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Human CARD12 Is a Novel CED4/Apaf-1 Family Member That Induces Apoptosis

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The CED4/Apaf-1 family of proteins functions as critical regulators of apoptosis and NF-κB signaling pathways. A novel human member of this family, called CARD12, was identified that induces apoptosis when expressed in cells. CARD12 is most similar in structure to the CED4/Apaf-1 family member CARD4, and is comprised of an N-terminal caspase recruitment domain (CARD), a central nucleotide-binding site (NBS), and a C-terminal domain of leucine-rich repeats (LRR). The CARD domain of CARD12 interacts selectively with the CARD domain of ASC, a recently identified proapoptotic protein. In addition, CARD12 coprecipitates caspase-1, a caspase that participates in both apoptotic signaling and cytokine processing. CARD12 may assemble with proapoptotic CARD proteins to coordinate the activation of downstream apoptotic and inflammatory signaling pathways.

Key Words: **CARD12 protein; CARD domain; nucleotide-binding site; leucine-rich repeats; apoptosis; CED4/Apaf-1 family member; caspase-1; ASC.**

The caspase recruitment domain (CARD) is a protein interaction domain through which CARD-containing proteins assemble into apoptosis and NF-κB signaling complexes (1). The CARD domain consists of six or seven antiparallel α-helices and is structurally related to the death domain (DD) and the death effector domain (DED), two apoptosis domains that function primarily to mediate the activation of caspase-8 and caspase-10 by death receptors (2). The CARD family of signal transduction proteins consists currently of 23 members with diverse structures and functions. Human members of this family include: caspases 1, 2, 4, 5, 9, and 13; Apaf-1, CARD4 (Nod1), Nod2, CARD7 (DEFCAP/NAC), cIAP-1, cIAP-2, RICK (RIP2/CARDIAK), ARC, BCL10, RAIDD, ASC, Iceberg, CARD8, CARD9, CARD10, CARD11 and CARD14 (3–19). Understanding the mechanisms by which upstream stimuli direct the assembly and/or activation of CARD–CARD signaling complexes will provide important insight into apoptosis and NF-κB signal transduction pathways.

Apoptosis in mammalian cells is mediated by large protein families that share sequence and structural similarity with the core apoptotic proteins of *Caenorhabditis elegans* (20). The nematode CED-4 protein and its human homolog Apaf-1 play central roles in apoptosis by transducing death signals to the activation of caspases. Both CED-4 and Apaf-1 contain an N-terminal CARD domain that mediates caspase binding and a centrally located nucleotide-binding site (NBS) domain. Unlike CED-4, Apaf-1 contains a C-terminal WD-40 domain that mediates protein activation in response to the release of mitochondrial cytochrome *c* (4, 21, 22). Three additional CED4/Apaf-1 family members have been recently identified (5–9). CARD4, Nod2, and CARD7 each contain NBS domains and effector CARD domains that mediate binding to their respective CARD-containing signaling partners. Both CARD4 and Nod2 assemble together with the CARD protein RICK and induce the activation of NF-κB. Recent evidence suggests that CARD7 may play a role analogous to Apaf-1 and directly mediate caspase activation. In addition, each protein contains extensive leucine-rich repeats (LRR) that have been proposed to function as binding sites for upstream regulators. The structure of CARD4, Nod2 and CARD7 is strikingly similar to the family of plant NBS/LRR proteins that induce gene expression and cell death in response to pathogen infection (5, 7, 23). Thus, CARD4, Nod2 and CARD7 likely play critical roles in stress-activated signaling pathways and may be components of the host innate immune response.

We report here that human CARD12 is a novel CED4/Apaf-1 family member that induces apoptosis when expressed in cells. The N-terminal CARD domain

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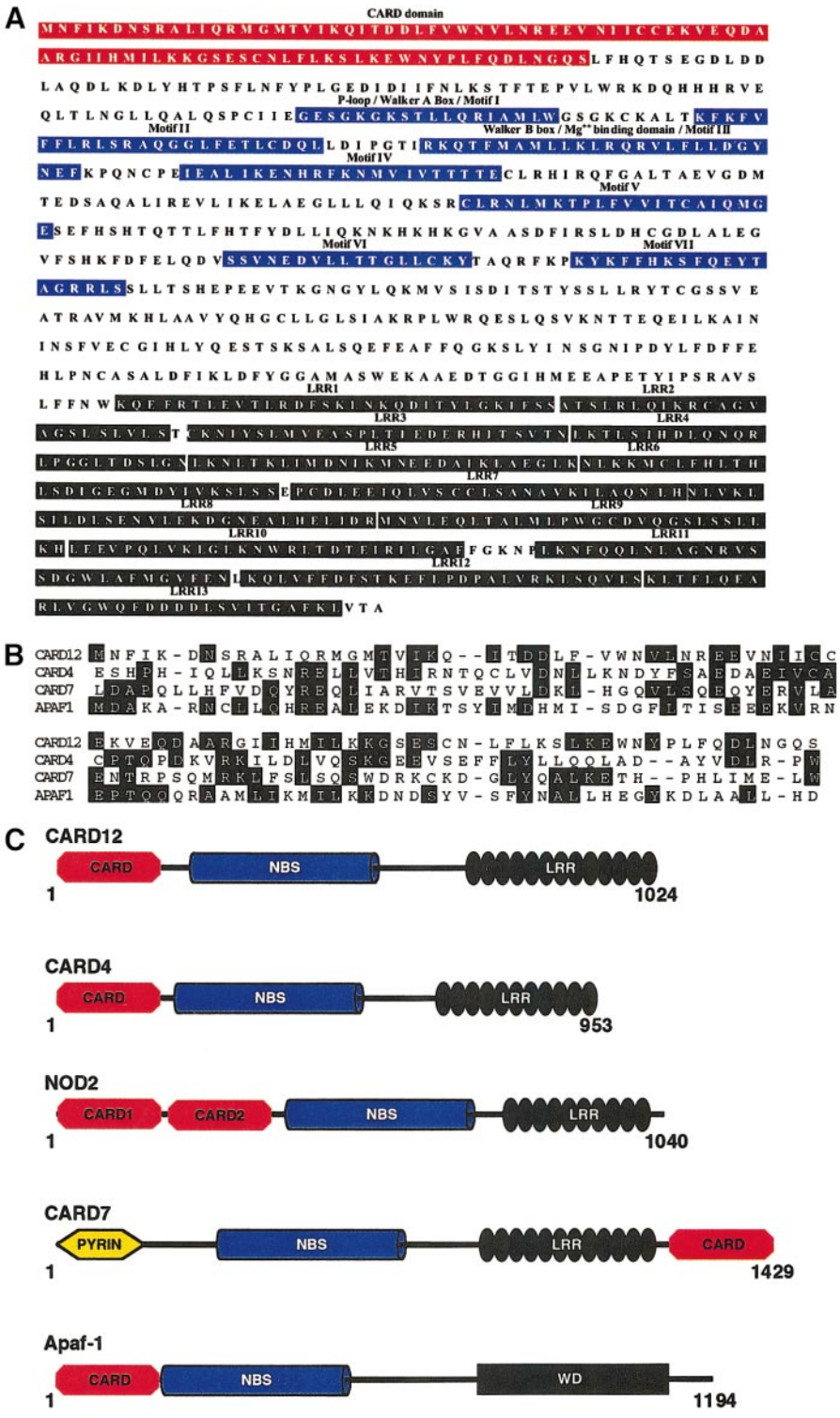

FIG. 1. Sequence and domain structure of human CARD12 protein. (A) Amino acid sequence of CARD12. CARD domain (residues 1–88, red shading), NBS domain (residues 163–457) with seven consensus motifs that are found in the NACHT subfamily of NTPases (blue shading), and C-terminal LRRs (residues 656–1024; LRR1–LRR13, black shading) are indicated. (B) Alignment of the CARD of CARD12 (residues 1–88) with CARD domains found in the CED4/Apaf-1 family members: CARD4 (residues 15–103), CARD7 (residues 1329–1416) and Apaf-1 (residues 1–89). Black shading indicates identical residues. (C) Domain structure of CARD12 compared with CARD4, Nod2, CARD7, and Apaf-1. CARD, NBS, PYRIN, LRR, and WD domains are indicated.

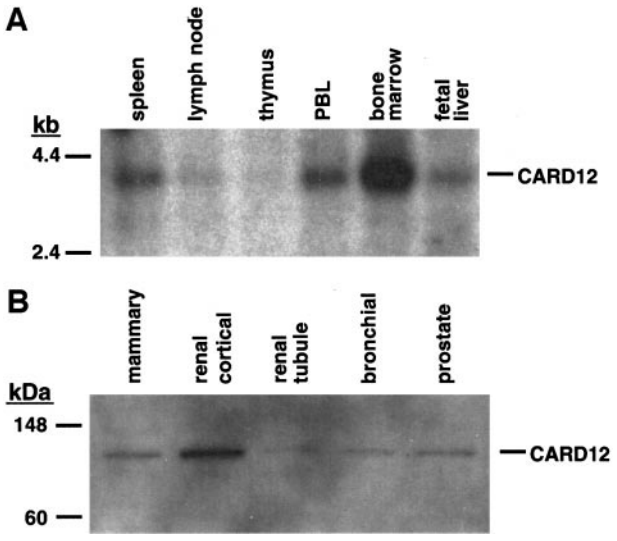

FIG. 2. Expression of CARD12 mRNA and protein. (A) The expression of CARD12 mRNA in immune tissues and cells was determined by Northern blot analysis using a Clontech human multiple tissue Northern blot. PBL, peripheral blood leukocytes. (B) The expression of endogenous CARD12 protein in human epithelial cells (Clonetics, EpiPanel) was determined by Western blot analysis using a peptide polyclonal antibody that specifically recognizes CARD12.

of CARD12 interacts with the CARD domain of the proapoptotic protein ASC. In addition, CARD12 binds to caspase-1 when coexpressed in cells. These findings suggest that CARD12 may be a novel regulator of apoptosis and inflammatory signaling pathways.

MATERIALS AND METHODS

Expression plasmids and CARD12 antibodies. A plasmid expressing CARD12 with a C-terminal Myc epitope was constructed using pCMV-Tag5A (Stratagene). Plasmids expressing either caspase-1 or caspase-9 with C-terminal T7 epitopes were a gift from Emad Alnemri (Kimmel Cancer Institute, Philadelphia). Both caspases are inactive due to a substitution mutation that replaces their catalytic cysteine residue with alanine. For mammalian two-hybrid assays, pCMV-CARD12-CARD/BD plasmid was constructed by inserting the CARD domain of CARD12 (residues 1–83) into pCMV-BD (Stratagene). The panel of CARD domains used for the mammalian two-hybrid screen was described previously (18). Affinity-purified CARD12 antibody was raised in rabbits injected with a 15-mer peptide (LWRQESLQSVKNTTE) corresponding to residues 528–542 of CARD12 (Research Genetics).

Mammalian two-hybrid assay. For mammalian two-hybrid assays, 293T cells in 6-well plates (35-mm wells) were transfected with the following plasmids: 750 ng of pCMV-CARD12-CARD/BD, 750 ng of pCMV-AD fused to individual CARD domains, 250 ng of pFR-Luc firefly reporter (Stratagene), and 250 ng of pRL-TK renilla reporter (Promega). Cells were harvested 24 h after transfection, and firefly luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega). In addition, renilla luciferase activity was determined and used to normalize transfection efficiencies.

Adenovirus and apoptosis assays. A tetracycline-regulated adenovirus expression system was used to express CARD12 with a C-terminal FLAG epitope (24). The open reading frame of CARD12 was cloned into the adenovirus transfer vector pLE11 (that coexpresses a modified green fluorescent protein (KGFP) using an internal ribosome entry site (Kelly Therieault, Millennium Pharmaceuticals Inc.). E1/E3-deleted adenovirus was then generated by homologous recombination in 911 cells (25). For apoptosis assays, Vero cells in 96-well dishes were transfected with recombinant adenovirus (20 plaque forming units/cell) and then fixed at 36 h in 4% paraformaldehyde. The nuclei were then stained with Hoescht 33342 and the percentage of apoptotic versus healthy nuclei in transfected cells was determined.

Coimmunoprecipitation assays. 293T cells transfected with plasmids were lysed in 50 mM Tris, pH 8.0, 120 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40 buffer and incubated with a monoclonal antibody that recognizes Myc (Oncogene). The immune complexes were precipitated with protein G–Sepharose (Amersham Pharmacia Biotech), washed extensively, and then subjected to SDS–polyacrylamide gel electrophoresis and immunoblotted with a mixture of polyclonal antibodies to caspase-9 and caspase-1 (Santa Cruz Biotechnology, Inc). The amount of CARD12-Myc, caspase-1-T7 and caspase-9-T7 proteins in total cell lysates was determined by immunoblot analysis using either anti-Myc or anti-T7 antibodies (VWR).

RESULTS AND DISCUSSION

Using the TBLASTN and GENESCAN programs, we searched the HTG database of genomic sequences and identified a novel human CARD-encoding gene. A cDNA corresponding to the predicted gene was isolated by PCR using sequence information obtained from Millennium Pharmaceuticals' and Incyte Genomics' EST databases. Sequencing of the 3.3-kilobase cDNA revealed an open reading frame encoding a protein of 1024 amino acids with a predicted molecular mass of 113 kDa (Fig. 1A). This protein was designated CARD12 (for CARD protein 12) because it was one of

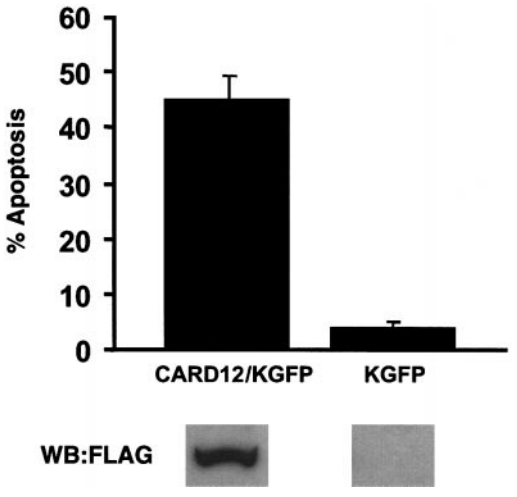

FIG. 3. CARD12 is a proapoptotic CED4/Apaf-1 family member. Vero cells were transfected with recombinant adenoviruses (20 plaque forming units/cell) expressing either full-length CARD12/KGFP or KGFP alone. CARD12 expression was verified by immunoblot analysis (WB) using a monoclonal antibody (Sigma) specific for its C-terminal FLAG epitope (lower panel). Transfected cells were fixed and stained at 36 h with the nuclear dye Hoescht 33342 and the percentage of apoptotic versus healthy nuclei in transfected cells determined.

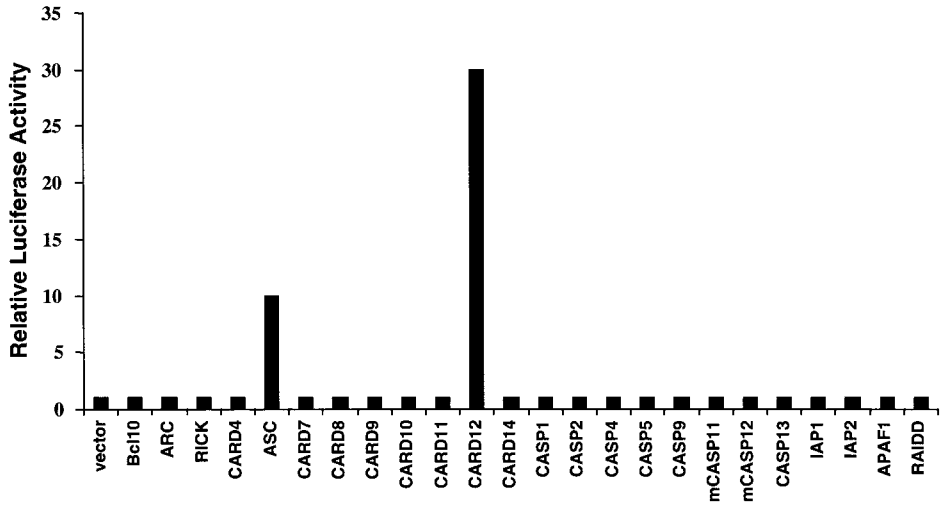

FIG. 4. The CARD domain of CARD12 interacts selectively with CARD of ASC by mammalian two-hybrid analysis. 293T cells were transfected with the mammalian two-hybrid reporter construct pFR-Luc (Stratagene). The CARD of CARD12 fused to the DNA-binding domain of Gal4 was then screened against a panel of individual CARDs fused to the activation domain of murine NF-κB. After 24 h, cells were collected and assayed for relative luciferase activity as a measure of protein–protein interaction.

many CARD-containing proteins that we identified from our searches of genomic and EST databases (GenBank/EBI Data Bank Accession No. AY032589). Analysis of its amino acid sequence identified at least three putative functional domains: an N-terminal CARD domain (residues 1–88), a central NBS domain (residues 163–457), and a C-terminal domain (residues 656–1024) comprised of at least 13 LRR motifs (Fig. 1A). LRRs are protein interaction motifs found in many proteins involved in signal transduction (26). The CARD domain of CARD12 shares significant sequence similarity with the CARD motifs found in other CED4/Apaf-1 family members, including those found in CARD4, Nod2, CARD7 and Apaf-1 (Fig. 1B). Further analysis of the NBS domain identified seven distinct motifs that are found in the NACHT subfamily of NTPases (27), including the ATP/GTPase-specific P-loop (Walker A box/motif I), the magnesium binding site (Walker B box/motif II) and 5 other conserved motifs (Fig. 1A). Other members of the NACHT NTPase subfamily include CARD proteins 4 and 7, Nod2 and the apoptosis inhibitor NAIP. The CARD/NBS/LRR domain structure of CARD12 justifies its inclusion as a new member of the CED4/Apaf-1 family of proteins (Fig. 1C). Northern blot analysis was performed and a 3.3-kilobase transcript corresponding to CARD12 was identified primarily in human immune tissues and cells, including spleen, peripheral blood lymphocytes, bone morrow and fetal liver (Fig. 2A). We also generated a polyclonal antibody that specifically recognizes the CARD12 protein. Immunoblot analysis of extracts derived from several primary epithelial cells revealed a predominant band of approximately 120 kDa corresponding to endogenous CARD12 (Fig. 2B). CARD12 protein was also detected in extracts derived from numerous endothelial and muscle primary cells (data not shown).

Many CARD proteins participate in signaling pathways that regulate apoptosis and/or the activation of NF-κB. To determine if CARD12 functions in apoptotic signaling, we expressed CARD12 in Vero cells using a recombinant adenovirus. Cells expressing CARD12 underwent extensive apoptosis with rounding-up, membrane blebbing and lifting off from the plate. Approximately 45% of the cells expressing CARD12 had apoptotic nuclei with condensed chromatin and pyknotic morphology (Fig. 3). In contrast, cells transfected with a control adenovirus remained flat and attached to the plate with only 4% of the nuclei having an apoptotic morphology. Although apoptotic signaling was induced potently by CARD12 in cells, it failed to activate a luciferase reporter gene with either NF-κB or AP-1 promoter elements (data not shown). Thus, CARD12 is a proapoptotic member of the CED4/Apaf-1 family of apoptosis proteins.

By analogy to other CED-4/Apaf-1 family members, the N-terminal CARD domain of CARD12 likely functions to mediate interactions with downstream CARD-containing signaling molecules. Homophilic CARD interactions between signaling partners are highly selective. For example, the CARD of Apaf-1 interacts with the caspase-9 CARD but not to other CARD proteins. To identify CARD domains that interact with the CARD of CARD12, we performed a mammalian two-hybrid analysis and screened for binding to the CARD domains of 24 family members (Fig. 4). The CARD of CARD12 interacted with the CARD of ASC, resulting in a 10-fold increase in relative luciferase activity. In addition, the CARD domain of CARD12 self-associated, resulting in a 30-fold increase in relative luciferase

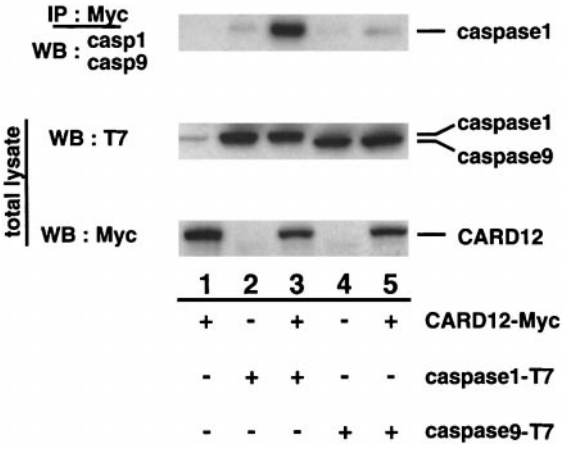

FIG. 5. CARD12 interacts with caspase-1 but not caspase-9. 293T cells were transfected with expression plasmids encoding Myc-tagged CARD12 and either T7-tagged caspase-1 or T7-tagged caspase-9. Cell extracts were immunoprecipitated (IP) with Myc antibodies and immunoblotted (WB) with antibodies that recognize caspase-1 and caspase-9 (upper panel). Protein levels in total cell lysates was determined by immunoblot analysis using either anti-T7 (middle panel) or anti-Myc (lower panel) antibodies.

activity. Coexpression of CARD12-CARD with other CARD domains failed to activate luciferase expression indicating that the CARD of CARD12 interacts selectively with the CARD of ASC, a protein that mediates apoptosis induced by chemotherapeutic agents (15). Because caspases play a central role in apoptosis, we further examined the interaction of CARD12 with either caspase-1 or caspase-9 when coexpressed in cells (Fig. 5). Immunoprecipitation of Myc-tagged CARD12 quantitatively coprecipitated T7-tagged caspase-1. This association was a selective interaction because Myc-tagged CARD12 failed to coprecipitate caspase-9. Furthermore, when the reverse experiment was performed caspase-1, but not caspase-9, coprecipitated CARD12 (data not shown). Taken together, these data suggest that ASC and caspase-1 are putative signaling partners of CARD12.

We have identified CARD12 as a novel CED4/Apaf-1 family member. In addition, CARD12 displays an intriguing homology to the NBS/LRR class of cytoplasmic, receptor-like proteins that mediate disease resistance in plants (5, 7). CARD12 and the other human NBS/LRR proteins (CARD4, Nod2, CARD7, NAIP) might therefore function to transduce upstream stress or pathogen signals to the activation of downstream apoptotic and/or proinflammatory signaling pathways. Our finding that CARD12 induces apoptosis suggests an association with downstream proapoptotic CARD proteins. The selective interaction of CARD12 with ASC and caspase-1 identifies these proteins as putative proapoptotic signaling partners of CARD12. CARD12 might activate both ASC and caspase-1 through an induced proximity mechanism, similar to that used by Apaf-1 for the activation of caspase-9 (28). Thus, CARD12 may function as a critical activator of specific apoptotic and proinflammatory signaling pathways.

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