Human CARD4 Protein Is a Novel CED-4/Apaf-1 Cell Death Family Member That Activates NF-κB*

(Received for publication, February 10, 1999, and in revised form, March 12, 1999)

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The nematode CED-4 protein and its human homolog Apaf-1 play a central role in apoptosis by functioning as direct activators of death-inducing caspases. A novel human CED-4/Apaf-1 family member called CARD4 was identified that has a domain structure strikingly similar to the cytoplasmic, receptor-like proteins that mediate disease resistance in plants. CARD4 interacted with the serine-threonine kinase RICK and potently induced NF-κB activity through TRAF-6 and NIK signaling molecules. In addition, coexpression of CARD4 augmented caspase-9-induced apoptosis. Thus, CARD4 coordinates downstream NF-κB and apoptotic signaling pathways and may be a component of the host innate immune response.

Apoptosis, or programmed cell death, is an evolutionarily conserved process of cell suicide critical for normal development and elimination of pathogen-infected cells (1). Genetic studies in the nematode Caenorhabditis elegans have identified components of the death pathway that show similarity to large families of mammalian proteins involved in apoptosis (2). Surprisingly, Apaf-1 has been the only CED-4-like cell death protein identified thus far in humans (3). Like CED-4, Apaf-1 has been the only CED-4-like cell death protein families of mammalian proteins involved in apoptosis (2). Surprisingly, Apaf-1 has been the only CED-4-like cell death protein known thus far in humans (3). Like CED-4, Apaf-1 has the caspase recruitment domain (CARD)1 as its N-terminal identified thus far in humans (3). Like CED-4, Apaf-1 has been the only CED-4-like cell death protein families of mammalian proteins involved in apoptosis (2). Surprisingly, Apaf-1 has been the only CED-4-like cell death protein known thus far in humans (3). Like CED-4, Apaf-1 has the CASPASE RECRUITMENT DOMAIN (CARD)1 as its N-terminal identified thus far in humans (3). Like CED-4, Apaf-1 has been the only CED-4-like cell death protein families of mammalian proteins involved in apoptosis (2). Surprisingly, Apaf-1 has been the only CED-4-like cell death protein known thus far in humans (3). Like CED-4, Apaf-1 has the CARD interaction with the prodomain of caspase-9, resulting in caspase-9 oligomerization and activation (4, 5). The presence of CARD motifs in a family of other effector and signaling molecules, including numerous caspases (6) and a recently identified NF-κB-activating kinase (7–9), suggests that other CED-4/Apaf-1 family members likely exist in humans to coordinate downstream stress responses. We report here the identity and characterization of a novel human CED-4/Apaf-1 family member that activates NF-κB and apoptotic signaling pathways.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Assay—The bait vector was constructed by cloning the CARD-containing N-terminal region of CARD4 (residues 1–145) into yeast vector pGBT9. The resulting plasmid, pGBT9-CARD4-CARD, was used to screen a human breast library (Millennium Pharmaceuticals, Inc.) according to the Matchmaker Two-hybrid System Protocol (CLONTECH). Clones positive for his and lacZ expression were isolated and sequenced to determine identity.

Expression Vectors—HA-CARD4, HA-CARD4–518 (residues 1–518), HA-CARD4-CARD (residues 1–126), HA-CARD4-NBS (residues 127–518), Myc-TRAF6-DN (residues 289–530), Myc-NIK-DN (residues 623–947), Myc-RICK, Myc-RICK-DN (residues 406–541), Myc-RICK/No CARD (residues 1–435), and Myc-Bcl-XL were amplified by polymerase chain reaction using oligonucleotides encoding either HA or Myc epitopes and cloned into the pCI expression plasmid (Promega). The expression plasmid encoding Apaf-1 was described previously (3).

Luciferase Reporter Assays—293T cells were plated in 6-well plates (35-mm wells) and transfected 2 days later (90% confluency) with 1 μg of NF-κB luciferase reporter plasmid (pNFκB-Luc, Stratagene), 200 ng of pCMV β-gal, 600 ng of pCI vector, and 200 ng of indicated expression plasmids using SuperFect transfection reagent (Qiagen). For dominant-negative experiments, 2 ng of CARD4 expressing plasmid and 800 ng of dominant-negative plasmid were used. Cells were harvested 48 h after transfection, and luciferase activity in 1000-fold diluted cell extracts was determined using the Luciferase Assay System (Promega). In addition, β-galactosidase activities were determined and used to normalize transfection efficiency.

Co-immunoprecipitation Assay—293T cells were plated in 10-cm dishes and transfected the following day (60% confluency) with 4 μg of each expression plasmid using LipofectAMINE (Life Technologies, Inc.). Cells were harvested 48 h after transfection, lysed in 0.6 ml of buffer (50 ml Tris, pH 8.0, 5 ml EDTA, 120 mEq NaCl, 0.5% Nonidet P-40 preheated with specific anti-HA or anti-Myc polyclonal antibodies (Berkeley Antibody Co.). Immune complexes were then precipitated with protein A-Sepharose beads (Amersham Pharmacia Biotech), subjected to 12% SDS-polyacrylamide gel electrophoresis and immunoblotted with polyclonal antibodies.

RESULTS AND DISCUSSION

To identify novel human members of the CED-4/Apaf-1 family, we searched Millennium Pharmaceuticals’ proprietary data base of expressed sequence tags (EST) for clones having sequence similarity to CARD motifs (6). A CARD-encoding EST was identified and used to screen a human umbilical vein endothelial cell cDNA library for full-length clones. A single cDNA of approximately 3.5 kilobases contained an open reading frame encoding a protein of 953 amino acids with a predicted molecular mass of 108 kDa (Fig. 1A). This molecule was designated CARD4 because it was one of several CARD-con-
taining proteins identified from the EST search. A BLAST search of protein data bases indicated that CARD4 was a new protein with at least three putative functional domains (Fig. 1, A and C). The N-terminal region of CARD4 (residues 14–104) shares significant sequence similarity with CARD motifs found in a variety of apoptotic signaling molecules, including those found in CED-4 and Apaf-1 (Fig. 1B). Combined within the central region of CARD4 (residues 199–398) is a NBS-like domain that shares approximately 33% sequence identity with the putative GTP-binding domain (residues 416–618) of CIITA, a transactivator of major histocompatibility complex class II genes (12). Within this region of homology are several NBS consensus sequences, including kinase 1a, 2, and 3a motifs that are also found within the NBS of CED-4 and Apaf-1 (13). The C-terminal region of CARD4 (residues 674–950) encodes at least 10 tandem leucine-rich repeats (LRR), a protein interaction motif found in a variety of proteins with diverse functions, including signal transduction (14). The 28-amino acid consensus sequence derived from the aligned LRRs of CARD4 belongs to the ribonuclease inhibitor-like subfamily of LRRs that are found exclusively in animal intracellular proteins. The CARD/NBS/LRR domain structure of CARD4 justifies its inclusion as a new member of the CED-4/Apaf-1 family of proteins (Fig. 1C). CARD4 is also structurally similar to members of the NBS/LRR class of plant disease resistance gene products whose N-terminal effector domains contain either a leucine zipper motif or a Toll/interleukin-1 receptor homology domain (15).

By analogy to CED-4 and Apaf-1 proteins, the N-terminal CARD motif of CARD4 likely functions as an effector domain that mediates specific homophilic interactions with downstream CARD-containing signaling molecules. To gain insight into signaling pathways engaged by CARD4, we used its N-terminal CARD (residues 1–145) as bait in a yeast two-hybrid screen of a human breast cDNA library to identify CARD-containing interactors. From approximately 8 million transformants, 12 positive clones showing activation of his and lacZ reporter genes were identified. Of these 12 interactors, six were partial-length cDNAs of RICK (RIP2, CARDIAK) (7–9), a recently identified serine-threonine kinase that contains a C-terminal CARD motif (Fig. 2A). In similar screens of human prostate and brain cDNA libraries, approximately 30–40% of all positive clones were found to be RICK (data not shown). Interestingly, the CARD of CARD4 shows the most similarity (26% identity) with the corresponding domain in RICK when compared with the family of known CARD motifs (Fig. 1B). This interaction was mediated by the CARD of RICK because this domain alone (residues 435–540) was sufficient for binding to the bait (data not shown). Furthermore, immunoprecipitation of epitope-tagged RICK expressed in mammalian cells quantitatively coprecipitated CARD4 (Fig. 2B). This association was mediated specifically by the CARD motif of RICK, because CARD4 did not coprecipitate with a truncated form of RICK lacking its CARD (RICK/No CARD). Taken together,
Recent studies have shown that RICK activates NF-κB signaling pathways (8, 9). Because the putative N-terminal effector domain of CARD4 was found to interact with the CARD of RICK, we determined whether CARD4 can activate NF-κB signaling using a luciferase reporter gene directed by an NF-κB-responsive promoter. Expression of CARD4 in 293T cells potently induced NF-κB activity in a concentration-dependent manner; maximum induction of luciferase activity was 40-fold compared with control vector (Fig. 3A). Induction of NF-κB activity was not a general activity of CED-4/Apaf-1 family members because expression of Apaf-1 failed to activate this signaling pathway (Fig. 3B). Although RICK also activates the Jun N-terminal kinase signaling pathway (Ref. 9 and data not shown), CARD4 expression in 293T cells failed to either induce phosphorylation of Jun N-terminal kinase or activate a luciferase reporter gene with AP-1 promoter elements (data not shown), CARD4 expression in 293T cells failed to either induce phosphorylation of Jun N-terminal kinase or activate a luciferase reporter gene with AP-1 promoter elements (data not shown). We next determined the domains of CARD4 that mediate the induction of NF-κB activity (Fig. 3B). A C-terminal truncated mutant (CARD4-Δ518) activated NF-κB at levels comparable with full-length CARD4, demonstrating that the LRR domain was not required for induction. The N-terminal CARD (CARD4-CARD), but not the NBS domain (CARD4-NBS), was found subsequently to be sufficient for induction, establishing the CARD motif as the NF-κB-activating domain of CARD4 (Fig. 3B). Induction of NF-κB activity by RICK is mediated in part by TRAF-6 and NIK kinase (8). Dominant-negative versions of these molecules (TRAF6-DN and NIK-DN) were also found to inhibit induction when coexpressed with CARD4, suggesting that TRAF-6 and NIK act downstream of CARD4 to activate NF-κB (Fig. 3B). Conversely, coexpression of the antiapoptotic protein Bcl-XL had no effect on NF-κB induction. Coexpression of the CARD of RICK (RICK-DN) also functioned as a dominant-negative mutant, consistent with this molecule being a potential downstream mediator of CARD4 function.

Besides having a role in NF-κB signaling, RICK also affects apoptotic signaling pathways by an unknown mechanism, because it increases apoptotic death induced by caspase-8 and caspase-10 and augments the processing of pro-caspase-1 (7, 9). Confirming the role of RICK as a general activator of caspases, we found that RICK coexpression also increases caspase-9 apoptotic activity (see below), even though RICK does not interact with caspase-9 (7, 9). We therefore determined whether CARD4 coexpression increases apoptotic death induced by caspase-9. 293T cells expressing caspase-9 underwent apoptotic activity (see below), even though RICK does not interact with caspase-9 (7, 9). We therefore determined whether CARD4 coexpression increases apoptotic death induced by caspase-9. 293T cells expressing caspase-9 underwent apoptosis without rounding-up, membrane blebbing, and lifting off from the plate, thereby reducing the number of viable cells (Fig. 4A). In contrast, expression of CARD4, RICK, or Apaf-1 had no effect on cell viability when expressed alone. However, CARD4 coexpression increased the apoptotic death induced by caspase-9 at levels comparable with those observed with either RICK or Apaf-1 (Fig. 4B). This activity was mapped to the N-terminal CARD motif of CARD4 because coexpression of this domain alone was sufficient to increase apoptotic death induced by caspase-9 (Fig. 4B). Interestingly, CARD4 coexpres-
a homophilic CARD-CARD interaction implicates the serine-threonine kinase as a potential downstream mediator of CARD4 signaling. It is possible, however, that other CARD-containing molecules are responsible for mediating CARD4 function in 293T cells. Although a role for the C-terminal LRRs remains uncertain, it is likely that this domain functions in a manner analogous to the Apaf-1 WD-40 domain to mediate CARD4 activation by upstream signaling molecules. Our finding that CARD4 also displays structural similarity to the NBS/LRR class of plant disease resistance gene products is intriguing. NBS/LRR-containing plant proteins initiate a complex defense response to pathogen infection, including changes in gene expression and localized cell death (15). Interestingly, tomato resistance response to bacterial speck disease involves both an NBS/LRR protein and a serine-threonine kinase. Recent studies suggest that plant defense mechanisms activated in response to pathogen infection are analogous to the innate immune response of vertebrates and insects (16). CARD4 may therefore be a component of the innate immune response that transduces upstream stress or pathogen signals to the activation of NF-κB and apoptotic signaling pathways.

Acknowledgments—We thank E. Alnemri for caspase-9 vector, X. Wang for Apaf-1 vector, T. Libermann and Y. Akbarali for luminometer help, and M. Jacobson, N. Roy, L. Chiang, R. Curtis, and R. Breitbart for comments and discussion.

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FIG. 4. CARD4 augmentation of a caspase-9 apoptotic activity. 293T cells were cotransfected with pCMV β-gal and indicated expression constructs. Cells were fixed and stained for β-gal expression and then viewed by phase contrast microscopy. The number of flat, blue-staining viable cells in representative fields of view was determined. A, apoptosis-inducing activity of CARD4. B, effect of CARD4 on caspase-9 death-inducing activity.