Molecular Cloning and Characterization of DEFCAP-L and -S, Two Isoforms of a Novel Member of the Mammalian Ced-4 Family of Apoptosis Proteins*

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We report the deduced amino acid sequences of two alternately spliced isoforms, designated DEFCAP-L and -S, that differ in 44 amino acids and encode a novel member of the mammalian Ced-4 family of apoptosis proteins. Similar to the other mammalian Ced-4 proteins (Apaf-1 and Nod1), DEFCAP contains a caspase recruitment domain (CARD) and a putative nucleotide binding domain, signified by a consensus Walker’s A box (P-loop) and B box (Mg2+-binding site). Like Nod1, but different from Apaf-1, DEFCAP contains a putative regulatory domain containing multiple leucine-rich repeats (LRR). However, a distinguishing feature of the primary sequence of DEFCAP is that DEFCAP contains at its NH2 terminus a pyrin-like motif and a proline-rich sequence, possibly involved in protein-protein interactions with Src homology domain 3-containing proteins. By using in vitro coimmunoprecipitation experiments, both long and short isoforms were capable of strongly interacting with caspase-2 and exhibited a weaker interaction with caspase-9. Transient overexpression of full-length DEFCAP-L, but not DEFCAP-S, in breast adenocarcinoma cells MCF7 resulted in significant levels of apoptosis. In vitro death assays with transient overexpression of deletion constructs of both isoforms suggest the following: 1) the nucleotide binding domain may act as a negative regulator of the killing activity of DEFCAP; 2) the LRR/CARD represents a putative constitutively active inducer of apoptosis; 3) the killing activity of LRR/CARD is inhibitable by benzoxycarbonyl-Val-Ala-Asp (OMe)-fluoromethyl ketone and to a lesser extent by Asp-Glu-Val-Asp (OMe)-fluoromethyl ketone; and 4) the CARD is critical for killing activity of DEFCAP. These results suggest that DEFCAP is a novel member of the mammalian Ced-4 family of proteins capable of inducing apoptosis, and understanding its regulation may elucidate the complex nature of the mammalian apoptosis-promoting machinery.

Apoptosis (programmed cell death) is the genetically determined cell suicide program resulting in distinct biochemical and morphological features. Some of the hallmark characteristics of apoptosis include plasma membrane blebbing, nuclear and cytosolic condensation, and ultimately the formation of membrane-bound apoptotic bodies primed for phagocytosis. Alterations in the ability of the cell to initiate and/or execute the proper apoptotic signaling cascade have been implicated in many diseases such as cancer, autoimmune diseases, viral infections, and neurodegenerative disorders. Identifying the key mediators of apoptosis and understanding the molecular mechanisms of programmed cell death is critical to understanding the pathogenesis of these diseases.

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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) NP_055737.

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‡ The abbreviations used are: CARD, caspase recruitment domain; NBBD, nucleotide binding domain; LRR, leucine-rich repeat; PR, proline-rich sequences; PLM, pyrin-like motif; EGF, enhanced green fluorescent protein; DR3, Death Receptor 3; ZVAD-fmk, benzoxycarbonyl-Val-Ala-Asp (OMe)-fluoromethyl ketone; DEVD-fmk; Asp-Glu-Val-Asp (OMe)-fluoromethyl ketone; RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pair; a.a., amino acids; PBS, phosphate-buffered saline; PMN, polymorphonuclear; PBMCs, peripheral blood mononuclear cells; IP, immunoprecipitation.

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Mammalian expression vector pcDNA3.1 was purchased from Invitrogen and modified to contain an NH2-terminal Myc epitope (pcDNA3.1 NmycH) by annealing sense and antisense oligonucleotides coding for the Myc epitope followed by digestion and ligation into the Nhel and HindIII sites. 5’-Sequences flanking the Myc epitope were converted to Kozak consensus translation initiation motifs.

Northern Analysis and Semi-quantitative RT-PCR—Human 12-lane MTN blot (catalog number 7780-1) and a human cancer cell line MTN blot (catalog number 7757-1) were obtained from CLONTECH. Full-length DEFCAP-L cDNA was radiolabeled with α-32PdCTP, hybridized, and washed according to the protocol described previously (15) and exposed to autoradiography film for 5 days. β-Actin cDNA was used as a control for equal loading of RNA. Random primed reverse transcription for semi-quantitative RT-PCR was performed using Superscript II reverse transcriptase (Life Technologies, Inc.) according to the manufacturer’s protocol. RNAs from human normal liver, spleen, polymorphonuclear cells (PMNs), peripheral blood mononuclear cells (PBMCs), K562 and Jurkat cancer cell lines, and a negative control without template were reversed-transcribed. RNAs from K562 and Jurkat cancer cell lines were isolated using the Trizol reagent (Life Technologies, Inc.). PMN and PBMC RNAs were isolated by a Ficolli-Paque (Amersham Pharmacia Biotech) gradient, following dextran sedimentation and hypotonic red blood cell lysis. 1 μl of the RT reaction was used for PCR with the following oligonucleotides: 5’-CGAGAACACGTGCTTCTCCAGGGGCTCTAGC (antisense) and 5’-TCCCCCTGGGGAGTCCTCGAGT (sense) yielding an amplified product of ~300 bp encoding amino acids 560–665. These primers were believed to be restricted to a single exon based on the identification of continuous genomic sequences from PAC clone J891a18 identified by a nucleotide search of the High Throughput Genomic Sequences database.

Site-directed Mutagenesis—An A box (K340S) point mutation for full-length DEFCAP-L and -S was created using a two-step PCR protocol with the following primer pairs: 5’-CGGGTTACCTCAGCCGCTGCTG (sense)/5’-GCCAGTGTCGACGAC (antisense) and 5’-GGCCAGTGTCGACGAC (sense)/5’-GCCAGTGTCGACGAC (antisense) followed by PCR amplification with the outside primers, digestion with AhdI/BstXI, and a four-part ligation into pcDNA3.1 NmycH using KpnI, AhdI, BstXI, and NotI restriction sites.

Expression Vectors—NH2-terminal Myc-tagged DEFCAP expression constructs were made in pCMV1 NmycH using KpnI restriction sites for the following: DEFCAP-L (a.a. 1–1473), DEFCAP-S (a.a. 1–1439), K340S-L (a.a. 1–1473), K340S-S (a.a. 1–1439), ΔCARD-L (a.a. 1–1355), ΔCARD-S (a.a. 1–1311), ΔPR-L (a.a. 309–1473), ΔPR-S (a.a. 309–1439), LRR/CARD-L (a.a. 696–1439), LRR/CARD-S (a.a. 696–1439), LRR-L (a.a. 696–1355), LRR-S (a.a. 696–1311), PR/NBD (a.a. 1–648), PR (a.a. 1–308), and DEFCAP-CARD (a.a. 1356–1473). Caspase-2 (Ich1-L) (16), caspase-3 (Yama, CPP32) (17), caspase-8 (FLICE) (18), caspase-9 (interleukin 1β-converting enzyme-LAP-6, Apaf-3), caspase-9 (C287A) (19), and caspase-10 (FLICE2) (20) constructs were obtained or created as described elsewhere.

Construction of EGFP Constructs and Fluorescence Microscopy—The multiple cloning site of pEGFPC1 (CLONTECH) was digested with BamHI, Klenow filled-in to create blunt ends, and ligated with NotI linkers (New England Biolabs) to create pEGFPC1-NotI. The NH2-terminal EGFP DEFCAP-CARD (a.a. 1356–1473) fusion construct was made in pEGFPC1-NotI by ligating DEFCAP sequences in frame using 5’ KpnI and 3’ NotI restriction sites. Cells were visualized at ×100 magnification by fluorescence microscopy using a PizCell II microscope (Amerham Biosciences).
were transfected using standard CaPO4 precipitation, and MCF7 cells were transiently transfected using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s protocol.

**Immunoprecipitation and Western Blotting**—293 cells were seeded at 10% confluency on 10-cm dishes, grown to 50% confluency, and prior to transfection fresh media was added. A total of 10 μg of DNA was transfected into each dish as stated previously. 6 h post-transfection, the transfected cells were given fresh media, and cells were harvested 12–24 h post-transfection. Cells were harvested by collecting and pooling floating cells with adherent cells on ice and washed once with ice-cold phosphate-buffered saline (PBS). The cells were resuspended in 1 ml of lysis buffer containing 1% Nonidet P-40, 20 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 5 mM sodium vanadate, 0.5 mM phenylmethylsulfonyl fluoride, and 1× protein inhibitors (complete, Mini, BMB 1 836 153) and incubated on ice for 15 min. The cell debris was spun down at 20,000 × g for 15 min, and the supernatant was transferred to a fresh tube. The pellet was washed once with ice-cold PBS, mixed with 25 μl of 1.5× SDS-polyacrylamide gel electrophoresis sample buffer containing dithiothreitol, boiled at 100 °C for 5 min, and saved at –20 °C for Western analysis. The supernatant was precleared with protein G beads (Sigma) overnight at 4 °C with gentle rotation. The following day, the protein G beads were spun down at 2000 × g, and the supernatant was transferred to a new tube. 35 μl of precleared supernatant was analyzed for Western analysis, and the remaining supernatant was used for immunoprecipitations conducted with gentle rotation. The appropriate antibody for 3 h. DEFCAP constructs were immunoprecipitated with 10 μl of Myc-AC. Following incubation, the immunoprecipitates were washed 4 times with PBS on ice, resuspended in 1.5× SDS-polyacrylamide gel electrophoresis sample buffer, boiled, and analyzed by Western blotting.

**Caspase Interaction Experiments**—Immunoprecipitation experiments with either full-length NH2-terminal Myc-tagged DEFCAP-L or -S and caspase-8, -9, -12, and -15 were performed in 293 cells. Briefly, 293 cells were cotransfected with 5 μg of Myc-DEFCAP-L or Myc-DEFCAP-S and with 3 μg of caspase construct. 18–24 h post-transfection, cells were harvested and immunoprecipitated with Myc-AC and immunoblotted with caspase specific antibodies. The caspase-2 and caspase-9 coimmunoprecipitation experiments with both DEFCAP isoforms were performed in three independent experiments.

**Cell Death Assays**—MCF7 cells were plated on 35-mm 6-well tissue culture dishes, and each well was cotransfected at ~60% confluency with 0.25 μg of the reporter plasmid pCMV β-galactosidase and 1 μg of either pcDNA3.1 alone or an NH2-terminal Myc-tagged DEFCAP construct. 24 h post-transfection, the cells were fixed in 0.5% glutaraldehyde and stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) for 4 h. The cells were visualized by phase contrast microscopy, and the percentage of apoptotic cells was determined by counting at least 600 blue cells (n ≥ 3). Round blue cells and/or blue cells exhibiting plasma membrane blebbing and cell shrinkage were scored apoptotic. The data presented were from at least two independent experiments conducted in duplicate or triplicate. A Student’s t test using the computer program SigmaStat (Jandel) comparing vector control with various DEFCAP constructs was performed to obtain p values.

**RESULTS AND DISCUSSION**

**Cloning of DEFCAP-L from a K562 cDNA Library and Chromosomal Assignment**—By homology search using BLASTP for CAR-containing proteins, we identified clone KIAA0926 (GenBank™ accession number NP_055737), which was kindly provided by the Kazusa DNA Research Institute (21). For simplicity, clone KIAA0926 will be referred to as DEFCAP-S. By using the DEFCAP-S nucleotide sequence, we generated oligonucleotides that were used to PCR-amplify the DEFCAP open reading frame from a K562 cDNA library. PCR-amplified products were cloned and restriction-mapped, and DNA sequencing was performed on both strands to confirm the sequence accuracy of the clones. DNA sequences with translated products of perfect identity to DEFCAP-S were obtained from a K562 human erythroleukemia cancer cell line cDNA library. In addition, we identified some clones that contained a 132-bp insert near the 3’ end of the DEFCAP open reading frame. DNA sequencing followed by a BLAST search of the nonredundant data base identified a perfect match for human hypothetical protein DKFZp58601822.1 (GenBank™ accession number T17255). Analysis of the 132-bp insertion revealed that these sequences encode for an extra LRR not found in DEFCAP-S. We designated the full-length DEFCAP sequence containing the additional 132-bp sequence as DEFCAP-L.

By using the Stanford G3 radiation hybrid panel, we assigned the DEFCAP chromosomal localization between sequence-tagged sites (STSs) D17S849 and D17S796 of chromosome 17p13 (LOD score 7.53–9.25). These results are in agreement with those obtained by Nagase et al. using the Genebridge4 radiation hybrid panel for clone KIAA0926 (21).

**The Amino Acid Sequences of DEFCAP-L and DEFCAP-S**

**Share Homology with Apaf-1, Nod1, and Ced-4.**—The deduced amino acid sequences for DEFCAP-L and DEFCAP-S encode proteins of 1473 and 1429 amino acids, respectively. Similar to the other mammalian Ced-4 homologues identified thus far, DEFCAP contains a CARD domain, a putative nucleotide binding domain (NBD), and a putative regulatory domain containing multiple repeat elements (LRRs). However, unlike the other mammalian Ced-4 homologues Apaf-1 and Nod1, the positioning of these protein domains in the primary sequence is not conserved. Both Apaf-1 and Nod1 contain NH2-terminal CARD domains followed directly by an NBD. The COOH terminus of Apaf-1 is composed of 12–13 WD-40 repeats due to alternate splicing, whereas that of Nod1 is composed of 10 LRRs. Without knowing the crystal structure of the full-length DEFCAP protein or that of any other Ced-4 family member, it is difficult to comment on the significance of the COOH-terminal CARD found in DEFCAP versus the NH2-terminal CARD found in all other Ced-4-like proteins. However, the juxtaposition of the LRRs and the CARD in DEFCAP may result in an overall structure that is unique among the Ced-4 family members.

Some features of DEFCAP’s primary sequence that distinguish it from all other Ced-4-like molecules are its NH2-terminal pyrin-like motif (PLM) (a.a. 1–95), reverse-highlighted in gray, and its proline-rich sequence (PR) (a.a. 40–257) containing 9 PXXP motifs, underlined in black (Fig. 1A). DEFCAP’s PLM shares 25% identity to pyrin or meroastrocin, a CARD-containing protein originally identified by positional cloning experiments in patients with Familial Mediterranean Fever disease, an inherited disease characterized by excessive neutrophil activity resulting in recurrent episodes of inflammation involving serosal and synovial spaces. The pyrin-like motif is conserved with other mammalian proteins such as ASC (apoptosis-associated speck-like protein containing a CARD), a COOH-terminal CARD-containing protein with an NH2-terminal PLM (22), which shares 28% identity with DEFCAP-PLM. Furthermore, the PLM is evolutionarily conserved as seen by a protein alignment with Danio rerio ASC1 (Fig. 1B). The recent identification of an emerging number of PLM and CARD-containing proteins suggests that the PLM may play a role in regulating the apoptotic machinery.

Directly following the PLM and PR of DEFCAP is a highly conserved Ced-4 homology domain or NBD (a.a. 309–648) containing a consensus A box (P-loop), B box (β3’ binding), and motif III, a conserved sequence with unknown function (all highlighted in red, Fig. 1A). Asterisks below the residues denote the conserved amino acids as determined by Walker and coworkers (12). A ψ-BLAST search of the nonredundant data base using amino acids 279–608 of DEFCAP identified the NBD of Nod1 and the mouse gene Mater, maternal-antigen-that embryos require (sequence not shown). A sequence alignment of the NBDs for all of the Ced-4 family members suggests that DEFCAP is most homologous to Nod1 with 29% identity. No significant similarities between the NBD of DEFCAP and Apaf-1, DARK, and Ced-4 were found when using the NCBI
Fig. 1. Deduced amino acid sequence of human DEFCAP-L and amino acid alignment of human DEFCAP and its related proteins. A, the deduced DEFCAP-L open reading frame encodes a protein of 1473 amino acids. PLM is indicated with reverse-highlighting in gray. Nine PR motifs (PXXP) are underlined in black. The consensus sequence of the Walker’s A box (P-loop), B box, and NBD conserved sequence III with unassigned function are indicated with red highlighting. Asterisks below indicate conserved amino acids. K/D LRRs are reverse-highlighted in blue and non-K/D LRRs are boxed in blue. The 44 amino acids unique to the DEFCAP-L isoform are underlined in blue. The CARD is indicated with green highlighting. B, DEFCAP PLM sequence alignment with pyrin, ASC1, and ASC. C, a comparison of the putative NBD of DEFCAP with the NBD of Nod1, Apaf-1, DARK, and CED4. A box, B box, and motif III are boxed in red. Asterisks denote highly conserved residues. D, DEFCAP-L LRR (LRRs 1–12) amino acid alignment. The putative β-sheet, β-turn, and α-helix are labeled on top according to the three-dimensional structure of the porcine ribonuclease inhibitor. E, alignment of CARDs for DEFCAP, ASC, Nod1, Apaf-1, Ced-3, and caspase-9. The residues identical and similar to those of DEFCAP are shown by reverse and dark highlighting, respectively. The putative α-helices, H1–H6, are shown according to the three-dimensional structure of the CARD of Apaf-1 and caspase-9. F, the domain structures of DEFCAP-L, Nod1, Apaf-1 xL, and Ced-4.
BLAST 2 Sequences tool for protein sequence alignment.

Amino acids 703–1280 of DEFCAP-L and amino acids 703–1220 of DEFCAP-S encode a domain containing 9 consensus LRR elements in DEFCAP-L and 8 LRRs in DEFCAP-S with the consensus sequence XAXXAXXX(N/C/T/Q)1/2XA (X indicates any residue, and A indicates residue with an aliphatic side chain) according to Kobe and Deisenhofer (K/D LRR) (reverse-highlighted in blue, Fig. 1A) (23). LRRs 8, 10, and 11 (boxed in blue, Fig. 1A) represent three non-K/D LRRs with the consensus sequence XAXXAXXX(N/C/T/Q)1/2XA. DEFCAP LRRs 2, 4, and 6 are prototypical ribonuclease inhibitor type B (RI type B) repeats, whereas LRRs 3 and 5 share similarity to the ribonuclease inhibitor type A (RI type A) repeats. The alternating nature of the asparagine and cysteine, also known as the asparagine-cysteine ladder, in residues at position 10 of LRRs 2–6 are similar to other proteins with multiple internal LRR repeats. With the exception of leucine at position 20, the putative α-helical sequences of the LRRs do not share significant homology, a characteristic found in many LRRs. However, the RI type B LRRs 2, 4, and 6 share significant homology among each other as depicted in purple (Fig. 1D).
The CARD domain of DEFCAP is located at the most carboxyl end of the protein and is depicted in Fig. 1A by green highlighting. A PSI-BLAST search of the nonredundant data base using the CARD of DEFCAP identifies 56% identity with ASC and 29% identity with Nod1. Less similarity is seen in an alignment comparing DEFCAP’s CARD with the CARD of Apaf-1, Ced-3, and caspase-9.

Human DEFCAP mRNA Is Expressed in Multiple Tissues but Has the Highest Level of Expression in Peripheral Blood Leukocytes and the Chronic Myelogenous Leukemia Cell Line K562—Northern blot analysis revealed DEFCAP to be expressed as at least two transcripts of ~7.0 and ~8.0 kilobase pairs in size (Fig. 2A). Both transcripts were found in a variety of human adult tissues with the highest levels of expression in peripheral blood leukocytes, heart, thymus, and spleen. Low levels of DEFCAP mRNA expression were found in skeletal muscle, colon (no mucosa), kidney, liver, small intestine, placenta, and lung. No detectable levels of DEFCAP expression were found in the adult brain. Equal loading of RNA was determined by probing the same blots with β-actin cDNA.

Northern analysis for DEFCAP in cancer cell lines revealed a high level of expression in the chronic myelogenous leukemia cell line K562. A weak ~7.0-kilobase pair band was seen in the Burkitt’s lymphoma Raji, colorectal adenocarcinoma SW-480, and melanoma G-361 cell lines. No significant DEFCAP transcripts were detected in the promyelocytic leukemia HL-60, cervical carcinoma HeLa S3, lymphoblastic leukemia MOLT-4, or lung carcinoma A549.

Both DEFCAP-L and DEFCAP-S Isoforms Are Expressed in mRNAs from Normal Tissue and Cancer Cell Lines—Since the long DEFCAP isoform was cloned from a K562 cancer cell line, we first wanted to determine whether the long isoform exists in RNAs from normal tissues, and second to gain an understanding of the relative abundance of the two isoforms in various RNAs by semiquantitative RT-PCR. Oligonucleotides flanking the alternately spliced sequences of DEFCAP-L were used to amplify the long isoform as a 322-bp fragment and the short isoform as a 190-bp fragment (Fig. 3B). Both 322- and 190-bp RT-PCR products were gel-purified and confirmed to be specific to DEFCAP by DNA sequencing (data not shown). Both long and short DEFCAP isoforms were identified in RNAs from K562 cells, Jurkat cells, normal human liver, spleen, PMNs, and PBMCs. Interestingly, DEFCAP-L mRNA levels were relatively constant in all RNAs with the exception of the Jurkat and spleen RNAs that were slightly diminished. DEFCAP-S mRNA expression was weakest in K562, Jurkat, and liver but was significantly increased in spleen, PMNs, and PBMCs. β-Actin mRNA levels served as a control for RNA integrity and RT-PCR efficiency and were relatively constant with the exception of the K562 RNA which is slightly diminished. The weak band at ~590 bp seen in the K562, spleen, PMN, and PBMC lanes may represent a PCR artifact. This band was gel-purified from spleen, PMN, and PBMC RT-PCR samples, subjected to PCR with the same oligonucleotides used in the RT-PCR, and did not yield a ~590-bp PCR product.

EGFP-DEFCAP-CARD Fusion Proteins Are Capable of Forming Death Effector Filament-like Structures in MCF7 Cells—To gain insight into the subcellular localization of DEFCAP, NH2-terminal EGFP constructs containing the full-length DEFCAP-L, DEFCAP-S, and DEFCAP-CARD were created and transfected into MCF7 cells. Overexpression of full-length EGFP-DEFCAP-L and EGFP-DEFCAP-S resulted in a mostly diffuse cytoplasmic subcellular localization (data not shown).
shown). However, an EGFP/DEFCAP-CARD fusion protein was capable of forming novel cytoplasmic filamentous structures similar to the death-effector-filaments (DEF) formed by FADD, the death-effector domain (DED-B) of procaspase-8 (14), the prodomain of caspase-2, and RAIDD (24). The formation of DEF-like structures by DEFCAP-CARD suggests that DEFCAP may have the ability to dimerize or oligomerize in a CARD-mediated manner. The fact that both full-length DEF-CAP isoforms cannot form DEFs while the CARD alone can suggests that the CARD of DEFCAP is normally in a conformation that prevents CARD-mediated oligomerization.

DEFCAP-L and DEFCAP-S Bind Caspase-2 and Weakly to Caspase—To identify DEFCAP/caspase interactions, 293 cells were transiently cotransfected with either full-length DEF-CAP-L or DEFCAP-S in combination with either pcDNA3.1, caspase-2, caspase-3, caspase-8, caspase-9, or caspase-10. DEF-CAP failed to coimmunoprecipitate caspase-3, -8, and -10 and the adaptor proteins FADD and RAIDD (data not shown). However, both DEF-CAP-L and DEFCAP-S were able to immunoprecipitate effectively caspase-2 (Fig. 4A). Def-CAP expression was determined by Western analysis with Myc-horseradish peroxidase antibodies shown at the bottom of each panel.

**Fig. 4.** DEF-CAP interacts with caspase-2 and weakly with caspase-9. 293 cells were transiently transfected as described under "Materials and Methods." Supernatants (S), pellets (P), and coimmunoprecipitated proteins (IP) were analyzed by Western analysis with caspase-2- (A) or caspase-9- (B)-specific antibodies. The pellet lane was included to show that caspase-2, caspase-9, and both DEF-CAP-L and -S are found in the membrane-insoluble fraction. A, arrows at −48, −37, and −33 kDa represent the caspase-2 proenzyme and two processed forms of the enzyme, respectively. IgG heavy and light chains are depicted with arrows at 55 and 18 kDa, respectively. B, a weak DEF-CAP interaction with caspase-9 is seen by a band at −35 kDa representing the processed caspase-9 protein. A and B, DEF-CAP expression was determined by Western analysis with Myc-horseradish peroxidase antibodies shown at the bottom of each panel.
were only able to detect the ~35-kDa band may be that the DEFCAP interaction is specific to the processed caspase-9 molecule. Furthermore, the weak DEFCAP/caspase-9 interaction may be indirect possibly requiring an unknown adaptor molecule. Another important observation is that DEFCAP expression levels were noticeably reduced when cotransfected with caspase-9 (Fig. 4B, bottom panels). Examination of the pellet lanes for caspase-9 alone, DEFCAP-L/caspase-9, and DEFCAP-S/caspase-9 suggests that coexpression of caspase-9 with either DEFCAP construct leads to a significant increase in the ~48 and ~35-kDa bands in the membrane-insoluble fraction. Non-specific bands seen above the ~48-kDa procaspase-9 are relatively equal suggesting that protein loading does not adequately explain these differences. The functional significance of this observation remains unclear; however, DEFCAP may play a role in targeting caspase-9 to different subcellular membrane fractions.

The fact that DEFCAP interacts strongest with caspase-2 and only weakly with caspase-9 raises some interesting questions. First of all, what is the functional consequence of the ability of DEFCAP-L and -S to bind caspase-2 and can DEFCAP interact with both long and short isoforms of caspase-2? Mice deficient of both isoforms of caspase-2 exhibit an increase in facial motor neuron apoptosis, a partial resistance of B lymphoblasts to granzyme B apoptosis, and a significant increase in the number of primordial follicles in the postnatal ovary (28). These results suggest that caspase-2 can have both a pro- and anti-apoptotic function depending on the cell type. These results raise the possibility that a DEFCAP/caspase-2 interaction can lead to either a pro- or anti-apoptotic outcome. Second, where in the cell do the DEFCAP/caspase interactions take place? Immunohistochemical and cell fractionation experiments show that caspase-2 and to a lesser degree caspase-9 have both a cytosolic and nuclear subcellular localization. Further studies are needed to investigate whether the DEFCAP/caspase-2 or DEFCAP/caspase-9 interactions are exclusively cytosolic or whether DEFCAP is able to bind these caspases in the nucleus to exert its apoptotic function. Moreover, is DEFCAP capable of being translocated to the nuclear membranes? This idea seems plausible given the data presented by Chen et al. (29) showing that Ced-4 translocates to the perinuclear membrane upon induction with a death stimulus.

Overexpression of DEFCAP-L, LRR/CARD-L, and LRR/CARD-S Kills MCF7 Cells—To determine if the ectopic expression of DEFCAP constructs alone could kill cells in culture, MCF7 breast carcinoma cells were transiently transfected with NH2-terminal Myc full-length and mutant DEFCAP constructs (Fig. 5C). The base-line apoptosis level as determined by transfection with pcDNA3.1 was 25% (n = 8, S.E. = 1.655). Full-length DEFCAP-L alone exhibited 36% (n = 4, S.E. = 0.958) killing, whereas full-length DEFCAP-S resulted in a 26% killing activity (n = 4, S.E. = 3.49), a level comparable to vector control. A lysine to serine (K340S-L) point mutation in the highly conserved P-loop of DEFCAP did not decrease the killing activity of DEFCAP-L that resulted in 38% (n = 6, S.E. = 2.611) apoptotic cells. These results suggest that full-length NBD of DEFCAP-L may not be functioning as an ATPase during apoptosis. Likewise, a K340S mutation in DEFCAP-S had no effect on its killing activity that remained at levels (24%, n = 4, S.E. = 2.743) similar to wild-type DEFCAP-S and

**FIG. 5.** Mutational analysis of DEFCAP-L and DEFCAP-S in β-galactosidase death assays in MCF7 cells. A, NH2-terminal Myc-tagged full-length and mutant expression constructs. PLM, PR, NBD, LRRs, and CARD are indicated by gray, black, red, blue, and green boxes, respectively. The additional LRR (LRR12) found in DEFCAP-L is shown within a yellow box representing the 44-a.a. insertion. B, phase contrast microscopy of MCF7 cells transiently transfected with CMV-β-galactosidase and pcDNA3.1 (a) or LRR/CARD-L (b). Black arrowheads depict live transfected cells, and red arrowheads depict apoptotic cells. C, killing activity of the various DEFCAP deletion constructs. A dashed line represents the basal level of killing as determined by transfection with pcDNA3.1 alone. DR3-transfected cells represent a positive control for apoptosis.
vector control. Deleting amino acids 1–309 of DEFCAP-L (APLM/PR-L) resulted in 44% (n = 3, S.E. = 1.649) apoptosis, a slight increase in apoptosis versus the wild-type full-length DEFCAP-L construct. A APLM/PR-S construct showed no significant killing activity, 26% (n = 3, S.E. = 3.936) versus vector control or full-length DEFCAP-S. A deletion construct containing only the leucine-rich repeats and the CARD (LRRCARD-L and -S) exhibited a significant level of killing activity at 63% (n = 3, S.E. = 0.627) for LRR/CARD-L and 41% (n = 4, S.E. = 1.54) for LRR/CARD-S. These results suggest that the LRR/CARD for both long and short isoforms may act as a constitutively active proapoptotic form of DEFCAP. In the presence of 25 μM ZVAD-fmk, the pan-caspase inhibitor, the killing activity of LRR/CARD-L and LRR/CARD-S were dramatically reduced to 21% (n = 4, S.E. = 1.319) and 18% (n = 4, S.E. = 1.624), respectively (data not shown), suggesting that the mechanism of killing is most likely caspase-dependent. Base-line apoptosis with pcDNA3.1 alone in the presence of ZVAD-fmk was 11% (n = 3, S.E. = 0.284) (data not shown). In the presence of DEVD-fmk, the killing activity of LRR/CARD-L and -S was slightly reduced to 40 and 34%, respectively (data not shown). These results suggest that the activation of both a caspase with DEVD specificity and one with a non-DEVD specificity are required for maximal apoptotic activity by the LRR/CARD of DEFCAP.

Deletion constructs eliminating the CARD for both DEFCAP isoforms (∆CARD-L and ∆CARD-S) were unable to induce apoptosis and exhibited killing activities of 25% (n = 3, S.E. = 1.737) and 23% (n = 3, S.E. = 1.085), respectively. These results suggest that the CARD is critical for the ability of DEFCAP-L to kill. This conclusion is further supported by a comparison of the LRR/CARD-L and -S constructs with the DEFCAP-L and the latter is composed of an LRR/CARD and the domain juxtaposed next to the CARD.

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