[CANCER RESEARCH 62, 2976–2981, May 15, 2002]

Identification and Characterization of Bimγ, a Novel Proapoptotic BH3-only Splice Variant of Bim[1]

Jun-Wei Liu, Dhyan Chandra, Shao-Hua Tang, Dharam Chopra, and Dean G. Tang[2]

Department of Carcinogenesis, the University of Texas M. D. Anderson Cancer Center, Science Park Research Division, Smithville, Texas 78957 [J-W. L., D. Cha., S-H. T., D. G. T.], and Institute of Chemical Toxicology, Wayne State University, Detroit, Michigan 48226 [D. Cho.]

ABSTRACT

BH3 (Bcl-2 homology 3)-only proteins of the Bcl-2 family play an essential role in apoptosis. In this study, a novel human BH3-only protein, Bcl-2-interacting mediator (Bim)γ, was identified during our study of regulation of prostate cancer cell death by Bcl-2 family proteins. Bimγ shares the highest amino acid sequence homology to BimEL and BimL, two proapoptotic BH3-only Bcl-2 proteins derived from alternative mRNA splicing. Genomic studies indicate that Bimγ is a novel splice variant of Bim and is generated as a result of the retention of a 126-bp intron of the *bim* gene. Bimγ mRNA displays a tissue-specific expression pattern distinct from those of the other Bim isoforms. Subcellular fractionation studies indicate that Bimγ is localized both in intracellular membranes and cytosol. Interestingly, Bimγ mRNA, similar to the BimEL protein, is up-regulated in the majority of the prostate cancer cell lines studied, whereas several other proapoptotic Bcl-2 proteins, including Bax, Bak, and Bad, are down-regulated in prostate cancer cells. Functional studies indicate that Bimγ inhibits clonal growth in prostate cancer cells and promotes apoptosis, which is inhibited by overexpressing Bcl-2. Because both Bimγ and BimEL are proapoptotic BH3-only proteins and both are up-regulated in prostate cancer cells, they may play a unique role in prostate cancer development.

INTRODUCTION

Homeostasis in multicellular organisms is maintained by balanced cell proliferation and programmed cell death or apoptosis (1). Impairment of this balance may contribute to a number of human diseases, such as cancer, autoimmune diseases, and neurodegenerative disorders (2). We have been studying how dysregulation of apoptosis may contribute to prostate cancer development.

Various Bcl-2 proteins play a central role in regulating apoptosis. They possess up to four conserved BH3[3] domains designated BH1 to BH4. More than 20 Bcl-2 family members have been reported, which are grouped into antiapoptotic and proapoptotic subfamilies. The antiapoptotic subfamily includes Bcl-2 itself and Bcl-X_L, Bcl-w, Mcl-1, A1, Boo/Diva, Bcl-B, and NR13, which contain at least BH1, BH2, and BH4 (3, 4). The proapoptotic subfamily of Bcl-2 proteins can be further subdivided into two groups. One group includes Bax, Bak, Bok/MTD (containing BH1–BH3) and Bcl-rambo (containing all four BH domains), Bcl-X_S (containing BH3 and BH4), and Bcl-G (containing BH2 and BH3). The other group of proapoptotic Bcl-2 proteins, which include Bim/Bod, Bid, Bad, Bik/NBK, Blk, Hrk, NIP3, NIX/BNIP3, Noxa, Bmf, and PUMA, contain only BH3 domain, thus the name "BH3-only" proteins (3, 5–9). It has become clear that BH3-only proteins are critical regulators of apoptosis and play an essential role in mammalian development.

Bim, a BH3-only protein, provokes apoptosis, and its BH3 domain is required for most of its cytotoxicity (10). There are three isoforms of Bim (BimEL, BimL, and BimS) generated by alternative splicing. All three Bim proteins possess a hydrophobic COOH terminus, which is thought to help the proteins to localize to intracytoplasmic membranes (10). Bim proteins bind to and antagonize Bcl-X_L or Bcl-w (10), and they play a critical role in development, as a significant number of *bim*-null mice, and even some of the *bim*$^{+/-}$ animals die *in utero* before E9.5 for unknown reasons (11).

In this study, we report the identification and characterization of a novel Bim splice variant, Bimγ. Bimγ is also a BH3-only protein, which induces cell death and is up-regulated at the mRNA level in most of the prostate cancer cell lines studied.

MATERIALS AND METHODS

Cells and Reagents. Five NHP epithelial strains, NHP1- NHP5, were primary cultures freshly isolated from five different donors. NHP1, NHP3, and NHP4 cells were obtained from Clonetics (Walkersville, MD), and NHP2 and NHP5 cells were derived and characterized by D. Chopra (12–14). All these primary strains were cultured in serum-free PrEBM (Clonectics) medium supplemented with insulin, epidermal growth factor, hydrocortisone, bovine pituitary extract, and cholera toxin (12–14) and used at passage 2–6. PPC-1 cells were isolated from primary prostate carcinoma (15). MDA PCa 2b was a cell line derived from primary cultures of bone marrow metastases (16). LNCaP (17) and its sublines, C4–2 and C5 (18), PC3 (19), Du145 (20), JCA-1 (21), and Tsu-Pr (22) were all established metastatic cell lines. All cancer cell lines were cultured in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum and antibiotics. HEK 293 cells, which were used in the transfection experiments, were purchased from American Type Culture Collection and cultured in DMEM supplemented with 5% fetal bovine serum and antibiotics.

Mouse monoclonal and rabbit polyclonal anti-GFP antibodies were purchased from Clontech (Palo Alto, CA). Rabbit polyclonal anti-Bim was bought from Calbiochem (San Diego, CA). Rabbit polyclonal anti-Bcl-X_L and -Bak and mouse monoclonal anti-Bad antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-Bax and -actin antibodies were purchased from BD PharMingen (San Diego, CA) and ICN Biomedicals (Aurora, OH), respectively. The secondary antibodies, goat anti-mouse or rabbit IgG conjugated to horseradish peroxidase, were purchased from Amersham (Piscataway, NJ). Liposome FuGENE 6 was bought from Roche (Indianapolis, IN).

Cloning of Bimγ cDNA and Construction of Expression Vectors. The Bimγ cDNA sequence was identified in GenBank human EST database using the TBLASTN search (see "Results" for details). Full-length Bimγ was obtained by RT-PCR amplification using forward primer 5′-GACAAGAATCCGACCAAATGGCAAA-3′ and reverse primer 5′-AAAAGGATCCATGAGAAATCCTTGTGG-3′, based on the Bimγ cDNA sequence. mRNA from NHP3 cells was used as template to synthesize cDNA with Superscript II reverse transcriptase (Life Technologies, Inc., Grand Island, NY). Two μl of cDNA were used for PCR. The PCR product (362 bp), which contained the full-length coding region of Bimγ except the stop codon, was cloned into pCRII-TOPO (Invitrogen, Carlsbad, CA) and sequenced.

To construct the Bimγ expression vector, the Bimγ coding sequence minus stop codon was released from pCR II-TOPO by *Eco*RI digestion and subcloned

Received 12/28/01; accepted 3/20/02.

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.

[1] Supported by NIH National Cancer Institute Grant CA-90297, National Institute of Environmental Health Sciences Center Grant ES07784, Burroughs-Wellcome Fund Award BWF-1122, and M.D. Anderson Cancer Center institutional grants.

[2] To whom requests for reprints should be addressed, at Department of Carcinogenesis, the University of Texas M. D. Anderson Cancer Center, Science Park Research Division, Park Road 1C, Smithville, TX 78957. Phone: (512) 237-9486; Fax: (512) 237-2475; E-mail: dtang@sprd1.mdacc.tmc.edu.

[3] The abbreviations used are: BH, Bcl-2 homology; aa, amino acid; Bim, Bcl-2-interacting mediator; EGFP, enhanced green fluorescence protein; EST, expressed sequence tag; HM, heavy mitochondria; LM, light mitochondria; NHP, normal human prostate; MTN, multiple tissue northern; RT-PCR, reverse transcriptase-PCR.

into the mammalian expression vector pEGFP-N2 (Clontech). The orientation of the gene in the vector (pBimγ-EGFP) was determined by sequencing.

Northern Blotting. Human MTN blot membranes containing 16 tissues (Clontech) were used for Northern blotting. Either the full-length Bimγ (362 bp) or the Bimγ-specific EST part (*i.e.*, the 126-bp intron sequence) was used as the hybridization probe. The probes were radiolabeled (α-^{32}P dCTP) using Ready-To-Go labeling kit (Pharmacia) and purified using Probe Quant G-50 Micro Columns (AP Biotech). After prehybridization in ExpressHyb solution (Clontech) for 1 h, the membranes were first hybridized overnight at 68°C in ExpressHyb solution with the full-length probe, followed by washing at room temperature (×2, 30 min each) in 0.1 × SSC buffer containing 0.1% SDS. After stripping, the membranes were rehybridized with the Bimγ-specific probe overnight at 42°C followed by washing at room temperature (×2, 30 min each) in 0.1 × SSC buffer containing 0.1% SDS.

Immunoprecipitation and Western Blotting. HEK 293 cells (2×10^6) were either untransfected or transfected with control vector (pEGFP-N2) or pBimγ-EGFP. After transfection (48 h), whole cell lysates were prepared in complete immunoprecipitation buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.5% Triton X-100, and 10 mM EDTA] containing protease inhibitor cocktail (Sigma), and protein concentrations were determined by MicroBCA kit (Pierce, Rockford, IL). The lysates (1 mg) were incubated overnight at 4°C with mouse monoclonal anti-GFP antibody preconjugated to protein A/G plus-agarose beads (Santa Cruz Biotechnology), followed by washing with PBS for five times. The immunoprecipitates were solubilized in 2 × sample buffer and, after boiling, loaded on a 15% precast SDS-polyacrylamide gel (Bio-Rad), and the fractionated proteins were transferred subsequently to nitrocellulose membrane. Then the membrane was sequentially probed with a polyclonal anti-GFP and polyclonal anti-Bim antibodies. The same anti-Bim antibody, whose epitope was mapped at aa 22–40 of BimEL/BimL, has been used by several other groups (23, 24). An aliquot of whole cell lysates from the same batch of samples was also directly used in Western blotting for actin (as a control for sample qualities) using a monoclonal antiactin antibody. Western blotting was performed as described previously (14, 25) using enhanced chemiluminescence.

Subcellular Fractionation. Subcellular fractionation was carried out using the differential centrifugation protocols described previously (26, 27), with minor modifications. Briefly, HEK 293 cells were either untransfected or transfected with empty vector or pBimγ-EGFP. Later (24 h), cells were dissociated, washed twice in ice-cold PBS, and resuspended in 600 μl of homogenizing buffer [20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, and 1 mM DTT] containing 250 mM sucrose and a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 1 mM leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml chymostatin). After a 20-min incubation on ice, cells were homogenized in the same buffer using a glass Pyrex homogenizer (type A pestle, 140 strokes). Unbroken cells, large plasma membrane pieces, and nuclei were removed by centrifuging the homogenates at 1600 rpm for 5 min at 4°C. The resulting supernatant was centrifuged at 1,000 × *g* for 5 min at 4°C to obtain the HM pellet. The supernatant was then subjected to centrifugation at 10,000 × *g* for 20 min at 4°C to obtain LM fraction. The remaining supernatant was further centrifuged at 100,000 × *g* for 1 h at 4°C to obtain cytosol and microsome pellet. The HM, LM, and microsomal pellets were washed three times in homogenizing buffer and then solubilized in 50 μl of TNC buffer [10 mM Tris-acetate (pH 8.0), 0.5% NP40, and 5 mM $CaCl_2$] containing protease inhibitors. Protein concentration was determined using MicroBCA kit (Pierce). Western blotting was performed using enhanced chemiluminescence.

mRNA Isolation and RT-PCR Analysis of Bimγ mRNA Expression. mRNA was isolated using Poly(A) Pure kit (Ambion, Austin, TX) according to the manufacturer's instructions. mRNA (0.5 μg) from each cell type was used in reverse transcription (42°C × 2 h) in a total of 20 μl of reaction containing random hexamers and Superscript II reverse transcriptase (Life Technologies, Inc.). The PCR primers used were the same as those used for cloning Bimγ (above). For PCR, 2 μl of cDNA from each cell type were used in a 50-μl reaction containing 1 μM primers, deoxynucleotide triphosphates, and Taq, using the cycling profile 94°C × 30 s, 60°C × 30 s, and 72°C × 30 s for 30 cycles. PCR products were analyzed by agarose gel electrophoresis. RT-PCR of glyceraldehyde-3-phosphate dehydrogenase was used as a control (28).

Overexpression of Bimγ in PPC1 Prostate Cancer Cells. PPC1 cells cultured in a six-well tissue culture plate (5×10^4 cells/well) were either untransfected or transfected with 1.5 μg of vector (pEGFP-N2) or pBimγ-EGFP using FuGENE 6. In another set of experiments, cells were cotransfected with a pCMV-bcl2 expression plasmid, which encodes murine Bcl-2 cDNA (25). After transfection (48 h), both floating and adherent cells were collected and counted under a phase-contrast microscope. Dead (both apoptotic and necrotic) cells were counted by their typical morphologies (25). The results are expressed as the percentage of cell death ± SE obtained from three independent experiments with triplicate wells per condition.

Clonal Growth Assays. PPC1 cells were plated in a 12-well tissue culture plate (1×10^4 cells/well) and transfected with 1 μg of empty vector (pEGFP-N2) or pBimγ-EGFP using FuGENE 6. After transfection (48 h), G418 (1 mg/ml) was added to the culture medium to select for transfected cells. Medium was changed, and fresh G418 was added every 3–4 days. After 2 weeks of selection, cells were rinsed with PBS and then stained with 0.4% Giemsa solution for 10 min, followed by gentle washing in PBS. The number of colonies in each well was quantified under an inverted microscope. The results are presented as mean ±SE from three individual experiments with triplicate wells per condition.

RESULTS AND DISCUSSION

Identification of Bimγ, a Novel BH3-only Molecule. We are studying regulation of prostate cancer cell apoptosis by Bcl-2 family proteins. One of the Bcl-2 proteins we studied is Bim, a BH3-only proapoptotic protein. Three alternatively spliced isoforms of Bim, BimEL (196 aa, M_r ~23,000), BimL (140 aa, M_r ~19,000), and BimS (110 aa, M_r ~15,000) have been reported (10). Western blotting using a polyclonal anti-Bim antibody (epitope being aa 22–40) demonstrated that all five NHP cell strains expressed low levels of BimEL and BimL, both of which were increased in most of the nine prostate cancer cell lines examined. In particular, BimEL protein level was significantly higher in six of the nine prostate cancer cell lines than in NHP strains (Fig. 1, *top panel*). Interestingly, the anti-Bim antibody also detected a M_r ~36,000 protein, which, in sharp contrast to BimEL, was expressed abundantly in all five strains of NHP cells but reduced or lost in all of the prostate cancer cell lines (Fig. 1, *top panel*).

In an attempt to determine what this M_r 36,000 protein might be, we searched the GenBank human EST database using as query the cDNA sequence encoding the epitope (AERPPQLRPGAPTSLQTEP) of the anti-Bim antibody. One clone (AI971169) was identified containing the full epitope sequence and showing significant homology to human

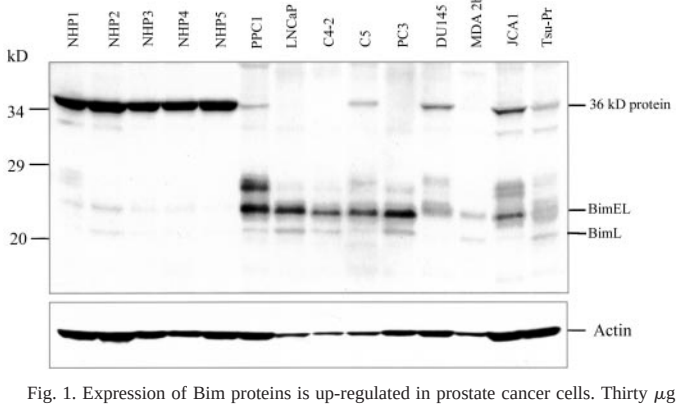

Fig. 1. Expression of Bim proteins is up-regulated in prostate cancer cells. Thirty μg of whole cell lysates from each cell type were loaded in each lane on a 15% SDS-polyacrylamide gel. The membrane was probed with a polyclonal anti-Bim antibody (*top panel*), stripped, and reprobed with a monoclonal antiactin antibody (loading control; *bottom panel*). The anti-Bim antibody detected BimEL (M_r ~23,000), BimL (M_r ~19,000), an unknown M_r ~36,000 protein, and some minor bands (*top panel*). Note that LNCaP cells and their derivatives C4-2, C5, and MDA 2b cells are known to express lower levels of actin (Refs. 14 and 16 and data not shown).

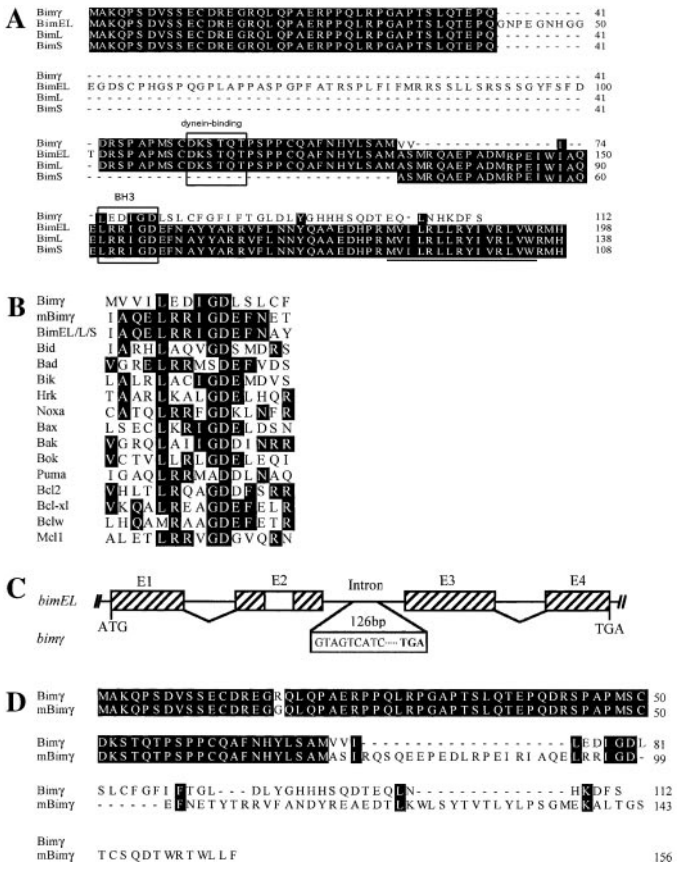

Fig. 2. Sequence characterization of Bimγ. *A*, alignment of aa sequences of BimEL/BimL/BimS and Bimγ (GenBank accession no. AI971169). Identical residues are *highlighted*. The BH3 and dynein-binding domains are *boxed*. The COOH-terminal hydrophobic region is *underlined*. The numbers of aa are indicated on the *right*. *B*, alignment of BH3 domains of Bimγ, murine Bimγ (mBimγ, GenBank accession no. BI555336), and other Bcl-2 family members. *C*, genomic organization of human *bim*, which contains four exons (E1, E2, E3, and E4) and three introns. Part of E2 (*blank box*) is spliced out in BimL (30). In BimS, the majority of E2 (*i.e.*, the blank box and the sequence behind it) is spliced out (30). *Bimγ* is composed of E1, E2, and the boxed sequence of intron 2. The premature in-frame stop codon (*TGA*) of Bimγ is indicated in *bold*. *D*, alignment of aa sequences of human Bimγ and murine Bimγ (*mBimγ*). Identical residues are *highlighted*. The cDNA of Bimγ was analyzed by MacVector 7.0 (Oxford Molecular, Ltd.), and the alignment of Bimγ with other genes was done using software DNASTAR MegAlign program.

Bim cDNA. Further analysis of its nucleotide sequence revealed an open reading frame that is predicted to encode a protein of 112 aa, whose first 71 aa were identical to the corresponding BimEL/BimL sequence (Fig. 2*A*). We named this molecule Bimγ for it is another isoform of Bim (Ref. 10; also see discussions below).

Similar to BimEL/BimL/BimS (10), Bimγ possesses a BH3 domain (LEDIGD; Fig. 2*A*, *boxed*; Fig. 2*B*) and a dynein-binding domain (DKSTQT; Fig. 2*A*, *boxed*), the latter of which is thought to mediate the interaction of Bim molecules with the cytoplasmic dynein light chain LC8 in healthy cells (29). On apoptotic stimulation, Bim proteins are released together with LC8 from the dynein motor complex and translocate to mitochondria to trigger apoptosis (29). Different from BimEL/BimL/BimS, however, Bimγ does not have the COOH-terminal hydrophobic region (Fig. 2*A*, *underlined*), which is thought to mediate Bim interaction with the intracellular membrane structures (10).

No BH1, BH2, or BH4 domain was identified in the Bimγ protein, suggesting that Bimγ is a new member of the BH3-only subfamily of proteins. Using cDNA sequence encoding Bimγ, we searched the human genomic database and found that its corresponding genomic sequence (NT015805) was located on the long arm of human chromosome 2, Chr 2q12–13, the same genomic locus as the human *bim* gene, suggesting that Bimγ may be another alternatively spliced product of *bim*. Human *bimEL* gene contains four exons and three introns (Fig. 2*C*; Ref. 30). Comparison of the Bimγ cDNA sequence with the human *bim* gene revealed that Bimγ was composed of exons 1 and 2 and part of the sequence from intron 2, suggesting that Bimγ indeed is another splice variant of *bim*. Retention of intron 2 (126 bp) in Bimγ introduced a premature in-frame stop codon (TGA; Fig. 2*C*), resulting in a molecule of 112 aa with an estimated molecular mass of M_r ~15,000, which was corroborated in our transfection experiments using Bimγ-GFP fusion constructs (see Fig. 5). Therefore, the newly identified Bimγ cannot be the M_r ~36,000 protein band identified by the polyclonal anti-Bim antibody (Fig. 1, *top panel*). Indeed, using proteomic approach, we identified it as Annexin II.[4]

We also searched the mouse EST database in the GenBank using Bimγ cDNA as query and found a clone (BI555336) that shared significant homology to Bimγ in its first half of the sequence (Fig. 2*D*), suggesting that aa 1–71 is highly conserved.

The above results suggest that the human *bim* gene has multiple splicing products. Indeed, while we were preparing this manuscript, Mami *et al.* (31) reported six other novel isoforms of human Bim (which they named Bimα1, α2, and β1-β4. In continuation with this naming system, we named our Bim splice variant Bimγ). Similar to Bimγ, none of these novel isoforms contains a COOH-terminal hydrophobic region. Among these six Bim isoforms, only Bimα1 and α2 contain a BH3 domain (31). Together, these studies suggest that human *bim* gene has ≥10 splice variants.

Bimγ Displays a Distinct Tissue-specific Expression Pattern in Normal Human Tissues. Some of the Bcl-2 family proteins show tissue-specific expressions, *e.g.*, Bcl-G is highly expressed in human testis (7), N-Bak in neuron (32), Bcl-B in adult human tissues (4), and Boo in ovary and epididymis (33). Bim is expressed in a variety of mouse tissues, such as hematopoietic, epithelial, neuronal, and germ cells (34). The expression profiles of most other Bcl-2 family members remain largely unknown.

To determine the expression of Bimγ in human tissues, we probed the human MTN blot membranes that contain 16 tissues using the full-length Bimγ cDNA as the hybridization probe. As shown in Fig. 3, *A* and *B*, three transcripts, a major (~5.7 kb) and two minor (3.8 and 1.4 kb) bands, were observed in all of the tissues studied, with the spleen, thymus, prostate, testis, placenta, liver, skeletal muscle, kidney, and pancreas expressing high levels. When using full-length mouse Bim cDNA as the hybridization probe, an identical expression pattern (*i.e.*, 5.7, 3.8, and 1.4 kb) was observed (10), suggesting that the full-length Bimγ probe we used detected the major mRNA transcripts of Bim. When we used the Bimγ-specific cDNA (*i.e.*, intron 2 sequence) encoding aa 72 to 112 of Bimγ, which shows no homology to all other sequences in the GenBank database, a major ~1.1 kb band and a minor ~1.8 kb band were detected (Fig. 3, *C* and *D*). By prediction, the 1.1-kb band probably represents the primary transcript of Bimγ, whereas the 1.8-kb band might be due to differential polyadenylation signal usage. Interestingly, Bimγ displays a tissue-specific expression pattern distinct from those of the other Bim isoforms (compare Fig. 3, *A* and *B* with Fig. 3, *C* and *D*). Specifically, Bimγ is most abundantly expressed in small intestine and colon with lower levels in spleen, prostate, testis, heart, liver, and kidney (Fig. 3, *C* and *D*). All other tissues examined expressed little or no Bimγ mRNA (Fig. 3, *C* and *D*). These results suggest that Bimγ and BimEL/BimL/BimS may play differential biological functions in tissue-specific manners.

[4] J-W. Liu *et al.*, Proteomic analysis identifies reduced or lost expression of annexins I and II in prostate cancer cells—Enforced expression of annexin II inhibits prostate cancer cell migration, submitted for publication.

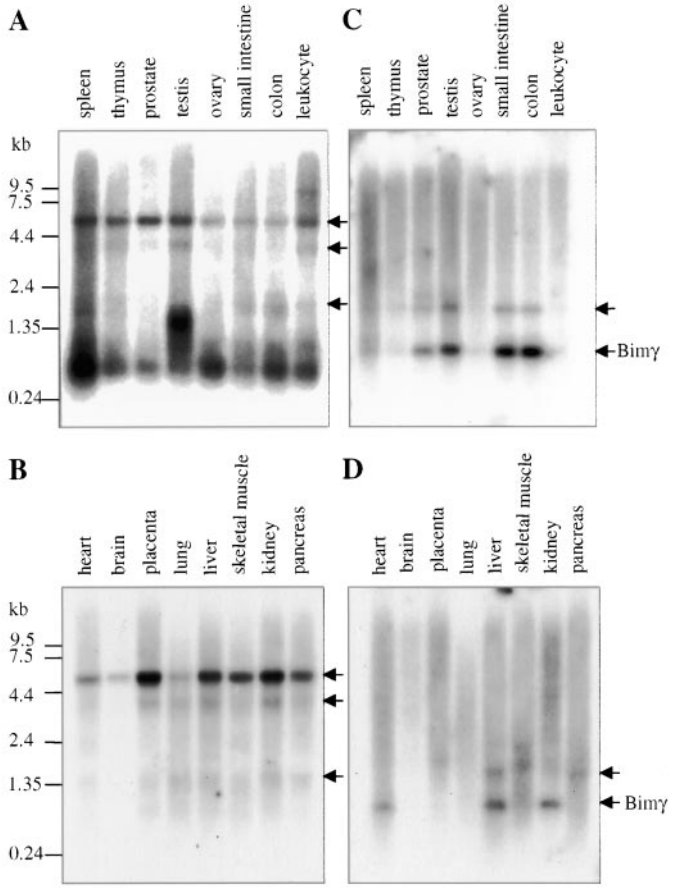

Fig. 3. Bimγ mRNA expression in normal human tissues. The MTN multitissue RNA membranes were probed with ^{32}P-labeled cDNA Bimγ (*A* and *B*) or Bimγ-specific COOH-terminal sequence (*C* and *D*). *Arrows* in *A* and *B*, the major 5.7-kb and the minor 3.8- and 1.4-kb bands. *Arrows* in *C* and *D*, the major 1.1-kb (Bimγ) and the minor 1.8-kb (upper) bands.

Bimγ mRNA Expression is Up-Regulated in Prostate Cancer Cells. Prostate cancer cells are generally more resistant to apoptosis induction (*e.g.*, by serum starvation) than NHP cells (14). One of the molecular mechanisms that prostate cancer cells use to bypass apoptosis induction is to up-regulate the expression of some antiapoptotic Bcl-2 family proteins (such as Bcl-X_L) and down-regulate or even lose the expression of some proapoptotic Bcl-2 proteins (such as Bax; Ref. 14). Even at unstimulated conditions, prostate cancer cells express lower levels of Bax, Bak and Bad, and a higher level of Bcl-X_L than NHP cells (Fig. 4*A*). In contrast to Bax, Bak, and Bad, Bim (*i.e.*, BimEL and BimL) is overexpressed in prostate cancer cell lines (Fig. 1).

To examine the expression pattern of Bimγ, we measured its mRNA in three NHP strains and nine human prostate cancer cell lines by RT-PCR (Fig. 4*B*). Bimγ mRNA was detected in one (*i.e.*, NHP3) of the three NHP cells but in six of the nine prostate cancer cell lines examined. Additionally, the Bimγ mRNA levels in some cancer cells (*i.e.*, PC3, Du145, JCA-1, and TSU-Pr) were higher than in NHP2 cells (Fig. 4*B*). These data suggest that, similar to BimEL, Bimγ is up-regulated in prostate cancer cells. On the other hand, Bimγ mRNA was not detected in PPC1 and LNCaP cells (Fig. 4*B*), whereas BimEL protein was abundantly expressed in both cells lines (Fig. 1), suggesting that the two alternatively spliced molecules are also differentially expressed in prostate cancer cells.

Bimγ Is Localized Both in Cytosol and Intracellular Membranes. Many Bcl-2 family proteins, such as Bak, Bcl-2, and Bcl-X_L, are localized in membranes of mitochondria, endoplasmic reticulum, or nuclear envelope (3, 35, 36), whereas several proapoptotic Bcl-2 family proteins, *e.g.*, Bax, Bad, and Bid, exist as inactive cytosolic proteins in normal conditions and translocate to mitochondria during apoptosis (37).

To study the potential biological function(s) of Bimγ in regulating apoptosis, we first studied its intracellular localization using a GFP-tagged Bimγ expression construct. To confirm the proper expression of Bimγ-GFP fusion protein, we transiently transfected HEK 293 cells with pBimγ-EGFP or the empty vector. As shown in Fig. 5, the Bimγ-GFP fusion protein (M_r ~44,000) was detected specifically, with both an anti-GFP antibody (*top panel*) and the anti-Bim antibody (*middle panel*), in cells transfected with pBimγ-EGFP but not in untransfected cells or cells transfected with the vector alone. As the GFP (*i.e.*, EGFP) protein was detected as a M_r ~29,000 protein in our experiments, the molecular weight of Bimγ was therefore M_r ~15,000, which is consistent with its predicted molecular weight (see preceding discussions).

To assess its subcellular localization, we expressed Bimγ-GFP fusion protein in 293 cells and prepared different subcellular fractions (see "Materials and Methods"). As shown in Fig. 6, Bimγ-GFP fusion protein localized in HM and cytosol, as well as in microsomes, which include cytoplasmic organelles, such as endoplasmic reticulum, with less Bimγ in the LM. These results suggest that Bimγ is probably normally distributed in both membranous and cytosolic compartments.

Many Bcl-2 family proteins are thought to localize to the organelle membranes through their COOH-terminal hydrophobic regions. It is thus interesting to note that Bimγ does not possess an identifiable COOH-terminal hydrophobic tail (Fig. 2*A*). Then how does Bimγ

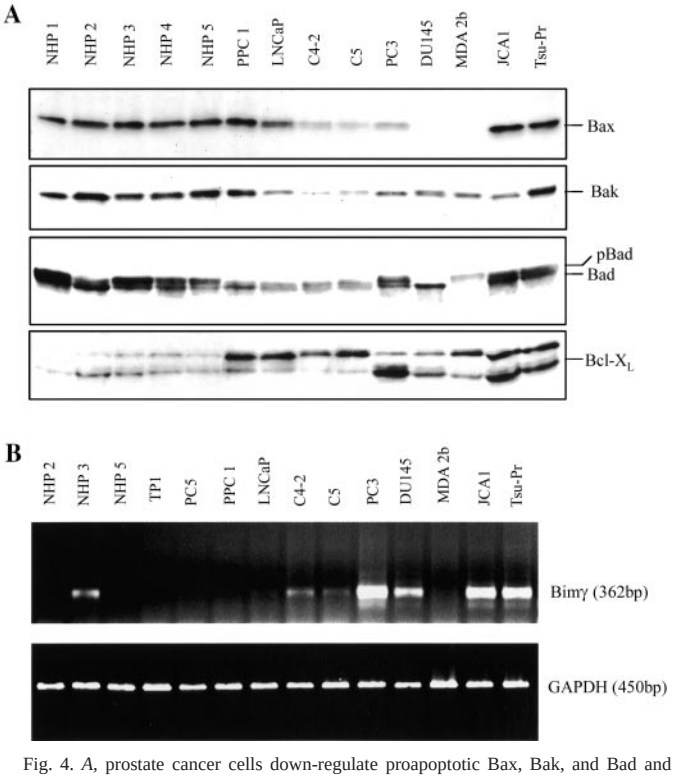

Fig. 4. *A*, prostate cancer cells down-regulate proapoptotic Bax, Bak, and Bad and up-regulate antiapoptotic Bcl-X_L. This Western blot membrane was the same as the one used in Fig. 1. Note that Bax protein was lost in DU145 and MDA 2b cells, because of a frameshift mutation in the *bax* gene (14, 16). Bad was detected as a doublet, and the upper band probably represents the phosphorylated Bad (pBad; Refs. 3 and 14). The polyclonal anti-Bcl-X_L antibody also detected an upper band of unknown identity, which was also up-regulated in the majority of prostate cancer cells. In *B*, expression of Bimγ mRNA is up-regulated in some prostate cancer cell lines.

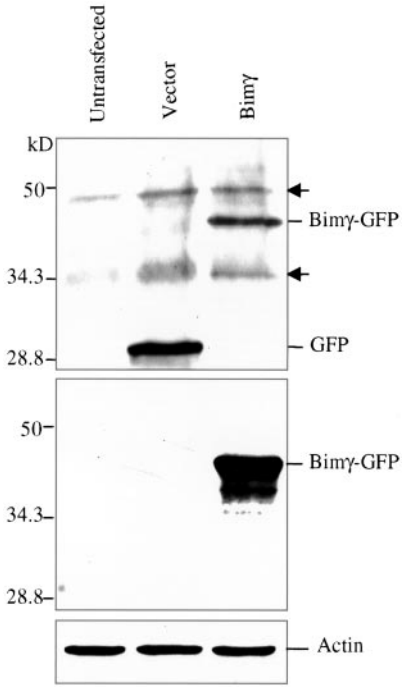

Fig. 5. Bimγ-GFP fusion protein is processed correctly and expressed in transfected cells. HEK 293 cells were either untransfected or transfected with pBimγ-EGFP or vector only. Cells were harvested 48 h after transfection, and the whole cell lysates were prepared using TNC buffer (see "Materials and Methods"). One mg of protein of each sample was used for immunoprecipitation using a monoclonal anti-GFP antibody. After SDS-PAGE and protein transfer, the membrane was probed with a rabbit polyclonal anti-GFP antibody (*top panel*). *Arrows*, the two nonspecific bands (the upper band probably is the Ig heavy chain). After stripping, the membrane was reprobed with the polyclonal anti-Bim antibody (*middle panel*). As a control for sample qualities, a Western blotting of actin was carried out using whole cell lysates (50 μg/lane) from the same batch of samples used for immunoprecipitation (*bottom panel*).

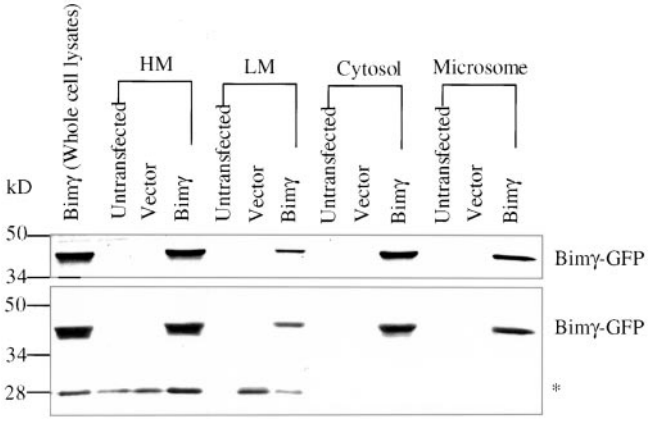

Fig. 6. Subcellular distribution of Bimγ. HEK 293 cells were untransfected or transfected with the empty vector (*Vector*) or pBimγ-EGFP (*Bimγ*). After transfection (24 h), cells were harvested and used to prepare individual cellular fractions, as detailed in "Materials and Methods." HM, 60 μg; LM, 58 μg; Cytosol, 56 μg; Microsome, 20 μg. The protein membrane was probed with anti-GFP peptide antibody (*top panel*) and after stripping, reprobed with polyclonal anti-Bim antibody (*bottom panel*). *, an unknown band of M_r ~26,000 that was detected with the polyclonal anti-Bim specifically in the mitochondrial fractions.

target itself to the intracellular membranes? Recent studies by Mami *et al.* (31) indicate that Bimα1 and α2, which lack the COOH-terminal hydrophobic region, are also located in mitochondria, whereas Bimβ1-β4 isoforms, which lack both BH3 domain and the hydrophobic regions, are located only in the cytosol. These data (31), together with our observations, suggest that the BH3 domain itself is probably sufficient for at least some membrane localization.

Bimγ Is a Proapoptotic Protein. Most BH3-only proteins, including Bim, Bid, Bad, Bik/NBK, Blk, Hrk NIP3, NIX/BNIP3, Noxa, Bmf, and PUMA, provoke apoptosis in various cell lines when highly expressed (3, 5–9). The BH3 domain was known to be essential for apoptosis induction by binding to a hydrophobic cleft on the surface of an antiapoptotic Bcl-2 family protein (38, 39). To study the biological function(s) of BH3-only Bimγ, we transiently transfected pBimγ-EGFP or the empty vector into PPC1 prostate cancer cells and then studied apoptosis in the transfected cells. We chose PPC-1 cells because they express undetectable endogenous Bimγ mRNA (Fig. 5) and are most susceptible to transfection in our pilot experiments. We first investigated whether Bimγ had any effect on the long-term clonal growth of stably transfected PPC1 cells, which survived presumably because of relatively lower levels of Bimγ expression. As shown in Fig. 7*A*, expression of Bimγ significantly reduced the number of PPC-1 colonies formed after 2 weeks of selection. These results suggest that Bimγ inhibits the clonal expansion of the transfected PPC-1 cells, probably as a result of increased cell death. Indeed, as shown in Fig. 7*B*, overexpression of Bimγ promoted cell death in PPC1 cells. The dead cells showed typical apoptotic morphologies (25), such as shrunken cell bodies and membrane blebbing (data not shown). The percentage of apoptotic cells in Bimγ-transfected cells was more than twice that of the vector-only transfected cells (Fig. 7*B*). The majority of the dead cells was bright GFP^+ cells (data not shown) and, thus, expressing high levels of Bimγ. As in other cell apoptotic systems triggered by BH3-only proteins (23, 24), Bcl-2 inhibited PPC-1 cell apoptosis induced by Bimγ (Fig. 7*B*).

In summary, in this study, we report the identification of another

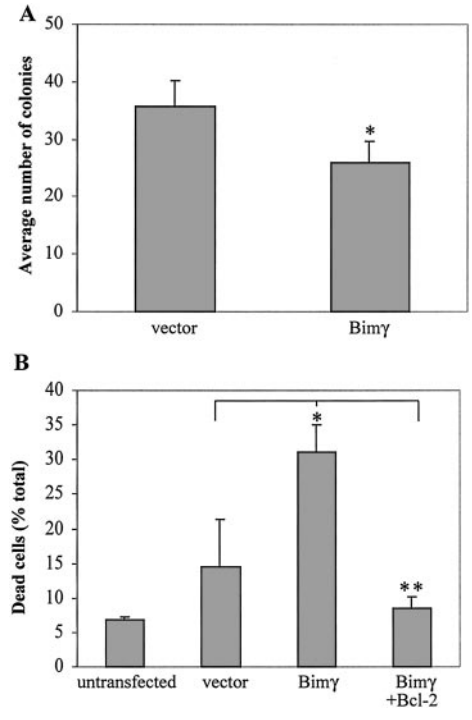

Fig. 7. *A*, Bimγ inhibits clonal growth of PPC-1 cells. PPC1 cells were transfected with either vector alone (*vector*) or pBimγ-EGFP (*Bimγ*). Forty-eight h post-transfection, transfected cells were selected with 1 mg/ml G418. Two weeks after transfection, the cells that formed colonies were stained with 0.4% Giemsa solution and counted under a phase-contrast microscope. Shown is the average number of colonies (mean ± SE). *, $P < 0.05$. *B*, Bimγ promotes cell death in PPC1 prostate cancer cells. PPC1 cells were either untransfected or transfected with empty vector (*vector*) or pBimγ-EGFP (*Bimγ*) or cotransfected with Bimγ and a Bcl-2 expression vector (pCMV-bcl-2; Ref. 25). After transfection (48 h), cells were collected and counted. Cells with apoptotic morphology (*e.g.*, cellular shrinkage and membrane blebbing; Ref. 25) were enumerated (mean ± SE). *, $P < 0.01$; **, $P < 0.001$.

BH3-only splice variant of Bim, Bimγ. Bimγ shows very distinct tissue-specific distribution patterns, and the protein is localized in both cytosolic and membranous compartments. Like most other BH3-only proteins, Bimγ is proapoptotic. The BH3 domain seems to be important and required for the proapoptotic function of all Bim isoforms (10, 31), because Bim isoforms lacking the BH3 domain, *e.g.*, Bimβ1-β4, also lack proapoptotic activities (31). It is reported that BimL induces apoptosis by binding to and antagonizing the antiapoptotic Bcl-2 family members, such as Bcl-X_L and Bcl-w (10). Bimγ may also trigger apoptosis through similar mechanisms because its apoptotic effect can be antagonized by Bcl-2, which also suggests that Bimγ may physically interact with Bcl-2 and/or other antiapoptotic Bcl-2 family proteins. The most intriguing finding of our study is that Bimγ mRNA is up-regulated in many of the prostate cancer cell lines examined. Very likely, the Bimγ protein, similar to BimEL/BimL, may also be up-regulated in these cancer cells. Because all these three Bim isoforms are proapoptotic, their up-regulation in prostate cancer cells, if confirmed *in vivo*, suggests that various Bim proteins may play a unique role in prostate cancer development as prostate cancer cells generally down-regulate proapoptotic (*e.g.*, Bax, Bak, and Bad) and up-regulate antiapoptotic (*e.g.*, Bcl-X_L) proteins. We are currently generating Bimγ-specific antibodies to study how this isoform, as well as BimEL/L, regulate prostate cancer cell death.

REFERENCES

1. Jacobson, M. D., Weil, M., and Raff, M. C. Programmed cell death in animal development. Cell, *88:* 347–354, 1997.
2. Thompson, C. B. Apoptosis in the pathogenesis and treatment of disease. Science (Wash. DC), *267:* 1456–1462, 1995.
3. Gross, A., McDonnell, J. M., and Korsmeyer, S. J. Bcl-2 family members and the mitochondria in apoptosis. Genes Dev., *13:* 1899–1911, 1999.
4. Ke, N., Godzik, A., and Reed, J. C. Bcl-B, a novel Bcl-2 family member that differentially binds and regulates Bax and Bak. J. Biol. Chem., *276:* 12481–12484, 2001.
5. Puthalakath, H., Villunger, A., O'Raeilly, L. A., Beaumont, J. G., Coultas, L., Cheney, R. E., Huang, D. C. S., and Strasser, A. Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. Science (Wash. DC), *293:* 1829–1832, 2001.
6. Kataoka, T., Holler, N., Micheau, O., Martinon, F., Tinel, A., Hofmann, K., and Tschopp, J. Bcl-rambo, a novel Bcl-2 homologue that induces apoptosis via its unique C-terminal extension. J. Biol. Chem., *276:* 19548–19554, 2001.
7. Guo, B., Godzik, A., and Reed, J. C. Bcl-G, a novel pro-apoptotic member of the Bcl-2 family. J. Biol. Chem., *276:* 2780–2785, 2001.
8. Yu, J., Zhang, L., Hwang, P. W., Kinzler, K. W., and Volgelstein, B. PUMA induces the rapid apoptosis of colorectal cancer cells. Mol. Cell, *7:* 673–682, 2001.
9. Nakano, K., and Vousden, K. H. PUMA, a novel proapoptotic gene, is induced by p53. Mol. Cell, *7:* 683–694, 2001.
10. O'Connor, L., Strasser, A., O'Reilly, L. A., Hausmann, G., Adams, J. M., Cory, S., and Huang, D. C. Bim, a novel member of the Bcl-2 family that promotes apoptosis. EMBO J., *17:* 384–395, 1998.
11. Bouillet, P., Metcalf, D., Huang, D. C. S., Tarlinton, D. M., Kay, T. W. H., Kontgen, F., Adams, J. M., and Strasser, A. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science (Wash. DC), *286:* 1735–1738, 1999.
12. Chopra, D. P., Grignon, D. J., Joiakim, A., Mathieu, P. A., Mohamed, A., Sakr, W. A., Powell, I. J., and Sarkar, F. H. Differential growth factor responses of epithelial cell cultures derived from normal human prostate, benign prostate hyperplasia, and primary prostate carcinoma. J. Cell. Physiol., *169:* 269–280, 1996.
13. Chopra, D. P., Sarkar, F. H., Grignon, D. J., Sakr, W. A., Mohamed, A., and Waghray, A. Growth of human nondiploid primary prostate tumor epithelial cells *in vitro*. Cancer Res., *57:* 3688–3692, 1997.
14. Tang, D. G., Li, L., Chopra, D., and Porter, A. T. Extended survivability of prostate cancer cells in the absence of trophic factors: increased proliferation, evasion of apoptosis and the role of apoptosis proteins. Cancer Res., *58:* 3466–3479, 1998.
15. Brothman, A. R., Wilkins, P. C., Sales, E. W., and Somers, K. D. Metastatic properties of human prostatic cell line. PPC-1, in athymic nude mice. J. Urol., *145:* 1088–1091, 1991.
16. Navone, N. M., Olive, M., Ozen, M., Davis, R., Tronsco, P., Tu, S-M., Johnston, D., Pollack, A., Pathak, S., von Eschenbach, A. C., and Logothetis, C. J. Establishment of two human prostate cancer cell lines derived from a single bone marrow metastasis. Clin. Cancer Res., *3:* 2493–2500, 1997.
17. Horoszewicz, J. S., Leong, S. S., Kawinski, E., Karr, J. P., Rosenthal, H., Chu, T. M., Mirand, E. A., and Murphy, G. P. LNCaP model of human prostatic carcinoma. Cancer Res., *43:* 1809–1818, 1983.
18. Wu, H-C., Hsieh, J-T., Gleave, M. E., Brown, N. M., Pathak, S., and Chung, L. W. K. Derivation of androgen-independent human LNCaP prostatic cancer cell sublines: role of bone marrow stromal cells. Int. J. Cancer, *57:* 406–412, 1994.
19. Kaighn, M. E., Narayan, K. S., Ohnuki, Y., Lechner, J. F., and Jones, L. W. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). Investig. Urol., *17:* 16–23, 1979.
20. Stone, K. R., Mickey, D. D., Wunderli, H., Mickey, G. H., and Paulson, D. F. Isolation of a human prostate carcinoma cell line (Du145). Int. J. Cancer, *21:* 274–281, 1978.
21. Muraki, J., Addonizio, J. C., Choudhury, M. S., Fischer, J., Eshghi, M., Davidian, M. M., Shapiro, L. R., Wilmot, P. L., Nagamatsu, G. R., and Chiao, J. W. Establishment of new human prostatic cancer cell line (JCA-1). Investig. Urol., *36:* 79–84, 1990.
22. Iizumi, T., Yazaki, T., Kanoh, S., Kondo, I., and Koiso, K. Establishment of a new prostatic carcinoma cell line (TSU-PR-1). J. Urol., *137:* 1304–1306, 1987.
23. Cheng, E. H-Y. A., Wei, M. C., Weiler, S., Flavell, R. A., Mak, T. W., Lindsten, T., and Korsmeyer, S. J. Bcl-2. Bcl-X_L sequester BH3 domain-only molecules preventing Bax- and Bak-mediated mitochondrial apoptosis. Mol. Cell, *8:* 705–711, 2001.
24. Zong, W-X., Lindsten, T., Ross, A. J., MacGregor, G. R., and Thompson, C. B. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev., *15:* 1481–1486, 2001.
25. Tang, D. G., Chen, Y. Q., and Honn, K. V. Arachidonate lipoxygenases as essential regulators of cell survival and apoptosis. Proc. Natl. Acad. Sci. USA, *93:* 5241–5246, 1996.
26. Tang, D. G., Li, L., Zhu, Z., and Joshi, B. Apoptosis in the absence of cytochrome *c* accumulation in the cytosol. Biochem. Biophys. Res. Commun., *242:* 380–384, 1998.
27. Joshi, B., Li, L., Taffe, B. G., Zhu, Z., Wahl, S., Tian, H., Ben-Josef, E., Taylor, J. D., Porter, A. T., and Tang D. G. Apoptosis induction by a novel anti-prostate cancer compound. BMD188 (a fatty acid-containing hydroxamic acid), requires the mitochondrial respiratory chain. Cancer Res., *59:* 4343–4355, 1999.
28. Tang, D. G., Tokumoto, Y. M., and Raff, M. C. Long-term culture of purified postnatal oligodendrocyte precursor cells: evidence for an intrinsic maturation programme that plays out over months. J. Cell Biol., *148:* 971–984, 2000.
29. Puthalakath, H., Huang, D. C., O'Reilly, L. A., King, S. M., and Strasser, A. The proapoptotic activity of the Bcl2 family member Bim is regulated by interaction with the dynein motor complex. Mol. Cell, *3:* 287–296, 1999.
30. Bouillet, P., Zhang L. C., Huang, D., Webb, G. C., Bottema, C. D. K., Shore, P., Eyre, H. J., Sutherland, G. R., and Adams, J. M. Gene structure, alternative splicing, and chromosomal localization of pro-apoptotic Bcl-2 relative Bim. Mamm. Genome, *12:* 163–168, 2001.
31. Mami, U., Miyashita, T., Shikama, Y., Tadokoro, K., and Yamada, M. Molecular cloning and characterization of six novel isoforms of human Bim, a member of the proapoptotic Bcl2 family. FEBS Lett., *509:* 135–141, 2001.
32. Sun, Y., Yu, L., Saarma, M., Timmusk, T., and Arumae, U. Neuron-specific Bcl-2 homology 3 domain-only variant of Bak is anti-apoptotic in neurons, but pro-apoptotic in non-neuronal cells. J. Biol. Chem., *276:* 16240–16247, 2001.
33. Song, Q., Kuang, Y., Dixit, V. M., and Vincenz, C. Boo, a novel negative regulator of cell death, interacts with Apaf-1. EMBO J., *18:* 167–178, 1999.
34. O'Reilly, L. A., Cullen, L., Visvader, J., Lindeman, G. J., Print, C., Bath, M. L., Huang, D. C. S., and Strasser, A. The proapoptotic BH3-only protein Bim is expressed in hematopoietic, epithelial, neuronal, and germ cells. Am. J. Pathol., *157:* 449–461, 2000.
35. Reed, J. C. Double identity for proteins of the Bcl-2 family. Nature (Lond.), *387:* 773–776, 1997.
36. Adams, J., and Cory, S. The Bcl-2 protein family: arbiters of cell survival. Science (Wash. DC), *281:* 1322–1326, 1998.
37. Antonsson, B., and Martinou, J. C. The Bcl-2 protein family. Exp. Cell Res., *256:* 50–57, 2000.
38. Huang, D. C., and Strasser, A. BH3-only protein: essential initiators of apoptotic cell death. Cell, *103:* 839–842, 2000.
39. Sattler, M., Liang, H., Nettesheim, D., Meadows, R. P., Harlan, J. E., Eberstadt, M., Yoon, H. S., Shuker, S. B., Chang, B. S., Minn, A. J., Thompson, C. B., and Fesik, S. W. Structure of Bcl-X_L-Bak peptide complex: recognition between regulators of apoptosis. Science (Wash. DC), *275:* 983–986, 1997.