RIP3, a Novel Apoptosis-inducing Kinase*

(Received for publication, March 24, 1999, and in revised form, April 19, 1999)

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RIP3 is a novel gene product containing a N-terminal kinase domain that shares extensive homology with the corresponding domain in RIP (receptor-interacting protein) and RIP2. Unlike RIP, which has a C-terminal death domain, and RIP2, which has a C-terminal caspase activation and recruitment domain, RIP3 has a unique C terminus. RIP3 binds RIP through its unique C-terminal segment and by virtue of this interaction is recruited to the tumor necrosis factor (TNF)-receptor-1 signaling complex. Previous studies have shown that RIP mediates TNF-induced activation of the anti-apoptotic NF-κB pathway. RIP3, however, attenuates both RIP and TNF receptor-1-induced NF-κB activation. Overexpression studies revealed RIP3 to be a potent inducer of apoptosis, capable of selectively binding to large prodomain initiator caspases.

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§ The abbreviations used are: TNF, tumor necrosis factor; TNFR-1, TNF receptor-1; TRAF, TNF receptor-associated factor; CARD, caspase activation and recruitment domain; IL, interleukin; PCR, polymerase chain reaction; kb, kilobase(s); FADD, Fas/Apo-1-associated death domain protein; TRADD, TNF receptor-associated death domain protein; RIP, receptor-interacting protein.

Tumor necrosis factor receptor-1 mediates both the proinflammatory and pro-apoptotic effects of the pleiotropic cytokine TNF (1, 2). The proinflammatory effects are mediated by activation of the transcription factor NF-κB. Recently it has been shown that NF-κB activation results in both the transcriptional activation of proinflammatory genes, including IL-8 and E-selectin (3–5), and in activation of a cell survival pathway mediated at least in part by induction of anti-apoptotic IAP family members (6–8). Therefore, quite paradoxically, two diametrically opposed pathways emanate from TNFR-1: a cell death pathway and a cell survival pathway mediated by activation of NF-κB.

The intracellular segment of TNFR-1 contains a 70-amino acid homophilic interaction domain, dubbed the "death domain," which is required for both signaling and NF-κB activation. Upon activation of TNFR-1, a multi-component signaling complex is assembled by a series of homophilic interactions (1). Initially, the death domain-containing platform adapter molecule TRADD is recruited to TNFR-1 by virtue of a homophilic death domain interaction (9). TRADD in turn binds the death adapter molecule FADD (10, 11), which interacts with the zymogen form of the initiator death protease caspase-8 (12). Subsequent activation of caspase-8 by an induced proximity mechanism leads to amplification of the death signal through proteolytic activation of downstream caspasezymogens (13).

Studies done with FADD-deficient embryonic fibroblasts suggest that this is the major but not the only pro-apoptotic pathway engaged by TNFR-1, because instead of being completely resistant to TNF-induced apoptosis, 30% of FADD-null cells are still sensitive (14, 15). Taken together, these studies suggest that there exists a subsidiary FADD-independent TNFR-1-initiated death pathway. Earlier biochemical studies indicated that the TNFR-1-associated adapter molecule RAIDD might fulfill this function by recruiting caspase-2 to the receptor signaling complex (16). However, caspase-2 null cells do not show any loss of sensitivity to TNF-induced cytotoxicity (17); therefore, the physiological significance of this interaction remains unclear. Initial biochemical studies also suggested a role for the TRADD-binding, RING finger containing adapter molecule TRAP2 as the major conduit for the flow of NF-κB-activating signals from TNFR-1 (18, 19). Here again, targeted gene deletion showed that contrary to expectations TRAF2 was not involved in mediating NF-κB activation but rather engaged the JNK (c-Jun N-terminal kinase) pathway (20, 21). The TRADD-associated adapter responsible for engaging NF-κB was shown to be the death domain containing Ser/Thr kinase RIP (22). Overexpression of RIP is sufficient to engage NF-κB, and RIP-deficient cells fail to activate NF-κB in response to TNF (23, 24). RIP possesses kinase activity as it autophosphorylates itself on Ser/Thr residues. Surprisingly, the kinase domain is not required for mediating NF-κB activation; rather, it is the intermediate domain that resides between the kinase and death domain that mediates this activity (25). It now appears that RIP is the prototypical member of an emerging family of related molecules. RIP2 (also known as CARDIAK/RICK) is a similar molecule possessing a remarkably conserved kinase domain (26–28). However, instead of having a C-terminal death domain, it contains a CARD motif, which, like the death domain, is a homophilic interaction domain found within the promdomains of caspase-1, caspase-2, and caspase-9 and a variety of adapter molecules including Apaf-1 (which uses its CARD segment to associate with caspase-9) and RAIDD (which similarly associates with caspase-2) (29). The CARD motif in RIP2 specifically binds to the promdomain of caspase-1 (27).

RIP2 promotes the processing of caspase-1 zymogen to generate the protease that cleaves and activates the proinflammatory cytokine pro-IL-1β. Additionally, because of its ability to interact with TRAF adapter molecules, RIP2 is recruited to TRAF-binding receptors, especially CD-40 (26). It is therefore tempting to speculate that RIP2 may be part of a link that connects certain proinflammatory receptors to the generation of the proinflammatory cytokine IL-1β.

Herein, we report the identification and characterization of a third RIP-related molecule designated RIP3. Like RIP2, its kinase domain is similar to that of RIP. However, unlike either RIP or RIP2, RIP3 possesses neither a death domain nor a
CARD motif at its unique C terminus. RIP3 overexpression is a potent inducer of apoptosis, an activity readily localizable to the unique C terminus. Additionally, RIP3 binds RIP within the TNFR-1 signaling complex and attenuates its ability to engage the NF-κB pathway. Inhibition of this survival pathway and binding to initiator caspases may contribute to the pro-apoptotic activity of RIP3. It is therefore possible that RIP3 represents the alternate death pathway engaged by TNFR-1, especially because a dominant negative version of RIP3 partially blocks TNFR-1-mediated cell death.

MATERIALS AND METHODS

Cloning of Human RIP3—A proprietary incyte lifeseq® data base was screened for sequences that encode homologues of RIP and RIP2. One expressed sequence tag (incyte accession number 1626570) was found, and a full-length cDNA encoding RIP3 was cloned from both a human fetal brain cDNA library and a human aortic endothelial cDNA library using standard PCR and hybridization protocols. Additional human multiple tissue poly(A) RNA were hybridized according to the manufacturer’s instructions using a 32P-labeled cDNA probe specific for RIP3. D, in vitro kinase assay. 293E cells were transiently transfected with empty vector (lane 1) or expression vectors encoding Myc-tagged native RIP3 (lane 2) or Myc-tagged RIP3 (K50A) point mutant (lane 3). Cell lysates were immunoprecipitated with Myc antibody 24 h following transfection. The immunoprecipitates were subjected to in vitro kinase assay (upper panel) or immunoblot analysis with anti-Myc monoclonal antibody (lower panel).

Northern Blot Analysis—Human multiple tissue poly(A) RNA blots (CLONTECH) containing 2 μg/lane poly(A) RNA were hybridized according to the manufacturer’s instructions using a 32P-labeled RIP3 probe encompassing amino acids 1–223 of the RIP3 open reading frame.

Expression Vectors—All eukaryotic expression vectors were constructed in pFLAG-CMV-2 (N-terminal FLAG tag) or pcDNA3.1/Myc-His (C-terminal Myc tag) using standard PCR techniques employing custom-designed primers containing appropriate restriction sites. Expression constructs encoding RIP, RIP2, TNFR-1, TRADD, CrmA, p35, and various caspases have been described previously (26). GD tag is an epitope generated at Genentech that has been described previously (30). Mutation of the catalytic lysine to an alanine in RIP3 (K50A) was accomplished by site-directed mutagenesis using the Quickchange™ kit from Stratagene. The presence of the introduced mutation and fidelity of PCR replication were confirmed by sequence analysis.

Co-immunoprecipitation and Western Blot Analysis—293E cells were transfected with the indicated constructs. Where necessary, a CrmA expression construct was included to suppress apoptosis. Cells were harvested 24–26 h post-transfection and lysed in 1 ml of lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40). Resulting lysates were subjected to immunoprecipitation with antibodies directed to the epitope tag as described previously (26). Immunoprecipitates were washed in lysis buffer, resolved by SDS-polyacrylamide gel electrophoresis, and subsequently analyzed by protein immunoblotting.

NF-κB Luciferase Assay—293E cells (1.5 × 10⁵ cells/well) were seeded onto 12-well plates and transfected with 0.25 μg of a dual luciferase reporter gene plasmid (10:1 ratio) and indicated amounts of each expression construct. Additionally, p35 was included in each transfection assay to suppress apoptosis. The total DNA concentration was kept constant by supplementation with empty vector to 1 μg. Cells were harvested 24 h post-transfection, and reporter gene activity was determined using the dual luciferase assay system (Promega).

In Vitro Kinase Assay—Immunoprecipitated RIP3 and RIP3 (K50A) were obtained from transfected 293E cells and subjected to an auto-kinase assay exactly as described previously for RIP2 (26).
RESULTS AND DISCUSSION

Cloning and Structure of RIP3—Data base searching revealed a partial cDNA that possessed substantial homology to the previously characterized Ser/Thr kinase domain of RIP and RIP2 (RICK/CARDIAK). A human fetal brain cDNA library was screened to obtain a full-length cDNA. It contained a 1554-base pair open reading frame encoding a novel 518-residue protein with a predicted molecular mass of 57 kDa (Fig. 1A). An in-frame stop codon was present upstream of the initiator methionine. Because it shared extensive homology to the kinase domain of RIP (34% identity, 60% similarity) and RIP2 (31% identity, 58% similarity) (Fig. 1B), it was termed RIP3. However, the C terminus of RIP3 contained neither a death domain (like RIP) nor a CARD domain (like RIP2) and had no discernible homology to any known protein.

Tissue Distribution of RIP3—Human tissue RNA blots were hybridized with a $^{32}$P-labeled cDNA probe specific for the RIP3 kinase domain. RIP3 transcript was present in a variety of human tissues, being especially prominent in the pancreas where two species of approximately 2.1 and 2.6 kb were clearly discernible (Fig. 1C). The 2.1-kb transcript corresponds in size to the cloned full-length cDNA.

RIP3 Is a Protein Kinase—To determine whether RIP3 was indeed a protein kinase, 293E cells were transfected with Myc epitope-tagged RIP3 or RIP3 (K50A) in which the invariant lysine conserved in all protein kinases was mutated (31). Immunoprecipitated RIP3 was subjected to an in vitro kinase assay followed by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. An approximately 60-kDa $^{32}$P-labeled band corresponding to RIP3 was observed only when the native sequence was expressed (Fig. 1D). As expected, mutant RIP3 (K50A), in which the lysine essential for enzymatic activity and ATP binding was altered to alanine, did not possess kinase activity. Taken together, these observations are consistent with RIP3 being a bona fide autophosphorylating protein kinase.

RIP3 Induces Apoptosis—During the course of transfection studies, it became evident that RIP3 induced dramatic apoptotic responses that required cotransfection with a caspase inhibitor to allow for cell survival and detectable RIP3 expression. This was surprising because as noted earlier RIP3 possesses none of the pro-apoptotic homophilic interaction domains involved in activation of the death pathway. RIP3 induced apoptosis in both 293E renal epithelial cells and MCF7 breast carcinoma cells. The transfectants rapidly developed morphological characteristics typical of adherent cells undergoing apoptosis, including membrane blebbing, becoming rounded and shrunken and detaching from the culture dish. To identify the pro-apoptotic region within RIP3, truncated versions were analyzed for their ability to induce cell death (Fig. 2B). Expression of a version encoding the first 431 residues, RIP3 (1–431), failed to induce apoptosis, suggesting that the missing 87 C-terminal residues were critical for pro-apoptotic function. Additionally, the kinase domain of RIP3 was not required for killing activity because expression of a C-terminal segment of RIP3 (224–518), which lacks the kinase domain, induced apoptosis at levels comparable with that of the intact molecule (Fig. 2B). As anticipated, RIP3-induced apoptosis was blocked by caspase inhibitors including CrmA, a cowpox virus encoded serpin and by p35, a baculovirus gene product that equipotently inhibits multiple caspases (Fig. 2C). RIP3-induced death was not inhibited by a dominant negative version of FADD, implying that it engages a pathway independent or downstream of this receptor-associated pro-apoptotic adapter molecule (data not shown).

An adapter-independent mechanism by which RIP3 may induce apoptosis would be binding and activating caspases. In keeping with this notion, co-precipitation studies revealed an association between transfected RIP3 and the large prodomain caspases 2, 8, 9, and 10, but no detectable binding to downstream small prodomain caspases, such as caspase-3 (Fig. 2D). A number of important caveats with regard to this experiment should be noted, including that the interaction is observed in an overexpression system and could still be indirect (for example, mediated by an endogenous intervening adapter molecule). Additional studies will be required to confirm the exact mechanism by which RIP3 engages the caspase death machinery.

RIP3 Does Not Activate NF-κB—Given the sequence homology between RIP3 and RIP, a known activator of NF-κB, we asked if it, too, could induce the NF-κB pathway (23, 25). NF-κB-dependent reporter constructs were co-transfected into 293E cells with RIP, TNFR-1, or RIP3 expression constructs. Despite being expressed to an extent no less than RIP or TNFR-1, RIP3 activated the NF-κB reporter gene to a negligible level (4.5-fold) when compared with RIP (460-fold) and...
TNFR-1 (124-fold) (Fig. 3A). Given this, we asked the converse question, namely, whether RIP3 could inhibit RIP- or TNFR-1-induced NF-κB activation. As shown in Fig. 3B, cotransfection studies revealed that RIP3 inhibited RIP and TNFR-1 induced NF-κB activation in a dose-dependent manner. It did not influence activation of this pathway by TRAF6 (32), an unrelated inducer that mediates IL-1-induced NF-κB activation (data not shown).

This result prompted us to look for a potential physical interaction between RIP and RIP3. Cotransfection studies deploying differentially epitope-tagged expression constructs revealed strong binding of RIP3 to RIP (Fig. 3C). RIP3 also bound RIP2, but to a much lesser extent, possibly because of overexpression unveiling a weak promiscuous interaction (data not shown). Truncated versions of RIP3 were co-expressed to delineate the segment that associated with RIP. Reminiscent of the studies mapping the pro-apoptotic activity of RIP3, an intact C terminus was found to be essential, as evidenced by the inability of RIP3 (1–431) to bind RIP. Additionally, the kinase domain was dispensable because RIP3 (224–518) still bound. Finer mapping studies will be needed to delineate the exact, possibly overlapping domains responsible for mediating apoptosis and RIP association.

**RIP3 Is Recruited to the TNFR-1 Signaling Complex in a RIP-dependent Manner**—Because RIP is recruited to the TNFR-1 signaling complex through interaction with the receptor-associated platform adapter molecule TRADD (25), we asked whether RIP3 could similarly be recruited to the TNFR-1 complex by virtue of its interaction with RIP. As shown in Fig. 3D, RIP3 is recruited to the TNFR-1 signaling complex at stoichiometric amounts in a RIP-dependent manner, consistent with the ability of these two related molecules to bind each other. Interestingly, exceedingly small, nonstoichiometric amounts of overexpressed RIP and RIP3 can also be recruited to the TNFR-1 signaling complex in the absence of co-transfected TRADD. This is presumably mediated by the low level of endogenous TRADD present in the transfected cells. Once recruited to the TNFR-1 signaling complex (or possibly to other receptor complexes that bind TRADD and RIP such as the TNFR-1 related receptor, DR3), RIP3 could exert a pro-apoptotic activity. This may in part be accomplished by attenuating the NF-κB survival pathway and/or by activating caspases. Regardless, a dominant negative version of RIP3 should be able to partially attenuate TNFR-1-induced cell death. The inhibition can only be partial because the TRADD-caspase-8 pathway would still function even in the presence of dominant negative RIP3 (12). In keeping with this notion, we found that RIP3 (1–431) could act as a dominant negative and partially inhibit TNFR-1-induced cell death (Fig. 3E). The partial nature of the inhibitory effect was evident, especially when compared with the potent caspase-8 inhibitor CrmA.

In summary, RIP3 is a pro-apoptotic molecule that binds RIP and antagonizes its ability and that of TNFR-1 to engage the NF-κB pathway.

**Acknowledgments**—We thank Sarah Schilbach for sequencing and Audrey Goddard for advice on sequence analysis. We are grateful to members of the laboratory including Minhong Yan, Karen O’Rourke, Domagoj Vucic, Somasekar Seshagiri, Haoran Zhao, Heike Arlt, and Eric Humke for reagents, helpful discussion, and encouragement.

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