THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 47, Issue of November 23, pp. 44069–44077, 2001
Printed in U.S.A.
© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.

CARDINAL, a Novel Caspase Recruitment Domain Protein, Is an Inhibitor of Multiple NF-κB Activation Pathways*

Received for publication, August 2, 2001, and in revised form, September 6, 2001
Published, JBC Papers in Press, September 10, 2001, DOI 10.1074/jbc.M107373200

Lisa Bouchier-Hayes‡§, Helen Conroy‡, Helen Egan‡, Colin Adrain‡, Emma M. Creagh‡, Marion MacFarlane¶, and Seamus J. Martin‡‖

From the ‡Molecular Cell Biology Laboratory, Department of Genetics, Smurfit Institute, Trinity College, Dublin 2, Ireland and the ¶Medical Research Council Toxicology Unit, University of Leicester, Leicester LE19HN, United Kingdom

Proteins possessing the caspase recruitment domain (CARD) motif have been implicated in pathways leading to activation of caspases or NF-κB in the context of apoptosis or inflammation, respectively. Here we report the identification of a novel protein, CARDINAL, that contains a CARD motif and also exhibits a high degree of homology to the C terminus of DEFCAP/NAC, a recently described member of the Apaf-1/Nod-1 family. In contrast with the majority of CARD proteins described to date, CARDINAL failed to promote apoptosis or NF-κB activation. Rather, CARDINAL potently suppressed NF-κB activation associated with overexpression of TRAIL-R1, TRAIL-R2, RIP, RICK, Bcl10, and TRADD, or through ligand-induced stimulation of the interleukin-1 or tumor necrosis factor receptors. Co-immunoprecipitation experiments revealed that CARDINAL interacts with the regulatory subunit of the IκB kinase (IKK) complex, IKKγ (NEMO), providing a molecular basis for CARDINAL function. Thus, CARDINAL is a novel regulator of NF-κB activation in the context of pro-inflammatory signals.

The caspase recruitment domain (CARD)[1] is a protein-protein interaction motif, comprising a bundle of six α-helices, that was first identified in caspases and their associated adaptor molecules (1). Caspases, cysteine aspartic acid-specific proteases, play a key coordinating role in apoptosis by targeting a subset of cellular proteins for limited proteolysis (2–6).

Caspases that participate in apoptosis appear to be organized into hierarchical cascades, with those containing CARD motifs (or the related death effector domain (DED) motif) within their pro-domain regions occupying positions at the apex of caspase activation cascades (3, 5, 7–9). Activation of apical (CARD- or DED-containing) caspases appears to be achieved through aggregation of the latter via interactions between adaptor molecules that contain similar CARD or DED motifs (5, 8, 10). This strategy serves to increase the local concentration of apical caspases and allow processing of adjacent caspase molecules in *trans*, a process that has been termed the "induced-proximity model" (10).

The CARD motif does not appear to be restricted to caspases and their adaptor proteins, however. Recently, several CARD-containing proteins have been identified (Bcl10, CARD4/Nod1, Nod2, CARD10, CARD11, CARD14), the function of which appears to be directed primarily toward activation of the NF-κB transcription factor, rather than caspases (11–19). The prototypical member of this family, Bcl10 (cE10, CIPER, CLAP, CARMEN), has recently been found to be essential for NF-κB activation in the context of ligation of the T or B cell receptors for antigen (20). The viral Bcl10 homologue, vCLAP (vE10), is also a potent activator of NF-κB and has been shown to interact with the regulatory subunit of the IκB kinase complex, IKKγ (21). Cellular Bcl10 may also drive NF-κB activation via recruitment of IKKγ, although this remains to be demonstrated.

Several other CARD family proteins have been described that also appear to drive NF-κB activation through association with Bcl10 (16, 18, 19). Thus, Bcl10 appears to be an important convergence point for CARD-containing proteins that regulate NF-κB activation (see Ref. 22 for a recent review). In the latter cases, CARD:CARD interactions between Bcl10 and its binding partners may facilitate activation of the IKK complex through recruitment of IKKγ, followed by the IKKα and IKKβ subunits.

More recently, CARD-containing homologues of Apaf-1 (CARD4/Nod1, Nod2) have also been shown to promote NF-κB activation through recruitment of the CARD-containing kinase RICK (CARDIAK/RIP-2; Refs. 17 and 23). Moreover, RICK has also been shown to interact with IKKγ providing a means whereby recruitment of RICK may result in NF-κB activation through downstream activation of the IKK complex (23).

Thus, CARD-containing proteins occur repeatedly in stress response pathways that lead to activation of either caspases or NF-κB. Activation of the latter molecules results in either apoptosis or transcription of pro-inflammatory genes, responses that are consistent with the preservation of the integrity of multicellular organisms. Here, we describe a novel CARD-containing protein, CARDINAL, which does not appear to be involved in activation of either caspases or NF-κB. Instead, CARDINAL was found to inhibit divergent NF-κB activation signals and to interact with the regulatory subunit of the IκB kinase complex, IKKγ/NEMO. Thus, CARDINAL may play a role in setting a threshold for NF-κB activation, or in limiting the duration of the NF-κB response in the context of pro-inflammatory signals.

EXPERIMENTAL PROCEDURES

Plasmids—All plasmid constructs were generated using standard polymerase chain reaction procedures with primers designed to incor-

* This work was supported in part by Wellcome Trust Senior Fellowship Award in Biomedical Science 047580 and EU Grant QLG1-1999-00739 (to S. J. M. and M. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF405558.

§ Supported by Wellcome Trust Prize Studentship Award 055295.

‖ To whom correspondence should be addressed. Tel.: 353-1-608-1289; Fax: 353-1-679-8558; E-mail: martinsj@tcd.ie.

[1] The abbreviations used are: CARD, caspase recruitment domain; DED, death effector domain; GST, glutathione S-transferase; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; IL, interleukin; TNF, tumor necrosis factor; PBS, phosphate-buffered saline; IKK, IκB kinase.

Downloaded from www.jbc.org at IReL (Trinity College Dublin), on January 23, 2012

porate appropriate restriction enzyme sites. Polymerase chain reaction products were then digested and cloned into pcDNA3 (Invitrogen), pEGFP-C3 (CLONTECH), or pGEX4TK2 (Amersham Pharmacia Biotech), as indicated in the text. Plasmids were sequenced on an ABI 310 automated sequencer using ABI PRISM dye terminator cycle sequencing kits (PerkinElmer Life Sciences), according to the manufacturer's instructions.

Antibody Generation and Affinity Purification—A polyclonal anti-CARDINAL antibody was generated by repeated immunization of rabbits with a GST-CARDINAL fusion protein (amino acids 321–431). Antiserum was purified by running over an Affi-Gel (Bio-Rad) affinity column containing the immobilized GST-CARDINAL-(321–431) fusion protein, followed by elution of bound antibody with 100 mM glycine, pH 2.8.

Western Blot Analysis of CARDINAL Expression on Human Tissues and Human Tumor Cell Lines—Total protein lysates (100 μg/lane) prepared from a range of normal human tissues (CLONTECH) were electrophoresed on 12% polyacrylamide gels under standard SDS-polyacrylamide gel electrophoresis conditions. Total protein lysates were also prepared from a panel of human tumor cell lines by lysing cells at 10^7/ml in SDS-polyacrylamide gel electrophoresis sample buffer, followed by electrophoresis of samples (~50 μg/lane) on 12% polyacrylamide gels. Proteins were then transferred onto 0.45-μm nitrocellulose membrane and were probed for CARDINAL or actin expression using specific antibodies.

Luciferase Reporter Assays—Typically, 2×10^5 HEK 293T cells were transfected with appropriate plasmid combinations using the standard calcium phosphate precipitation method. For NF-κB reporter assays, cells were transfected with 100 ng of pGL35XκB-luc, 50 ng of pCMVβgal, and amounts of the relevant expression plasmids as described in the figure legends. Total plasmid amounts per well were made equal using pcDNA3 empty vector. The p53 reporter assays were set up in the same way using 100 ng of p53-luciferase reporter plasmid (Stratagene) and 50 ng of pCMVβGal. In some experiments, because of the pro-apoptotic effects associated with some NF-κB-inducing molecules (RIP, TRADD, TRAIL-R1, etc.), z-VAD-fmk (5 μM) was added to all wells to minimize loss of cells during the experiment.

24–48 h after transfection, medium was removed from the transfected cells which were then washed with PBS, pH 7.2. Cells were lysed by addition of 150 μl of PBS, followed by an equal volume of luciferase reporter/lysis reagent (luciferase constant light signal reporter gene assay kit; Roche Molecular Biochemicals). The light emitted from triplicate 50-μl aliquots of cell lysates was then measured in black 96-well plates by luminometry. Transfection efficiencies were normalized by measuring β-galactosidase activities, as follows. Briefly, 50 μl of cell lysate was incubated with 0.66 mg/ml *O*-nitrophenyl β-D-galactopyranoside in 1 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7) at 37 °C until a color change developed (typically 30 min). The reaction was stopped by addition of 300 μl of 1 M Na_2CO_3 followed by measurement of β-galactosidase activity at A_{420}.

Transient Transfection and Co-immunoprecipitation Assays—Cells (HEK293T) were seeded at a density of 2×10^6 cells/10-cm plate the day before transfection. Cells were transfected with 5–10 μg of appropriate plasmids according to the established calcium phosphate precipitation method, and DNA complexes were typically allowed to remain on cells until harvesting. Cell lysates were made 24–48 h after transfection by resuspending cells in 800 μl of IP lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40) containing 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1 μg/ml aprotinin. Following centrifugation, clarified lysates were subjected to immunoprecipitation using 1 μg of the appropriate antibody and 30 μl of agarose-coupled protein A/G (Santa Cruz). Samples were incubated with rotation for 3–6 h at 4 °C, and complexes were washed three to four times in IP lysis buffer containing 0.1% Nonidet P-40. Immunoprecipitates were then analyzed by immunoblotting using appropriate primary and secondary antibodies.

Subcellular Localization—MCF-7 cells were plated on eight-well chamber slides at a density of 1×10^5 cells/well. The following day, cells were transfected by lipofection using Fugene-6 transfection reagent (Roche Molecular Biochemicals), according to the manufacturer's instructions. Typically, 200 ng of pEGFP-C3-based plasmids encoding GFP or GFP-CARDINAL and its derivatives were transfected per treatment. Approximately 18 h after transfection, cells were stained with Mitotracker red CMXRos (100 nM) for 15 min at 37 °C before fixation in 3.8% formaldehyde in PBS, pH 7.2, for 20 min at room temperature. Cells were then rinsed in PBS, and nuclei were stained with Hoechst 33258 (0.25 g/ml) for 20 min before mounting onto glass coverslips using Vectashield (Vector Laboratories). Optical sections were taken using argon-krypton, UV lasers, and a Leica TCS-4D confocal imaging system.

Apoptosis Assays—MCF-7 cells were plated at a density of 10^5 cells/well in six-well plates and transfected the following day with Fugene-6 (2 μl/well). Typically, 800 ng of pcDNA3-based plasmids were transfected along with 50 ng of pCMVβgal reporter plasmid (CLONTECH). Cells were washed briefly in serum-free medium 6 h after transfection, and medium was replaced. 24–48 h after transfection, cells were fixed and stained for β-galactosidase expression as described previously (24). A minimum of 300 blue (transfected) cells/well were assessed for features of apoptosis using standard criteria.

RESULTS

Identification of CARDINAL—To identify novel CARD-containing proteins, we searched the public expressed sequence tag data bases for clones encoding CARD motifs using the N-terminal prodomain of human caspase-1 (amino acids 1–119) as the query sequence. This search identified a predicted protein encoded by expressed sequence tag clone KIAA0955 (GenBank™ accession no. BAA76799) in the KDRI brain genomic data base, with significant homology to the CARD of caspase-1 within its C terminus. Sequence analysis of this clone revealed an open reading frame that encoded a predicted protein of 431 amino acids (Fig. 1, *A* and *B*). BLASTP searches with the predicted protein sequence encoded by this clone revealed significant homology with the CARD domains of several other CARD proteins (Fig. 1*C*). Furthermore, CARDINAL also exhibited a high degree of homology with the C terminus of the recently described Apaf-1/Nod-1 family member DEFCAP/NAC (Fig. 1*D*). We have designated this protein CARDINAL, for CARD inhibitor of NF-κB-activating ligands (see below).

Tissue Expression of CARDINAL—To explore the tissue distribution of CARDINAL, we generated a polyclonal antibody by immunizing rabbits with a GST-CARDINAL (amino acids 321–431) fusion protein. This antibody specifically recognized CARDINAL migrating as a single band of ~50 kDa, which is close to the predicted molecular mass of 49 kDa (Fig. 2*A*). Western blot analysis using affinity-purified anti-CARDINAL antibody revealed that CARDINAL was expressed in several normal human tissues, including; heart, kidney, liver, lung, ovary, placenta, and testis (Fig. 2*B*). The highest levels of CARDINAL expression were found in lung, ovary, testis, and placenta with low or absent expression in brain, skeletal muscle, and spleen. Western blot analysis of CARDINAL expression in a panel of transformed human cell lines revealed a high level of expression in the MCF-7 breast carcinoma cell line, with intermediate levels of expression in the monocytic THP.1 and U937 cell lines and low levels of expression in the T and B lymphoblastoid cell lines CEM and BJAB (Fig. 2*C*).

CARDINAL Subcellular Distribution—To explore the subcellular localization of CARDINAL, an N-terminally EGFP-tagged CARDINAL expression plasmid was transiently transfected into MCF-7 cells. CARDINAL exhibited a mainly diffuse cytoplasmic distribution pattern, with a fraction of CARDINAL expression also detectable within the nucleus (Fig. 3). No significant overlap between CARDINAL and Mitotracker staining was observed. A similar subcellular distribution pattern of CARDINAL expression was also observed in 293T and HeLa cells (data not shown).

CARDINAL Fails to Promote Apoptosis or NF-κB Activation—As discussed in the Introduction, most CARD-containing proteins that have been described to date have been implicated in pathways that lead to activation of caspases (and consequent apoptosis), or activation of the NF-κB transcription factor. Thus, we explored whether CARDINAL could promote either apoptosis or NF-κB activation upon transient overexpression. Fig. 4*A* illustrates that, whereas transient overexpression of the well established pro-apoptotic proteins FADD, TRADD,

Downloaded from www.jbc.org at IReL (Trinity College Dublin), on January 23, 2012

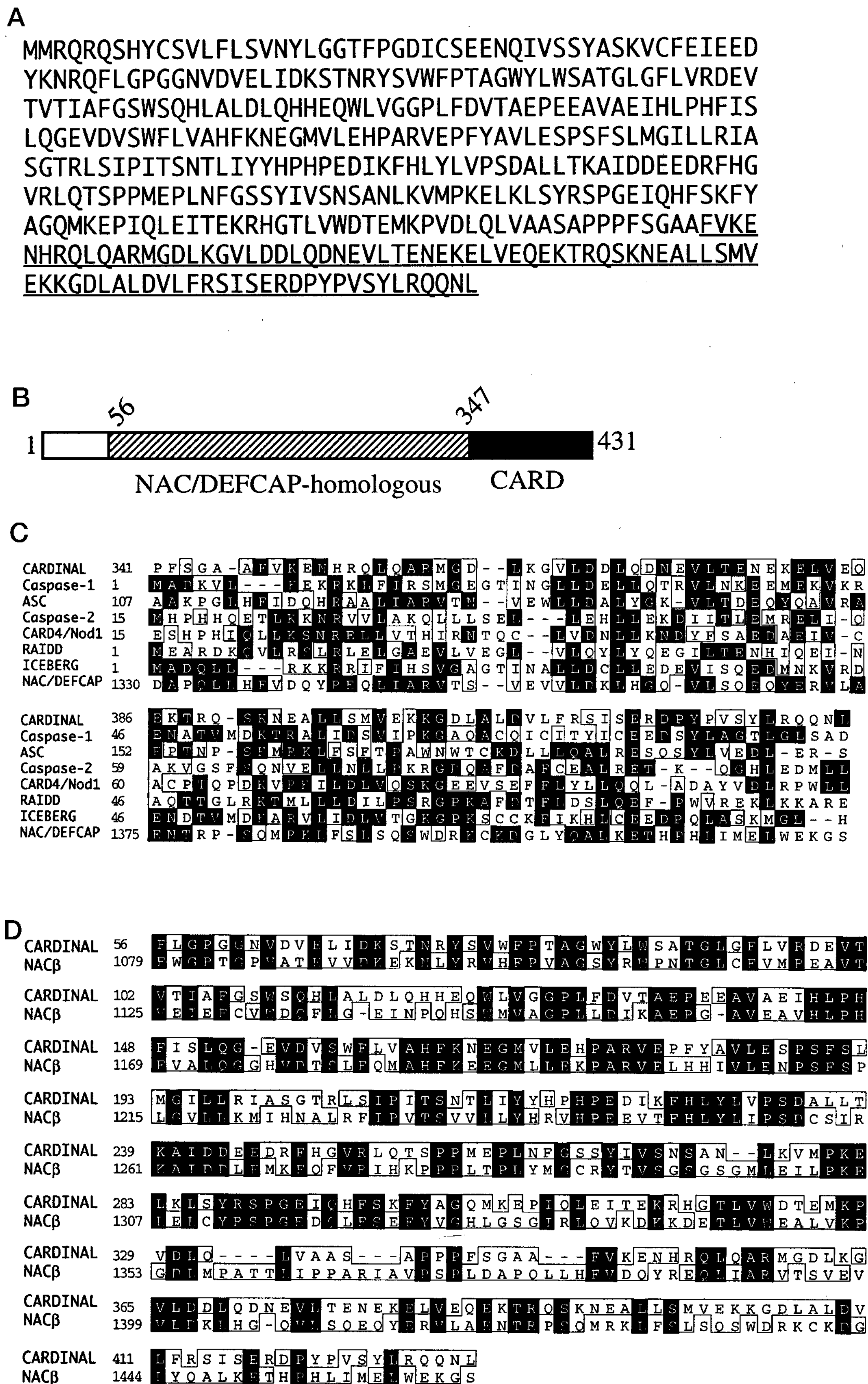


FIG. 1. **Sequence analysis of CARDINAL and alignment with proteins possessing similar motifs.** *A*, the CARDINAL open reading frame encodes a predicted protein of 431 amino acids. The region encompassing the CARD motif (amino acids 347–431) is *underlined*. *B*, schematic representation of the domain structure of human CARDINAL. *Numbers* represent amino acid positions. *C*, alignment of the CARD region of CARDINAL (amino acids 341–431) with that of the CARD-containing proteins caspase-1, ASC, caspase-2, CARD4/Nod1, RAIDD, ICEBERG, NAC/DEFCAP. Amino acid positions are indicated to the *left* of the alignment. *D*, alignment of CARDINAL with DEFCAP/NACβ. Amino acids identical to the consensus are *shaded black*; conservative substitutions are *boxed*.

Downloaded from www.jbc.org at IReL (Trinity College Dublin), on January 23, 2012

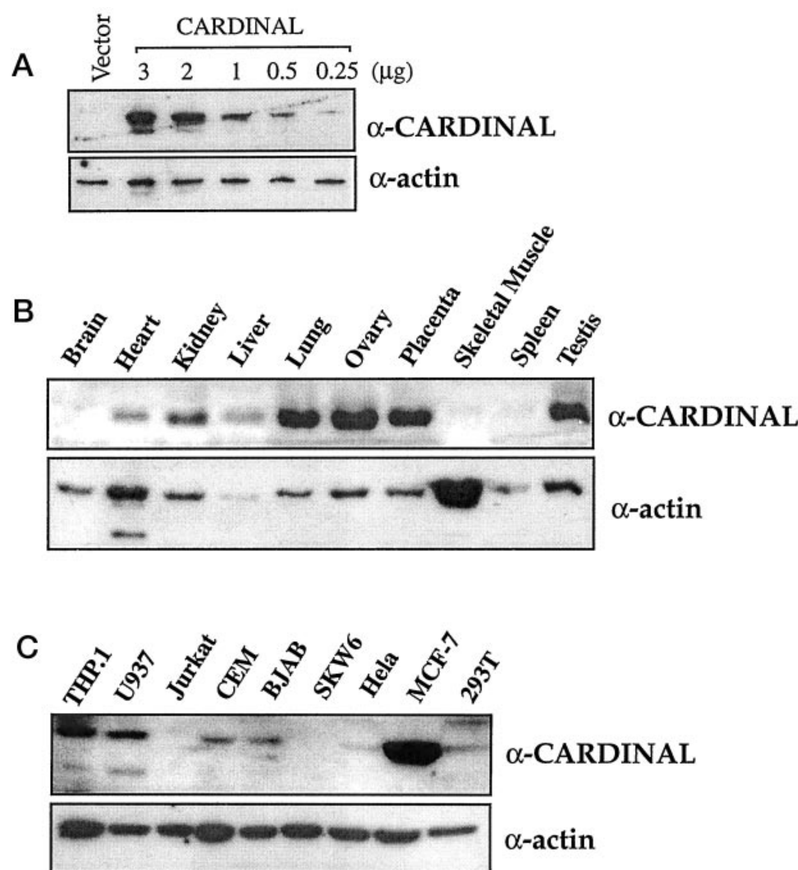


FIG. 2. **CARDINAL protein expression in human tissues and transformed cell lines.** *A*, validation of anti-CARDINAL antibody. A rabbit polyclonal antibody against CARDINAL was generated and affinity-purified as described under "Experimental Procedures." HEK 293T cells were transfected with the indicated amounts of pcDNA3 empty vector or pcDNA3-CARDINAL, and lysates made 24 h later. Equal amounts of total protein (~50 μg) were then analyzed by immunoblotting for CARDINAL expression, or actin as a loading control, as indicated. *B*, CARDINAL protein expression in adult human tissues. Whole cells lysates (100 μg/lane) from the indicated human tissues were analyzed for CARDINAL expression by immunoblotting. Blots were stripped and re-probed for actin. *C*, CARDINAL protein expression in human tumor cell lines. Total protein lysates (50 μg/lane) were prepared from the indicated human cell lines and assessed for CARDINAL expression by immunoblot. Duplicate blots were probed for β-actin as a loading control, as indicated.

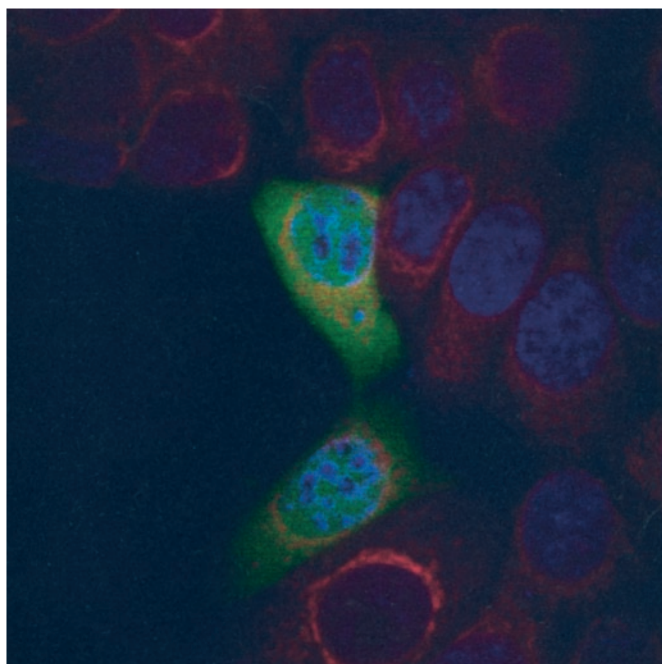


FIG. 3. **CARDINAL subcellular distribution.** An expression plasmid encoding EGFP-tagged CARDINAL was transfected into MCF-7 cells, as described under "Experimental Procedures." 18 h after transfection, cells were stained with Mitotracker/Hoechst 33342, followed by examination under confocal microscopy. CARDINAL expression is indicated in *green*, mitochondria appear *red*, and nuclei are stained *blue*.

Bax, or RIP resulted in extensive apoptosis of MCF-7 cells, overexpression of CARDINAL failed to promote apoptosis under the same conditions. Dose-response experiments performed over a wide range of pcDNA3-CARDINAL plasmid concentrations yielded similar results (data not shown).

We next explored whether CARDINAL could promote activation of NF-κB. Expression plasmids encoding CARDINAL, RIP, TRADD, TRAIL-R2 (DR5), or RICK were transfected into HEK293T cells along with a luciferase reporter construct under the control of five tandemly repeated NF-κB binding elements. Using this reporter system, significant NF-κB activity was detected upon transfection with RIP, TRADD, TRAIL-R2, and RICK, as expected (Fig. 4*B*). However, CARDINAL failed to activate NF-κB at any of the plasmid concentrations tested, despite high levels of CARDINAL protein expression being readily detectable in these cells (Fig. 4*B*, *inset*).

CARDINAL Is an Inhibitor of Multiple Pathways to NF-κB Activation—While investigating the ability of CARDINAL to activate NF-κB, we noticed that CARDINAL-transfected cells consistently produced NF-κB-driven luciferase reporter gene activity below the basal level of NF-κB activity seen with the empty vector control (data not shown). This suggested that CARDINAL may act to suppress rather than promote NF-κB activation. Thus, we co-transfected expression plasmids for CARDINAL along with a panel of established NF-κB activators (RIP, TRADD, DR4/TRAIL-R1, DR5/TRAIL-R2, Bcl10) to assess whether CARDINAL could suppress the ability of the latter to activate NF-κB. Fig. 5*A* illustrates that CARDINAL substantially attenuated NF-κB-dependent luciferase reporter gene activity associated with transient overexpression of RIP, TRADD, TRAIL-R1 (DR4), TRAIL-R2 (DR5), and Bcl10. CARDINAL also dose-dependently inhibited RICK-induced NF-κB activation (Fig. 5*B*). In contrast, CARDINAL did not influence activation of a luciferase reporter gene placed under the control of p53-responsive promoter elements, demonstrating that the effects of CARDINAL were specific to NF-κB (Fig. 5*C*).

Inhibition of IL-1- and TNF-associated NF-κB Activity by CARDINAL—We next explored whether CARDINAL could in-

Downloaded from www.jbc.org at IReL (Trinity College Dublin), on January 23, 2012

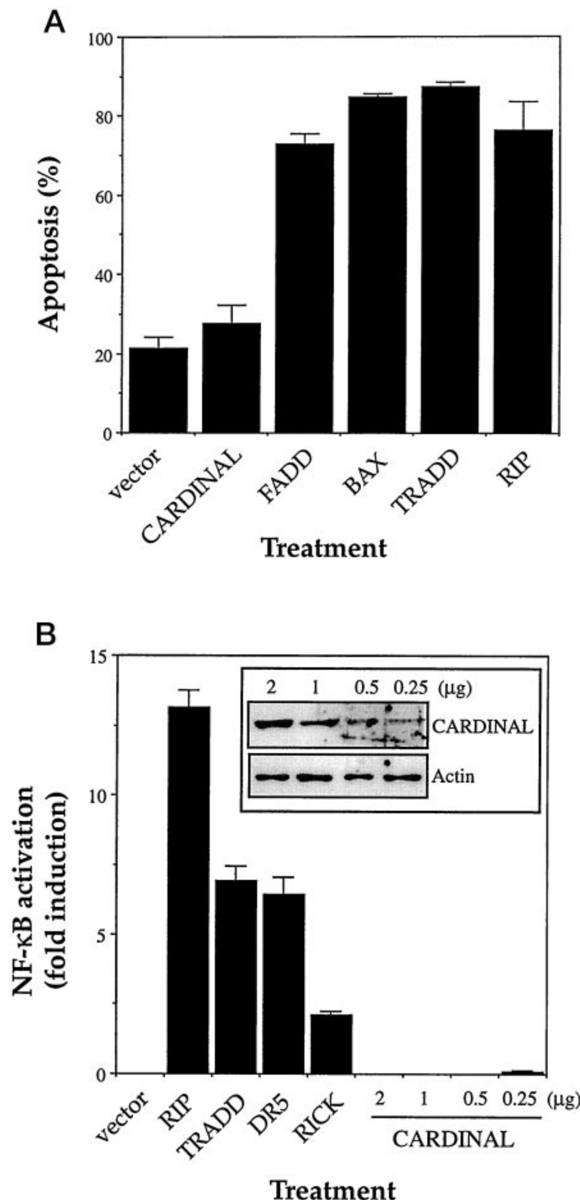


FIG. 4. **Assessment of apoptosis and NF-κB activation associated with CARDINAL overexpression.** *A*, MCF-7 cells were transfected with pcDNA3 empty vector (800 ng), or the same amount of expression plasmids encoding CARDINAL, FADD, Bax, TRADD, or RIP as indicated, along with 50 ng of β-galactosidase reporter plasmid (pCMVβ). 48 h after transfection, the percentage of β-galactosidase-positive (blue) cells exhibiting apoptotic features was determined from a minimum of 300 cells/well. Results are representative of three separate experiments. *B*, using standard calcium phosphate precipitation, HEK293T cells were transfected either with 1 μg of empty vector or with 1 μg of plasmids encoding RIP, TRADD, DR5, RICK, alongside the indicated amounts of pcDNA3-CARDINAL plasmid. All wells also received 200 ng of pGL35XκB-luc and 100 ng of pCMVβ reporter plasmids and were normalized to the same amount of total DNA using pcDNA3 empty vector. 24 h after transfection, cell lysates were prepared and luciferase activities associated with activation of the NF-κB-driven luciferase reporter were measured, in triplicate, as described under "Experimental Procedures." Luciferase activity values were normalized to β-galactosidase activity values to correct for variability in transfection efficiency between wells. Cell lysates prepared from CARDINAL-transfected cells were also assessed for CARDINAL expression by immunoblotting (*inset*). Results are representative of at least six separate experiments.

hibit NF-κB activation associated with engagement of the IL-1 or TNF receptors with their natural ligands. Thus, HEK293T cells were transiently transfected with either empty vector (pcDNA3), pcDNA3-CARDINAL, or the poxvirus-derived caspase-inhibitor CrmA, along with a NF-κB-driven luciferase reporter plasmid. Transfected cells were incubated for 24 h to allow expression of the transfected genes, followed by stimulation with either recombinant IL-1β or TNFα for 6 h. Fig. 6 shows that, whereas CrmA expression failed to block NF-κB activation associated with IL-1β or TNFα-treatment as expected, CARDINAL substantially suppressed NF-κB activation in both contexts.

Deletional Analysis of CARDINAL—To explore the region within CARDINAL responsible for inhibition of NF-κB activation, we constructed EGFP-tagged versions of full-length CARDINAL, a deletion mutant lacking the C-terminal CARD motif (EGFP-CARDINAL-(1–320)), and a mutant lacking the N-terminal NAC/DEFCAP-homologous region (EGFP-CARDINAL-(321–431); Fig. 7*A*). As in previous experiments, neither EGFP-tagged CARDINAL nor its deletion mutants were capable of spontaneously activating the NF-κB-driven luciferase reporter plasmid (Fig. 7*B*, *left panel*).

However, relative to the EGFP control, EGFP-tagged full-length CARDINAL potently inhibited NF-κB-dependent reporter gene activity associated with exposure of HEK293T cells to either recombinant IL-1β or TNFα (Fig. 7*B*). This inhibition was eliminated by removal of the N-terminal region of CARDINAL (amino acids 1–320), as the EGFP-CARDINAL-(321–431) mutant encoding the CARD region failed to suppress NF-κB activation associated with IL-1β or TNFα treatment (Fig. 7*B*). The failure of EGFP-CARDINAL-(321–431) to suppress IL-1- or TNF-associated NF-κB activation signals was not because of decreased expression levels of the latter, as this mutant was expressed at levels comparable with, or even higher than, that of the other EGFP-tagged constructs (Fig. 7*C*). Moreover, in keeping with a role for the N terminus of CARDINAL as the region responsible for the observed effects on NF-κB activation, the EGFP-CARDINAL-(1–320) mutant was as potent as full-length EGFP-CARDINAL in repressing IL-1β- or TNFα-driven NF-κB reporter gene activity under the same conditions (Fig. 7*B*).

CARDINAL Interacts with IKKγ (NEMO)—Because CARDINAL could antagonise NF-κB activation associated with engagement of multiple independent receptor pathways (IL-1, TNF, TRAIL-R1/DR4, TRAIL-R2/DR5), we considered it likely that CARDINAL intervened in these pathways at their point of convergence, or beyond. Many studies have shown that activation of the IKK complex is a key convergence point in divergent signaling pathways that result in NF-κB activation (25–27). In addition, recent studies have demonstrated that both RIP and RICK promote NF-κB activation by direct binding to the regulatory subunit of the IKK complex, IKKγ/NEMO (23, 28, 29). Because CARDINAL could antagonize NF-κB activation associated with transient overexpression of either RIP or RICK (Fig. 5, *A* and *B*), this suggested that CARDINAL may act at the level of IKKγ/NEMO recruitment by RICK or RIP, or at a point downstream of this.

Thus, we asked whether CARDINAL could directly bind to IKKγ, providing a means whereby this CARD protein could antagonize multiple independent NF-κB activation pathways. To explore this possibility, HEK293T cells were co-transfected with expression plasmids encoding IKKγ, in combination with EGFP or EGFP-CARDINAL. 24 h after transfection, cell lysates were made and CARDINAL was immunoprecipitated using an anti-CARDINAL polyclonal antibody. Immune complexes were then probed for the presence of co-precipitated IKKγ. Fig. 8*A* illustrates that IKKγ was readily detectable in CARDINAL precipitates, suggesting that CARDINAL directly interacts with this subunit of the IKK complex. Additional

Downloaded from www.jbc.org at IReL (Trinity College Dublin), on January 23, 2012

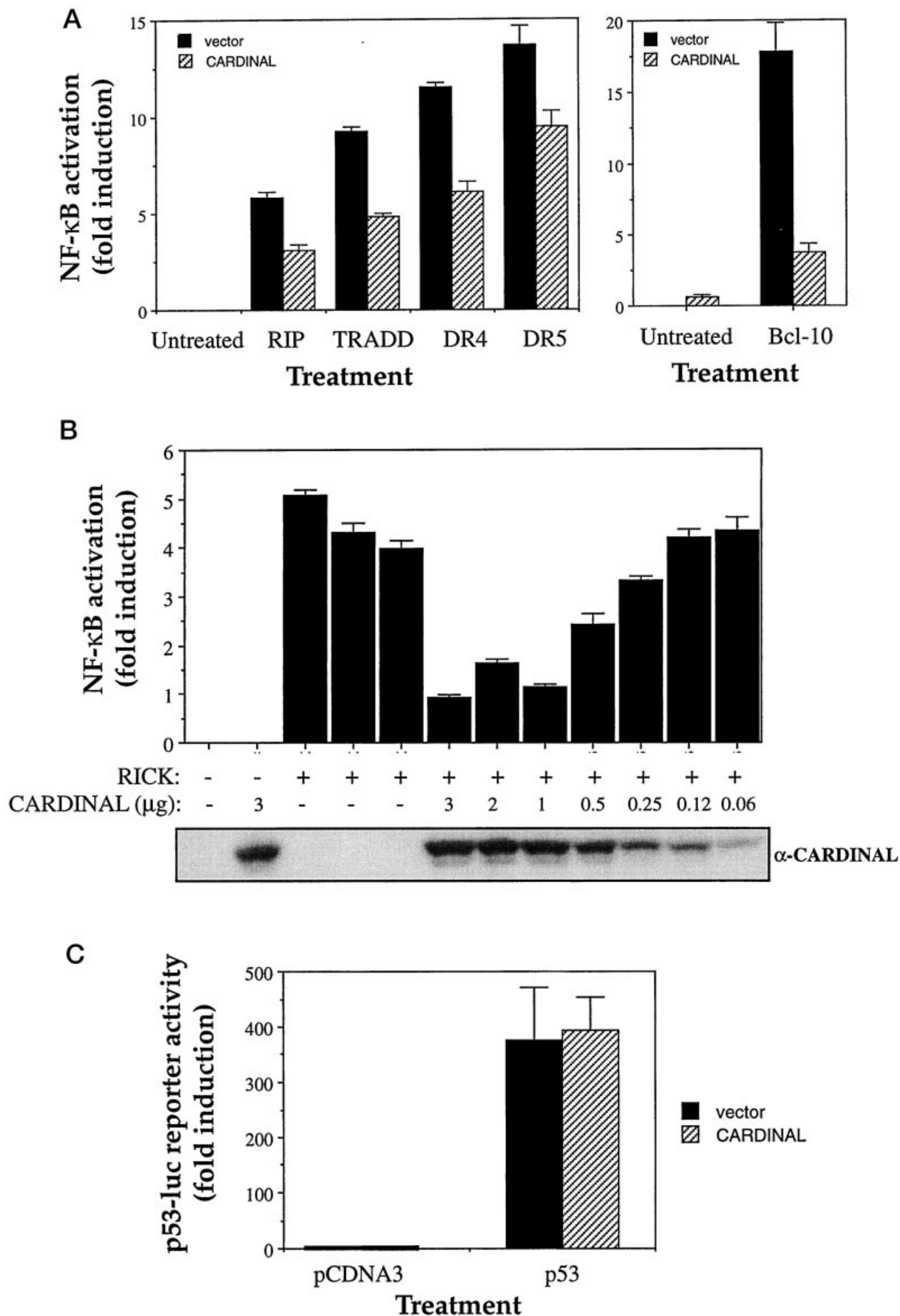


FIG. 5. **CARDINAL is an inhibitor of multiple pathways to NF-κB activation.** *A*, *left panel*, HEK293T cells were transfected with 1 μg of expression plasmids encoding, RIP, TRADD, DR4, or DR5, along with 2 μg of either empty vector (*black shading*) or 2 μg of pcDNA3-CARDINAL (*hatched shading*). *Right panel*, HEK293T cells were transfected with 1 μg of an expression plasmid encoding Bcl10, along with 1 μg of either empty vector (*black shading*) or 1 μg of pcDNA3-CARDINAL (*hatched shading*). In both cases, all wells also received 100 ng of pGL35XκB-luc and 50 ng of pCMVβ reporter plasmids and were normalized to the same amount of total DNA using pcDNA3 empty vector. 24 h after transfection, cells were lysed and NF-κB-driven luciferase reporter activities were measured, in triplicate, as described under "Experimental Procedures." Luciferase activity values were normalized to β-galactosidase activity values to correct for variability in transfection efficiency between wells. Results are representative of three separate experiments. *B*, HEK293T cells were transfected with 1 μg of RICK expression plasmid, along with the indicated amounts of pcDNA3-CARDINAL. All wells also received the pGL35XκB-luc (100 ng) and pCMVβ (50 ng) reporter plasmids. Luciferase assays were performed 24 h after transfection and normalized to correct for transfection efficiency as described above. Lysates were also assessed for CARDINAL expression by immunoblot using anti-CARDINAL rabbit polyclonal antibody (*lower panel*). *C*, CARDINAL does not inhibit transactivation of a p53-responsive luciferase reporter gene. HEK 293T cells were transfected with 500 ng of empty vector or 500 ng of a p53 expression plasmid, along with 1 μg of empty vector (*black shading*) or 1 μg or CARDINAL expression plasmid (*hatched shading*). All wells also received 100 ng of p53-luc and 50 ng of pCMVβ reporter plasmids. 24 h after transfection, cells were lysed and p53-driven luciferase reporter activities were measured (in triplicate) and normalized to β-galactosidase activity values. Results are representative of three separate experiments.

Downloaded from www.jbc.org at IReL (Trinity College Dublin), on January 23, 2012

co-immunoprecipitation experiments with the other components of the IKK signalsome (IKKα, IKKβ) revealed that CARDINAL selectively co-precipitated IKKγ (Fig. 8*B*). Furthermore, co-immunoprecipitation experiments with CARDINAL and several CARD-containing caspases (caspase-1, caspase-2, caspase-4, caspase-5, and caspase-9) failed to find association between CARDINAL and the latter molecules (data not shown). The ability of CARDINAL to interact with IKKγ/NEMO provides a molecular basis for the observed NF-κB inhibitory effects of CARDINAL. Thus, CARDINAL may compete with other CARD proteins, such as RICK and Bcl10, for recruitment of IKKγ thereby antagonizing NF-κB activation in multiple independent signaling pathways.

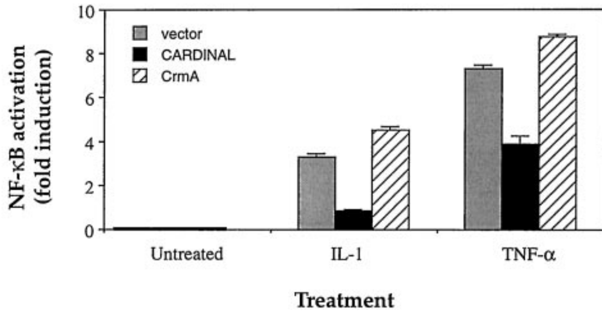
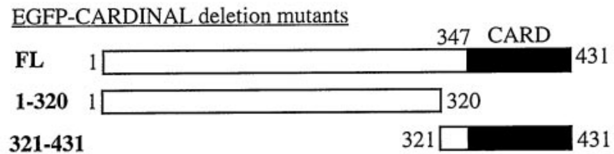
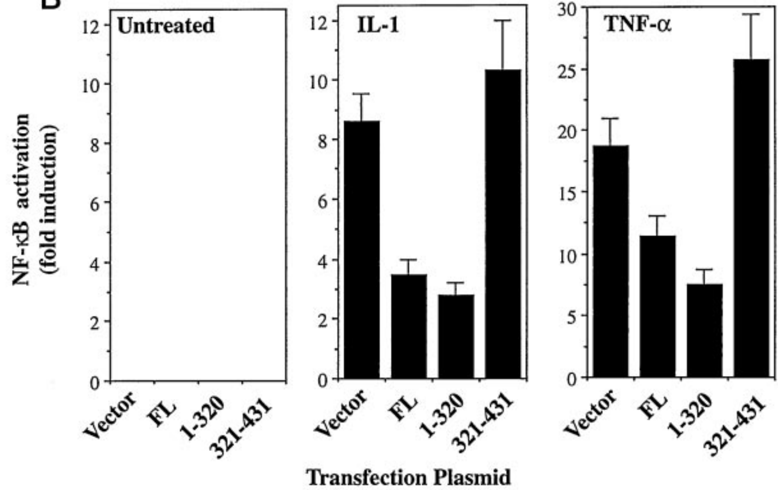


FIG. 6. **CARDINAL inhibits IL-1- and TNF-associated NF-κB activation.** HEK293T cells were transfected with either pCDNA3 empty vector (2 μg) or the same amounts of pcDNA3-CARDINAL, or pcDNA3-CrmA expression plasmids, as indicated. All wells also received the pGL35XκB-luc (100 ng) and pCMVβ (50 ng) reporter plasmids. 24 h after transfection, cultures were stimulated for 6 h with 20 ng/ml IL-1 or TNF, or were left untreated as shown. Cells were then lysed and NF-κB activation assays performed as described under "Experimental Procedures."

DISCUSSION

Here we report the identification of CARDINAL, a CARD-containing protein that exhibits potent NF-κB-inhibitory activity. CARDINAL was found to associate with IKKγ but not with several CARD-containing caspases. Consistent with this, CARDINAL failed to promote apoptosis but was found to antagonize NF-κB activation signals initiated by a variety of receptors or their adaptors. During the preparation of this report, CARDINAL was also reported by Reed and colleagues as TUCAN (30). Although there is broad agreement on the domain structure, tissue distribution, and failure of TUCAN to promote apoptosis, Reed's group report that TUCAN can act as an antagonist of caspase-9 and can block apoptosis associated with transient overexpression of Bax or caspase-9/Apaf-1 (30). In contrast, our investigations along these lines failed to find a role for CARDINAL/TUCAN as an inhibitor of apoptosis, or an interaction partner for caspase-9 (data not shown). Rather, we have found that CARDINAL/TUCAN antagonizes NF-κB activation, a role





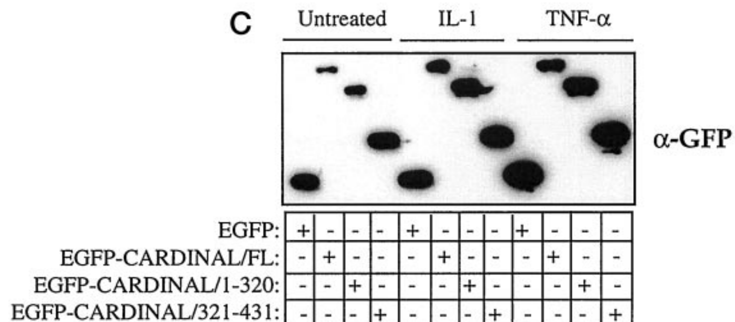


FIG. 7. **Deletional analysis of CARDINAL.** *A*, schematic representation of EGFP epitope-tagged CARDINAL deletion mutants. The CARD region is *shaded* in *black*. *B*, HEK293T cells were transfected with either EGFP vector (2 μg), or the same amount of the indicated EGFP-tagged CARDINAL expression plasmids, as indicated. All wells also received the pGL35XκB-luc (100 ng) and pCMVβ (50 ng) reporter plasmids. 24 h after transfection, cultures were stimulated for 6 h with 20 ng/ml IL-1 or TNF, or were left untreated as shown. Cells were then lysed and NF-κB activation assays performed as described under "Experimental Procedures." *C*, expression of EGFP and EGFP-CARDINAL deletion mutants under the conditions described in *panel B*. Total cell lysates (50 μg/lane) were analyzed by immunoblot with an anti-GFP monoclonal antibody.

Downloaded from www.jbc.org at IReL (Trinity College Dublin), on January 23, 2012

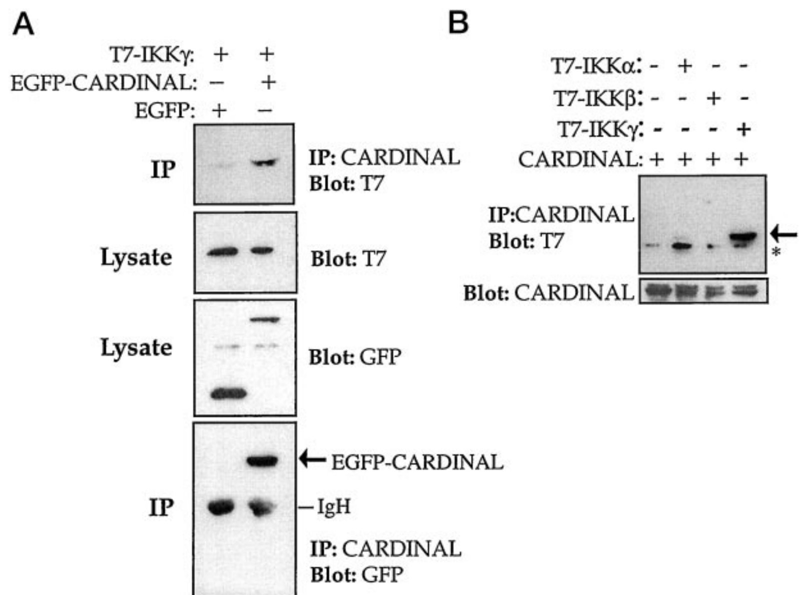


FIG. 8. **CARDINAL interacts with IKK-γ (NEMO).** *A*, HEK293T cells were transfected with 5 μg of T7 epitope-tagged IKKγ, along with 5 μg of plasmids encoding EGFP or EGFP-CARDINAL, as indicated. 24 h after transfection, cells were lysed and CARDINAL was immunoprecipitated with anti-CARDINAL polyclonal antibody, followed by probing with horseradish peroxidase-linked anti-T7 monoclonal antibody (Novagen) or anti-GFP monoclonal antibody (CLONTECH), as shown. *B*, HEK293T cells were transfected with 5 μg of the indicated T7 epitope-tagged IKK expression plasmids, along with 5 μg of pcDNA3-CARDINAL, as indicated. 24 h after transfection, cells were lysed and CARDINAL was immunoprecipitated with anti-CARDINAL polyclonal antibody, followed by probing with anti-T7 or anti-CARDINAL antibodies, as shown. The *asterisk* represents immunoglobulin heavy chain; the *arrow* indicates T7-tagged IKKγ.

that was not explored by Pathan *et al.* (30). Some of the disagreement between the two groups may relate to the expression systems/assays used. Clearly, further investigation of CARDINAL/TUCAN, particularly at constitutive protein levels, will be required to resolve these issues.

As outlined in the Introduction, many recent studies have implicated CARD-containing proteins as signaling components of pathways that result in NF-κB activation (see Ref. 22 for a recent review). The Bcl10 CARD protein appears to be a common convergence point for several recently described CARD proteins that activate NF-κB (16, 18, 19). Moreover, using *BCL10*-null mice, it has also been found that Bcl10 is essential for NF-κB activation associated with stimulation of the T or B cell receptors for antigen (20).

A different route to NF-κB activation appears to involve a distinct set of CARD proteins, which includes members of the Nod1/Apaf-1 family. Apaf-1 is a well established caspase-activating molecule that exhibits homology with the *Caenorhabditis elegans* caspase-activating protein CED-4 (31). Apaf-1 possesses an N-terminal CARD motif, a nucleotide-binding domain, and a domain rich in WD repeats that acts as a sensor for cellular damage through binding of cytosolic cytochrome *c* (9, 31). Upon binding of cytosolic cytochrome *c* that has escaped from mitochondria through cell stress/damage, Apaf-1 acts as an oligomerization and activation platform for caspase-9. Recently, two CARD-containing proteins with significant homology to Apaf-1 (Nod1/CARD4 and Nod2) have been described that, in contrast to Apaf-1, appear to be primarily involved in NF-κB activation through recruitment of the RICK kinase (17, 23). Because RICK has been shown to interact with IKKγ, this suggests that aggregation of RICK by the Apaf-1/Nod1-like family members may result in NF-κB activation through downstream activation of the IKK complex via an induced proximity mechanism (23).

Strikingly, Nod1/CARD4 and Nod2 have recently been implicated as intracellular sensors for bacterial lipopolysaccharide, through binding of this conserved component of Gram-negative bacteria through their leucine-rich repeat regions (32). Furthermore, Nod1/CARD4 and Nod2 also share significant homology with plant disease resistance (R) proteins that also act as intracellular sensors of pathogen products. Thus, the Nod1/Apaf-1 family appear to be involved in host defense responses to different forms of cellular stress (bacterial infection, cell damage) where the outcome is either caspase activation and apoptosis (in the case of Apaf-1), or NF-κB activation and a pro-inflammatory response (in the case of Nod1/CARD4 and Nod2).

Given the emerging role for CARD-family proteins as signaling intermediaries in multiple pathways that result in NF-κB activation, CARDINAL may act to counteract some of the latter molecules to set a threshold for NF-κB activation. Alternatively, CARDINAL may play a role in limiting the duration of NF-κB activation, through competition with other proteins for IKKγ/NEMO recruitment. Further work is clearly necessary to explore how CARDINAL expression/stability is regulated in response to pro-inflammatory stimuli and to determine the specific biological context(s) in which CARDINAL operates.

The significance of the extensive homology between the N terminus of CARDINAL and the C terminus of DEFCAP/NAC also remains unclear. DEFCAP/NAC is a member of the Apaf-1/Nod1 family of CARD/nucleotide binding domain proteins (33, 34). There is disagreement as to the specific binding partners of DEFCAP/NAC (caspase-2/caspase-9 *versus* Apaf-1) and whether this protein promotes caspase activation and apoptosis via direct or indirect means (33, 34). The domain structure of NAC/DEFCAP would strongly suggest that this protein is likely to act as a sensor for pathogen products, akin to Nod1/CARD4 and Nod2, although this remains to be determined. The latter possibility introduces a scenario where CARDINAL may act to antagonise signals routed through DEFCAP/NAC by competing for the same C-terminal binding partners of the latter.

Clearly, there are many interesting questions concerning CARDINAL function that require further investigation. In the present study, we have provided data to suggest that CARDINAL can antagonize NF-κB activation signals in diverse contexts. This represents a novel function for a CARD family protein and adds to the growing body of evidence that proteins containing CARD motifs play diverse roles within the overall context of host defense.

Acknowledgments—We thank Drs. Emad Alnemri, Vishva Dixit, David Goeddel, Doug Green, Gabriel Nunez, Jurg Tschopp, and Henning Walczak for valuable reagents.

REFERENCES

1. Hofmann, K., Bucher, P., and Tschopp, J. (1997) *Trends Biochem. Sci.* **22,** 155–156
2. Martin, S. J., and Green, D. R. (1995) *Cell* **82,** 349–352
3. Earnshaw, W. C., Martins, L. M., and Kaufmann, S. H. (1999) *Annu. Rev. Biochem.* **68,** 383–424

Downloaded from www.jbc.org at IReL (Trinity College Dublin), on January 23, 2012

4. Nicholson, D. W. (1999) *Cell Death Differ.* **6,** 1028–1042
5. Slee, E. A., Adrain, C., and Martin, S. J. (1999) *Cell Death Differ.* **6,** 1067–1074
6. Slee, E. A., Adrain, C., and Martin, S. J. (2001) *J. Biol. Chem.* **276,** 7320–7326
7. Aravind, L., Dixit, V. M., and Koonin, E. V. (1999) *Trends Biochem. Sci.* **24,** 47–53
8. Kumar, S., and Colussi, P. A. (1999) *Trends Biochem. Sci.* **24,** 1–4
9. Adrain, C., and Martin, S. J. (2001) *Trends Biochem. Sci.* **26,** 390–397
10. Salvesen, G. S., and Dixit, V. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 10964–10967
11. Willis, T. G., Jadayel, D. M., Du, M. Q., Peng, H., Perry, A. R., Abdul-Rauf, M., Price, H., Karran, L., Majekodunmi, O., Wlodarska, I., Pan, L., Crook, T., Hamoudi, R., Isaacson, P. G., and Dyer, M. J. (1999) *Cell* **96,** 35–45
12. Koseki, T., Inohara, N., Chen, S., Carrio, R., Merino, J., Hottiger, M. O., Nabel, G. J., and Nunez, G. (1999) *J. Biol. Chem.* **274,** 9955–9961
13. Srinivasula, S. M., Ahmad, M., Lin, J. H., Poyet, J. L., Fernandes-Alnemri, T., Tsichlis, P. N., and Alnemri, E. S. (1999) *J. Biol. Chem.* **274,** 17946–17954
14. Bertin, J., Nir, W. J., Fischer, C. M., Tayber, O. V., Errada, P. R., Grant, J. R., Keilty, J. J., Gosselin, M. L., Robison, K. E., Wong, G. H., Glucksmann, M. A., and DiStefano, P. S. (1999) *J. Biol. Chem.* **274,** 12955–12958
15. Inohara, N., Koseki, T., del Peso, L., Hu, Y., Yee, C., Chen, S., Carrio, R., Merino, J., Liu, D., Ni, J., and Nunez, G. (1999) *J. Biol. Chem.* **274,** 14560–14567
16. Bertin, J., Guo, Y., Wang, L., Srinivasula, S. M., Jacobson, M. D., Poyet, J. L., Merriam, S., Du, M. Q., Dyer, M. J., Robison, K. E., DiStefano, P. S., and Alnemri, E. S. (2000) *J. Biol. Chem.* **275,** 41082–41086
17. Ogura, Y., Inohara, N., Benito, A., Chen, F. F., Yamaoka, S., and Nunez, G. (2001) *J. Biol. Chem.* **276,** 4812–4818
18. Wang, L., Guo, Y., Huang, W. J., Ke, X., Poyet, J. L., Manji, G. A., Merriam, S., Glucksmann, M. A., DiStefano, P. S., Alnemri, E. S., and Bertin, J. (2001) *J. Biol. Chem.* **276,** 21405–21409
19. Bertin, J., Wang, L., Guo, Y., Jacobson, M. D., Poyet, J. L., Srinivasula, S. M., Merriam, S., DiStefano, P. S., and Alnemri, E. S. (2001) *J. Biol. Chem.* **276,** 11877–11882
20. Ruland, J., Duncan, G. S., Elia, A., del Barco Barrantes, I., Nguyen, L., Plyte, S., Millar, D. G., Bouchard, D., Wakeham, A., Ohashi, P. S., and Mak, T. W. (2001) *Cell* **104,** 33–42
21. Poyet, J. L., Srinivasula, S. M., and Alnemri, E. S. (2001) *J. Biol. Chem.* **276,** 3183–3187
22. Martin, S. J. (2001) *Trends Cell Biol.* **11,** 188–189
23. Inohara, N., Koseki, T., Lin, J., del Peso, L., Lucas, P. C., Chen, F. F., Ogura, Y., and Nunez, G. (2000) *J. Biol. Chem.* **275,** 27823–27831
24. Adrain, C., Slee, E. A., Harte, M. T., and Martin, S. J. (1999) *J. Biol. Chem.* **274,** 20855–20860
25. Yamaoka, S., Courtois, G., Bessia, C., Whiteside, S. T., Weil, R., Agou, F., Kirk, H. E., Kay, R. J., and Israel, A. (1998) *Cell* **93,** 1231–1240
26. Rothwarf, D. M., Zandi, E., Natoli, G., and Karin, M. (1998) *Nature* **395,** 297–300
27. Rudolph, D., Yeh, W. C., Wakeham, A., Rudolph, B., Nallainathan, D., Potter, J., Elia, A. J., and Mak, T. W. (2000) *Genes Dev.* **14,** 854–862
28. Poyet, J. L., Srinivasula, S. M., Lin, J. H., Fernandes-Alnemri, T., Yamaoka, S., Tsichlis, P. N., and Alnemri, E. S. (2000) *J. Biol. Chem.* **275,** 37966–37977
29. Zhang, S. Q., Kovalenko, A., Cantarella, G., and Wallach, D. (2000) *Immunity* **12,** 301–311
30. Pathan, N., Marusawa, H., Krajewska, M., Matsuzawa, Si S., Kim, H., Okada, K., Torii, S., Kitada, S., Krajewski, S., Welsh, K., Pio, F., Godzik, A., and Reed, J. C. (2001) *J. Biol. Chem.* **276,** 32220–32229
31. Zou, H., Henzel, W. J., Liu, X., Lutschg, A., and Wang, X. (1997) *Cell.* **90,** 405–413
32. Inohara, N., Ogura, Y., Chen, F. F., Muto, A., and Nunez, G. (2001) *J. Biol. Chem.* **276,** 2551–2554
33. Chu, Z. L., Pio, F., Xie, Z., Welsh, K., Krajewska, M., Krajewski, S., Godzik, A., and Reed, J. C. (2001) *J. Biol. Chem.* **276,** 9239–9245
34. Hlaing, T., Guo, R. F., Dilley, K. A., Loussia, J. M., Morrish, T. A., Shi, M. M., Vincenz, C., and Ward, P. A. (2001) *J. Biol. Chem.* **276,** 9230–9238

Downloaded from www.jbc.org at IReL (Trinity College Dublin), on January 23, 2012