a luciferase reporter controlled by promoters containing either AP-1 or SRE elements. The AP-1 complex is composed of members of the c-Jun and c-Fos families and is regulated by the phosphorylation of c-Jun by the JNK kinase and c-Fos by c-Fos-regulating kinase (41). The SRE element is found in the promoter region of c-Fos and is activated by the transcription factor Elk1, a ternary complex factor protein that binds SRE through direct DNA interaction and cooperation with the serum response factor. Elk1 can be phosphorylated and activated by either ERK or JNK kinases.

Fig. 1A shows that RIP2 can activate both the AP-1 and SRE promoters. Expression of a kinase-deficient variant D146A, which eliminates the conserved catalytic base in the kinase subdomain VI, has a significantly reduced activity in both assays.

RIP2 expression may increase AP-1 activity by directly activating the JNK pathway. Alternatively, RIP2 can activate SRE promoters, AP-1 may be activated indirectly by the increased expression of c-Fos through the activation of the ERK pathway. To distinguish between these possibilities, we examined whether RIP2 could activate Elk1 or c-Jun in vivo. We measured the ability of Elk1 or c-Jun (expressed as GAL4 fusion proteins) to activate a GAL4-regulated reporter in response to the increased expression of RIP2. Fig. 1B shows that the expression of RIP2 led to the activation of Elk1 but not c-Jun indicating that RIP2 directly activates the ERK but not the JNK pathway. The extent of Elk1 activation is dependent on the level of RIP2 expression (Fig. 1C) and also on the kinase activity of RIP2 (Fig. 1D). The residual Elk1 activation resulting from increased expression of RIP2 (D146A) may be due in part to contributions made by other regions of RIP2 (see Fig. 7B).

RIP2 Phosphorylates and Activates ERK Directly—to determine how RIP2 may specifically activate Elk1, we examined whether RIP2 can activate and phosphorylate any of the major components of the ERK signaling pathway. Fig. 2A shows that RIP2 activated exogenously added ERK2 to phosphorylate MBP in vitro. Similar results were obtained with the addition of ERK1 (data not shown). No significant increase in MBP phosphorylation was observed subsequent to the addition of MEK1, suggesting that RIP2 activated ERK2 directly. In the
FIG. 1. Expression of RIP2 activates ERK. A, RIP2 expression activates AP-1 and SRE-regulated gene expression. CHO cells were transfected with 1.0 μg of FLAG-RIP2, FLAG-RIP2(D146A), or empty vector together with 0.5 μg of either pAP1-Luc (AP1-luciferase) or pSRE-Luc (SRE-luciferase) reporter plasmids and 0.1 μg of pRL-TK (Renilla luciferase) as an internal control for gene expression. Plasmids expressing constitutively active MEKK (360–672) (pFC-MEKK) and MEK1 (S218E/S222E, D32–51) (pFC-MEK1) were used as positive controls. Bar graphs represent fold induction of luciferase expression (see “Experimental Procedures”) and represent the mean ± S.E. of duplicate experiments. B, RIP2 expression leads to the phosphorylation of Elk1 in vivo. CHO cells were transfected with 1.0 μg of FLAG-RIP2 or control vector together with 1.0 μg of pFR-Luc (GAL4-luciferase) and 50 ng of either pFA-Elk1 (GAL4-Elk1) or pFA-c-Jun (GAL4-c-Jun). pFC-MEK1 and pFC-MEKK were used as positive controls for Elk1 and c-Jun-mediated activation, respectively. pGAL4(dbd) (expressing only GAL4 DNA binding domain [dbd]) was used to control for GAL4-independent gene expression. The amount of DNA was adjusted equally using a GFP plasmid. A total of 0.75 μg of vector, FLAG-RIP2, or control vector together with 1.0 μg of pAP1-Luc (AP1-luciferase) or pFC-MEK1 were used as negative and positive activator controls, respectively. The relative luciferase units represent the mean ± S.E. of triplicate experiments. C, RIP2-mediated Elk1 phosphorylation is dose-dependent. Indicated amounts of a FLAG-RIP2 expression vector were transfected in CHO cells together with 0.05 μg of GAL4-Elk1 and 1.0 μg of GAL4-luciferase reporter plasmid adjusted to a total amount of 2.0 μg with a GFP plasmid. A total of 0.75 μg of vector and 0.05 μg of pFC-MEK1 were used as negative and positive activator controls, respectively. The relative luciferase units represent the mean ± S.E. of duplicate experiments. D, RIP2-mediated Elk1 phosphorylation is dependent on the kinase activity of RIP2. A similar assay described in C was performed but using 1.0 μg of vector, FLAG-RIP2, or FLAG-RIP2(D146A) as activators. Each bar graph represent the mean ± S.E. of duplicate experiments. Western blot shows the total amount RIP2 immunoprecipitated from the cell lysates using anti-FLAG antibody.

control experiment, Raf1 induced the phosphorylation of MBP in vitro only with the addition and subsequent activation of purified unactivated MEK1 and ERK2. RIP2 was also able to phosphorylate ERK2 directly (Fig. 2A, middle panel) but not MEK1 (data not shown but see Fig. 6D). The activation and phosphorylation of ERK2 appears to be mediated directly by the kinase activity of RIP2 since the RIP2(D146A) mutant failed to phosphorylate or activate ERK2 in a similar assay (Fig. 2B). Western analysis of ERK2 with an antibody raised specifically against phospho-p44/42 MAP kinase (Thr-202/Tyr-212) revealed that RIP2, but not the D146A mutant, phosphorylated ERK2 on the activating TEF sites in vivo (Fig. 2C). Furthermore, RIP2 was able to phosphorylate a kinase-deficient GST-ERK1(K71A) in vitro also in a manner dependent on the kinase activity of RIP2, suggesting that that increased phosphorylation of ERK was not due to its enhanced autophosphorylation activity (Fig. 2D). These results indicate that RIP2 is an ERK kinase that can directly phosphorylate and activate ERK1 and ERK2.

FIG. 2. RIP2 phosphorylates and activates ERK2 in vitro. A, comparison of the kinase activity and substrate specificity between RIP2 and Raf1. 293 cells were transfected with empty vector or with plasmids expressing either FLAG-RIP2 or Raf1 as indicated. After 24 h, RIP2 and Raf1 were immunoprecipitated (IP) using anti-FLAG and anti-Raf1 antibodies, respectively, and then subjected to in vitro kinase assays using [γ-32P]ATP and with the addition of purified unactivated MEK1 and/or ERK2, and MBP substrate as indicated. Top panel shows MBP phosphorylation, and the middle panel shows phosphorylation of ERK2. Bottom panel indicates the total amounts of FLAG-RIP2 and Raf1 determined by Western blotting. B, ERK2 activation is dependent on the kinase activity of RIP2. 293 cells were transfected with empty vector or with plasmids expressing FLAG-RIP2 or FLAG-RIP2(D146A). After 24 h, RIP2 was immunoprecipitated from cell lysates with anti-FLAG antibody and subjected to a kinase assay with [γ-32P]ATP and with the addition of purified unactivated ERK2 and MBP. Top panel shows MBP phosphorylation, and the middle panel shows ERK2 phosphorylation. Bottom panel indicates the total amounts of FLAG-RIP2 and Raf1 determined by Western blotting. Similar results were obtained using COS cells. C, RIP2 phosphorylates ERK2 on the activating TEF sites. 293 cells were transfected as in B. ERK2 was immunoprecipitated using anti-ERK2 antibody and immunoblotted with a phospho-specific p44/42 MAP kinase (Thr-202/Tyr-212) antibody (top panel) and subsequently with ERK2 antibody (bottom panel). Similar results were obtained using COS cells. D, RIP2 phosphorylates ERK1(K71A) in vitro. 293 cells were transfected with 5.0 μg of empty vector or with plasmids expressing FLAG-RIP2 or FLAG-RIP2(D146A). After 24 h, RIP2 was immunoprecipitated from cell lysates with anti-FLAG antibody and subjected to a kinase assay with [γ-32P]ATP using GST-ERK1(K71A) as substrate (top) or to Western blot analysis with anti-FLAG antibody (bottom).
(Fig. 3A) or with Raf1 (Fig. 3B) further induced ERK2 activity. The cooperative interaction between RIP2 and Ras or between RIP2 and Raf1 suggests an activating interaction between these molecules. Since RIP2 activates ERK directly, Ras and Raf1 could possibly play a role in enhancing RIP2 activity toward ERK.

**RIP2 Interacts Directly with Raf1 in a Manner Enhanced by GTP-Ras**—The cooperative interactions between RIP2 and Ras or Raf1 suggest the possibility that RIP2 could physically interact with either of these molecules. Transient transfection and co-immunoprecipitation using anti-Raf1 antibodies revealed that Raf1 interacted with FLAG-RIP2 and RIP2(D146A) in vivo (Fig. 4A). The reduction in the amount of RIP2(D146A) that co-immunoprecipitated with Raf1 (as compared with RIP2) in Fig. 4A may reflect a requirement for this region of the molecule for full interaction with Raf1. The specificity of this interaction was also confirmed by immunoprecipitating RIP2 using FLAG beads and performing Western analysis with Raf1 antibody (data not shown). Furthermore, Raf1 was found to interact only with FLAG-RIP2 but not with an unrelated protein FLAG-Nsp1 (42) (Fig. 4B). The binding of RIP2 to Raf1 was enhanced by the co-expression of constitutively active Ha-Ras (GTP-Ras), which contains an activating substitution (L61Q) that was previously shown to increase nucleotide exchange (GTP for GDP) as well as decrease GTP hydrolysis in Ras (43). As shown in Fig. 4C, the amount of RIP2 present in Raf1 immunoprecipitates increased with the amount of GTP-Ras that coincidently interacted with Raf1. A conformational change in Raf1 as a consequence of its interaction with GTP-Ras (44) may induce the increased affinity for RIP2. Conversely, increased expression of the dominant negative RasN17 decreased the amount of RIP2 that interacted with Raf1 (Fig. 4D) consistent with the possibility that an active Raf1 is required to bind RIP2. The presence of RIP2 in the Ras-Raf1-RIP2 complex does not appear to be mediated by a Ras-RIP2 interaction as we could not detect any direct interaction between RIP2 and Ras by co-immunoprecipitation (data not shown).

**Ras-activated Raf1 Phosphorylates and Activates RIP2**—The Raf1-RIP2 interaction led us to examine whether Raf1 could directly phosphorylate and activate RIP2. First, we determined whether co-expression and co-immunoprecipitation of Raf1 with the kinase-inactive RIP2(D146A) would induce the phosphorylation of RIP2(D146A) in vitro when Raf1 is activated by GTP-Ras. Raf1 activation achieved through co-expression with increasing amounts of GTP-Ras led to an increased phosphorylation of RIP2(D146A) (Fig. 5A, panel a, lanes 3–5). The phosphorylation of RIP2(D146A) appears to be due to the kinase activity of Raf1 itself since the amount of phosphorylation associated with RIP2(D146A) was significantly reduced when the kinase-inactive Raf1(K375M) was used (Fig. 5A, panel d).

We also determined whether increasing Raf1 activation enhances the ability of RIP2 to activate ERK in vitro.Transiently expressed FLAG-RIP2 and Raf1 were co-immunoprecipitated in the presence or absence of GTP-Ras and assayed for ERK2 activation. As shown on Fig. 5B, Raf1 expression and activation by GTP-Ras further increased ERK2 activation by RIP2. This effect also appears to be mediated directly by the kinase activity of Raf1 since expression of Raf1(K375M) was significantly less effective in activating RIP2. Expression of GTP-Ras in the absence of Raf1 did not directly lead to an
increase in RIP2 activity (Fig. 5C) nor RIP2 expression (data not shown). These results therefore suggest that Raf1, when activated by GTP-Ras, can phosphorylate RFP2 and can further stimulate it to activate ERK2.

RIP2 Does Not Phosphorylate or Activate Raf1—Despite the interaction with Raf1, RIP2 does not appear to phosphorylate Raf1. Fig. 6A shows that transiently expressed Raf1 does not possess any discernible autophosphorylation activity (lane 2), but it becomes phosphorylated when constitutively active Ras L61Q (GTP-Ras) is co-expressed (lane 7). Although RIP2 appears to phosphorylate itself in Raf1 immunoprecipitates, increased binding of RIP2 to Raf1 did not cause any concomitant increase in the phosphorylation of Raf1 in vitro (lanes 3–6).

The failure to phosphorylate Raf1 suggests that RIP2 does not activate Raf1. To examine this, we assayed Raf1 immunoprecipitates containing increasing amounts of RIP2 for their ability to activate exogenously added ERK2 in the presence (Fig. 6B) or absence (Fig. 6C) of MEK1. There was significant phosphorylation of MBP by Raf1 only after Raf1 was activated by GTP-Ras and when assayed in the presence of both MEK1 and ERK2 (compare lanes 2 and 7 in Fig. 6, B and C). In contrast, increased binding of RIP2 did not increase phosphorylation of Raf1 (Fig. 6A) or the activation of Raf1 toward MEK1 (Fig. 6D). The slight increase in MBP phosphorylation detected with increasing RIP2 in Fig. 6, B and C, can be accounted for by the increase in the amount of RIP2 that co-immunoprecipitated with Raf1 and that activated ERK2 directly. That Raf1 is not activated is most clearly seen in Fig. 6D where the Raf1 immunoprecipitates, in the presence of RIP2 but absence of GTP-Ras, have no detectable MEK1 phosphorylation activity. These results therefore suggest that RIP2 does not activate Raf1.

Kinase-deficient RIP2 Mutants Block the Activation of ERK2 by TNFα—RIP2 was earlier reported to interact with the TRAF1 and TRAF2 adaptor molecules that associate with members of the TNF receptor superfamily (38, 39). TRAF2, in particular, has been shown to signal the activation of the JNK pathway from both TNFR1 and TNFR2 (28). Results presented above indicate that RIP2 activates and interacts with members of the ERK pathway. As TNFα also activates ERK (4–6), it is possible that RIP2 could be involved in the activation of ERK by TNFα.

To examine this, we expressed various kinase-deficient point and deletion mutants of RIP2 (Fig. 7A) and determined whether these would block the activating phosphorylation of ERK2 by TNFα through Western analysis using a phosphospecific p44/42 MAP kinase (Thr-202/Tyr-212) antibody. To determine specificity, we also examined whether the same RIP2 mutants could block the activation of ERK2 by EGF or of c-Jun by TNF. Fig. 7B shows that stimulation of the cells by TNFα or EGF led to an increase in the activation or phosphorylation of ERK2 or c-Jun in vivo (lanes 1 and 2). Expression of the different RIP2 variants alone did not induce ERK2 activation in unstimulated cells (lanes 3, 7, and 11). However, the increased expression of these variants significantly reduced the activation of ERK2 upon TNF stimulation (upper panel) but had only minimal effect on activation induced by EGF (middle panel). In addition, the dominant negative effects of these RIP2 mutants were only observed for TNF-induced ERK activation.
RIP2 is a Raf1-activated ERK Kinase—RIP2 was originally isolated based on its homology to CARD and was shown to associate with members of the TNFR-1, CD40, and Fas signaling complexes and to induce NF-κB, JNK, and apoptosis. Like RIP, the induction of these responses by RIP2 does not appear to involve its kinase activity as kinase-inactive variants of RIP2 are still capable of engaging these pathways (38, 39).

We independently isolated RIP2 and have demonstrated that RIP2 functions as a novel activating MAPK kinase that may modulate ERK signaling by TNFα. First, RIP2 expression induces SRE-regulated gene expression (Fig. 1A), Elk1 activation (Fig. 1D), and ERK phosphorylation and activation (Fig. 2). All of these activities were significantly reduced when a kinase-inactive RIP2(D146A) variant was used. Second, RIP2 interacts with components of the ERK signaling pathway (Fig. 4) and cooperates with these molecules in promoting the activation of ERK2 (Fig. 3). Third, RIP2 mutants lacking a functional kinase activity can significantly reduce the activation of ERK2 by TNFα (Fig. 7).

Despite having a high sequence similarity to the kinase domain of Raf1, RIP2 apparently behaves more like MEK1. RIP2 binds to and is activated by Ras-activated Raf1 (Fig. 5), and RIP2 appears to phosphorylate and activate ERK1 and ERK2 directly (Fig. 2). In contrast, RIP2 does not phosphorylate or activate Raf1 (Fig. 6) or MEK1 (Fig. 2A). Although RIP2 possesses an active kinase activity even in the absence of any exogenous stimuli, it can be further activated by Ras-activated Raf1 (Fig. 5). This activation is also reflected in the association between Raf1 and RIP2 which is enhanced by activated Ras (Fig. 4C) and is diminished by a dominant negative mutant of Ras (Fig. 4D).

RIP2 in TNF Signaling to ERK—Although it is clear that TNFα can activate the ERK pathway, the role of Raf1 in this process remains controversial (33). Yao et al. (31) have proposed that the secondary messenger ceramide, produced by TNF stimulation, can activate ceramide-activated protein kinase to phosphorylate and activate Raf1 directly. More recent studies, however, have shown that ceramides binds Raf1 directly but induces Raf1 to form an inactive complex (relative to MEK1 activation) with GTP-Ras (33). Furthermore, the ceramide-activated protein kinase-induced phosphorylation of Raf1 was mapped to Thr-268/269 which are Raf1 autophosphorylation sites rather than the known MEK1-activating phosphorylation sites (45). It has been suggested that the phosphorylation of these sites may serve to redirect the substrate specificity of Raf1 to yet unknown substrates different from MEK1. This substrate may be RIP2. That kinase-deleted variants of RIP2 can significantly block ERK activation in response to TNFα suggests that RIP2 could be a major transducer of this pathway (Fig. 7C). As Raf1 can phosphorylate and activate RIP2, it is possible that in the case of TNF signaling, either Raf1 bound to ceramide or Raf1 phosphorylated by ceramide-activated protein kinase could have its specificity shifted toward RIP2.

It was recently shown that the adaptor protein Grb2 directly interacts with the TNF receptor in a TNF-dependent fashion (35). This complex consequently recruits SOS which leads to the activation of Ras and Raf1. This process is similar to that activated by receptor tyrosine kinases, although in this case, additional steps are apparently required to achieve full ERK activation. Our data suggest the possibility that recruitment of RIP2 by TRAFs, coupled to its activation by Raf1 within the TNF receptor complex, may fulfill at least some of the missing requirements. Furthermore, the use of RIP2 as an alternative to MEK1 may be to contribute to a faster and more specific signal transmission in TNF signaling to ERK. There are multiple MAPK cascades within a cell and the same subset of kinases can be used, yet different effector proteins are activated depending on the stimulus. There is increasing support for the concept that specific adaptor molecules are used to assemble and route these proteins to prevent or facilitate cross-talk between pathways (46). For example, kinase suppressor of Ras was shown to facilitate Ras signal transmission possibly by shutting MEK1 from the cytoplasm to activated Raf1 at the membrane (47–49). Similarly, the adaptor protein MP1 ap-
pears to associate selectively with ERK1 and MEK1, and not MEK2 or ERK2, to enhance ERK1 activation (50). Whereas the kinase suppressor of Ras facilitates MEK1 activation by Ras and Raf1, a similar protein may be involved in RIP2 activation by Ras and Raf1.

Coordination of Apoptosis, NF-κB, and ERK Activation by RIP2—Although distinct signaling responses are induced by TNFα, functional interactions exist between these pathways. For example, both NF-κB and apoptosis are induced by TNFα, and one consequence of NF-κB activation is the prevention of apoptosis, perhaps through the TRAF-mediated suppression of caspase 8 (51–53). A similar dichotomy may exist for RIP2. Thus RIP2 is pro-apoptotic via the interaction of its CARD domain with CLARP, caspase-1, or caspase-8 (37) and anti-apoptotic via activation of the ERKs (this report). The activation of the RIP2 kinase activity could positively regulate cell proliferation and survival, whereas negative regulators of RIP2 may favor caspase activation and promote cell death. Whether and how RIP2 may coordinate the balance between life and death in response to TNF stimulation is an important question that will be addressed in future experiments.

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