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BRCC2, a Novel BH3-like Domain-containing Protein, Induces Apoptosis in a Caspase-dependent Manner*[S]

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We report here the structure-functional characterization of a novel intronless gene, *BRCC2*, located on human chromosome 11q24.1. *BRCC2* open reading frame (327 bp) codes for an ~12-kDa protein (108 amino acids (aa)) localized predominantly in the cytosol and to a lesser extent in the mitochondria. Ectopic expression of *BRCC2* cDNA also was found in both the cytosol and mitochondria. Exogenous expression of *BRCC2* caused apoptotic cell death in three different cell lines as evidenced by enhanced chromatin condensation, DNA fragmentation, or an enhanced number of cells in the sub-G_1 phase. In human prostate cancer cells (PC-3), BRCC2-induced DNA fragmentation was blocked efficiently by coexpression of the anti-apoptotic molecule, Bcl-X_L. Transient transfection of *BRCC2* cDNA into PC-3 cells in the presence of a broad-range caspase inhibitor, Z-VAD-fmk (100 μM, 24 h), abrogated DNA fragmentation. Consistently, BRCC2 expression correlated with the activation of caspase-3 and caspase-9. An N-terminal deletion mutant of BRCC2 (10.2 kDa, Δ1–16 aa) lacking a BH3-like domain (5–12 aa, LPIEGQEI) or BRCC2 containing a mutant BH3-like domain (leucine 5→glutamate) failed to induce apoptosis, whereas a C-terminal deletion mutant (6.8 kDa, Δ62–108 aa) retained the apoptotic activity comparable to the full-length BRCC2. Finally, the treatment of HeLa cells with doxorubicin or hydrogen peroxide (H_2O_2) led to an increase in the mitochondrial (heavy membrane) level of endogenous BRCC2 (doxorubicin (100 ng/ml), 5 h, ~2-fold; H_2O_2 (200 μM), 2 h, ~2-fold). These findings demonstrate that BRCC2 functions as a proapoptotic molecule and suggest that BRCC2 induces a caspase-dependent mitochondrial pathway of cell death.

Programmed cell death or apoptosis is a physiological process that is essential in development and tissue homeostasis, and deregulation of apoptosis has been associated with cancer and degenerative diseases (1). Two signal transduction pathways of apoptosis have been studied extensively (2). The "extrinsic pathway" is induced by the binding of a "death ligand" (tumor necrosis factor or FasL) to its receptor on the plasma membrane (tumor necrosis factor receptor 1 or Fas) and activation of a cysteine protease, caspase-8. In response to a variety of cytotoxic/stress-related signals, apoptosis is triggered through an "intrinsic pathway" involving the Bcl-2 family of proteins, the release of apoptogenic molecules from the mitochondria into the cytosol, and activation of caspase-9 (3). Caspase-8 and caspase-9 activate the "effector caspases" such as caspase-3, caspase-6, and caspase-7 that cleave and inactivate a number of substrates including poly(ADP-ribose) polymerase, inhibitor of caspase-activated DNA nuclease, lamins, and others, ultimately leading to chromatin condensation, DNA fragmentation, and cell death (4). Although the effector caspases are critical components of the apoptotic pathways, caspase-independent apoptosis also has been demonstrated. For example, apoptosis inducing factor and endonuclease G may be released from the damaged mitochondria, and these can directly act in the nucleus, inducing chromatin condensation and DNA fragmentation (5, 6).

The Bcl-2 family of proteins plays a pivotal role in the control of intrinsic pathway of apoptosis and consists of both anti-apoptotic and proapoptotic members (7, 8). The anti-apoptotic proteins including Bcl-2, Bcl-X_L, and Bcl-w share homology within three or four Bcl-2 homology (BH)[1] domains (BH1–4). The proapoptotic members contain either two or three BH domains (multi-BH domain proapoptotic proteins: Bax, Bak, Bcl-GL, and Bcl-X_S) or only the short (9–16 amino acids) BH3 domain (BH3-only proteins: Bid, PUMA, Bim, Noxa, Bad, Bmf, and so on) (9). More recently, a new class of proapoptotic molecules has emerged that contains a BH3-like domain with completely conserved leucine but not aspartate in the BH3 domain (BH3-like-only protein, modulator of apoptosis-1) (10). The BH3-only proteins are believed to act as early damage sensors that bind to other Bcl-2-like molecules. Aggregation of Bax or Bak molecules in the mitochondria is an equally obligatory step in the initiation of apoptosis. The signals upstream of the BH3-only proteins remain elusive. Because apoptosis also appears to occur in the absence of a known key initiator(s) of the intrinsic pathway, an unidentified adapter molecule has been proposed to interact with the aggregated Bax/Bak and activate an initiator caspase (9). In addition, cross-talk among the different apoptotic pathways and redundancy of the apoptogenic signals challenge many of the existing paradigms of this relatively complex cascade of events.

BRCC2 (breast cancer cell 2) gene was originally identified as an ~1.2-kb transcript in MDA-MB 231 human breast car-

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[S] The on-line version of this article (available at http://www.jbc.org) contains Fig. S1.

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[1] The abbreviations used are: BH, Bcl-2 homology; *BRCC2*, breast cancer cell 2; ORF, open reading frame; HA, hemagglutinin; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; FACS, fluorescence-activated cell sorter; LS-HB, low salt homogenization buffer; HA, hemagglutinin; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; L5E, leucine 5→glutamate; AFC, 7-amino-4-trifluoromethylcoumarin.

cinoma cells, other human cancer cell lines, and in normal tissues (GenBank™ accession numbers AF220061 and AF303179) (11). The longest predictive open reading frame (ORF) of *BRCC2* cDNA (862 bp) codes for a protein consisting of 108 amino acids.[2] *BRCC2* is an intronless gene localized on human chromosome 11q24.1. The loss of heterozygosity at chromosome 11q24.1–11q25 has been associated with early onset breast cancer and poor prognosis of breast and ovarian neoplasias (12, 13). The goals of this study were to determine the subcellular localization and function of BRCC2. We provide the first evidence that BRCC2 is a cytosolic and mitochondrial protein, and it has a negative impact on cell survival. BRCC2 expression correlates with caspase activation, chromatin condensation, and DNA fragmentation, hallmarks of cells undergoing apoptosis. Our data demonstrate that the N-terminal region of BRCC2 including a BH3-like domain (amino acids at positions 5–12) is important for BRCC2-induced apoptosis. In addition, the translocation of endogenous BRCC2 to the mitochondria (heavy membrane) is increased in response to doxorubicin and hydrogen peroxide, which are known to be cytotoxic agents.

EXPERIMENTAL PROCEDURES

Cell Cultures and Transient Transfections

All of the cell lines used in this study were obtained from the Tissue Culture Shared Resource of the Lombardi Cancer Center and grown in a humidified atmosphere at 37 °C in the presence of 5% CO_2 and 95% air in Dulbecco's minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine (all from Invitrogen), and 100 mg/ml penicillin/streptomycin (Biofluids). Transient transfections were performed using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol.

Antibodies and Chemicals

Rabbit polyclonal anti-serum was generated by Zymed Laboratories Inc. against a BRCC2-specific custom peptide, KARLPLEALLGSNKEPMLPK(C) (amino acids 42–61; the last cysteine was added for conjugation purposes). The following antibodies, reagents, and chemicals were obtained commercially: anti-β-actin polyclonal antibody (Sigma); anti-poly(ADP-ribose) polymerase polyclonal antibody (Upstate Biotechnologies); anti-cytochrome oxidase IV monoclonal antibody (Molecular Probes); anti-hemagglutinin (HA) (12CA5) monoclonal antibody (Roche Applied Science); anti-Myc (9E10) monoclonal antibody (Santa Cruz Biotechnology); anti-β-tubulin monoclonal antibody (Santa Cruz Biotechnology); anti-Bax (N-20) polyclonal antibody (Santa Cruz Biotechnology); anti-caspase-9 polyclonal antibody against activated, cleaved caspase-9 (Cell Signaling); horseradish peroxidase-conjugated secondary antibody to mouse or rabbit (Jackson ImmunoResearch Laboratories); pan-caspase inhibitor Z-VAD-fmk (Calbiochem); proteinase K (Ambion); DNA molecular weight markers (Denville); and Doxorubicin (Sigma).

Plasmid Constructions

Myc-BRCC2(Val)—Myc-BRCC2(Val) is referred to as Myc-BRCC2 in Fig. 1, *C* and *D*, *bottom panel* (also see Table I). BRCC2 cDNA (862 bp) was amplified using total mRNA from MDA-MB 231 breast cancer cells and cloned into the pCR2.1 vector (Invitrogen).[2] N-terminal Myc-tagged BRCC2 ORF (357 bp) was amplified subsequently by PCR using pCR2.1Myc-BRCC2(Val) as template. The forward primer sequence containing the Kozak sequence, translation initiation codon, and the Myc epitope (*underlined*) was 5′-GCCGCCATGGAGCAGAAACTCATCTCTGAAGAGGATCTGATGGTGACTTTGTTGCCTATAGAG-3′, and the reverse primer sequence was 5′-GCCTCATTACAGGGCCTCAGCAGAGGAATTGCATAA-3′. The PCR conditions were as follows: 94 °C for 2 min; 25 cycles of denaturation at 94 °C for 30 s; annealing at 65 °C for 1 min; extension at 72 °C for 1 min; and a final extension at 72 °C for 7 min. The amplified product was visualized by 1.5% agarose gel electrophoresis and cloned into the pCR3.1 expression vector (Invitrogen). *BRCC2* cDNA sequence was verified by automated DNA sequencing of both strands using the vector primers (T7 and bovine growth hormone reverse).

[2] P. C. Gokhale *et al.*, manuscript in preparation.

Myc-BRCC2(Ile)—Myc-BRCC2(Ile) is referred to as Myc-BRCC2 in Fig. 1*D*, *top panel*, and Figs. 2–4 (also see Table I). N-terminal Myc-tagged BRCC2 ORF (357 bp) was amplified using 100 ng of normal human trachea mRNA (Clontech) and the Titan one-tube reverse transcription-PCR kit (Roche Applied Science) followed by cloning into pCR3.1 vector and sequencing. The gene-specific 5′- and 3′-primer sequences used for reverse transcription-PCR were as described for Myc-BRCC2(Val).

Myc-BRCC2(ΔN-16)—A deletion mutant of Myc-tagged BRCC2 lacking the first 16 amino acids was generated by PCR and cloning using pCR3.1-Myc-BRCC2(Ile) as template and PCR conditions as described for Myc-BRCC2(Ile) followed by verification of the truncated cDNA sequence (309 bp). For PCR, the forward primer was 5′-GCCGCCATGGAGCAGAAACTCATCTCTGAAGAGGATCTGATCCTAGAATCTGAGTGT-3′ (a Kozak sequence and initiation codon at the 5′-end are followed by Myc epitope (*underlined*)), and the reverse primer was 5′-GCCTCATTACAGGGCCTCAGCAGAGGAATTGCATAA-3′.

Myc-BRCC2(ΔC-47)—A deletion mutant of Myc-tagged BRCC2 lacking the last 47 amino acids was generated by PCR (216 bp) and cloning using pCR3.1-Myc-BRCC2(Ile) as template and PCR conditions as described for Myc-BRCC2(Ile) followed by sequence verification. The forward primer was as described for Myc-BRCC2(Val), and the reverse primer was 5′-CTATTACTTAGGCAACATAGGTTCTTTGT-3′ (two-stop codons incorporated are *underlined*).

Myc-BRCC2(L5E)—Myc-tagged BRCC2 containing mutated BH3-like domain was generated by the substitution of leucine at position 5 with glutamate. This change was incorporated in the design of the 5′-primer sequence replacing natural leucine codon (TTG) with a glutamate codon (GAG). First, N-terminal Myc-tagged BRCC2(Ile) ORF was cloned into pCR2.1 and sequenced as described for Myc-BRCC2(Ile). Subsequently, N-terminal Myc-tagged BRCC2 ORF containing a single amino acid change (leucine 5→glutamate) was amplified using pCR2.1Myc-tagged BRCC2(Ile) as a template followed by cloning into pCR3.1 and sequence verification. The forward primer was 5′-GCCGCCATGGAGCAGAAACTCATCTCTGAAGAGGATCTGATGGTGACTTTG**GAG**CCTA-3′ (Myc epitope (*underlined*) is flanked by Kozak sequence and initiation codon at the 5′-end; codon substitution is shown in boldface). The reverse primer was as described for Myc-BRCC2(Val).

HA-p53 and HA-Bcl-X_L—HA-tagged cDNAs representing ORFs of p53 (1179 bp, GenBank™ accession number X60013) and Bcl-X_L (699 bp, GenBank™ accession number Z23115) and the ORF of Bax (576 bp, GenBank™ accession number L22473) were generated by PCR using human placenta Marathon cDNA as template (Clontech). The following pairs of primers were used (HA tag is *underlined* and is flanked by Kozak sequence and initiation codon at the 5′-end): p53, 5′-GCCATGGCTTACCCATACGACGTCCCAGACTACGCTATGGAGGAGCCGC-3′ (forward) and 5′-TCATTAGTCTGAGTCAGGCCCTTCTGTCTTGAACATGAGTTTTTTATGGC-3′ (reverse); and Bcl-X_L, 5′- GCCATGGCTTACCCATACGACGTCCCAGACTACGCTATGTCTCAGAGCAACCGGGA-3′ (forward) and 5′-ATTTCCGACTGAAGAGTGAGCCCAGCAGAACCACGCCGGCCACAGTCATG-3′(reverse). All of the amplified cDNAs were cloned into the pCR3.1 vector and verified by sequencing of both strands.

Baxα—The following pairs of primers were used: 5′-GCCATGGCTTACCCATACGACGTCCCAGACTACGCTGACGGGTCCGGGGAGCA-3′ (forward) and 5′-TCAGCCCATCTTCTTCCAGATGGTGAGCGAGGCGGTGAGCACTCCCGCCACAAAGA-3′ (reverse). The Bax construct was found to code for p18 Bax (amino acids 20–192).

Apoptosis Assays

For β-galactosidase assay, COS-1 cells were seeded on coverslips and, a day later, transfected with 2 μg of Myc-BRCC2 or empty vector along with 0.2 μg of pcDNA3.1/LacZ (Invitrogen). In certain cases, 0.5 μg of BRCC2 construct (Myc-BRCC2(L5E)) or empty vector along with 0.05 μg of pcDNA3.1/lacZ was used for transfection. Twenty-four hours later, adherent cells were collected, fixed in 10% formaldehyde, and stained using an X-gal staining kit (Invitrogen). The morphology of transfected cells (*blue*) was visualized with an Olympus AH2 Vanox microscope (×25 objective). DNA fragmentation assay was carried out as follows. Cells were (~2×10^6) transfected transiently with various cDNA constructs (5–10 μg). At various times posttransfection (0–36 h), adherent and floating cells were collected and lysed in 1 ml of 100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 100 mM NaCl, and 0.5% SDS and incubated with 100 μg/ml proteinase K overnight at 37 °C. Genomic DNA was extracted sequentially with 1 ml of phenol, 1 ml of phenol: chloroform, and 1 ml of chloroform followed by ethanol precipitation in

the presence of 0.3 M potassium acetate. DNA pellet was rinsed with 70% ethanol, dried, and resuspended in nuclease-free water containing 100 μg/ml RNase A (Ambion). The solution was incubated at 37 °C for 1 h, and 20–25 μg of DNA were loaded onto a 2% agarose gel followed by electrophoresis at 50 V for 2 h in 1× TAE buffer (Eppendorf) and ethidium bromide staining. The percentage of cells in the sub-G_1 phase was determined by FACS analysis using FACSort (BD Biosciences). Cells were transfected transiently for various times, and then both floating and adherent cells were collected and fixed in absolute ethanol for FACS analysis.

Subcellular Fractionation

Approximately 5×10^6 cells were seeded/150-mm dish. On the next day, cells were collected by trypsinization and washed once with ice-cold phosphate-buffered saline. Low salt homogenization buffer (LS-HB) containing 100 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 5 mM EDTA, and 1 mM dithiothreitol was added (300 μl of LS-HB/dish for 10 min on ice). An equal volume of 0.6 M sucrose in LS-HB was added, and the cells were homogenized either by 40 strokes with a Dounce homogenizer equipped with a tight-fitting pestle B or by passing 25–30 times through a 26-G needle. The homogenate was centrifuged twice at 700 × *g* for 10 min. The pellet represented the initial nuclear fraction. The supernatant was centrifuged at 10,000 × *g* for 10–20 min, resulting in the supernatant and the mitochondria-enriched or heavy membrane (HM) fractions (pellet). The supernatant was centrifuged at 120,000 × *g* for 1 h to collect the cytosolic fraction (supernatant). The mitochondria-enriched fraction was resuspended twice in isotonic homogenization buffer (0.3 M sucrose in LS-HB) and centrifuged twice at 10,000 × *g* for 15 min each time. Mitochondria either were resuspended in isotonic buffer or extracted in radioimmune precipitation assay buffer (14) for 20 min on ice and centrifuged at 16,000 × *g* for 10 min to remove residual pellet. The initial nuclear fraction was washed twice in LS-HB, and the proteins were extracted by resuspending the pellet in high salt homogenization buffer (*i.e.* LS-HB containing 750 mM KCl, 200 μl/dish), incubation with mild agitation at 4 °C for 20 min, and centrifugation at 16,000 × *g* for 1 h. The supernatant representing the nuclear fraction was diluted with an equal volume of LS-HB. The cytosolic, mitochondrial (heavy membrane), and nuclear fractions were stored at −80 °C until use.

Doxorubicin or Hydrogen Peroxide Treatment

HeLa cells (6×10^6) were seeded in 150-mm tissue culture dishes, and ~20 h later, cells were treated with 100 ng/ml doxorubicin (Sigma) prepared as a 2 mg/ml stock in Me_2SO or vehicle (Me_2SO) in serum-containing medium. Cells were incubated for 5 or 16 h and subsequently harvested by trypsinization and fractionated into cytosolic (*C*) and mitochondria-enriched fractions (*HM*) as described above. HeLa cells grown as above in serum-containing medium were treated with hydrogen peroxide (Sigma, 30% w/w) at a final concentration of 200 μM for 2 h, and subcellular fractions were isolated as described above.

Western Blot Analysis and Caspase-3 Activity Assay

Immunoblotting was performed essentially as described previously (14). At various times posttransfection, adherent and floating cells were collected. Whole cell extracts (total cell homogenates) were prepared by cell lysis in radioimmune precipitation assay buffer (14), and proteins were separated on a 4–12% gradient SDS gel (NuPAGE, Invitrogen). Following SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes (0.45 μm) immunoblotted with the appropriate primary antibody and peroxidase-conjugated secondary antibody. The antigen-antibody complex was determined using the ECL detection assay (PerkinElmer Life Sciences). The signals were scanned and quantified using the NIH ImageJ 1.31 software program (rsb.info.nih.gov/ij/). Caspase-3 activity assay was carried out using the fluorometric assay (ApoAlert caspase assay kit, Clontech) according to the manufacturer's instructions. Both adherent and floating cells were collected and lysed. Fluorescence was measured with a TECAN Ultra 384 fluorometer.

Immunofluorescence Assay

COS-1 cells were grown on coverslips and transfected with 1 μg of Myc-BRCC2 or empty vector. Twenty-four hours after transfection, the cells were fixed with 3.7% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 for 5 min. Nonspecific binding was blocked with 5% nonfat dry milk for 1 h. The cells were stained with anti-Myc antibody (1:250 dilution, overnight at 4 °C) followed by washing with phosphate-buffered saline and incubation with anti-mouse secondary antibody conjugated to Alexa Fluor 488 (Molecular Probes) (1:1,000 dilution, 45 min). During the last 10 min of the incubation, 2 μg/ml 4′,6-diamidino-2-phenylindole was added to stain the nuclei. Images were acquired with a camera mounted on a Nikon Eclipse E600 fluorescence microscope. For mitochondrial localization studies, transfected COS-1 cells were first incubated with 100 nM of a mitochondrion-specific dye Mitotracker Red CMXRos (Molecular Probes, Inc.) in normal growth medium for 20 min at 37 °C, washed with phosphate-buffered saline and fixed, and then stained as above.

TABLE I

BRCC2 open reading frame shows a single nucleotide polymorphism at nucleotide 34 (A or G) resulting in the change of a single amino acid at position 12 (isoleucine or valine of the protein or) (GenBank™ accession number AF303179)

Reverse transcription-PCR was performed using one-tube Titan RT-PCR kit (Roche Applied Science) and the primers and PCR conditions as detailed under "Experimental Procedures." Amplified products were purified using a High Pure PCR kit (Roche Applied Science) and sequenced by automated DNA sequencing using the BRCC2-specific 3′-primer.

Source of human tissue or cell line	
BRCC2 (Ile) (codon 12, ATA)	BRCC2 (Val) (codon 12, GTA)
Normal placenta[a]	Normal prostate[a]
Normal trachea[b]	Normal brain[a]
ME-180 cervical cancer cells[c]	MDA-MB 231 breast cancer cells[c]
PC-3 prostate cancer cells[c]	

[a] Marathon-cDNAs.
[b] mRNA were obtained commercially (Clontech).
[c] Total RNA was isolated using the Trizol reagent (Invitrogen) according to the manufacturer's recommendations. Total RNA was initially treated with RNase-free DNase I (Ambion) to remove any residual genomic DNA. The DNase I was inactivated subsequently by heat inactivation at 70 °C for 15 min or by using DNase-inactivation slurry (Ambion).

RESULTS AND DISCUSSION

BRCC2 Is a Novel Proapoptotic Protein—The sequence alignment of the *BRCC2* cDNA (GenBank™ accession number AF303179) with the genomic DNA (GenBank™ accession numbers XM_208544 and NT_033899; www.ncbi.nlm.nih.gov/blast/Blast.cgi) revealed that *BRCC2* is an intronless gene and that the cDNA sequence contains a guanine, whereas the genomic DNA sequence has an adenine at nucleotide position 34 of the predicted longest ORF (327 bp). To verify the BRCC2 ORF sequence, we analyzed the cDNA sequence in four normal human tissues and three human cancer cell lines. Our results indicated a single nucleotide polymorphism at nucleotide 34 (A or G) of the ORF, resulting in the change of a single amino acid at position 12 (Ile or Val) (Table I). As shown below (Fig. 2*E*), this functionally conserved substitution does not appear to modify the function of the protein. The BRCC2 ORF codes for ~12-kDa protein (108 amino acids). Manual inspection of the amino acid sequence identified a putative BH3-like domain (amino acids 5–12) retaining the highly conserved leucine but not aspartate characteristic of the BH3 domain of proapoptotic proteins (see Supplementary Fig. S1) (15).

A polyclonal anti-peptide antibody was generated against a BRCC2 epitope (amino acids 42–61). The anti-BRCC2 antibody recognized a 12-kDa protein in all of the human cell lines tested (Fig. 1*A*). In COS-1 cells, subcellular fractionation and Western blotting procedures indicated that endogenous BRCC2 was predominantly a cytosolic protein and that a relatively low level of expression was seen in the mitochondria (Fig. 1*B*). Similar observations also were made in HeLa cells (data not shown). The BRCC2 ORF tagged with a N-terminal Myc epitope (Myc-BRCC2) was cloned into the pCR3.1 expression vector. In transient transfection experiments using pCR3.1-Myc-BRCC2, anti-Myc epitope antibody and anti-BRCC2 antibody detected the same band (12 kDa) in COS-1 cells, confirming the expression of full-length exogenous BRCC2 in these transfectants

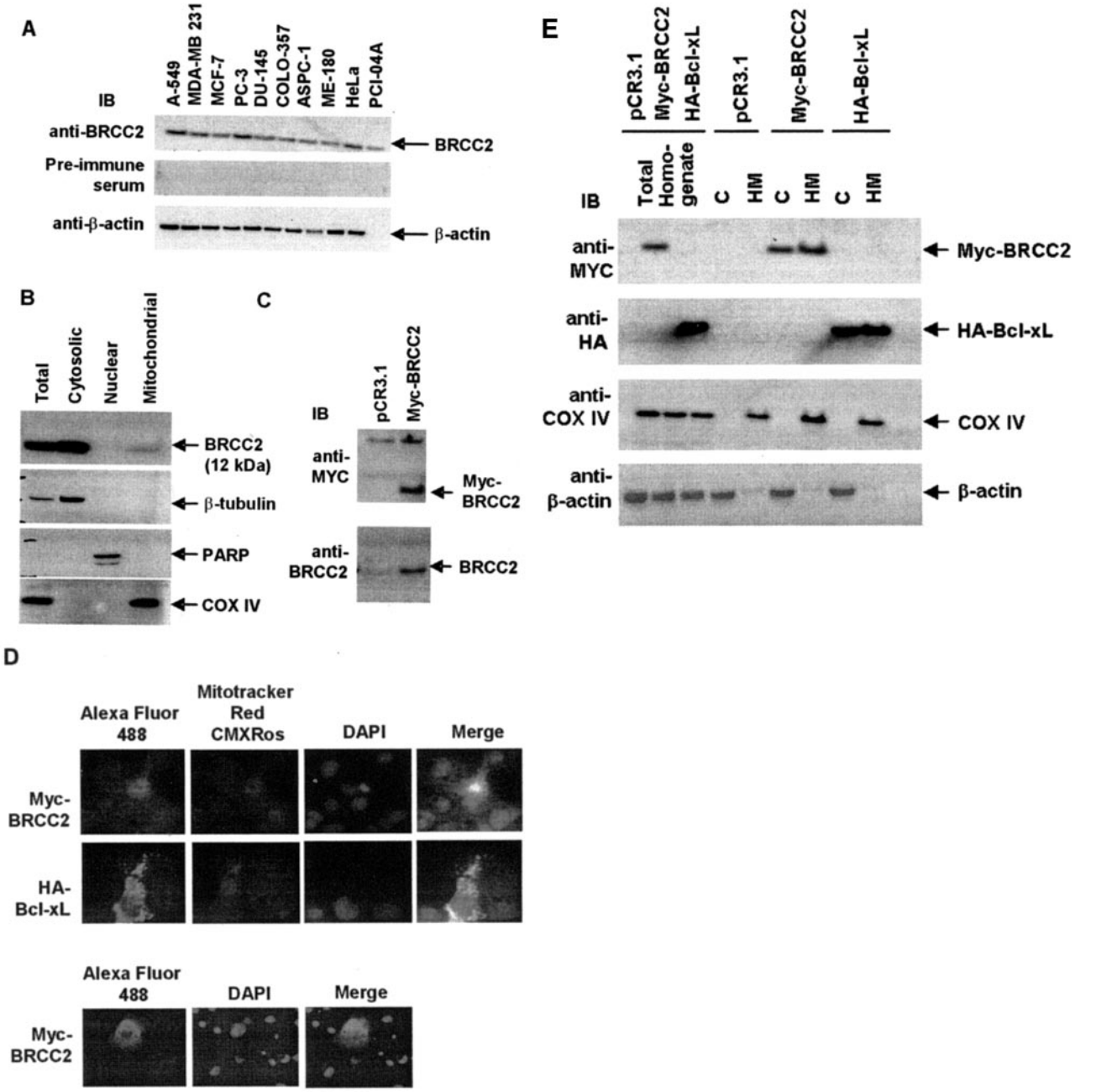

FIG. 1. **BRCC2 is primarily localized to the cytosol.** *A*, expression of BRCC2 in various human cancer cell lines: lung (A549); breast (MDA-MB 231, MCF-7); prostate (PC-3, DU-145); pancreatic (COLO-357, ASPC-1); cervical (ME-180, HeLa); and head and neck (PCI-04A). 20 μg of total cell lysates were run on a 4–12% gradient gel (NuPAGE) and immunoblotted sequentially with anti-BRCC2 antiserum (1:10,000), anti-β-actin polyclonal antibody (1:3,000), and preimmune serum (1:5,000). *B*, expression of endogenous BRCC2 in cytosol and mitochondria. COS-1 cells were fractionated into cytosolic, nuclear, and mitochondrial fractions. 20 μg of protein were separated on a 4–12% gradient gel and immunoblotted sequentially with anti-BRCC2 antiserum, anti-β-tubulin monoclonal antibody (cytosolic marker), anti-poly(ADP-ribose) polymerase (*PARP*) polyclonal antibody (nuclear marker), and anti-cytochrome oxidase IV (*COX*) monoclonal antibody (mitochondrial marker). *C*, transient transfection and expression of Myc-BRCC2. COS-1 cells were transiently transfected with either 1 μg of Myc-BRCC2 or empty vector (pCR3.1). 24 h later, cells were lysed and 50 μg of total cell extracts were subjected to 4–12% SDS-PAGE and immunoblotted sequentially with anti-Myc epitope monoclonal antibody (9E10) and anti-BRCC2 antiserum. *D*, immunofluorescence analysis. COS-1 cells grown on coverslips were transfected transiently with Myc-BRCC2 (*top* and *bottom panels*) or HA-Bcl-X_L (*middle panels*), and stained with Mitotracker Red CMXRos (*Red*) followed by staining with anti-Myc or anti-HA epitope antibody (Alexa Fluor 488) (*top* and *middle panels*) and 4′,6-diamidino-2-phenylindole (*DAPI*) (*blue*) (*top*, *middle*, and *bottom panels*). *E*, immunoblotting analysis of subcellular distribution of the exogenous BRCC2. COS-1 cells were seeded at 1.7×10^6 cells/100-mm dish, and on the next day, the cells were transfected with 10 μg of empty vector (pCR3.1), Myc-BRCC2, or HA-Bcl-X_L in the presence of 40 μM Z-VAD-fmk caspase inhibitor. Approximately 24 h later, adherent cells were collected by trypsinization. Total homogenates (whole cell extracts) and cytosolic (*C*) and mitochondria-enriched or HM fractions were isolated as described under "Experimental Procedures." 50 μg of total cell homogenate and 25 μg of either C or HM were loaded onto a 4–12% NuPAGE gel, transferred to polyvinylidene difluoride membrane, and immunoblotted sequentially with the indicated antibodies at a 1:500 (anti-Myc), 1:1,000 (anti-HA), 1:2,000 (anti-COX IV), or 1:3,000 (anti-β-actin) dilution. COX IV and β-actin signals are shown verifying the purities of the HM and C fractions, respectively.

(Fig. 1*C*). The subcellular localization of the exogenous BRCC2 (Myc-BRCC2) in COS-1 cells was examined by indirect immunofluorescence. Control experiments were performed using COS-1 cells transiently transfected with HA-tagged Bcl-X_L, a known mitochondrial anti-apoptotic protein (16). The transfectants were sequentially stained with Mitotracker Red CMXRos

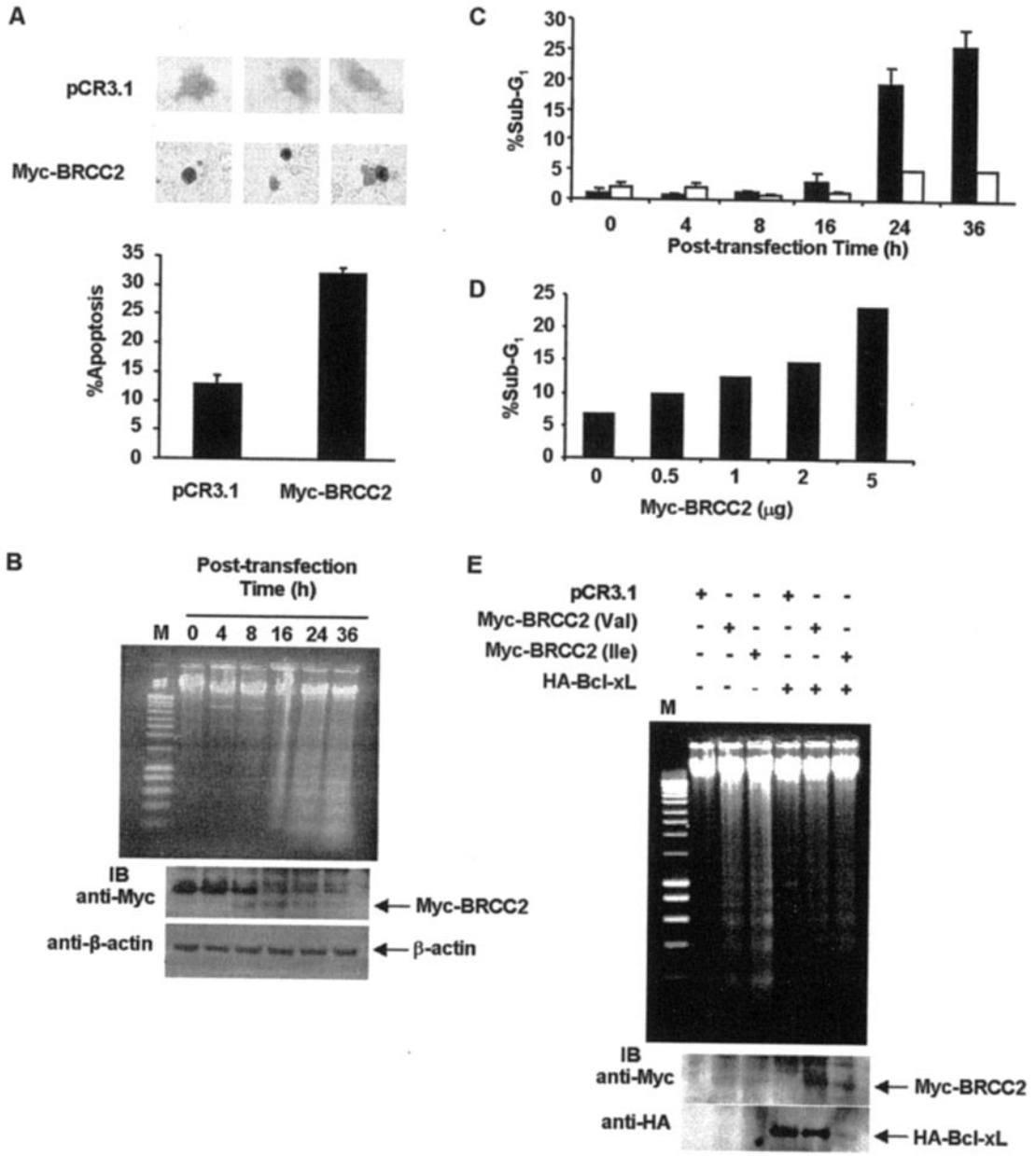

FIG. 2. **Ectopic expression of BRCC2 induces apoptosis.** *A*, β-galactosidase assay. COS-1 cells grown on coverslips were transfected transiently with Myc-BRCC2 or empty vector (pCR3.1) along with pcDNA3.1/LacZ vector followed by staining with the X-gal (*top*). Two hundred blue cells (X-gal-positive) were scored randomly and the percentage of apoptotic cells relative to the total number of transfectants (X-gal-positive cells, 100%) were calculated. Quantification data (mean ± S.D.) from three independent experiments are shown (*bottom*). *B*, DNA fragmentation assay in ME-180 cells. ME-180 cells were grown in 100-mm culture dishes (2×10^6/dish) and transfected transiently with 10 μg of Myc-BRCC2/dish. Genomic DNA was isolated from floating and adherent cells that were pooled per dish per time point, and 25 μg of DNA per time point was electrophoresed followed by ethidium bromide staining. The expression of Myc-BRCC2 at various times posttransfection was determined by immunoblotting with anti-Myc antibody (9E10). The blot was reprobed with anti-β-actin antibody. *C*, the time course of apoptosis by the FACS method. ME-180 cells were transfected with Myc-BRCC2 (*solid bars*) or empty vector (*empty bars*) as in *B*. Floating and adherent cells were pooled from two dishes per time point and subjected to FACS analysis. *D*, dose response of apoptosis by FACS method. ME-180 cells grown in six-well plates were transfected with the indicated amounts of Myc-BRCC2 plasmid. Floating and adherent cells were pooled from 3 wells/dose and analyzed 24 h later by FACS. Empty vector DNA was added where necessary to maintain the total amount of transfected DNA at 5 μg/well. *E*, cotransfection with Bcl-X_L suppresses BRCC2-induced DNA fragmentation. PC-3 cells grown in 100-mm culture dishes were cotransfected with Myc-BRCC2(Val) (5 μg) or Myc-BRCC2(Ile) (5 μg) (see Table I) and HA-Bcl-X_L (5 μg). Control cells were transfected with empty vector at 10 μg (pCR3.1, Myc-BRCC2(Val)), empty vectors at 5 μg each (Myc-BRCC2(Val) and Myc-BRCC2(Ile)), empty vector at 5 μg each (Myc-BRCC2(Ile)) or HA-Bcl-X_L, and empty vector at 5 μg each (HA-Bcl-X_L). Floating and adherent cells were pooled at 24 h posttransfection and used for DNA fragmentation and immunoblotting assays. Genomic DNA fragmentation was examined by agarose gel electrophoresis (25 μg/lane) and ethidium bromide staining. Whole cell lysates were subjected to 4–12% SDS-PAGE and sequential immunoblotting with anti-Myc and anti-HA antibodies.

(a mitochondrial marker), monoclonal anti-Myc, or anti-HA antibody followed by Alexa Fluor 488-conjugated anti-mouse secondary antibody and 4′,6-diamidino-2-phenylindole. Consistent with localization of endogenous protein, Myc-BRCC2 was localized to cytosol and mitochondria in COS-1 transfectants (Fig. 1*D*). To quantify the relative distribution of exogenous BRCC2 in cytosolic *versus* mitochondria-enriched/HM fractions, subcellular fractions were isolated using COS-1 transfectants followed by sequential immunoblotting with various antibodies as shown in Fig. 1*E*. The expression of exogenous Bcl-X_L (a positive control) was detected in the cytosolic and heavy membrane fractions (Fig. 1*E*). In addition, exoge-

nous myosin binding subunit of myosin phosphatase, a cytosolic and cytoskeletal protein (14), was seen only in the cytosolic fraction in COS-1 transfectants (data not shown). The expression of exogenous BRCC2 was observed in both the cytosolic (*C*) and mitochondria-enriched/HM fractions (Fig. 1*E*). The expression of Myc-BRCC2 in these fractions was quantified and normalized against the corresponding cell equivalents loaded. Approximately 10% of the exogenous BRCC2 was expressed in the mitochondria-enriched fraction, and 90% was present in the cytosolic compartment.

Transient transfection of *BRCC2* cDNA into COS-1 cells invariably resulted in the loss of cell viability as evidenced by the trypan blue dye exclusion assay (data not shown). We examined the link between BRCC2 expression and apoptosis in three cell lines, COS-1 cells, human cervical carcinoma cells (ME-180), and human prostate carcinoma cells (PC-3). Three different assays were used to correlate the expression of BRCC2 with apoptotic cell death (Fig. 2). First, β-galactosidase assay was used to quantify apoptosis in COS-1 cells transiently cotransfected with Myc-BRCC2 (or control vector) and LacZ vector. Blue cells with fragmented or condensed nuclei were scored as apoptotic. Myc-BRCC2 transfectants revealed an approximately three-times higher percentage of apoptotic cells as compared with vector transfectants (percentage of apoptotic cells: pCR3.1, 12%; Myc-BRCC2, 33%) (Fig. 2*A*). Second, DNA fragmentation was examined in ME-180 cells transiently transfected for various times with Myc-BRCC2. Western blot analysis showed the expression of Myc-BRCC2 at 8 h posttransfection. DNA laddering was seen first at 16 h posttransfection and increased within 24–36 h posttransfection (Fig. 2*B*). Third, FACS analysis was used to compare the number of ME-180 cells in the sub-G_1 phase posttransfection with Myc-BRCC2 *versus* control vector. At 24 or 36 h posttransfection, a significantly higher percentage of Myc-BRCC2 transfectants were in the sub-G_1 phase as compared with vector transfectants (percentage sub-G_1 at 24 h posttransfection: vector, ~5%; Myc-BRCC2, ~20%) (Fig. 2*C*). A progressive increase in the number of ME-180 cells in sub-G_1 phase at 24 h posttransfection was noted with increasing amounts of Myc-BRCC2 plasmid DNA (0.5 μg, 10%; 5.0 μg, 24%) (Fig. 2*D*). As mentioned above (Table I), BRCC2 ORF shows a single nucleotide polymorphism at codon 12, resulting in a conserved switch in amino acid (valine or isoleucine). The apoptotic effects of the two BRCC2 isoforms were examined in PC-3 cells. DNA fragmentation assay revealed that both isoforms (Myc-BRCC2(Val) and Myc-BRCC2(Ile)) induced apoptosis in PC-3 cells (Fig. 2*E*). PC-3 cells transiently transfected with epitope-tagged wild type p53 (HA-p53) showed a comparable level of DNA fragmentation (data not shown). We also show that the cotransfection of HA-Bcl-X_L (26 kDa) and Myc-BRCC2(Val) or Myc-BRCC2(Ile) led to a partial disappearance of DNA laddering in PC-3 transfectants (Fig. 2*E*), implying a negative regulatory role of Bcl-X_L in BRCC2-mediated apoptosis in PC-3 cells.

BRCC2 Induces Apoptosis in a Caspase-dependent Manner—To address the possibility that some of the known caspases may be downstream effectors of BRCC2, DNA fragmentation assay was carried out in PC-3 cells transiently transfected with BRCC2 (or empty vector) in the presence or absence of the pan-caspase inhibitor, Z-VAD-fmk. DNA laddering was diminished greatly in BRCC2 transfectants treated with the inhibitor, suggesting that BRCC2 exerts its apoptotic effect through a caspase-dependent pathway (Fig. 3*A*). Myc-BRCC2 expression in PC-3 transfectants was more pronounced in the presence of the caspase inhibitor, indicative of decreased apoptotic potential in these cells (Fig. 3*A*, *lower panel*). In addition, a 2-fold higher caspase-3 activity was observed in BRCC2 transfectants as compared with control vector, and pretreatment of cell lysates with DEVD-CHO (Clontech), a caspase-3-specific inhibitor (17), abolished the activation of caspase-3 (Fig. 3*B*).

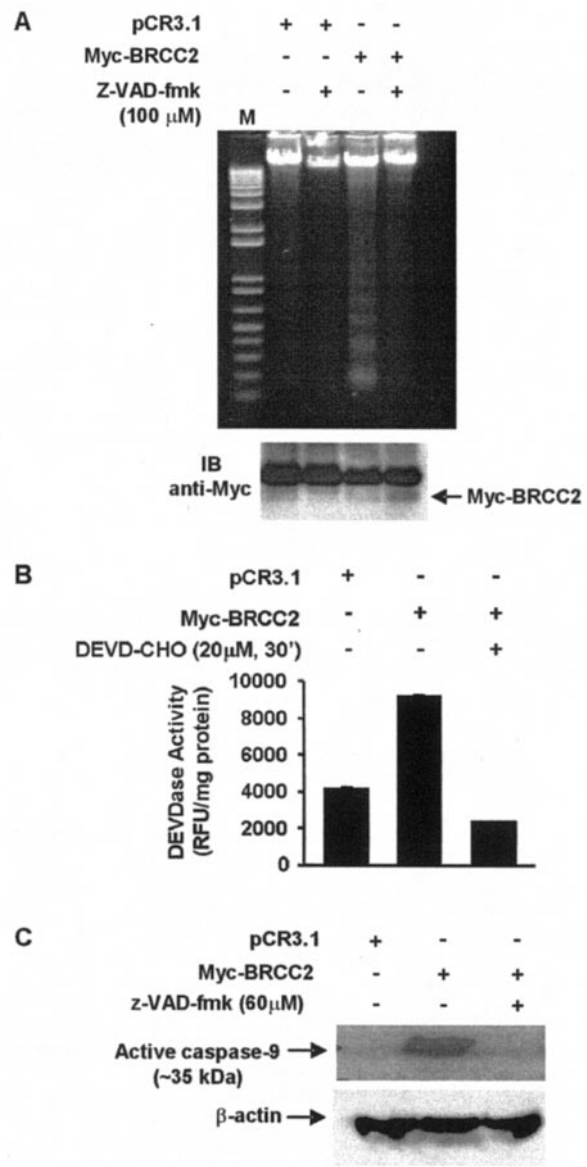

FIG. 3. **BRCC2 induces apoptosis in a caspase-dependent manner.** *A*, caspase inhibitor Z-VAD-fmk blocks BRCC2-induced apoptosis. PC-3 cells grown in 100-mm culture dishes were transiently transfected with 10 μg of Myc-BRCC2 or empty vector in the presence or absence of 100 μM of pan-caspase inhibitor Z-VAD-fmk. Twenty-four hours later, DNA fragmentation was assessed by agarose gel electrophoresis. Myc-BRCC2 expression was verified by immunoblotting with anti-Myc antibody. *B*, BRCC2 transfection correlates with activated caspase-3. PC-3 cells grown in six-well plates were transfected with 1 μg of Myc-BRCC2 or 1 μg of pCR3.1, and 24 h later, the cells were lysed and assayed for caspase-3 activity using DEVD-AFC (Clontech) as substrate in the presence or absence of caspase-3 inhibitor (DEVD-CHO). Data shown are mean ± S.D. from three independent experiments. *RFU*, relative fluorescence units. *C*, BRCC2 transfection correlates with activated caspase-9. HeLa cells were transfected with Myc-BRCC2 or empty vector (1 μg of plasmid) in the presence or absence of caspase inhibitor Z-VAD-fmk. Cells were lysed 24 h posttransfection. The lysates were analyzed by 4–12% SDS-PAGE and sequential immunoblotting with anti-active caspase-9 and anti-β-actin antibodies.

The cleavage and activation of procaspase-9 lead to the activation of caspase-3, and activation of caspase-9 has been correlated with the mitochondrial pathway of apoptosis (2). We employed a caspase-9 antibody (Cell Signaling) that specifi-

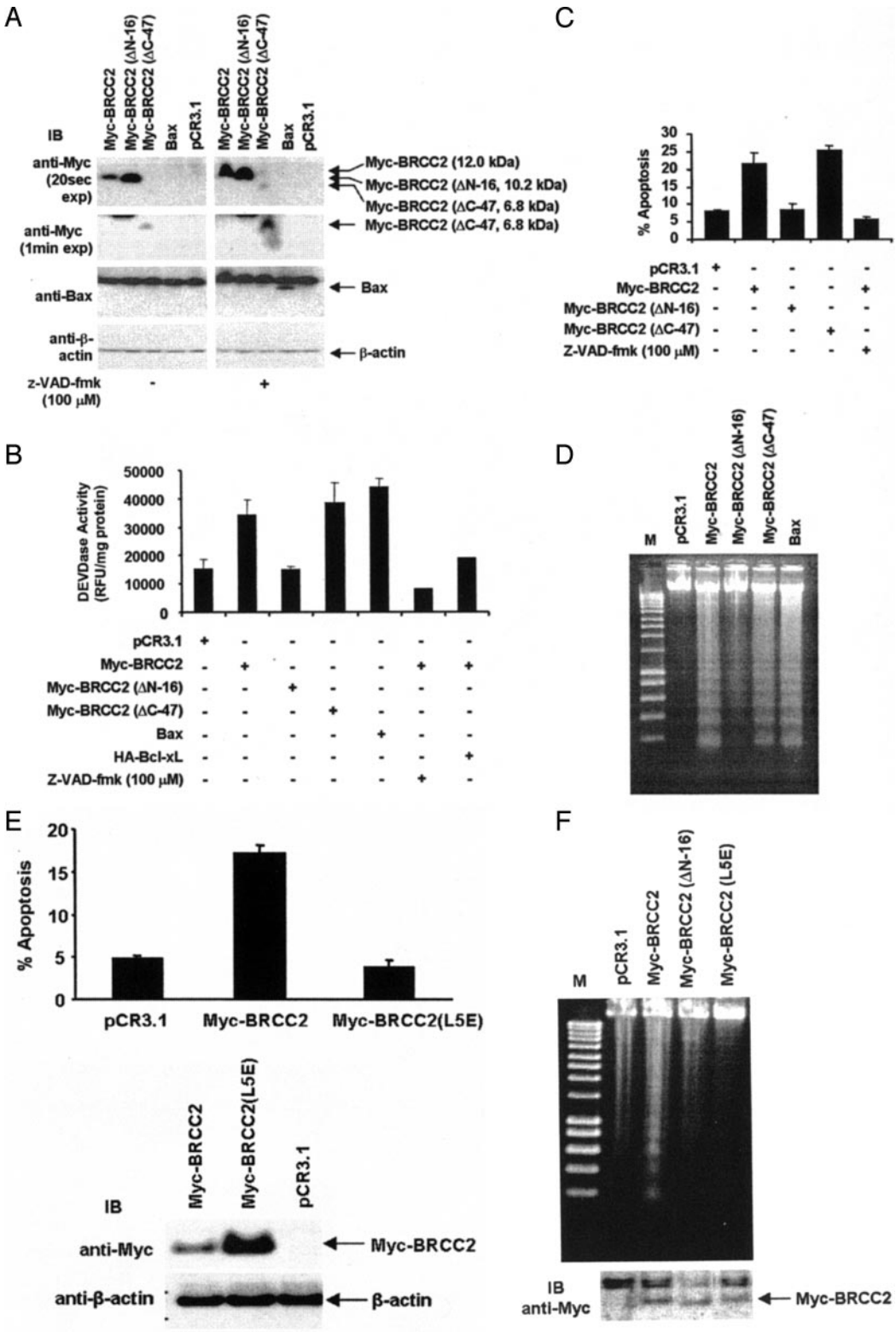

FIG. 4. **An N-terminal deletion mutant of BRCC2 (Δ1–16 amino acids) or BRCC2 containing a mutated BH3-like domain (leucine 5→glutamate) fails to induce apoptosis.** *A*, expression of full-length BRCC2 (Myc-BRCC2), N-terminal deletion mutant (Myc-BRCC2(ΔN-16)), or C-terminal deletion mutant (Myc-BRCC2(ΔC-47)) in COS-1 cells (also see Fig. S1*b*). COS-1 cells were seeded into six-well plates (300,000 cells/well in duplicate) and, on the next day, were transfected with the indicated constructs (0.5 μg/well) in the presence or absence of 100 μM Z-VAD-fmk. Twenty-four hours later, adherent cells were directly lysed in radioimmune precipitation assay buffer. The lysates (50 μg of protein) were subjected to sequential immunoblotting with the indicated antibodies. Bax plasmid (0.5 μg/well) was used as a control. *B*, basal caspase-3 activity in COS-1-Myc-BRCC2(ΔN-16) transfectants. COS-1 cells were grown in six-well plates and transfected with various plasmids as in *A*. Twenty-four hours later, both floating and adherent cells were collected, lysed, and assayed for caspase-3 activity using DEVD-AFC as substrate. Results shown are the mean ± S.D. from two independent experiments. Controls were transfections with Bax plasmid (0.5 μg/well), cotransfection of Myc-BRCC2 (0.5 μg/well) and HA-Bcl-X_L (1 μg/well), and Myc-BRCC2 transfection in the presence of caspase inhibitor Z-VAD-fmk. *C*, basal level of apoptosis in COS-1-Myc-BRCC2(ΔN-16) transfectants. COS-1 cells were transfected transiently with various constructs as in *A* along with one-tenth the amount of β-galactosidase plasmid in the presence or absence of the caspase inhibitor, and 24 h later, the cells were fixed and stained for X-gal and the percentage of apoptotic cells relative to the total number of X-gal-positive blue cells (100%) were calculated. Results shown are

cally recognizes the active/cleaved caspase-9 but not procaspase-9. Active caspase-9 (molecular mass ~35 kDa) was undetectable in HeLa cells transfected with control vector. In contrast, a significant level of active caspase-9 was detected in HeLa cells transiently transfected with Myc-BRCC2 (either isoform) (Fig. 3*C* and data not shown). Similar results were obtained in HeLa cells transiently transfected with Bax, a known activator of caspase-9 (data not shown). Transient transfection of Myc-BRCC2 into HeLa cells in the presence of a caspase inhibitor, Z-VAD-fmk, prevented the appearance of active caspase-9 (Fig. 3*C*). These data demonstrate that BRCC2-induced apoptosis involves a caspase-dependent mechanism of cell death.

The N terminus of BRCC2 (amino acids 1–16) Including a BH3-like Domain Is Essential for Apoptosis—The N terminus of BRCC2 contains a putative BH3-like domain (amino acids at positions 5–12) (see Supplemental Fig. S1). To determine the functional significance of the N-terminal sequence including the BH3-like region, a Myc-tagged deletion mutant of BRCC2 lacking the first 16 amino acids, Myc-BRCC2(ΔN-16), was constructed. In addition, a deletion mutant lacking the last 47 amino acids, Myc-BRCC2(ΔC-47), was constructed, and Bax expression vector was used as a positive control. COS-1 cells were transfected transiently with various constructs in the presence or absence of the caspase inhibitor (Z-VAD-fmk). Western blot analysis confirmed the expression of various cDNAs (Fig. 4*A*). The anti-Bax antibody detected two bands: ~21 (endogenous Bax) and 18 kDa (exogenous Bax) in COS-1-Bax transfectants (Fig. 4*A*) and PC-3-Bax transfectants (data not shown). COS-1 cells expressing full-length Myc-BRCC2, Myc-BRCC2(ΔC-47), or Bax revealed a 2.5–3-fold higher caspase-3 activity as compared with control vector transfectants. In contrast, Myc-BRCC2(ΔN-16) transfectants exhibited caspase-3 activity comparable to the control (Fig. 4*B*). As anticipated, the Myc-BRCC2 and HA-Bcl-X_L cotransfectants revealed no change in caspase-3 activity. Likewise, transfection of full-length Myc-BRCC2 in the presence of Z-VAD-fmk resulted in the base-line level of caspase-3 activity. In the β-galactosidase assay, full-length Myc-BRCC2 and Myc-BRCC2(ΔC-47) transfectants showed higher numbers of apoptotic cells, whereas Myc-BRCC2(ΔN-16) transfectants and control vector transfectants had comparable levels of apoptotic cells (percentage of apoptotic cells: pCR3.1, ~6%; Myc-BRCC2, ~22%; Myc-BRCC2(ΔN-16), ~6%; Myc-BRCC2(ΔC-47), ~25%; Myc-BRCC2 plus Z-VAD-fmk, ~5%). Transfection of Myc-BRCC2(ΔN-16) but not full-length Myc-BRCC2, Myc-BRCC2(ΔC-47), or Bax into ME-180 cells failed to induce DNA fragmentation (Fig. 4*D*). Similar observations were made in PC-3 cells (data not shown).

The prototype BH3 domain of proapoptotic molecules (for example Bak) consists of 16 amino acids with the completely conserved leucine and aspartate at positions 7 and 12, respectively, of the BH3 domain (15). To unequivocally define a role of the BH3-like domain in apoptosis, a Myc-tagged BRCC2(L5E) carrying a single amino acid substitution (leucine 5→glutamate) within the BH3-like domain was constructed. In β-galactosidase assay, COS-1 Myc-BRCC2(L5E) transfectants and control vector transfectants had comparable levels (percentage of apoptotic cells: pCR3.1, ~5%; Myc-BRCC2(L5E), ~4%; Myc-BRCC2, ~17%) (Fig. 4*E*). In addition, the transfection and expression of Myc-BRCC2(L5E) or Myc-BRCC2(ΔN-16) but not full-length Myc-BRCC2 into ME-180 cells failed to induce DNA fragmentation (Fig. 4*F*).

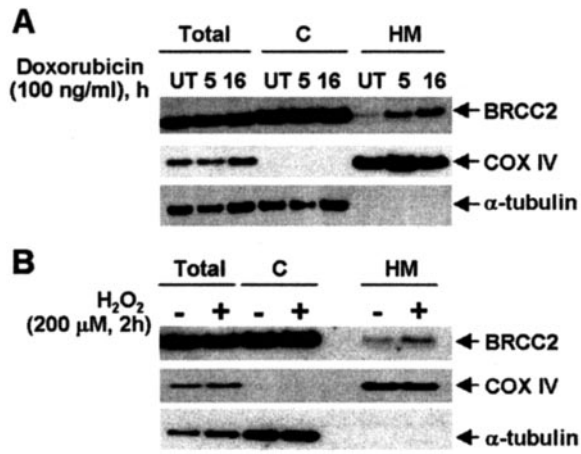

FIG. 5. **Expression of endogenous BRCC2 in mitochondria is enhanced upon treatment of HeLa cells with doxorubicin or hydrogen peroxide.** *A*, HeLa cells (6×10^6 cells/150-mm dish) were left untreated (*UT*) or treated with doxorubicin (100 ng/ml) for the indicated times. Total cell homogenates (*Total*) and cytosolic (*C*) and mitochondria-enriched/HM fractions were isolated as described under "Experimental Procedures." 15 μg of total cell homogenate and C and HM fractions were separated by 4–12% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and sequentially immunoblotted with anti-BRCC2 antiserum (1:3,500), anti-COX IV (1:2,000), and anti-α-tubulin (1:50,000) antibodies. BRCC2 signal in the various mitochondrial fractions was normalized against COX IV expression in the corresponding lane, and fold increase *versus* UT was calculated. The data shown are a representative of at least two independent experiments performed. *B*, HeLa cells were treated with 200 μM H_2O_2 for 2 h and total cell homogenates and C and HM fractions were collected as described under "Experimental Procedures." 15 μg of total cell homogenate and C and HM fractions were separated by 4–12% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and sequentially immunoblotted as in *A*. BRCC2 expression in the mitochondrial fractions was normalized against COX IV expression in the corresponding lane and fold increase *versus* minus treatment (−) was determined. COX IV and α-tubulin signals are shown verifying the purities of the HM and C fractions, respectively, and as loading controls.

Doxorubicin or Hydrogen Peroxide Stimulates Mitochondrial Accumulation of Endogenous BRCC2—Doxorubicin, a cytotoxic agent, has been shown to induce the mitochondrial pathway of apoptosis in a variety of cells including HeLa cells (18–21). Doxorubicin treatment of cells leads to the activation of BH3-only proteins, Bax, Bak, and caspases (22, 23). In untreated HeLa cells, ~2% of endogenous BRCC2 expression was seen in the mitochondria-enriched/HM fraction and 98% was seen in the cytosolic fraction determined by normalization of the BRCC2 signal against the corresponding number of cell equivalents loaded (Fig. 5*A*, *UT lanes* in *C* and *HM* fractions). Doxorubicin treatment of HeLa cells led to an increase in the mito-

the mean ± S.D. from two independent experiments. *D*, inhibition of DNA fragmentation in ME-180-Myc-BRCC2(ΔN-16) transfectants. ME-180 cells were transfected with 5.0 μg of empty vector (pCR3.1), wild type Myc-BRCC2, Myc-BRCC2(ΔN-16), Myc-BRCC2(ΔC-47), or Bax plasmid (positive control). Twenty-four hours posttransfection, floating and adherent cells were pooled and genomic DNA was examined for oligonucleosomes. *E*, basal level of apoptosis in COS-1-Myc-BRCC2(L5E) transfectants. COS-1 cells were transfected transiently with indicated constructs along with one-tenth the amount of β-galactosidase plasmid, and 24 h later, the adherent cells were fixed and stained for X-gal and the percentage of apoptotic cells relative to the total number of X-gal-positive blue cells (100%) were calculated. Results shown are the mean ± S.D. from two experiments (*top*). The expression of exogenous BRCC2 in lysates from the adherent cells was detected by immunoblotting with anti-Myc antibody. The blot was reprobed with anti-β-actin antibody (*bottom*). *F*, inhibition of DNA fragmentation in ME-180-Myc-BRCC2(L5E) transfectants. ME-180 cells were transfected with 5.0 μg of empty vector (pCR3.1), wild type Myc-BRCC2, Myc-BRCC2(ΔN-16), or Myc-BRCC2(L5E) plasmid, and genomic DNA was examined for oligonucleosomes as in *D*. The expression of exogenous BRCC2 in lysates from adherent cells was detected by immunoblotting with anti-Myc antibody.

chondrial level of endogenous BRCC2 as determined by normalization of the BRCC2 signal against cytochrome oxidase IV signal in the corresponding HM lane (100 ng/ml *versus* UT: 5 h, 2.1-fold; 16 h, 3.6-fold) (Fig. 5*A*). It should be also noted that all three HM lanes (Fig. 5*A*, *UT*, *5* and *16 h*) represent an equal number of cell equivalents loaded (~4×10^5 cells/lane). Doxorubicin treatment (50 ng/ml, 20 h) of HeLa cells caused the activation of caspase-3 judged by the appearance of the cleaved/active caspase-3 (~20 kDa) (data not shown). In addition, a ~50% loss of cell viability was observed in HeLa cells treated with doxorubicin (50 ng/ml, 40 h) as indicated by the WST-1 assay (Roche Applied Science) (data not shown). Several reports indicate the generation of reactive oxygen species including oxygen radicals and hydrogen peroxide (H_2O_2) in cells treated with doxorubicin (24, 25). In other studies, the H_2O_2 treatment of HeLa or U937 cells was shown to induce caspase-dependent apoptosis (26, 27). Hydrogen peroxide treatment of HeLa cells led to an increase in BRCC2 expression in the mitochondria (heavy membrane fraction) (200 μM, 2 h; 1.92-fold) (Fig. 5*B*). The *HM lanes* (Fig. 5*B*, *lanes* − and +) represent an equal number of cell equivalents loaded (~4×10^5 cells/lane). These results demonstrate that mitochondrial translocation of BRCC2 occurs in response to certain cytotoxic agents.

Enhanced expression of an apoptotic protein following exposure of cells to a damaging agent has been perceived as a potential cell death signal. In PC-3 cells, endogenous BRCC2 protein expression level (12 kDa) did not seem to alter by stress (ionizing radiation, 15 Gy, 8–48 h postirradiation; UV, 50 J/m2, 8–24 h postirradiation; or tumor necrosis factor-α, 20 ng/ml, 8–24 h).[3] A sequence homology search for putative transcription factor binding sites within 2 kb upstream of the start codon of BRCC2 indicated clusters of DNA binding sites of FREAC-7 and FREAC-3, members of the forkhead superfamily of transcription factors (28, 29). Future studies of the effects of FREAC-7 or FREAC-3 on BRCC2 expression and regulation of cell death may offer insight into a physiological significance of BRCC2-mediated apoptosis.

The mechanism of caspase activation via BRCC2 has not been addressed in this study. Mitochondrial translocation and dimerization of the BH3-only proteins and the multi-BH domain proteins have been associated with a relatively complex mechanism of caspase activation (9). The aggregation of Bax or Bak molecules causes disruption of the mitochondrial membrane, presumably in a cell type-dependent manner, resulting in the release of cytochrome *c*, assembly of the apoptosome (Apaf-1 and cytochrome *c* complex), and activation of caspase-9 and caspase-3 (30, 31). Smac/DIABLO, another molecule released from mitochondria into the cytosol, binds to and interferes with the action of inhibitors of apoptosis that inhibit active caspases (7, 32). In preliminary studies of COS-1 cotransfectants, Myc-BRCC2 did not coimmunoprecipitate with HA-Bcl-X_L.[3] These observations may be explained by the presence of an imperfect BH3 domain in BRCC2 lacking the highly conserved aspartate that is purported to form electrostatic bonds with a highly conserved arginine (Arg-139) in the BH1 domain of Bcl-X_L (15). In addition, the technical limitations of the coexpression of BRCC2 and Bax in COS-1 cells in the presence or absence of a caspase inhibitor (100 μM, Z-VAD-fmk) made it difficult at this time to determine a potential interaction between these two apoptotic molecules.[3] The interaction between BRCC2 and the other members of the Bcl-2 family is currently under investigation. Alternatively, BRCC2 may utilize an adapter molecule to promote caspase activation as described earlier (9, 33, 34). In conclusion, this report provides the first evidence of a proapoptotic role of BRCC2 involving the BH3-like domain of BRCC2 and activation of caspase-9 and caspase-3. Further investigations of the effects of BRCC2 expression and localization on mitochondrial and nonmitochondrial effectors should advance our understanding of the mechanism of caspase activation and cell death.

[3] C. G. Broustas and U. Kasid, unpublished observations.

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BRCC2, a Novel BH3-like Domain-containing Protein, Induces Apoptosis in a Caspase-dependent Manner

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