Identification of a Novel Homotypic Interaction Motif Required for the Phosphorylation of Receptor-interacting Protein (RIP) by RIP3*

Received for publication, October 2, 2001, and in revised form, November 27, 2001
Published, JBC Papers in Press, December 4, 2001, DOI 10.1074/jbc.M109488200

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Receptor-interacting protein (RIP), a Ser/Thr kinase component of the tumor necrosis factor (TNF) receptor-1 signaling complex, mediates activation of the nuclear factor-κB (NF-κB) pathway. RIP2 and RIP3 are related kinases that share extensive sequence homology with the kinase domain of RIP. Unlike RIP, which has a C-terminal death domain, and RIP2, which has a C-terminal caspase activation and recruitment domain, RIP3 possesses a unique C terminus. RIP3 binds RIP through this unique C-terminal segment to inhibit RIP- and TNF receptor-1-mediated NF-κB activation. We have identified a unique homotypic interaction motif at the C terminus of both RIP and RIP3 that is required for their association. Sixty-four amino acids within RIP3 and 88 residues within RIP are sufficient for interaction of the two proteins. This interaction is a prerequisite for RIP3-mediated phosphorylation of RIP and subsequent attenuation of TNF-induced NF-κB activation.

Tumor necrosis factor receptor-1 (TNFR1)1 is a potent activator of nuclear factor-κB (NF-κB), a transcription complex that drives the synthesis of a number of pro-inflammatory gene products (1, 2). The intracellular segment of TNFRI responsible for NF-κB activation has been mapped to a discrete 70-amino acid homotypic interaction domain termed the “death domain” (DD). The DD is one of four recognized homotypic interaction motifs that form the “molecular glue” holding together components of the apoptotic machinery. The DD, caspase activation and recruitment domain (CARD), death effector domain, and PYRIN motifs are variants of a common death fold composed of six α-helical bundles with Greek key topology (3–5). Upon ligation of TNFR1, a multicomponent signaling complex is assembled through a series of homotypic interactions (1). The DD-containing adapter molecule TRADD (TNF receptor-associated death domain protein) is recruited to TNFR1, followed by the DD-containing Ser/Thr kinase RIP. RIP-deficient cells from knockout mice and a human mutant Jurkat cell line fail to activate NF-κB in response to TNF,

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‡ The abbreviations used are: TNFR1, tumor necrosis factor receptor-1; TNF, tumor necrosis factor; NF-κB, nuclear factor κB; DD, death domain; CARD, caspase activation and recruitment domain; RIP, receptor-interacting protein; GST, glutathione S-transferase; RHIM, RIP homotypic interaction motif.

indicating that RIP is required for TNFRI-induced NF-κB activation (6, 7). Indeed, overexpression of RIP in cell lines increases NF-κB activation (6, 8). RIP kinase autophosphorylates itself at Ser/Thr residues, although the precise residues that are phosphorylated have not been identified. Surprisingly, the kinase domain is not required for activation of NF-κB. The crucial NF-κB-activating domain resides between the kinase and death domains and is termed the intermediate domain (6, 8). However, the RIP kinase domain is reported to be essential for caspase-independent cell death triggered by FasL (9).

The NF-κB pathway is strictly regulated, as persistent activation is associated with chronic inflammatory syndromes and the development of certain malignancies such as mucosa-associated lymphoid tissue lymphoma (10). Factors that limit NF-κB activation are only beginning to be understood. One such factor is the TNF-inducible zinc finger protein A20 (11). Mice lacking A20 display constitutive NF-κB activation in lymphoid organs, leading to fatal systemic inflammation (12).

RIP belongs to a family of related kinases that includes RIP2 (also known as CARDIAK/RICK) and RIP3 (13). The kinases share significant homology in their amino-terminal kinase domains, but possess distinct C termini. RIP has a DD at its C terminus, whereas RIP2 has a related homotypic interaction domain called CARD (14–17). The CARD mediates the interaction of RIP2 with CARD-containing caspase-1 (15, 18). Unlike RIP and RIP2, RIP3 has no DD or CARD motif at its C terminus (19–22), nor does it resemble any other protein in the database. Previously, we reported that RIP3 physically associates with RIP to inhibit RIP- and TNFR1-induced NF-κB activation (22). Here, we report the identification of a new homotypic interaction motif required for the association of RIP3 with RIP. Furthermore, we found that the kinase activity of RIP3 is important for inhibition of NF-κB activation.

MATERIALS AND METHODS

Expression Vectors, Mutagenesis, and Antibodies—All eukaryotic expression vectors were constructed in pCMV2-FLAG (N-terminal FLAG tag), pcDNA3.1/Myc-His (C-terminal Myc tag), or pGST (N-terminal GST tag) using standard PCR techniques employing custom-designed primers containing appropriate restriction sites. Alanine cluster mutations were done using the QuikChange™ site-directed mutagenesis kit from Stratagene. The presence of the introduced mutation and the fidelity of PCR replication were confirmed by sequence analysis. The pGEX-2T-1 vector (Amersham Biosciences, Inc.) was used for expression of GST-tagged RIP3 and RIP truncations in Escherichia coli. Mouse anti-RIP3 monoclonal antibody was raised against a His-tagged C-terminal 218-amino acid segment of RIP3, which was then expressed in BL21(DE3) plysE cells. Anti-RIP3 polyclonal antibody was raised against a His-tagged RIP3 (Amersham Biosciences, Inc.) using standard PCR techniques employing custom-designed primers containing appropriate restriction sites. Alamine cluster mutations were done using the QuikChange™ site-directed mutagenesis kit from Stratagene. The presence of the introduced mutation and the fidelity of PCR replication were confirmed by sequence analysis. The pGEX-2T-1 vector (Amersham Biosciences, Inc.) was used for expression of GST-tagged RIP3 and RIP truncations in Escherichia coli. Mouse anti-RIP3 monoclonal antibody was raised against a His-tagged C-terminal 218-amino acid segment of RIP3, which was then expressed in BL21(DE3) plysE cells. Anti-RIP3 polyclonal antibody was raised against a synthetic peptide composed of the last C-terminal 20 amino acids of RIP3.

Cell Cultures, Transfections, and Luciferase Assays—HEK293E, human U937, and Jurkat cells were cultured as previously described (6, 8). Constructs expressing deletions and mutations of RIP and RIP3 were transiently transfected into HEK293E cells using FuGENE reagent
Fig. 1. Defining the RHIM required for RIP-RIP3 association. A: left, schematic representation of the different Myc-tagged RIP truncations. The red boxes represent the conserved core segment within RHIM. Right, coexpression of FLAG-tagged RIP3 with different Myc-tagged RIP truncations in HEK293E cells. Following transfection, cells were lysed, immunoprecipitated (IP) with anti-FLAG M2 beads, and immunoblotted with anti-Myc antibody (Ab). 10% of the input cell lysate was analyzed by immunoblotting as indicated in the lower two panels. The expression of RIP3 appeared as a doublet, which is likely due to autophosphorylation of RIP3. B: left, schematic representation of the different FLAG-tagged RIP3 truncations. The red boxes indicate the conserved core of RHIM in RIP3. Right, coexpression of FLAG-tagged RIP3 truncations with wild-type Myc-RIP in HEK293E cells. Samples were analyzed as described for A. C: left, schematic representation of alanine cluster mutations in RIP3 (upper) and RIP (lower). Right, immunoprecipitation of alanine cluster mutant versions of RIP3 (upper two panels) and RIP (lower two panels), followed by immunoblotting to detect binding to cotransfected wild-type (WT) RIP or RIP3.
FIG. 2. Mapping of the minimal binding region within RIP and RIP3. A, schematic representation of the different GST-RIP3 and GST-RIP constructs and corresponding binding activities with cotransfected partners. B, coexpression of Myc-RIP and the indicated GST-RIP3 truncations, followed by immunoprecipitation (IP) with GST-glutathione-Sepharose and immunoblotting with anti-Myc monoclonal antibody. C, coexpression of FLAG-RIP3 and the indicated GST-RIP truncations, followed by immunoprecipitation with GST beads and immunoblotting with anti-FLAG monoclonal antibody. D, coexpression of FLAG-RIP3-(411–474) (f-RIP3(411–474)) with either GST-RIP-(501–588) or control GST-E10, followed by immunoprecipitation with GST beads and immunoblotting with anti-GST and anti-FLAG antibodies. Input cell lysates were analyzed by immunoblotting as indicated in the left two panels. E, recombinant RIP and RIP3 coelute as a complex upon gel filtration chromatography. GST-RIP3-(388–518) and GST-RIP-(496–583) were coexpressed in E. coli, bound to glutathione-Sepharose, eluted with reduced glutathione, cleaved with thrombin, and resolved by size-exclusion chromatography. Column fractions were analyzed by SDS-PAGE and Coomassie Blue staining.
FIG. 3. RIP3 is recruited to the TNFR1 complex, and RIP3 phosphorylates RIP. A, cellular localization of RIP3. COS-7 cells were transfected with green fluorescent protein (GFP)- and FLAG-tagged RIP3 and visualized by confocal microscopy (upper right panel). The same cells were co-stained for endogenous RIP using anti-RIP monoclonal antibody (Pharmingen). RIP staining (red) was revealed by a Cy3-conjugated secondary antibody (Molecular Probes, Inc.) (upper left panel). The lower panel shows merged images.

B, recruitment of RIP to the TNFR1 complex in a TNF-dependent manner. U937 cells were treated with TNF (100 ng/ml) for 15 min (lanes 3 and 4) or left untreated (lanes 1 and 2). Cell lysates from 10⁶ cells were immunoprecipitated (IP) with anti-TNFR1 monoclonal antibody (lanes 2 and 4) or with mouse control IgG (lanes 1 and 3). Coprecipitating RIP was detected by immunoblot analysis. C, RIP3 recruitment to the TNFR1 signaling complex. The TNFR1 signaling complex was immunoprecipitated from 10⁹ U937 cells and subjected to an in vitro kinase assay using [γ³²P]ATP. The TNFR1 complex was then dissociated...
Con

mixture of DNAs containing a 3:1 ratio of RIP or RIP3 plasmid versus the SV40

performed using the program ProFit (ProCeryon Biosciences, Inc.).

293E cells were subjected to an

complex and various RIP and RIP3 proteins obtained from transfected

microscopy (Leica SP). Cells were examined and photographed by confocal

(Pharmingen) was detected using a Cy5-conjugated secondary antibody

stained with anti-RIP monoclonal antibody. Bound anti-RIP antibody

was immunoprecipitated from U937 cells with or without prior

exposure to TNF. Endogenous RIP complexed with TNFR1 in a

signalosome that was immunoprecipitated from U937 cells was detected by

immunoblotting (lower panel). Abs, antibodies; WT, wild-type; Mut, mutant; Con, alanine cluster control.

RESULTS AND DISCUSSION

Defining the RIP Homotypic Interaction Motif (RHIM)—

Analysis of the C terminus of RIP3 revealed a stretch of 16

amino acids that was highly homologous to a region in the

intermediate domain of RIP: 8 of 16 contiguous residues were

identical, and 14 of 16 were conserved. To elucidate the func-
tional significance of this region, we generated a series of

C-terminal RIP truncations. Cotransfection studies revealed

that 20 amino acids encompassing the core 16 residues were

required for binding of RIP to RIP3 (Fig. 1A). Only truncated

forms of RIP that contained this conserved region bound RIP3

(Fig. 1A, lanes 1 and 5–7). Similar truncation analysis of RIP3

revealed that an equivalent stretch of 24 residues containing

the 16 core residues was required for association with RIP (Fig.

1B, lanes 1, 3, and 4). To further characterize this novel homo-
typic interaction motif, we generated alanine cluster substitu-
tions within the conserved and flanking regions. Alanine sub-
stitutions within the core 16 residues significantly disrupted

the association between RIP and RIP3, whereas alanine sub-
stitutions outside the core region did not (Fig. 1C). Therefore,

this stretch of 16 residues forms the essential core of a novel

homotypic interaction motif, the RHIM.

Mapping the Minimal Region in RIP and RIP3 Sufficient for

Their Association—To elucidate the minimal region of RIP3

that is sufficient for homotypic binding, a series of GST-RIP3

fusion proteins were tested for their ability to bind to RIP.

GST-RIP3-(411–474) retained the ability to bind RIP, indicat-
ing that this stretch of 64 amino acids is sufficient to confer RIP

binding (Fig. 2B). Similar experiments were conducted using

GST-RIP fusion proteins. GST-RIP-(501–588) bound RIP3,

whereas a more truncated version, GST-RIP-(501–551), bound

RIP3 only weakly (Fig. 2C). To address the question of whether

these two minimal binding segments can bind each other,

FLAG-RIP3-(411–474) was coexpressed with GST-RIP-(501–

588) or, as a negative control, GST-E10 (25). Only GST-RIP-

(501–588) bound FLAG-RIP3-(411–474), consistent with the

notion that the two RHIMs in RIP and RIP3 bind each other

(Fig. 2D). To confirm a direct association between the two

RHIMs, GST-RIP3-(388–518) and GST-RIP-(496–583) were

expressed in E. coli and were found to copurify on glutathione-Sepharose as a soluble complex. RIP3 was then cleaved from GST by thrombin and separated on a sizing column. RIP and RIP3, both containing the core RHIM, coeluted during gel filtration, indicating that these fragments of RIP and RIP3 can interact directly (Fig. 2E). These results also indicate that residues outside the core regions are required for mutual inter-

action of the RHIMs.

Interestingly, secondary structure predictions using two dif-

ferent methods suggested that the minimal binding fragments

of RIP and RIP3 are predominantly coil-like. However, the

RHIMs were predicted to have β-hairpin structures with turns

centered around NSTG (residues 534–537) of RIP and NCSG (residues 454–457) of RIP3. These predictions were supported by “threading” analysis (data not shown).

RIP3 Is Recruited to the TNFR1 Signaling Complex—Confo-
cal microscopy was used to ascertain the cellular localization of

RIP and RIP3. Ectopically expressed green fluorescent protein-

tagged RIP3 colocalized with endogenous RIP in punctate

structures (Fig. 3A), distinct from labeled mitochondria (data not shown). To detect association of endogenous RIP and RIP3 within the TNFR1 signaling complex, a monoclonal antibody against RIP3 was generated. Previous studies have shown that RIP is recruited to the TNFR1 signaling complex upon TNF treatment (8). Additionally, we have reported that upon over-

expression, RIP3 can be recruited to the TNFR1 complex by

RIP3. To determine whether endogenous RIP3 is recruited to the TNFR1 complex in a TNF-dependent manner, TNFR1 was immunoprecipitated from U937 cells with or without prior exposure to TNF. Endogenous RIP complexed with TNFR1 in a

TNF-dependent manner (Fig. 3B), but endogenous RIP3 could not be detected by immunoblotting due to poor reactivity of the anti-RIP3 antibody. Because RIP3 is a kinase, in vitro kinase reactions offered a more sensitive method of detection. Follow-
ing immunoprecipitation with anti-TNFR1 antibody, the

TNFR1 signaling complex was subjected to an in vitro kinase

reaction, and the complex was disrupted and subjected to a second round of immunoprecipitation using antibody against RIP or RIP3. Using this protocol, we could detect recruitment of both RIP and RIP3 to the TNFR1 complex after TNF treatment (Fig. 3C). Many other proteins in the TNFR1 complex were also phosphorylated (Fig. 3C, lane 4), some of which likely represent components of the TNFR1 signalosome (26).

RIP3 Phosphorylates RIP—Because RIP3 binds RIP, we de-
determined whether RIP and RIP3 could phosphorylate each other. An in vitro kinase assay showed RIP3 to be the stronger autophosphorylating kinase. Furthermore, RIP3 could phosphorylate RIP, but the converse was not observed (data not shown). To address whether the phosphorylation of RIP by RIP3 was specific, kinase-dead RIP(K45A) and the indicated

**FIG. 4.** The kinase domain and RHIM of RIP3 are required to inhibit TNF-induced NF-κB activation. **A,** schematic representation of the various RIP3 and RIP constructs. **B,** RIP3 inhibits TNF-induced NF-κB activation. The indicated RIP3 expression constructs together with a NF-κB luciferase reporter plasmid were transfected into HEK293E cells. Following transfection, cells were exposed to TNF (20 ng/ml) for 8 h. Cell lysates were prepared, and a luciferase assay was carried out using the Promega dual-luciferase assay kit. **C,** RIP3 functions through RIP to inhibit NF-κB activity. RIP-deficient Jurkat cells were transfected with control vector (Vec), wild-type (WT) RIP, or mutant (Mut) RIP (alanine cluster disruption of RHIM) in the presence of vector, wild-type RIP3, or mutant RIP3 (disrupted RHIM). Luciferase activity was measured to assess NF-κB activation. **D,** RIP3 kinase activity is required for inhibition of NF-κB activation. Native Jurkat cells were transfected with the indicated RIP3 constructs, and a luciferase assay was performed as described for **B.** E, RIP3 rapidly inhibits NF-κB activation. The indicated RIP3 expression constructs together with a NF-κB luciferase reporter plasmid were transfected into HEK293E cells. 24 h after transfection, cells were exposed to TNF (20 ng/ml) for the indicated time periods. Cell lysates were prepared, and a luciferase assay was performed as described for **B.** All graphs are averages of at least three independent experiments. K-A, K45A; Con, alanine cluster control.
RIP3 constructs were expressed in HEK293E cells, immunoprecipitated, and subjected to an in vitro kinase assay. The use of catalytically inert RIP(K45A) in this assay eliminated interference from autophosphorylation. RIP(K45A) was phosphorylated in the presence of wild-type RIP3, but not in the presence of a kinase-dead RIP3 mutant (Fig. 3E, lanes 2 and 3). Importantly, mutation of the RIP3 RHIM abrogated RIP phosphorylation by RIP3 (Fig. 3E, compare lanes 4 and 5), indicating that RIP phosphorylation by RIP3 is dependent on the formation of a RIP-RIP3 complex.

**RIP3 Inhibits TNF-induced NF-κB Activation through RIP Phosphorylation**—We have previously shown that RIP3 attenuates RIP- and TNFR1-mediated NF-κB activation (22). To determine whether the kinase and homotypic interaction motifs of RIP3 are required to attenuate RIP-mediated NF-κB activation, HEK293E cells expressing TNFR1 and RIP, but not RIP3 (data not shown), were transfected with wild-type and mutant RIP3, and NF-κB activation was assessed following TNF treatment. Cells expressing wild-type RIP3 showed markedly reduced NF-κB activity upon TNF treatment. Mutant RIP3 containing a disrupted RHIM did not inhibit NF-κB activation (Fig. 4B), indicating that the ability of RIP3 to inhibit TNF-induced NF-κB activation is dependent on its homotypic interaction with RIP. To confirm this finding, we used a RIP-deficient Jurkat cell line in which TNF-induced NF-κB activity is rescued by RIP (6). Wild-type and mutant RIPs with a disrupted RHIM were able to stimulate NF-κB activation equally well in these cells. However, although the ability of wild-type RIP to stimulate NF-κB activation was attenuated by RIP3, NF-κB activation by a RIP mutant with a disrupted RHIM was not inhibited by RIP3. Disruption of the RHIM in RIP3 also prevented inhibition of RIP-mediated NF-κB activation (Fig. 4C). Therefore, an intact RHIM in both molecules is required for RIP3 to inhibit RIP-mediated NF-κB activation. Like the RIP3 RHIM mutant, a kinase-dead RIP3 mutant did not inhibit NF-κB activation by RIP (Fig. 4D). This result suggests that phosphorylation of RIP by RIP3 inhibits its ability to activate NF-κB. The kinetics of attenuation of TNF-induced NF-κB activation by RIP3 were analyzed over a 24-h period (Fig. 4E). In the presence of transfected wild-type RIP3, NF-κB activation in response to TNF was rapidly extinguished; however, RHIM-deficient mutant RIP3 did not influence NF-κB activation (Fig. 4E). We propose that when cells are exposed to TNF, RIP is recruited to the TNFR1 signaling complex to activate NF-κB. Subsequent recruitment of RIP3 through a RHIM-mediated interaction leads to phosphorylation of RIP by RIP3, and this inhibits the ability of RIP to further engage the NF-κB pathway. Such a desensitization mechanism would limit persistent activation of the NF-κB pathway.

**Acknowledgments**—We thank Theresa Shek and Jin Kim for antibody production; Wenlu Li and Gilbert-Andre Keller for microscopy; and the Genentech sequencing core, Andreas Strasser, and members of the Dixit laboratory for discussion, advice, and reagents.

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