CARD9 Is a Novel Caspase Recruitment Domain-containing Protein That Interacts With BCL10/CLAP and Activates NF-κB*

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BCL10/CLAP is an activator of apoptosis and NF–κB signaling pathways and has been implicated in B cell lymphomas of mucosa-associated lymphoid tissue. Although its role in apoptosis remains to be determined, BCL10 likely activates NF–κB through the IKK complex in response to upstream stimuli. The N-terminal caspase recruitment domain (CARD) of BCL10 has been proposed to function as an activation domain that mediates homophilic interactions with an upstream CARD-containing NF–κB activator. To identify upstream signaling partners of BCL10, we performed a mammalian two-hybrid analysis and identified CARD9 as a novel CARD-containing protein that interacts selectively with the CARD activation domain of BCL10. When expressed in cells, CARD9 binds to BCL10 and activates NF–κB. Furthermore, endogenous CARD9 is found associated with BCL10 suggesting that both proteins form a pre-existing signaling complex within cells. CARD9 also self-associates and contains extensive coiled-coil motifs that may function as oligomerization domains. We propose here that CARD9 is an upstream activator of BCL10 and NF–κB signaling.

Protein modules play an important role in defining the specificity of signal transduction pathways by mediating protein-protein interactions between upstream and downstream signaling components (1). Death domains (DDs), death effector domains (DEDs), and caspase recruitment domains (CARDs) are protein modules found extensively in proteins that mediate apoptotic signaling (2). These protein module families are similar in structure with each consisting of six or seven antiparallel α-helices that form highly specific homophilic interactions between signaling partners. Whereas DD and DED motifs function primarily in the activation of caspase-8 and -10 by death receptors, CARD domains function in diverse signaling pathways that regulate apoptosis. Examples of CARD-containing proteins include the majority of class I caspases, the ced-4-like proteins Apaf-1 and CARD4 (NOD1), IAP family members cIAP-1 and cIAP-2, RICK kinase, ARC, BCL10, RAIDD, and ASC (2, 3–7). Because CARD-CARD interactions are highly selective, it is generally accepted that most CARD-containing proteins will segregate with discrete binding partners and modulate intracellular signaling pathways.

Besides functioning as mediators of apoptosis, the CARD-containing proteins RICK, CARD4, and BCL10 also induce activation of the NF–κB transcription factor suggesting that CARD-CARD signaling complexes regulate activation of the IKK complex (3, 4, 6, 7). NF–κB plays a critical role in the stress response of cells by activating genes that control immune defense mechanisms (8). In unstimulated cells, NF–κB is found sequestered in the cytoplasm through interactions with inhibitory IκB proteins. Inhibition is relieved by the phosphorylation and proteosomal degradation of IκB proteins by proinflammatory cytokines. Phosphorylation is mediated by the IKK complex, which is comprised of at least three major proteins, two kinases designated IκKα and IκKβ that directly phosphorylate the IκB inhibitory proteins and a noncatalytic subunit called IκKγ that functions to link the IKKs to upstream regulatory molecules (9, 10, 11). Recently, RICK and RIP have been found to function as upstream regulatory molecules of the IKK complex (10, 11). Both proteins interact directly with IκKγ suggesting that they function as signaling adaptors between the IKK complex and upstream NF–κB activators. Indeed, CARD4 forms a CARD-CARD signaling complex with RICK that induces activation of the IKK complex and subsequent release of NF–κB (3, 4, 11).

BCL10 (also called CLAP/CIPER/cE10/CARMEN) has been proposed to play a role in the development of B cell lymphomas of mucosa-associated lymphoid tissues (6, 12–16). BCL10 has a bipartite structure consisting of an N-terminal CARD domain and a C-terminal effector domain that mediates activation of NF–κB. Upstream CARD-containing proteins that bind to BCL10 and activate NF–κB have not yet been identified. We report here the identity and characterization of a CARD-con-
taining protein that binds selectively to BCL10 and activates NF-κB.

EXPERIMENTAL PROCEDURES

Expression Plasmids and Antibodies—Plasmids expressing CARD9 with either FLAG or Myc epitopes were constructed by inserting the open reading frame of CARD9 into expression vectors pFLAG CMV-2 (Eastman Kodak Co.), pMyc CMV-2 (Stratagene), and pCI (Promega), respectively. Constructs encoding epitope-tagged BCL10 were described previously (12). Plasmids expressing GFP fusions were constructed using pEGFP (CLONTECH). For mammalian two-hybrid assays, pCMV-CARD9/AD and pCMV-CARD9/BD plasmids were constructed by inserting the CARD domain (residues 1–110) of CARD9 into pCMV-AD and pCMV-BD vectors, respectively (Stratagene). pCMV-CARD/AD and pCMV-CARD/BD plasmids were constructed by inserting individual CARD domains into pCMV-CARD/AD and pCMV-BD, respectively (Stratagene): BCL10 (residues 1–104), ARC (residues 1–119), RICK (residues 417–540), CARD4 (residues 1–119), ASC (residues 92–195), caspase-1 (residues 1–110), caspase-2 (residues 1–122), caspase-4 (residues 1–108), caspase-9 (residues 1–111), caspase-12 (residues 1–108), IAP-1 (residues 423–543), IAP-2 (residues 450–557), Apaf-1 (residues 1–108), and RAIDD (residues 1–108). The BCL10 monoclonal antibody was described previously (17). Affinity-purified CARD9 antibody was raised in rabbits injected with a 15-mer peptide (QKGWRQGEEDRENTT) corresponding to residues 512–526 of CARD9 (Research Genetics).

Reporter Gene Assays—For mammalian two-hybrid assays, 293T cells in 6-well plates (35-mm wells) were transfected with the following plasmids: 750 ng of pCMV-CARD9/AD or pCMV-CARD9/BD, 750 ng of pCMV-AD or BD fused to individual CARD domains, 250 ng of pFR-Luc firefly reporter (Stratagene), and 250 ng of pRL-TK Renilla reporter (Promega). For NF-κB assays, 293T cells were transfected with the following plasmids: 900 ng of pNF-κB luciferase reporter (Stratagene), 1000 ng of pRL-TK Renilla reporter (Promega), and 1000 ng of indicated expression plasmids. Cells were harvested 24 h after transfection, and 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.

Coimmunoprecipitation Assays—293T cells transfected with plasmids were lysed in 50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Nonidet P-40 buffer and incubated with anti-FLAG-M2 monoclonal antibody (Sigma Co.) or BCL10 monoclonal antibody. The immune complexes were precipitated with protein G-Sepharose (Amersham Pharmacia Biotech) or mouse IgG-agarose (Sigma Co.), washed extensively, subjected to SDS-polyacrylamide gel electrophoresis, and immunoblotted with polyclonal antibodies.

In Vitro Binding Assays—In vitro binding assays between BCL10 and CARD9 proteins were performed as described previously (18). In brief, BCL10 wild type and L41R mutant were expressed in DH5-alpha bacteria as GST fusion proteins, and equal amounts of protein were

Fig. 1. Sequence, domain structure, and expression of CARD9. A, amino acid alignment of human and rat CARD9. Residues in rat CARD9 that are not identical to human CARD9 are boxed. B, domain structure of CARD9 showing CARD domain (residues 7–98) and coiled-coil domain (residues 140–420). C, amino acid alignment of the CARD of CARD9 with CARDs found in BCL10, RAIDD, caspase-2, and caspase-9. Black shading indicates identical residues. D, expression of CARD9 mRNA in adult human tissues was determined by Northern blot analysis using CLONTECH multiple tissue Northern blots. PBL, peripheral blood leukocytes.

Fig. 2. The CARD domain of CARD9 selectively interacts with the CARD of BCL10 by mammalian two-hybrid analysis. 293T cells were transfected with the mammalian two-hybrid reporter construct pFR-Luc (Stratagene) and combinations of plasmids expressing CARD domains fused to either the activation domain of the mouse protein NF-κB or the DNA-binding domain of the yeast protein GAL4. After 24 h, cells were collected and assayed for relative luciferase activity. A, CARD of CARD9 fused to the activation domain and screened against a panel of individual CARDs fused to the DNA-binding domain. B, CARD of CARD9 fused to the DNA-binding domain and screened against the panel of individual CARDs fused to the activation domain.
immobilized on glutathione-Sepharose (Amersham Pharmacia Biotech). An equal amount of CARD9 protein labeled with \(^{35}\text{S}\)methionine was incubated with the protein-bound-Sepharose beads in 100 \(\mu\)l of binding buffer (50 mM Tris-Cl, pH 7.6, 120 mM NaCl, 0.5% Brij, and protease inhibitors) for 3 h. The beads were washed four times with the same buffer and boiled in SDS sample buffer. The proteins were then resolved on a 10% SDS gel and visualized by autoradiography.

**RESULTS AND DISCUSSION**

To identify novel CARD-containing proteins, we searched Millennium Pharmaceuticals proprietary database of expressed sequence tags (ESTs) for clones encoding CARD motifs (2). Full-length sequencing of one of the CARD-encoding ESTs revealed a cDNA that encodes a 536-amino acid protein with a predicted molecular mass of 62.3 kDa (Fig. 1A). This protein was designated CARD9 (for CARD protein 9) because it was one of many novel CARD-containing proteins that were identified from our EST search. A search for rodent orthologs also identified a rat homolog of CARD9 (for \(r\)CARD9) that is 88% identical in sequence to human CARD9 (Fig. 1A). A BLAST search of the protein database indicated that human CARD9 was a novel protein with at least two putative functional domains (Fig. 1B). The N-terminal region of CARD9 (residues 7–98) shares significant similarity with CARD motifs found in many apoptosis proteins, including those found in BCL10 (29% identity, 44% similarity) and RAIDD (28% identity, 40% similarity, Fig. 1C). The central region of CARD9 (residues 140–420) con-
contains heptad repeats characteristic of coiled-coil structures that function in protein oligomerization (19). The COILS2 program (20) predicts the existence of at least three coiled-coil regions with a probability of greater than 80% (residues 140–230, 243–277, and 332–419) that are interrupted by regions predicted to have low coiled-coil potential. Correspondingly, BLAST analysis of this region showed strong similarity to coiled-coil regions of other proteins, including myosins and plectins. Northern blot analysis was performed and a 2.1-kilobase transcript corresponding to CARD9 was identified in a variety of human adult tissues, including spleen, liver, placenta, lung, PBL, and brain (Fig. 1D). CARD9 was also expressed abundantly in the HL60 cancer cell line and showed some expression in fetal liver tissue (data not shown).

Homophilic interactions between CARD domains have been shown to be selective. For example, the CARD of Apaf-1 binds to caspase-9 CARD but not to other CARD-containing caspases. To identify the binding partner of CARD9, we performed a mammalian two-hybrid analysis and screened a panel of CARD domains for their ability to interact with the CARD of CARD9. Transfection of 293T cells with plasmids expressing the CARD of CARD9 fused to the transcriptional activation domain of mouse NF-κB (CARD9-CARD/AD) and the CARD of BCL10 fused to the DNA-binding domain of the yeast protein GAL4 (BCL10-CARD/BD) activated the mammalian two-hybrid reporter plasmid resulting in a 250-fold increase in relative luciferase activity (Fig. 2A). Likewise, expression of CARD9-CARD/BD and BCL10-CARD/AD increased luciferase activity 75-fold (Fig. 2B). Coexpression of CARD9-CARD with other CARD domains failed to activate luciferase expression indicating that the CARD of CARD9 interacts selectively with the CARD of BCL10.

Our finding that the CARD of CARD9 interacts with the activation CARD domain of BCL10 prompted us to further examine the interactions between these two proteins when overexpressed in cells. Immunoprecipitation of FLAG-tagged CARD9 quantitatively coprecipitated T7-tagged BCL10 (Fig. 3A). This association was dependent on the N-terminal CARD domain of BCL10 because CARD9 failed to coprecipitate a variant BCL10 with a point mutation (L41R) that disrupts CARD-mediated homodimerization (Fig. 3B and Ref. 12). In addition, CARD9 self-associated when expressed in cells suggesting that oligomerization may play a role in protein function (Fig. 3C). We were unable to perform immunoprecipitations with the individual domains of CARD9 because the CARD and coiled-coil domains were either unstable or insoluble when overexpressed. To rule out the possibility that other proteins were necessary for the CARD9-BCL10 interaction, we exam-
ined the interaction of radiolabeled CARD9 with GST-BCL10 in vitro and found that CARD9 associated directly with BCL10 (Fig. 3D). The amount of CARD9 that associated with GST-BCL10 (L41R) was greatly reduced confirming the importance of the BCL10 CARD domain in mediating interactions between these two proteins.

Components of signaling pathways are frequently found pre-assembled together within the cell to ensure a rapid response to upstream stimuli. To examine the interactions between endogenous CARD9 and BCL10, we generated a polyclonal antibody that specifically recognizes CARD9. Immunoblot analysis of extracts derived from human monocyte Thp1 cells revealed a predominant band of ~70 kDa corresponding to endogenous CARD9 (Fig. 3E, lane 1), and a 40-kDa band corresponding to endogenous BCL10 (Fig. 3F, lane 1). Immunoprecipitation of BCL10 coprecipitated CARD9 indicating that both endogenous proteins are associated with each other within Thp1 cells (Fig. 3E, lane 2).

To determine the cellular localization of CARD9, and to confirm that CARD9 associated intracellularly with BCL10 when the two proteins were coexpressed, we transfected Rat-1 cells with a Myc-tagged vector encoding full-length CARD9 and with an HA-tagged vector encoding full-length BCL10. The expressed proteins were detected using a mixture of a monoclonal anti-Myc antibody and a polyclonal rabbit anti-HA antibody. As shown in Fig. 4A, the two proteins exhibited distinctly different patterns of cellular localization when either vector was transfected alone. Whereas BCL10 exhibited either a clear pattern of discrete cytoplasmic filaments (Ref. 21 and Fig. 4A, left panel) or a diffuse whole-cell distribution (Fig. 4A, center panel), CARD9 displayed a somewhat punctate cytoplasmic or whole-cell distribution (Fig. 4A, right panel) but was not observed to form filament-like structures. When the two proteins were coexpressed in the same cell however, some of the CARD9 was found to colocalize with the BCL10 filaments (Fig. 4B). This finding is consistent with the interaction of CARD9 with BCL10 observed in immunoprecipitation experiments (Fig. 3A) and suggests that CARD9 is recruited to a cytoplasmic signaling complex with BCL10.

Our finding that CARD9 interacts with BCL10 prompted us to determine whether CARD9 can activate NF-κB using a luciferase reporter gene directed by an NF-κB-responsive promoter. Expression of CARD9 in 293T cells induced NF-κB activity by 8-fold compared with empty vector (Fig. 5A). Activation was specific for NF-κB signaling because CARD9 failed to activate a luciferase reporter gene with AP-1 promoter elements (data not shown). Activation of NF-κB by CARD9 correlated with binding to endogenous BCL10 suggesting that CARD9 forms a CARD9-BCL10 signaling complex within the transfected cells (Fig. 5B). We next determined the domains of CARD9 that mediate the activation of NF-κB (Fig. 5C). Because individual domains of CARD9 were either unstable or insoluble, we fused the CARD (residues 1–98), coiled-coil (residues 140–418), and C-terminal (residues 99–536) domains of CARD9 to GFP and expressed the fusion proteins in 293T cells. The CARD-GFP fusion activated NF-κB signaling to levels similar to that obtained with CARD9-GFP. The coiled-coil and C-terminal domains when fused to GFP failed to activate reporter gene expression establishing the CARD domain as the NF-κB activating domain of CARD9.

The data presented suggest that CARD9 is a specific regulator of BCL10 function. CARD9 could play a role as an upstream signaling molecule that recruits BCL10 through CARD-CARD interactions. The resulting signaling complex may interact directly or indirectly with components of the IKK complex resulting in its activation. One possible mechanism by which BCL10-CARD9 could activate the IKK complex is through oligomerization of IKK (10, 11). Indeed our data shows that both CARD9 and BCL10 form large oligomeric complexes (filaments) when overexpressed in mammalian cells. Furthermore, enforced oligomerization of the C terminus of BCL10 induces NF-κB activation (12), suggesting that the CARD domain of BCL10 functions as an oligomerization domain that transduces the activation signal to the IKK complex through its C-terminal domain. The ability of CARD9 to form a complex with BCL10 via CARD-CARD interactions supports the idea that BCL10 functions as an adaptor between the effector IKK complex and the proximal signaling complexes that interact with CARD9. We do not currently know the nature of the signaling molecules upstream of CARD9, but we predict that they transduce their signals to BCL10 through direct interactions with the C-terminal coiled-coil domain of CARD9. Taken together, these results identify CARD9 as an important mediator of NF-κB signaling through BCL10.

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