RICK, a Novel Protein Kinase Containing a Caspase Recruitment Domain, Interacts with CLARP and Regulates CD95-mediated Apoptosis*

Naohiro Inohara, Luis del Peso‡, Takeyoshi Koseki§, Shu Chen, and Gabriel Núñez¶

From the Department of Pathology and Comprehensive Cancer Center, The University of Michigan Medical School, Ann Arbor, Michigan 48109

Signaling through the CD95/Fas/APO-1 death receptor plays a critical role in the homeostasis of the immune system. RICK, a novel protein kinase that regulates CD95-mediated apoptosis was identified and characterized. RICK is composed of an N-terminal serine-threonine kinase catalytic domain and a C-terminal region containing a caspase-recruitment domain. RICK physically interacts with CLARP, a caspase-like molecule known to bind to Fas-associated protein with death domain (FADD) and caspase-8. Expression of RICK promoted the activation of caspase-8 and potentiated apoptosis induced by Fas ligand, FADD, CLARP, and caspase-8. Deletion mutant analysis revealed that both the kinase domain and caspase-recruitment domain were required for RICK to promote apoptosis. Significantly, expression of a RICK mutant in which the lysine of the putative ATP-binding site at position 38 was replaced by a methionine functioned as an inhibitor of CD95-mediated apoptosis. Thus, RICK represents a novel kinase that may regulate apoptosis induced by the CD95/Fas receptor pathway.

The surface CD95 death receptor (also known as Fas or APO-1), a member of the tumor necrosis factor superfamily, is widely expressed and plays a critical role in the regulation and homeostasis of the immune system (1). Activation of CD95 by Fas ligand (FasL), a trimeric cell surface protein, leads to rapid induction of apoptosis (1). The intracellular domain of CD95 and related death receptors contains a death domain that was originally described in the tumor necrosis factor receptor-1 (2). The death domain of CD95 and tumor necrosis factor receptor-1 are responsible for signaling cell death (1).

A major step forward in understanding early events in CD95 signaling was the discovery of molecules that are recruited to the CD95 receptor complex, following ligand-induced receptor oligomerization. The Fas-associated protein with death domain (FADD) (also known as MORT-1), is a cytoplasmic adapter protein that contains a C-terminal death domain that interacts with the death domain of CD95 (2, 3). CD95 utilizes FADD to link cytoplasmic receptor sequences to caspase-8 (also known as FLICE, MACH, and Mch5) (4, 5). Both FADD and caspase-8 interact through conserved death effector domains (DED) located in the pro-domain of caspase-8 and N-terminal region of FADD (4, 5). Following ligand-induced oligomerization, the CD95 receptor recruits caspase-8 to the receptor signaling complex through FADD, an event that leads to the processing and release into the cytosol of caspase-8 (6). Active caspase-8 induces a cascade of caspases and the rapid demise of the cell (1).

RIP is a protein kinase that was identified by its ability to interact with the intracytoplasmic region of CD95 (7). RIP contains a death domain that was shown to mediate the interaction with CD95 (7). However, it has proven difficult to show interaction between RIP and CD95 in vivo (7–9). Furthermore, in some systems RIP mediates activation of NF-κB but not CD95-initiated apoptosis (9). These results suggest that a kinase other than RIP could be involved in the regulation of CD95-mediated apoptosis.

In this report, we describe the identification and initial characterization of RICK, a novel protein kinase. While the N terminus of RICK contains a serine/threonine catalytic kinase domain, its C-terminal region contains a caspase recruitment domain (CARD) with amino acid homology to the pro-domains of CED-3 related mammalian caspases, Caenorhabditis elegans CED-4 and its mammalian homologue, Apaf-1.

EXPERIMENTAL PROCEDURES

Isolation of the RICK cDNA—The partial nucleotide sequences of cDNAs encoding peptides with homology to RIP were found in EST data bases of GenBank using the TBLASTN program. The entire nucleotide sequence of EST clone 591081, 239957, and 633044 was determined by dideoxy sequencing.

Northern Blot Analysis—The entire cDNA insert of EST clone 591081 was radiolabeled by random priming using a commercial kit (Boehringer Mannheim) and applied for analysis of human poly(A) RNA blots from various tissues (CLONTECH Laboratories) according to the manufacturer’s instructions.

Construction of Expression Plasmids—The entire cDNA insert (1.8 kilobase pairs) of EST clone 591081 was cloned into the EcoRI and NotI sites of pcDNA3 (Invitrogen) to produce pcDNA3-RICK. To produce tagged proteins, we constructed pcDNA3-Flag, pcDNA3-myc, pcDNA3-AU1, and pcDNA3-HA which are derivatives of pcDNA3 and share common restriction enzyme sites for cloning. The entire open reading frame of RICK was inserted into the XbaI and ApaI sites of pcDNA3-AU1.

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§ Recipient of Research Career Development Award CA-64421 from the National Institutes of Health. To whom correspondence should be addressed: Dept. of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109; Tel.: 313-764-8514; Fax: 313-647-9654; E-mail: Gabriel.Nunez@umich.edu.

1 The abbreviations used are: FADD, Fas-associated protein with death domain; DED, death effector domain; CARD, caspase recruitment domain; EST, expressed sequence tag; HA, hemagglutinin; mAb, monoclonal antibody.

2 N. Inohara and G. Nunez, unpublished results.
pcDNA3-Flag to produce N-terminal Flag-tagged RICK. Deletion mutants of RICK (Δ53, Δ247, Δ364, and Δ366–531) were constructed by digestion of the pcDNA3-Flag-RICK construct with restriction enzymes Apal, Ndel, XbaI, and Xbal/ApiI, respectively. The human caspase-10 cDNA was cloned into the BamHI and XhoI sites of pcDNA3-Flag-AU1 to produce C-terminal AU1-tagged caspase-10. pcDNA3-p35, pcDNA3-caspase-8-AU1, and pcDNA3-caspase-8-mt-AU1 were previously described (10). The human caspase-1, caspase-2, and caspase-10 cDNAs were cloned into the BamHI and XhoI sites of pcDNA3-AU1 to C-terminal AU1-tagged AU1 proteins. The human CLARP cDNA, its alternative splicing form CLARP-N, and the mutant CLARP-C were cloned into the BamHI and XhoI sites of pcDNA3-HA to produce C-terminal HA-tagged proteins.

Transfection, Expression, Immunoprecipitation, and Immunodetection of Tagged Proteins—5 × 10^5 human 293T cells were transfected with expression plasmids by a calcium phosphate method as described (10). The total amount of transfected plasmid DNA was adjusted with pcDNA3 plasmid to be the same within individual experiments. After transfection, 293T cells were harvested at different times and lysed with 0.2% Nonidet P-40 isotonic lysis buffer (10). The B lymphocyte-derived BJAB cell line was stably transfected by electroporation with pcDNA3 or pcDNA3-Flag-RICK (K38M). After selection with puromycin (1 μg/ml), bulk cell lines were tested for RICK protein expression with anti-Flag antibody by flow cytometric analysis. For immunoprecipitation, 1 mg of soluble protein was incubated with 10 μg anti-Flag, anti-Myc, or control antibody overnight at 4 °C and precipitated with 50 μl protein A-Sepharose 4B (Zymed Laboratories Inc.). Immunoprecipitated proteins or total lysates were subjected to 12% SDS-polyacrylamide electrophoresis and immunoblotted with monoclonal antibodies (mAb) to HA (Boehringer Mannheim) or Flag (Kodak).

Results and Discussion

Identification of RICK—To identify potential RIP-related genes, we searched public databases of expressed sequence tags (ESTs) for clones with homology to the catalytic domain of RIP (7). Three ESTs encoding novel overlapping peptides were identified with significant amino acid homology to the kinase domain of RIP. Sequence analysis of the three cDNAs demonstrated that the three clones represent fragments of the same gene. The longest cDNA clone had an 1.8-kilobase amino acids with an estimated molecular size of 60,332 Da (Fig. 1A). We have designated this protein RICK (RIP-like interacting CLARP kinase). Analysis of the RICK amino acid sequence revealed that it contains an N-terminal serine-threonine kinase catalytic domain with significant amino acid similarity to the kinase catalytic domain of RIP (Fig. 1, B and C). Unlike RIP, the C-terminal region of RICK had significant similarity to the pro-domain of several caspases including caspase-1 and -2 (Fig. 1D). In addition, the same C-terminal fragment of RICK (residues 426–517) had significant amino acid similarity to regions from other apoptosis regulatory proteins including RAIDD, cIAP-1, the N terminus of Apaf-1 (13), and its C. elegans homologue CED-4 (Fig. 1D). The region of homology between RICK and CED-3-like caspases/ced-4/Apaf-1/RAIDD/cIAP corresponds to a domain previously named CARD (14).

RICK Is Expressed in Multiple Human Tissues—We performed Northern blot analysis to determine the distribution of RICK RNA transcripts in various human tissues. RICK was detected in heart, brain, placenta, lung, pancreas, spleen, lymph node, and peripheral blood lymphocytes as two transcripts of 2.5 and 1.8 kilobases (Fig. 2). Further analysis revealed that the difference in RICK mRNA transcripts is due to differential polyadenylation.²

RICK Potentiates Apoptosis Induced by Caspase-8 and Caspase-10—To begin to elucidate the physiological function of RICK, expression constructs producing Flag-tagged RICK, native RICK, or control proteins were introduced into 293T which were subsequently observed for features of apoptosis. Expression of caspase-8 and -10, two caspases known to be activated during CD95 signaling (5, 6, 15), induced significant apoptosis but RICK did not (Fig. 3A). Significantly, expression of RICK augmented apoptosis promoted by caspase-8 and caspase-10 (Fig. 3A). The enhancement of caspase-8-mediated apoptosis induced by RICK required a catalytic active caspase-8 since RICK did not augment the level of apoptosis induced by caspase-8-mt, a mutant caspase-8 protein with a single amino acid change (Cys777 to Ser) in the conserved active pentapeptide (Cys364–Asp365–Asp366–Asp367–Glu368) (Fig. 3A). Furthermore, caspase-8-induced apoptosis potentiated by RICK was inhibited by the broad-based caspase inhibitors zVAD-fmk and baculovirus p35 (Fig. 3A).

RICK Enhances the Enzymatic Activity of Caspase-8—The potentiation of caspase-8-mediated apoptosis by RICK suggested that RICK could enhance the activation of caspase-8. To measure the protease activity associated with caspase-8, lysates from 293T cells co-transfected with AU1-tagged caspase-8 and Flag-tagged RICK were incubated with anti-AU1 antibody to immunoprecipitate caspase-8, and the immunoprecipitates were assayed for enzymatic activity using the Ac-DEVDA-MCA (acetyl-Asp-Glu-Val-Asp-amido-4-methylcoumarin) fluorogenic substrate (10). The results showed that RICK enhanced the enzymatic activity of wild-type (wt) caspase-8 but not that of caspase-8-mt (Fig. 3B). Moreover, the caspase promoting activity induced by RICK was similar to that observed when caspase-8 was co-expressed with FADD (Fig. 3B), a protein known to activate caspase-8 (4, 5).

A RICK ATP-binding Site Mutant as a Dominant-negative Inhibitor of CD95-mediated Apoptosis—The results presented in Fig. 3 suggested that RICK could be involved in the regulation of apoptosis induced by CD95 receptor stimulation. Catalytically inactive mutants of serine threonine kinases often act as dominant-negative inhibitors of the active kinase (16). Therefore, we engineered a mutant form of RICK (RICK-K38M) in which the lysine of the putative ATP-binding site at position 38 was replaced by a methionine to test if this mutant could regulate CD95-mediated apoptosis. Expression constructs producing Flag-tagged RICK-K38M or empty vector were stably transfected into BJAB, a human B cell line that undergoes apoptosis upon CD95 stimulation (3). To minimize clonal variation, bulk BJAB cell lines expressing RICK-K38M or control were selected with puromycin and analyzed 2 weeks later (Fig. 3A).
after transfection. BJAB expressing the RICK-K38M mutant were significantly less sensitive to anti-APO1-induced apoptosis than BJAB cells transfected with control plasmid (Fig. 3C, p < 0.001). Protein analysis revealed that BJAB transfected with RICK-K38M expressed the Flag-tagged RICK mutant protein (Fig. 3C, inset). These results indicate that the RICK-K38M mutant inhibits apoptosis induced by CD95 stimulation and suggests that RICK is involved in regulation of the CD95 signaling pathway. To confirm defective kinase function of RICK-K38M mutant, the wild type and mutant RICK proteins were expressed in 293T cells and immunoprecipitated with mAb to Flag. The K38M mutation in RICK dramatically decreased its autophosphorylation in vitro (Fig. 3D).

Deletion Mutant Analysis of RICK—We engineered deletion mutant forms of RICK to further characterize their abilities to regulate apoptosis (Fig. 4A). Deletion mutants of the N-terminal region spanning the catalytic kinase domain (Δ53, Δ247, and Δ364) failed to potentiate apoptosis of 293T cells induced by Fas, Fas plus FasL, or caspase-8 (Fig. 4A). In addition, a deletion mutant missing the C-terminal 165 amino acids that span the CARD domain did not enhance apoptosis (Fig. 4B). Immunoblotting analysis revealed that wt and mutant forms of RICK were expressed ruling out loss of function due to inappropriate levels of expression (Fig. 4C). These results indicate that both the kinase catalytic domain and the C-terminal region containing the CARD domain are required for enhancement of apoptosis by RICK.

RICK Interacts with the C-terminal Domain of CLARP, a Caspase-like Protein with Homology to Caspase-8—The presence of the CARD domain suggested that RICK could promote apoptosis by interacting with signaling molecules of the CD95 death pathway. To test this, expression constructs...
producing several HA-tagged apoptosis-regulatory proteins and Flag-tagged RICK were transiently co-transfected into 293T cells. Cell lysates were immunoprecipitated with anti-Flag antibody and co-immunoprecipitated proteins were analyzed by immunoblotting with anti-HA antibody. The analysis shown in Fig. 4 revealed that RICK co-immunoprecipitated with CLARP, a caspase-related protein also known as Casper, c-FLIP\textsubscript{p}, I-FLICE, FLAME-1, CASH\textsubscript{L}, and MRIT that interacts with FADD and caspase-8 (10, 17–22). The interaction between CLARP and RICK was specific in that RICK did not associate with multiple apoptosis regulatory proteins including caspase-1, caspase-2, caspase-3, caspase-4, caspase-8, CED-3, CED-4, Bcl-X\textsubscript{L}, TRAF2, c-IAP-1, c-IAP-2, or RAIDD.\textsuperscript{2}

CLARP is comprised of two N-terminal DEDs fused to a C-terminal caspase-like domain (10, 17–22). We engineered two mutant forms of CLARP to determine the regions of CLARP required for its interaction with RICK. One mutant containing the N-terminal DED modules (CLARP-N) corresponds to c-FLIP\textsubscript{p}/CASH\textsubscript{L}/MRIT\textsubscript{81}, a protein form generated by alternative RNA splicing (17, 19, 22), whereas the other mutant (CLARP-C) contained the C-terminal caspase-like do-

FIG. 3. Regulation of apoptosis by RICK. A, 293T cells were transfected with 2 \( \mu \)g of vector control, RICK, or Flag-RICK expression plasmids in the presence and absence of 0.5 \( \mu \)g of AU1-tagged caspase-8 or caspase-8-mt. 0.2 \( \mu \)g of caspase-10 or 1 \( \mu \)g of p35 expression plasmids were transfected in indicated lanes. After 8 h, 20 \( \mu \)m zVAD-fmk was added in indicated lanes. The percent of apoptotic cells was calculated in triplicate as described (10). B, RICK facilitates the DEVD-AMC cleavage activity of caspase-8. 293T cells were co-transfected with vector control (○), pcDNA3-caspase-8-AU1-mt and pcDNA3-Flag-RICK (△), pcDNA3-caspase-8-AU1 alone (□), pcDNA3-caspase-8-AU1 and pcDNA3-Flag-RICK (▲), or pcDNA3-caspase-8-AU1 and pcDNA3-Flag-RICK (●). Tagged caspase-8 was immunoprecipitated with mAb to AU1 and the enzymatic activity was determined in triplicate with the fluorogenic substrate DEVD-AMC as reported (10). The DEVD-AMC cleavage is given in arbitrary units (10). To confirm equal expression of caspase-8-AU1, whole protein fractions were analyzed by Western blotting with anti-AU1 mAb. The levels of caspase-8 in each sample are shown in inset. A nonspecific band is shown by a star. C, inhibitory effect of mutant RICK on apoptosis induced by anti-CD95 antibody. 7.5 \( \times \) \( 10^3 \) BJAB cells stably expressing pcDNA3 (○) or pcDNA3-Flag-RICK-K38M (△) were incubated with indicated amounts of anti-CD95 antibody plus 5 \( \mu \)g/ml protein G or 5 \( \mu \)g/ml protein G alone at 37 °C for 14 h after 18 h preincubation. The percent of apoptotic cells were determined by propidium iodide staining in triplicate cultures as described (11). The expression level of BJAB-Flag-RICK-K38M (closed profile) or control BJAB-pcDNA3 (open profile) stained with anti-Flag mAb as detected by flow cytometric analysis are shown (inset). D, RICK is a autophosphorylating kinase. 293T cells were transfected with pcDNA3, pcDNA3-Flag-RICK, or pcDNA3-Flag-RICK-K38M. Flag-RICK proteins immunoprecipitated with mAb to Flag was incubated with [\( \gamma-\text{32P} \)]ATP for 20 min at room temperature (upper panel). Total lysate was immunoblotted with mAb to Flag (lower panel).
main (residues 246–480). RICK interacted with the C-terminal caspase-like domain but not with the N-terminal DED-containing domain of CLARP (Fig. 4D). Thus, RICK interacts with the C-terminal caspase-like domain of CLARP.

Upon activation, the CD95 receptor recruits FADD and caspase-8 into a death-inducing signaling complex that is induced or enhanced by CD95 receptor oligomerization and activation (4, 5). CLARP has been shown to bind to caspase-8 and FADD (10, 17–22). Thus, CLARP could function as an adapter molecule to link RICK to proximal components of the CD95 signaling complex. RICK-K38M, a mutant in which the lysine molecule to link RICK to proximal components of the CD95 signaling complex. RICK-K38M, a mutant in which the lysine residue was replaced by a methionine, functioned as an inhibitor of CD95-mediated apoptosis. Identical mutations in the ATP-binding site of several serine threonine kinases lead to catalytically inactive proteins that act as dominant-negative mutants (16). Thus, these results suggest that RICK is a kinase that is involved in the regulation of apoptosis induced by the CD95 receptor pathway. The protein substrate(s) that are targets of RICK are presently unknown. One candidate substrate for RICK is CD95.

The intracytoplasmic region of CD95 can be phosphorylated (24) and this phosphorylation event might regulate CD95-induced apoptosis. Another potential substrate is FADD since RICK could associate with FADD-containing complexes via CLARP. The C-terminal caspase-like domain of CLARP associates with RICK, while the N-terminal domain that contains the DED modules is involved in the interaction with FADD (17, 19–22). Upon cross-linking and activation of CD95, FADD is phosphorylated on serine residues in vivo (23, 24), although the role of this phosphorylation is unknown. However, it is possible that phosphorylation of FADD may lead to increased activation of caspase-8 which is consistent with our observation that RICK increases the activity of caspase-8.

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