

CARD6 Is a Modulator of NF- κ B Activation by Nod1- and Cardiak-mediated Pathways*

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We cloned a novel cDNA derived from the *CARD6* gene locus on chromosome 5p12 of 311 amino acids in length. By immunoprecipitation we detected specific binding of this *CARD6*-encoding protein to Nod1 (*CARD4*), Cardiak (*Rip2/Rick*), NAC (*NALP1/DEFCAP/CARD7*), and TUCAN (*CARD8/Cardinal/NDPP/Dakar*), caspase recruitment domain (CARD)-containing proteins implicated in NF- κ B and caspase-1 activation but not to other CARD family proteins. Cardiak and Nod1 (but not other CARD proteins) also exhibited opposing effects on *CARD6* protein phosphorylation and expression, providing further evidence of functional interactions among these proteins in cells. In transfection experiments, the *CARD6* protein suppressed NF- κ B induction by Nod1 or Cardiak but did not interfere with NF- κ B activation by the CARD-containing adapter protein Bcl10 or the cytokine tumor necrosis factor- α , demonstrating specificity of *CARD6* for Nod1- and Cardiak-dependent pathways. In contrast to its effects on Nod1- and Cardiak-dependent NF- κ B activation, *CARD6* did not interfere with caspase-1-dependent interleukin-1 β secretion induced by Cardiak or Nod1. *CARD6* also did not affect caspase activation and apoptosis induced by overexpression of Fas, Bax, or other pro-apoptotic stimuli. Thus, *CARD6* represents a selective modulator of NF- κ B activation by Cardiak and Nod1, adding to the repertoire of CARD-family proteins implicated in inflammatory responses and innate immunity.

Proteins containing the caspase recruitment domain (CARD)¹ are critically involved in assembly of signal complexes leading to activation of caspase family proteases as well as induction of NF- κ B family transcription factors. The CARD represents a protein interaction motif consisting of a bundle of usually six antiparallel α -helices. This protein fold defines a

branch of a larger group of closely related domains, comprising four families that include the CARDS, “death domains,” “death effector domains,” and the “Pyrin, AIM, ASC, and death domain-like” (PAAD) (also known as PYRIN, PYD, or DAPIN) domains (1–5).

Homotypic interactions among CARD-containing adapter proteins occur with the inactive zymogen forms of caspases that possess N-terminal pro-domains containing complementary CARDS, resulting in formation of multiprotein complexes and leading to protease cleavage and activation by an induced proximity mechanism (6). Caspases represent a family of intracellular cysteine proteases that play important roles in apoptosis and cytokine processing by virtue of their ability to cleave protein substrates involved in cell death or inflammation. Various CARD-containing proteins have also been identified that compete for binding to these pro-caspases or adapter proteins, thereby suppressing caspase activation (for review, see Ref. 7).

Besides caspase activation, several CARD-containing proteins have been implicated in NF- κ B activation, including the Nod1 (*CARD4*) and Nod2 (*CARD15*) proteins, Cardiak (*Rick/Rip2*), Bcl10 (*Clap/Ciper/Carmen/mE10/cE10*), *CARD9*, the cIAP1 and cIAP2 proteins, and the MAGUK family of CARD proteins (*CARD10*, *CARD11*, and *CARD14*). These NF- κ B-inducing CARD family proteins function in signaling pathways of the adaptive or innate immune system, thus leading to the activation of the transcription factor NF- κ B and promoting inflammatory responses (for review, see Ref. 5). NF- κ B represents a family of dimeric transcription factors containing the Rel homology domain, which play critical roles in regulating expression of genes involved in inflammation, immunity, and apoptosis (for review, see Ref. 8). NF- κ Bs are sequestered in the cytoplasm by inhibitory proteins called I κ Bs. In the classical pathway for NF- κ B induction, I κ B molecules are removed by proteasome-dependent degradation after phosphorylation by the I κ B kinase complex (IKK) complex, thus releasing NF- κ B family proteins to enter the nucleus. The IKK complex consists of two related kinases, IKK α /IKK1 and IKK β /IKK2, and a scaffold subunit IKK γ /Nemo (for review, see Ref. 9).

Nod1/*CARD4*, a widely expressed protein, and Nod2 (*CARD15*), a monocyte-specific protein, have a domain organization reminiscent of the pro-caspase-9-activating protein Apaf1, the pro-caspase-1-activating protein CLAN (*Ipaf/CARD12*), plant disease resistance “R” proteins, and a large family of PAAD/Pyrin domain-containing proteins, which have been implicated in NF- κ B and caspase-1 regulation (10–16) (for review, see Refs. 17 and 18). This family of related proteins is characterized by the presence the NACHT module, a nucleotide binding oligomerization domain (19), typically followed by a series of leucine-rich repeats (LRRs). The LRRs of these intracellular proteins are analogous to the LRRs found in the extracellular domains of Toll family receptors expressed on the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY196783.

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¹ The abbreviations used are: CARD, caspase recruitment domain; LPS, lipopolysaccharide; IKK, I κ B kinase complex; TNF, Tumor Necrosis Factor; IL, Interleukin; LRR, leucine-rich repeat; wt, wild type.

cell surface, which recognize extracellular pathogen-derived molecules such as lipopolysaccharide (LPS), bacterial peptidoglycans, CpG DNA, and other structures that create so-called "pathogen-associated molecular patterns" (PAMPs) (20). In this regard Nod1 has been reported to function as an intracellular receptor for LPS, whereby LPS binding to the LRRs of Nod1 is envisioned as a signal for inducing oligomerization of the NACHT domains of this protein, triggering NF- κ B activation by the induced proximity mechanism by virtue of the ability of the CARD of Nod1 to interact with the corresponding CARD of the IKK-binding protein Cardiak/Rick/Rip2 (21–23) (for review, see Refs. 24 and 25). Nod1 was also recently reported to induce caspase-1 activation in conjunction with Cardiak (26), probably reflecting the ability of the CARD of Cardiak to bind the corresponding CARD within the pro-domain of pro-caspase-1. Caspase-1 is responsible for proteolytic processing of pro-inflammatory cytokines, including pro-interleukin (IL)-1 β and pro-IL-18 (27).

In addition to its CARD domain, Cardiak contains a serine/threonine kinase domain of uncertain function (28–30). Interestingly, Cardiak is recruited to certain Toll-like receptor-signaling complexes after stimulation with their cognate ligands (e.g. LPS, peptidoglycan, and double-stranded RNA). Recently, Cardiak ($-/-$) mice were also shown to exhibit defects in Toll-like receptor, IL-1, IL-18, and Nod signaling. Moreover, T-cells from Cardiak ($-/-$) mice displayed defects in NF- κ B activation, IL-2 production, proliferation, and differentiation to T_H1 cells, suggesting Cardiak functions as a signaling component of relevance to both adaptive and innate immune responses (31, 32).

Here we report cloning and characterization of a cDNA encoding a CARD-containing 311-amino acid protein encoded in the *CARD6* gene locus. This *CARD6*-encoded protein interacts with Cardiak and Nod1 and specifically impairs their ability to induce the transcription factor NF- κ B. The *CARD6* protein, thus, might regulate the duration of the signaling initiated by these pro-inflammatory proteins or help to set thresholds for their activation so that unintended inflammation is avoided.

EXPERIMENTAL PROCEDURES

Bioinformatics—Starting from the sequence of the CARD domain of Apafl (33), a cascade of PSI-BLAST (34) searches was performed using new "hits" as queries for subsequent searches until no new hits were found, as described elsewhere (35). Lower significance hits from this procedure ("saturated BLAST") were confirmed (or rejected) using the profile-to-profile alignment algorithm FFAS (36) against a library of apoptosis-related domains. The detailed analysis of three-dimensional models of the putative CARD domains was a final criterion in accepting/rejecting distant members of the family. After the complete list of proteins in the CARD family was assembled, representative members of the family were used to scan genomic and EST databases with the TBLASTN program. Genomic regions where similarity to the CARD domain was found were again verified against the FFAS server and later analyzed with gene-finding programs (37) for the presence of other domains until a complete protein sequence was assembled. Three-dimensional models of CARD domains were generated using the MODELLER program (38), based on FFAS alignments. Among the predicted CARD family genes thus identified was a locus previously given the name "*CARD6*" (GenBank™ accession number NM_032587).

cDNA Cloning and Plasmid Construction—High fidelity PCR (Stratagene) was used to clone cDNAs encoded by the *CARD6* gene from a skeletal muscle cDNA library (Clontech), obtaining a single PCR product of 1107 bp, which was cloned into pcDNA3 plasmids (Invitrogen) and determined by DNA sequencing to contain the complete open reading frame for a 311-amino acid protein followed by a stop codon, a polyadenylation signal, and a poly(A) sequence. Deletion mutants were generated by high fidelity PCR. The Cardiak (K47M) mutant was generated using the QuikChange mutagenesis kit (Stratagene). Expression plasmids for Apafl, ASC, Bcl10, Cardiak, Clan, pro-caspase-1, pro-caspase-9, Nac, Nod2, and TUCAN have been described previously (10, 39–41). CARD10, cIAP1, cIAP2, and Nod2 were amplified by re-

verse transcription-PCR from cDNAs (Clontech, Origene) and cloned into pcDNA3 plasmids (Invitrogen). Pro-caspase-4, pro-caspase-5, Nod1, and RAIDD expression plasmids were kindly provided by Yoshihide Tsuimoto, Alan G. Porter, Gabriel Nunez, and Emad S. Alnemri, respectively. Authenticity of all constructs was confirmed by DNA sequencing.

Cell Culture and Transfection—HEK293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum. Where indicated, cells were treated with 10 ng/ml TNF α for 8 h. Transfection of HEK293 cells was accomplished using Superfect (Qiagen), whereas HeLa cells were transfected with LipofectAMINE Plus (Invitrogen), holding total DNA content constant.

Co-immunoprecipitation Assays—Cells (5×10^5 – 1×10^6) were lysed in isotonic lysis buffer (150 mM NaCl, 20 mM Tris/HCl (pH 7.4), 0.2% Nonidet P-40, 12.5 mM β -glycerophosphate, 2 mM NaF, 200 μ M Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, and 1 \times protease inhibitor mix (Roche Applied Science)). Clarified lysates were subjected to immunoprecipitation using agarose-conjugated anti-c-Myc, anti-hemagglutinin (Santa Cruz Biotechnology), or anti-FLAG M2 (Sigma) antibodies. After incubation at 4 °C for 4–12 h, immune complexes were washed 3 times in lysis buffer, separated by SDS/PAGE, and analyzed by immunoblotting using anti-c-Myc, anti-hemagglutinin, anti-FLAG, or anti-caspase-4 antibodies as indicated in conjunction with ECL detection system (Amersham Biosciences). Where indicated, cell lysates (10% volume) were run alongside immune complexes. Alternatively, lysates were directly analyzed by immunoblotting after normalization for total protein content.

In Vitro Kinase Assays—HEK293T cells were transiently transfected with plasmids expressing FLAG-Cardiak. After 2 days, cell lysates were prepared, and FLAG-Cardiak protein was immunoprecipitated with agarose-conjugated anti-FLAG M2 antibody. Kinase reactions were initiated by resuspending washed immune complexes in 1 μ M of [γ -³²P]ATP in 10 μ l of kinase buffer (50 mM Tris (pH 7.4), 100 mM NaCl, 6 mM MgCl₂, 1 mM MnCl₂, and 1 mM EDTA) containing CARD6 protein as an exogenous substrate, where Myc-tagged CARD6 protein was generated by transient transfection in HEK293T cells and recovered by immunoprecipitation. After 20 min at 37 °C, reactions were stopped by the addition of an equal volume of SDS sample buffer and analyzed by SDS-PAGE and autoradiography.

Phosphatase Treatment—HEK293T cells were transiently transfected with plasmids encoding Myc-CARD6 with or without plasmids encoding FLAG-Cardiak. Cleared lysates were diluted 1:20 with 20 μ l of reaction buffer (25 mM Tris (pH 8.0), 50 mM NaCl, 5 mM MgCl₂) and treated with 2 units of calf intestine alkaline phosphatase (Invitrogen) for 30 min at 37 °C. The reaction was terminated by adding SDS sample buffer and following with SDS-PAGE/immunoblotting.

NF- κ B Reporter Gene Assay—Typically, 10^5 HEK293 cells, cultured in 24-well plates in 5% serum, were transiently transfected with a total of 1 μ g of plasmid DNA (normalized for total DNA) including 144 ng of pNF- κ B-LUC (Clontech) and 6 ng of a *Renilla luciferase* gene driven by a constitutive thymidine kinase (TK) promoter (pRL-TK; Promega). Lysates were analyzed using the dual luciferase kit (Promega). Where indicated, cells were treated with 10 ng/ml TNF α for 8 h before analysis.

IL-1 β Secretion Assay—IL-1 β secreted into culture supernatants of HEK293N cells transiently transfected in 24-well plates was measured by enzyme-linked immunosorbent assay using a commercial kit (R&D Systems); data were normalized for cell numbers, and assays were performed triplicate (42).

Caspase Activity Assay—HEK293N cells were transiently transfected in 6-well dishes, and at 24 h post-transfection, cells were suspended in caspase lysis buffer (10 mM HEPES (pH 7.4), 25 NaCl, 0.25% Triton X-100, and 1 mM EDTA) and normalized for protein content. Typically, 15–25 μ g of lysates were analyzed for protease activity by continuously measuring the release of fluorogenic Ac-DEVD-AFC (Bachem) at 37 °C, as described (43).

Immunofluorescence Analysis—HeLa cells were transfected with plasmids encoding green fluorescent protein green fluorescent protein-CARD6 fusion proteins, transferred to 4-well polylysine-coated chamber slides (LabTec) the following day, fixed in 4% paraformaldehyde 1 day later, permeabilized with Triton X-100, stained with Topro-3 (Molecular Probes) for detection of nuclei, and analyzed by confocal laser-scanning microscopy (Bio-Rad).

RESULTS

Cloning of cDNAs Encoding a CARD-containing Protein Encoded in the *CARD6* Gene Locus—Using bioinformatics ap-

lacks significant homology to other proteins but contains several potential phosphorylation sites, as predicted by NetPhos 2.0 (www.cbs.dtu.dk/services/NetPhos). Recently, the predicted cDNA sequence of a longer, 1037-amino acid protein with an identical CARD domain was deposited in GenBank™ (GenBank™ accession number NM_032587) under the name CARD6; however, we did not obtain such cDNAs by our cloning method. Besides being shorter, the last 30 amino acids are different in our predicted protein compared with accession number NM_032587.

The nucleotide sequence of human cDNA we identified displays significant homology to an automatic prediction of an open reading frame in the mouse by GenBank™ (Fig. 1D). The predicted human and mouse CARD6 proteins share 53.2% amino acid sequence identity (63.4% similarity), although the human *CARD6*-encoded protein we identified is 26 amino acid residues longer. In addition, the structures of the human *CARD6* and mouse *CARD6* genes differ in their 3'-ends, with cDNAs derived from the human gene demonstrating sequence identity to regions encompassed in three exons, compared with two exons in the mouse gene (Fig. 1E). The human gene therefore spans at least 18.9 kilobases on chromosome 5p12 (based on genomic clone NT_034769), whereas the mouse *Card6* gene spans at least 3.5 kilobases on band a1 of mouse chromosome 15 (based on genomic clone NW_000102). Interestingly, the region of the mouse genome where *Card6* resides is syntenic to the chromosome 5p12 region of humans.

Tissue Expression of *CARD6* mRNA and Subcellular Location of *CARD6* Protein—To explore the tissue distribution of *CARD6*-encoded mRNAs, we hybridized a Northern blot containing poly-A-selected mRNA from multiple human tissues with a ³²P-labeled *CARD6*-specific probe. Two transcripts were detected of ~4.4 and 6.3 kilobases, which could represent differentially spliced transcripts or utilization of different polyadenylation sites (Fig. 2A). The smaller transcript is found in almost all tissues except for brain, whereas the larger transcript was present only in heart and skeletal muscle.

To explore the intracellular location of the *CARD6* protein, we transiently transfected HeLa cells with a plasmid encoding a green fluorescent protein-CARD6 fusion protein and analyzed the location of the resulting protein by confocal microscopy. Green fluorescent protein-CARD6 exhibited a diffuse cytoplasmic pattern and in occasional cells was also found in the nucleus, as confirmed by counterstaining of nuclear DNA with Topro-3 (Fig. 2B).

***CARD6* Interacts with Other *CARD* Proteins**—The CARD is a homotypic interaction domain. Therefore, we tested the ability of the *CARD6* protein to associate with several of the known *CARD* family proteins by co-immunoprecipitation assays after transient co-transfection in HEK293T cells. As demonstrated in Fig. 3, among the 18 *CARD* family members tested, only Nod1, Cardiak, TUCAN, and NAC preferentially interacted with *CARD6* (Fig. 3A). However, we could not detect any association between *CARD6* and the other *CARD* proteins, including *CARD*-carrying caspases (Fig. 3B). Also we failed to detect *CARD6* self-association, although many *CARD* family members show this ability.

Effects of *Nod1* and *Cardiak* on *CARD6* Protein Levels—During our studies, we observed that co-expression of *Cardiak* with *CARD6* in HEK293T cells markedly increased *CARD6* protein levels and also resulted in a shift in molecular weight of a portion of the *CARD6* protein (Fig. 4A), suggestive of a post-translational modification. In contrast, *Nod1* did not influence the levels of *CARD6* protein (Fig. 4, A and B). However, co-expression of a *Nod1* mutant lacking its CARD domain, *Nod1* (Δ CARD), resulted in reduced expression of the *CARD6*

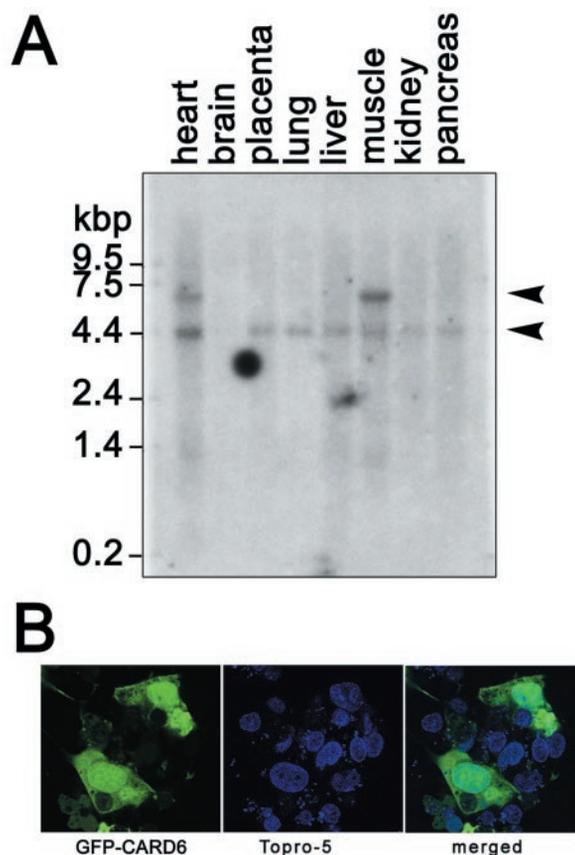


FIG. 2. Expression of *CARD6* in human tissues and subcellular location of *CARD6* protein. A, *CARD6* mRNA expression in various adult human tissues was determined by Northern blot analysis using a ³²P-labeled human *CARD6* cDNA probe. The two hybridizing transcripts are marked by arrows. B, confocal laser microscopy analysis was performed using HeLa cells that were transiently transfected with plasmids encoding green fluorescent protein-CARD6 fusion protein. Cells were also counterstained with Topro-3 (Molecular Probes) for visualization of nuclei. Cells were imaged by two-color fluorescence microscopy. *kbp*, kilobase pairs.

protein (Fig. 4B). When we expressed the three proteins of *Nod1*, *Cardiak*, and *CARD6* together, we observed that *Nod1* abolished the *Cardiak*-mediated effect on *CARD6* protein expression and modification. In contrast, co-expression of just the CARD of *Nod1* (*Nod1*-CARD) with *Cardiak* and *CARD6* did not alter the *Cardiak* effect on the *CARD6* protein (Fig. 4A). In contrast to *CARD6*, gene transfer-mediated overexpression of *Nod1* and *Cardiak* had no effects on the expression of a variety of other control proteins in similar co-transfection assays (not shown), thus demonstrating the specificity of these results.

Investigation of *Cardiak* Effects on *CARD6* Phosphorylation—Because *Cardiak* possesses a kinase domain (28, 29), we investigated whether the modification of *CARD6* observed upon co-expression of *Cardiak* might represent phosphorylation. Accordingly, we co-expressed *CARD6* with or without *Cardiak* in HEK293T cells, prepared cell lysates, and then treated half of the lysates with alkaline phosphatase for comparison with untreated lysates. Indeed, treatment of lysates with phosphatase reversed the modification of the *CARD6* protein, which was seen when *Cardiak* had been co-expressed. Thus, *Cardiak* appears to induce phosphorylation of the *CARD6* protein (Fig. 5A).

The effect of *Cardiak* on *CARD6* protein levels made it difficult to determine whether *Cardiak* directly induces phosphorylation of *CARD6* or merely allows sufficient *CARD6* protein accumulation so that the modified form of *CARD6* becomes

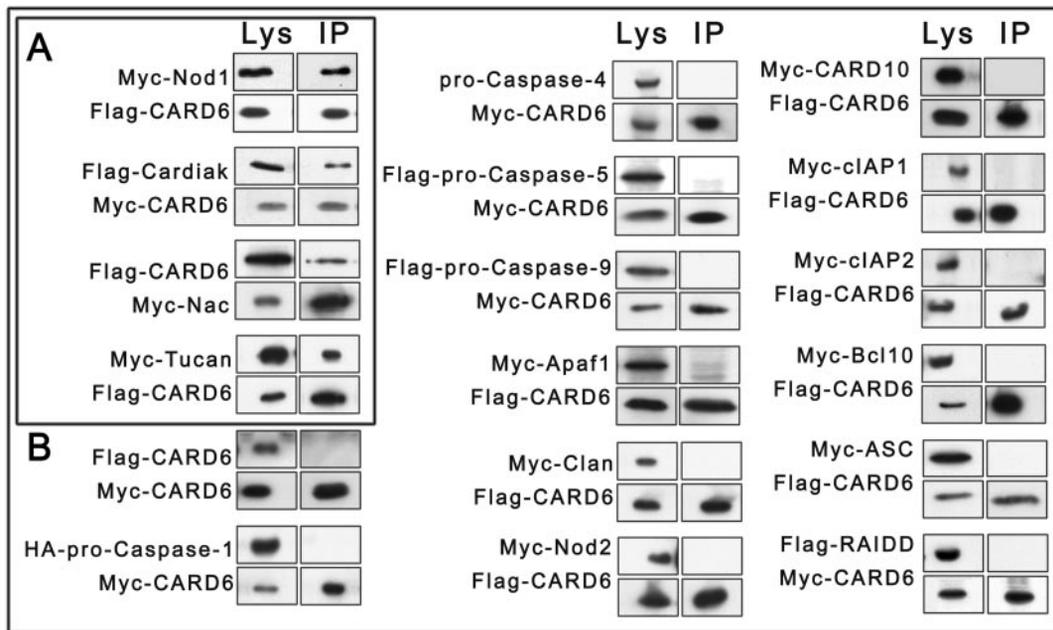


FIG. 3. CARD6-associated proteins. Co-immunoprecipitation assays were performed using lysates from HEK293T cells that had been transiently transfected with plasmids encoding epitope-tagged proteins as indicated. Lysates were immunoprecipitated (IP) with agarose-conjugated antibodies directed to the epitope tag of the protein shown in the lower panel of in pair of blots, and the resulting immune complexes were analyzed by SDS-PAGE/immunoblotting using antibodies to the epitope tag of the protein listed on top of each pair of blots, except for non-tagged pro-caspase-4, which was detected by an antibody to pro-caspase-4 itself. Alternatively, cell lysates were analyzed directly by immunoblotting (Lys) for expression of both proteins. Shown are CARD6-containing proteins that interact with CARD6 by transient overexpression (A) and CARD6-containing proteins that do not interact with CARD6 (B).

detectable. We therefore performed experiments in which Cardiak was immunoprecipitated from transiently transfected HEK293T cells and used for *in vitro* kinase assays, employing CARD6 as a substrate (Fig. 5B). Autophosphorylation of Cardiak (*P-Cardiak*) was detected in these kinase assays but not phosphorylation of CARD6 (*P-CARD6*) (Fig. 5B), suggesting that Cardiak does not directly phosphorylate CARD6.

Finally, to resolve the issue of the role of Cardiak in phosphorylation of CARD6, we compared the effects of co-expressing CARD6 with either wild type Cardiak or a kinase-dead mutant of Cardiak, where the lysine 47 was mutated to methionine. Co-expression of CARD6 with Cardiak (K47M) did not inhibit the phosphorylation of CARD6 compared with wild-type Cardiak. Both wild type and K47M Cardiak also resulted in increased levels of CARD6 protein, indicating that the kinase activity of Cardiak is not required for the Cardiak-mediated effect on CARD6 protein levels (Fig. 5C).

CARD6 Selectively Suppresses NF- κ B Activity Induced by Nod1 and Cardiak—Several Card family proteins have been reported to induce the transcription factor NF- κ B, including Nod1 and Cardiak. To explore the effects of CARD6 on NF- κ B, HEK293N cells were transiently transfected with control plasmid or an expression plasmid encoding epitope-tagged CARD6 protein and then analyzed for NF- κ B reporter gene activity. As shown in Fig. 6, CARD6 does not activate NF- κ B when expressed in cells by itself (Fig. 6C). Because it was shown that Nod1 and Cardiak strongly induce NF- κ B activation, we investigated the effect of CARD6 on NF- κ B activation in cells expressing Nod1, Cardiak, or various other NF- κ B-activators, such as Bcl10, a CARD-containing adaptor protein that links signals from B-cell and T-cell antigen receptors to NF- κ B activation (44). Accordingly, HEK293N cells were transiently transfected with plasmids encoding Cardiak, Nod1, or Bcl10 either in the presence (Fig. 6A, *hatched bars*) or absence (*black bars*) of CARD6. Cells were also induced by TNF α , a strong NF- κ B activating cytokine, either in the presence (*hatched bars*) or the absence of CARD6-encoding plasmid (*black bars*)

(Fig. 6A). Lysates were analyzed for NF- κ B reporter gene activity.

CARD6 blocked NF- κ B activity induced by expression of Nod1 or Cardiak but did not affect NF- κ B activity induced by Bcl10 or TNF α (Fig. 6). To demonstrate the specificity of the CARD6-mediated effect on NF- κ B activity, we transiently transfected HEK293N cells with plasmids encoding Cardiak together with either wild type (wt) CARD6 (*hatched bars*) or a mutant of CARD6 lacking its CARD domain, CARD6 (Δ CARD) (*white bars*) (Fig. 6B). Although CARD6 (wt) suppressed Cardiak-mediated NF- κ B activation, CARD6 (Δ CARD) did not (Fig. 6B). Immunoblotting analysis confirmed expression of all proteins, excluding differences in the levels of CARD6 (wt) and CARD6 (Δ CARD) as a trivial explanation for the results (Fig. 6D).

Investigation of CARD6 Effects on Caspase Activation—Because Nod1 and Cardiak are capable of activating caspase-1 in addition to inducing NF- κ B activity, we tested the effects of CARD6 overexpression on secretion of IL-1 β , a caspase-1-dependent event (45). A co-transfection scheme modeled after prior studies of caspase-1-regulating proteins was employed (42, 46) in which cells were transiently transfected with plasmids encoding pro-IL-1 β and pro-caspase-1 alone or in combination with various CARD-containing proteins. As shown in Fig. 7A, expression of pro-IL-1 β and pro-caspase-1 resulted in modest levels of IL-1 β secretion, which were enhanced by co-expression of either Cardiak or Nod1, consistent with prior reports (29, 46, 47). When CARD6 was co-expressed with Cardiak and Nod1 under the same conditions in which NF- κ B suppression was observed, IL-1 β secretion was unimpaired (Fig. 7A). Even when transfecting CARD6-producing plasmid DNA at ratios ranging up to 4:1 relative to Cardiak or Nod1-encoding plasmid DNA, we did not observe any changes in IL-1 β secretion (data not shown). We conclude, therefore, that the inhibitory effects of CARD6 on Nod1 and Cardiak are predominantly directed toward their NF- κ B-inducing activity, with little influence on their caspase-1-activating functions.

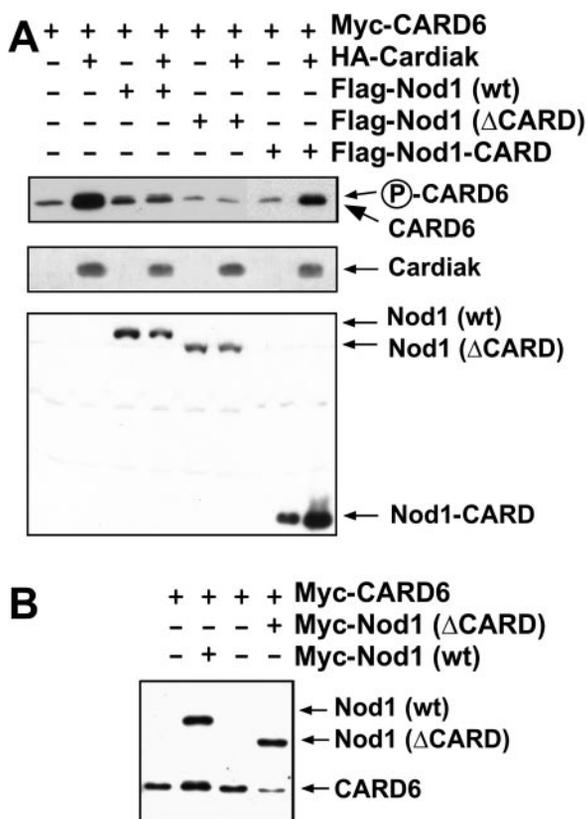


FIG. 4. Cardiak regulates CARD6 protein levels. *A*, lysates were prepared from HEK293T cells that had been transiently transfected with plasmids encoding myc-CARD6, hemagglutinin (HA)-Cardiak, Myc-Nod1 (wt), Myc-Nod1 (Δ CARD), and Myc-Nod1-CARD (amino acids 1–128) as indicated. Lysates were directly analyzed by SDS-PAGE/immunoblotting using anti-Myc (*top panel*), anti-hemagglutinin (*middle panel*), and anti-FLAG (*bottom panel*) antibodies. *B*, lysates were prepared from HEK293T cells that had been transiently transfected with plasmids encoding Myc-CARD6 with either wild type Myc-Nod1 (wt) (amino acids 1–953) or Myc-Nod1 lacking its CARD domain (Δ CARD) (amino acids 160–953). Lysates were directly analyzed by SDS-PAGE/immunoblotting using anti-Myc antibody with ECL-based detection.

Because the CARD domain was originally described as a motif linking various signaling pathways to pro-caspase activation and apoptosis, we also tested whether CARD6 might modulate apoptosis. For these experiments, we tested the effects of CARD6 on proto-typical activators of the mitochondrial (intrinsic) and Fas/TNF (extrinsic) pathways for apoptosis by transiently transfecting cells with plasmids encoding either the intrinsic pathway activator, Bax (for review, see Ref. 48), or the extrinsic, caspase-9-independent activator, Fas (for review, see Ref. 49 and 50). As controls, we also employed dominant-negative versions of caspase-9 and caspase-8 (in which the active site cysteine had been converted to alanine), representing the apical proteases in the intrinsic and extrinsic pathways, respectively. Activation of apoptosis-associated effector caspases that cleave the tetrapeptide substrate Ac-DEVD-AFC was then measured (Fig. 7*B*) in addition to the percentage of cells undergoing apoptosis (not shown).

When expressed by itself, CARD6 did not induce activation of DEVD-cleaving caspases (Fig. 7*B*) or apoptosis (not shown). CARD6 also did not suppress effector caspase activation induced by Bax or Fas even though the CARD6-encoding plasmid was used at a 4-fold excess relative to the Bax- or Fas-encoding plasmids. In contrast, dominant-negative caspase-9 and caspase-8 proteins effectively suppressed activation of effector caspases (Fig. 7*B*) and apoptosis (not shown) induced by Bax

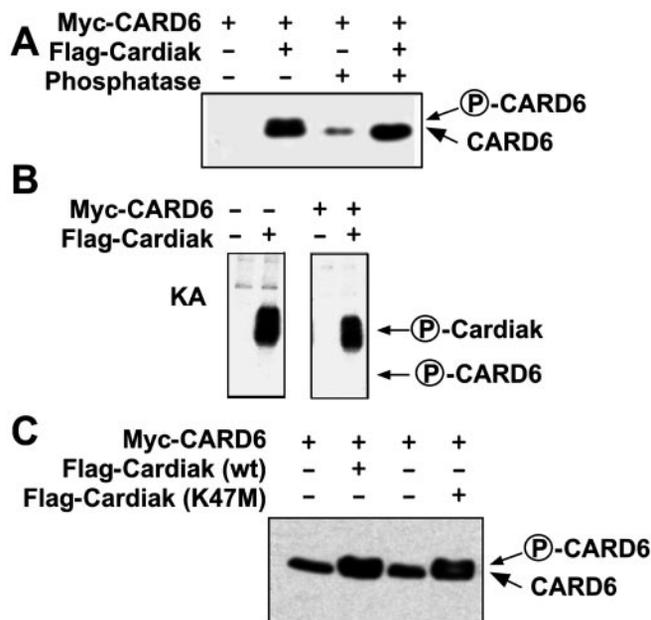


FIG. 5. Investigation of Cardiak effects on CARD6 phosphorylation. *A*, lysates from HEK293T cells expressing myc-CARD6 with or without FLAG-Cardiak were either treated with alkaline phosphatase or left untreated and analyzed by SDS-PAGE/immunoblotting using anti-Myc antibody with ECL-based detection. The phospho-CARD6 (*P-CARD6*) exhibits slower migration in SDS-PAGE than unphosphorylated CARD6. *B*, *in vitro* phosphorylation assay of CARD6. Lysates from HEK293T cells expressing FLAG-Cardiak were immunoprecipitated with agarose-conjugated anti-FLAG M2 antibody and subjected to *in vitro* kinase assay with or without purified Myc-CARD6 as a substrate. Autophosphorylation of Cardiak (*P-Cardiak*) but not phosphorylation of CARD6 (*P-CARD6*) was detected after autoradiography (*KA*, *in vitro* kinase assay). *C*, comparison of wild type and kinase-dead Cardiak. Lysates from HEK293T cells that had been transiently transfected with plasmids encoding Myc-CARD6 and either wild type FLAG-Cardiak (wt) or a kinase-deficient mutant, FLAG-Cardiak (K47M), were analyzed by SDS-PAGE/immunoblotting using anti-Myc antibody and ECL-based detection. Phosphorylated (*P-CARD6*) and unphosphorylated CARD6 are indicated by arrows.

and Fas, respectively. Thus, CARD6 does not appear to modulate activation of apoptotic caspases, unlike many other CARD family proteins.

DISCUSSION

We have identified and characterized cDNAs encoding a CARD-containing protein derived from the *CARD6* gene locus. Recently, a cDNA sequence encoding a similar protein with identical CARD was recorded in GenBank™ as CARD6 (GenBank™ accession number NM_032587), although no prior published reports have described the expression or function of this gene and encoded protein heretofore. Interestingly, this alternative form of the CARD6 protein is longer at its C terminus by 726 amino acids compared with our predicted CARD6 protein, although containing no recognizable protein motifs that are not present in the shorter protein. In addition, the last 30 amino acids of the shorter CARD6 protein are also different. These isoforms of the CARD6 protein might arise as a result of alternatively spliced transcripts, as supported by the presence of two transcripts detected by Northern blotting.

The CARD6 protein identified here binds specifically to Nod1 and Cardiak and suppresses their activation of NF- κ B while having comparatively little effect on their ability to induce activation of pro-caspase-1. The only recognized domain in CARD6 is the N-terminal CARD. After the CARD is a region that lacks obvious secondary structural elements that contains interspersed α -helix-disrupting glycine and proline residues.

We presume that the mechanism by which CARD6 disrupts

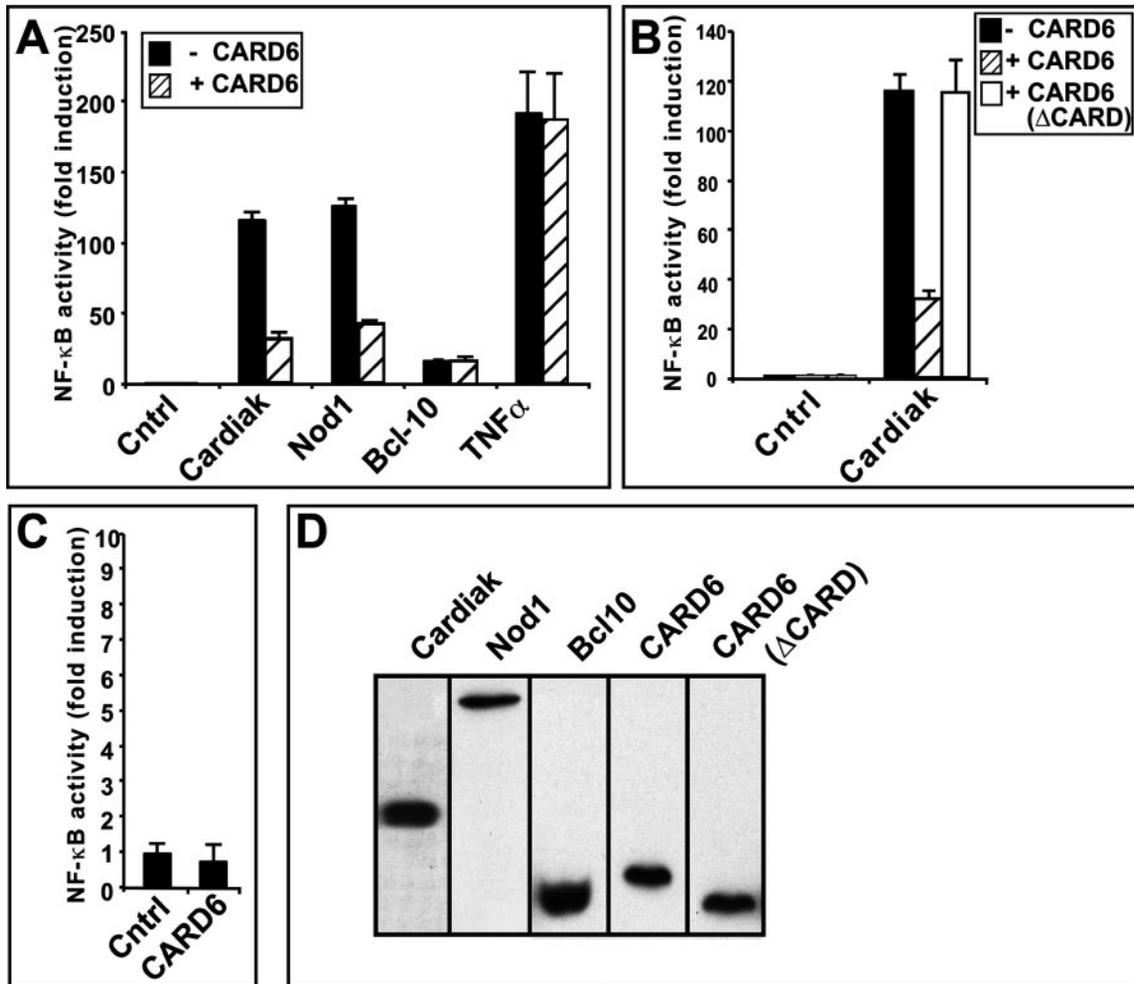


FIG. 6. CARD6 selectively suppresses NF- κ B activity induced by Nod1 and Cardiak. *A*, HEK293T cells were transfected in 24-well plates with either 850 ng of control pcDNA3 plasmid or 50 ng of plasmids encoding Cardiak, Nod1, or Bcl10 either in the absence or the presence of 100 ng of CARD6-encoding plasmid. Transfections also included 144 ng of pNF- κ B and 6 ng of pRL-TK, and total DNA of transfections was maintained at 1 μ g by the addition of control plasmid DNA. Data are expressed as fold-induction of NF- κ B compared with cells transfected with control DNA (mean \pm S.D.; $n = 3$). *B*, cells were transfected as above using either 850 ng of control plasmid or 50 ng of Cardiak-encoding plasmids and either 100 ng of CARD6 or 100 ng of CARD6 (Δ CARD)-encoding plasmids and maintaining the total DNA of transfections at 1 μ g by the addition of control plasmid DNA. *C*, cells were transfected as above using either control plasmid or plasmid encoding CARD6. Where indicated, cells were stimulated 36 h post-transfection for 8 h with 10 ng/ml TNF α , and samples were analyzed for NF- κ B activity by reporter gene assay. Data are represented as fold-induction relative to cells transfected with pcDNA3 control plasmid and are representative of several experiments (mean \pm S.D.; $n = 3$). *Cntrl*, control. *D*, lysates were also analyzed by SDS-PAGE/immunoblotting with ECL-based detection to verify expression of all proteins.

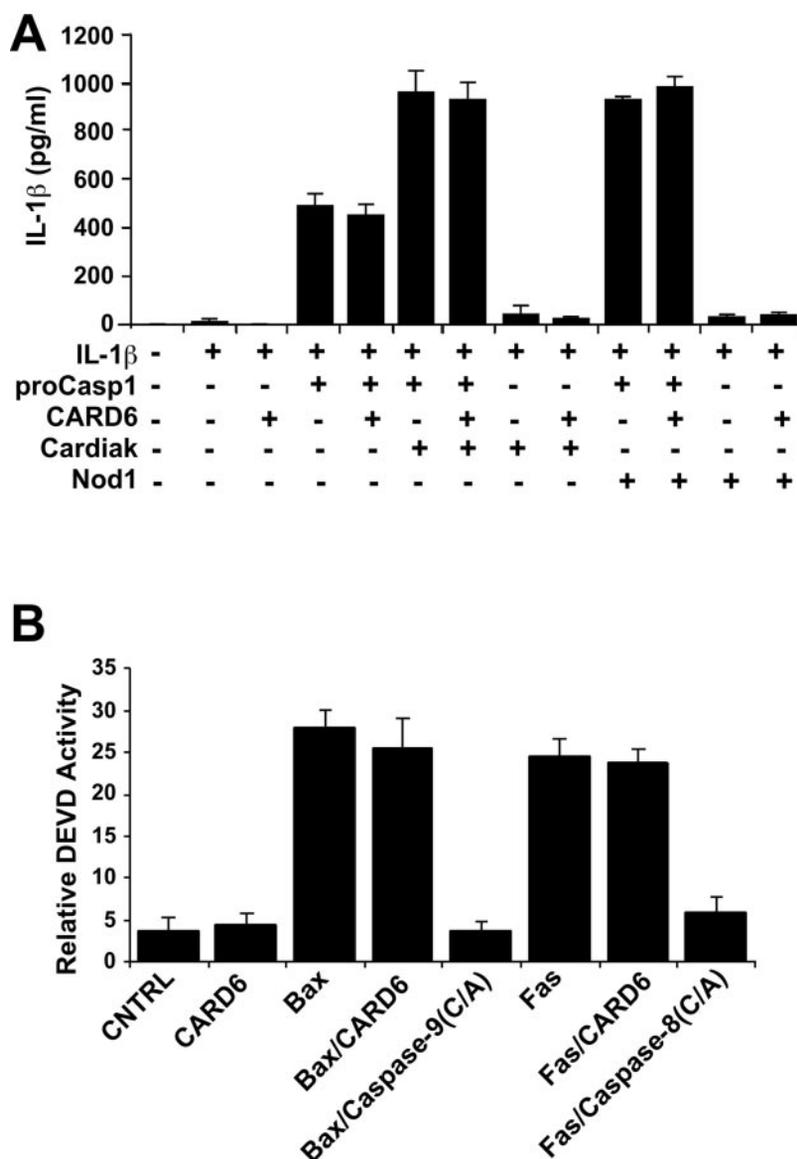
NF- κ B induction by Nod1 and Cardiak involves interference with their association with the IKK complex. In this regard, Cardiak reportedly associates directly with the IKK γ subunit of the IKK complex, whereas Nod1 is thought to activate NF- κ B indirectly by virtue of the ability of its CARD domain to bind the CARD of Cardiak. Thus, Cardiak functions as an adaptor that links Nod1 to the IKK complex (18, 21–23, 25). Other CARD family proteins that activate NF- κ B may also employ Cardiak as an adapter, such as Nod2 (51). While blocking NF- κ B activation induced by Cardiak-dependent pathways, CARD6 did not interfere with alternative routes of NF- κ B induction, as demonstrated by experiments using Bcl-10 and TNF α . In this regard, TNF α induces IKK activation through activation of upstream kinases that impinge on the IKK complex (52), whereas Bcl-10 links a class of CARD family proteins known as MAGUK family proteins to a caspase-like protein, Malt-1, that has been implicated in NF- κ B induction (53) (for review, see Ref. 54).

Nod1 and Nod2 are members of a family of proteins containing the NACHT domain that are thought to self-oligomerize in response to largely undefined signals (19). Some of these family

members are exclusively involved in activation of caspases, whereas others are implicated in NF- κ B induction (17, 55). Nod1 and Nod2 contain LRRs that reportedly bind LPS, functioning as intracellular receptors for PAMPs (pathogen-associated molecular patterns), analogous to the Toll receptors that bind PAMPs extracellularly (22, 23) (for review, see Ref. 24). Thus, Nod1 and Nod2 presumably play roles in innate immunity, similar to the Toll receptors. Interestingly, hereditary mutations in the Nod2-encoding gene have been implicated in some families with Crohn's disease, an inflammatory bowel disease and Blau syndrome, a rare granulomatous disorder (56–58). In as much as CARD6 functions as a suppressor of NF- κ B activation induced by Nod1 and Cardiak, it will be interesting to explore whether defects in CARD6 expression or function are associated with inflammatory bowel disease, Blau syndrome, or other inflammatory and autoimmune diseases.

Cardiak modulated the levels of CARD6 protein production in transfection experiments. Because we expressed CARD6 from a plasmid containing a strong promoter, we presume that Cardiak regulates the stability of the CARD6 protein in cells, accounting for the rise in CARD6 levels. Furthermore, given

FIG. 7. Effects of CARD6 on IL-1 β secretion and apoptosis. A, HEK293T cells were transiently transfected in 24-well plates with plasmids encoding pro-IL-1 β (200 ng), pro-caspase-1 (50 ng), CARD6 (300 ng), Cardiak (100 ng), and Nod1 (100 ng), as indicated, with total DNA maintained at 1 μ g by the addition of control (empty) plasmid. Cell culture supernatants were analyzed by enzyme-linked immunosorbent assay for secreted IL-1 β at 36 h post-transfection. Data shown represent pg/ml IL-1 β , normalized for cell number, and are representative of several experiments (mean \pm S.D.; $n = 3$). B, HEK293N cells were transiently transfected in 6-well dishes with either control plasmid or plasmids encoding CARD6 (1 μ g), Bax (0.3 μ g), Fas (0.5 μ g), caspase-9(C/A) (1 μ g), or caspase-8(C/A) (1 μ g), as indicated, with total DNA of transfections maintained at 2 μ g by the addition of control plasmid DNA. Lysates were normalized for protein content and analyzed for Asp-Glu-Val-Asp(DEVD)ase activity by continuously monitoring the release of fluorogenic AFC from substrate, Ac-DEVD-AFC, as described (43). Data represent relative fluorescence units/mg of protein (mean \pm S.D.; $n = 3$). *Ctrl*, control.



that CARD6 interacts with Nod1 and that expression of Nod1 (Δ CARD) reduced CARD6 protein levels, it could be that full-length Nod1 protein is required endogenously to stabilize CARD6 protein. Finally, we hypothesize further that because Nod1 interacts with Cardiak (23, 59, 60), competition between these proteins for CARD6 binding might influence CARD6 stability as well, accounting for CARD6 protein levels returning to base line upon co-expression of Nod1 with Cardiak and CARD6.

The accumulation of CARD6 protein observed in Cardiak-expressing cells also revealed a post-translationally modified form of CARD6. Based on *in vitro* phosphatase treatments, we deduced that this modified form of CARD6 represents a phosphoprotein. Although Cardiak possesses a protein kinase domain, *in vitro* kinase assay experiments failed to demonstrate a direct role for Cardiak in phosphorylation of the CARD6 protein. Thus, we presume that another kinase is responsible for the phosphorylation of CARD6. Also, a kinase-dead mutant of Cardiak was still able to induce increases in CARD6 levels.

In addition to Nod1 and Cardiak, we also detected interaction of CARD6 with TUCAN (CARD8/Cardinal/NDPP1/Dakar) and NAC (DEFKAP/NALP1/CARD7), two other CARD family members. TUCAN and NAC have been implicated in regulation of apoptosis by modulating pro-caspase-9 activation (39,

40) and also in NF- κ B regulation (10, 61, 62). Moreover, both proteins have been recently implicated in regulation of pro-caspase-1 and IL-1 β secretion (16, 63). The functional significance of CARD6 interaction with these proteins remains to be investigated. However, we did not detect an influence of CARD6 on caspase-9-dependent activation of DEVD-cleaving effector caspases in Bax overexpressing cells nor did we detect changes in IL-1 β secretion. CARD6 also did not modulate the caspase-8-dependent pathway for apoptosis induced by Fas, a member of the apoptotic TNF family cytokine receptors.

In summary, we have identified and characterized cDNAs encoding a CARD-containing protein encoded in the *CARD6* gene locus. This previously unstudied protein can selectively suppress NF- κ B induction mediated via Cardiak-dependent mechanisms. Further work will be necessary to determine the physiological roles of *CARD6*-encoded proteins in inflammatory responses *in vivo*, including use of gene ablation methods in mice.

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