Three Novel Bid Proteins Generated by Alternative Splicing of the Human Bid Gene*

Bid, a BH3-only Bcl-2 protein, is activated by proteolytic cleavage exposing the BH3 domain, which then induces apoptosis by interacting with pro-apoptotic Bcl-2 family proteins (e.g. Bax and Bak) at the mitochondrial surface. The arrangement of domains within Bid suggested that Bid function might be regulated in part by alternative splicing. We have determined the gene structure of human Bid and identified a number of novel exons. We have also demonstrated endogenous mRNA and protein expression for three novel isoforms of Bid, generated using these exons. BidΔ contains the N-terminal regulatory domains of Bid without the BH3 domain; BidEL corresponds to full-length Bid with additional N-terminal sequence; and BidES contains only the Bid sequence downstream of the BH3 domain. Expression of these isoforms is regulated during granulocyte maturation. In functional studies BidEL induces apoptosis, whereas BidΔ abrogates the pro-apoptotic effects of truncated Bid and inhibits Fas-mediated apoptosis. BidES induces apoptosis but is also able to partially inhibit the pro-apoptotic effects of truncated Bid. These three novel endogenously expressed isoforms of Bid are distinct in their expression, their cellular localization, and their effects upon cellular apoptosis. Differential expression of these novel Bid isoforms may regulate the function of Bid following cleavage and thus influence the fate of cells exposed to a range of pro-apoptotic stimuli.

Apoptosis, or programmed cell death, is an evolutionarily conserved program of changes in cell biochemistry and structure (1) leading to the loss of cellular functions and to engulfment and removal by phagocytes (2). This process is essential during normal development, in homeostasis, and also in disease pathogenesis (3). Apoptosis occurs following activation of specific caspases that amplify apoptotic signals in a cascade of proteolysis leading to cleavage of specific substrates. Caspase activation may be initiated in a number of ways, including ligation of cell surface death receptors (4) or activation of the apoptosome (a large multiprotein complex containing caspase-9) by cytochrome c released from mitochondria (5). Cytochrome c release from mitochondria is in turn regulated by the interaction of pro- and anti-apoptotic members of the Bcl-2 family of proteins at the mitochondrial surface (6).

Mammalian Bcl-2 family members share homology with the prototypic protein Bcl-2 and with the nematode CED-9 protein, across four conserved regions, termed the Bcl-2 homology domains (BH1–4). Bcl-2 proteins can be broadly divided into three groups according to their ability to either induce or inhibit apoptosis and their structural relationship to Bcl-2 (7). In addition to the BH3 domain, all anti-apoptotic Bcl-2 proteins described to date contain BH1, BH2, and sometimes BH4 domains. Pro-apoptotic family members either share the multidomain structure (e.g. Bax and Bak) or contain only the BH3 domain (e.g. Bim and Bid). Bcl-2 proteins containing only the BH3 domain have been suggested to play an important role in initiating mitochondrial-mediated apoptosis.

Bid is a 22-kDa “BH3-only” protein with similarity to other Bcl-2 family proteins only within the death-inducing BH3 region (8). Bid has a unique role in signaling of apoptosis, because it links the death receptor signaling pathway to the mitochondrial signaling pathway mediated by Bcl-2 proteins. Death receptor ligation activates caspase-8, which cleaves Bid, freeing the C-terminal moiety (t,Bid) to cooperate in the release of cytochrome c from mitochondria. Examination of the crystal structure of Bid reveals that the hydrophobic face of the BH3 domain, which is exposed in other family members, is enclosed by the N-terminal of Bid and only exposed following proteolytic cleavage of Bid (9, 10). There appears to be a second functional domain in Bid, in the N terminus, termed the BH3B domain (11), which has activity to suppress the apoptogenic activity of the BH3 domain. The region between these two opposing domains is extremely sensitive to proteolytic cleavage, containing cleavage sites for caspases, granzyme B, and various lysosomal proteases. The action of these proteases leads to the creation of two cleavage products (termed tΔ,Bid and tBS,Bid). tΔ,Bid translocates to mitochondria, where it interacts with other Bcl-2 family proteins to bring about the release of cytochrome c (12, 13).

We and others have shown that alternative gene splicing may regulate the function of other Bcl-2 family members (14–17). The structure of Bid, with opposing Bcl-2 homology domains, would potentially be readily regulatable by alternative gene splicing. We therefore investigated the functional regulation of this gene at the molecular level.
EXPERIMENTAL PROCEDURES

Novel Isoforms of Bid

Antibodies, Reagents, and Cell Lines—All chemicals were of analytical grade unless stated otherwise. Culture media (Hanks’ balanced salt solution, RPMI 1640, and Dulbeccos modified Eagle’s medium), LipofectAMINE, and OptiMEM were from Invitrogen. The antibody against Bid has been described previously (12) and was the kind gift of Dr. X. Wang (Howard Hughes Medical Institute, Dallas, TX). zVAD.fmk was from Bachem (St. Helens, OR). Preprepared protein samples were from the BioChain Institute (Hayward, CA). CH-11 was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Cos, HeLa, Jurkat, HL 60, 293T, and HepG2 cells were from American Type Culture Collection (Manassas, VA) and were cultured in RPMI 1640 supplemented with 10% fetal calf serum (Invitrogen). mRNA from NB4 cells was kindly supplied to us by Dr. P. D. Fearon (University of California, San Francisco) and cultured and stimulated as previously described (18). mRNA from neutrophil precursor populations was kindly supplied to us by Dr. J. Cowland (Copenhagen, Denmark) from samples purified from bone marrow and peripheral blood as previously described (19).

PCR—RNA was extracted using the RNaseasy kit (Qiagen). PCR was performed according to standard protocols. The primers used were BidF3 (exon 2, CGG GGA GAC GCT GCC TCG), BidF7 (exon 2, TGC TGG GAA ACT GTT GTA GAG TA), BidF10 (exon 6, CAA GTG CTG AGG AAG AAA CG), BidR2 (exon 9, AGG CAC TCA CAC TGC TGG AAC), GAPDH F forward (AAC TTT GGT ATC GTG GAA GGA C), and GAPDH R reverse (TGG TCG TTT AGG GCA ATG).

RACE was performed using the SMART™ RACE kit (BD Biosciences Clontech, Palo Alto, CA), using a modification of the protocol to include reduction of nonspecific base pair interaction (GC-Melt™; BD Biosciences Clontech). Gel extraction (Qiaquick; Qiagen), cloning (pCR-HI-TOPO; Invitrogen), and isolation of plasmid DNA (Concert Rapid; Invitrogen) were performed using commercially available kits according to the manufacturers’ instructions. Preprepared mRNA (Premium RNA) was from BD Biosciences Clontech, and cDNA was prepared using standard reverse transcription techniques.

Generation of Bid Expression Constructs—To generate the pCR3.1 (Invitrogen) expression constructs, PCR fragments were generated using the reverse primer, GGA TCC TCA GTC CAT CCC ATT CCT GGC TAA (exon 9), and the relevant forward primers, AAG CTT AGC GAC CAT GGA CTG TGA GGT CAA CAA C (Bid and BidS, exon 4), AAG CTT AGC CAC CAT GGA CTG CAG CGG TGC TGG GGT GA (Bid ES, exon 3), and AAG CTT AGC CAC CAT GGA CTG CAG CGG TGC TGG GGT GA (Bid ES, exon 7) using the appropriate construct in pCR-HI-TOPO as a template. The PCR product obtained was purified, and was cloned directly into the expression vector, a sixth noncoding exon is present, which lies 5’ of the genomic sequence of chromosome 22 identified five coding exons analogous to those coding for murine Bid (23). In addition, a sixth noncoding exon is present, which lies 25 kilobase pairs 5’ of the gene, which we have designated as exon 2 (Fig. 1). This exon has been previously recognized to be part of the Bid gene (24). A second previously undescribed 5’ exon of the gene exists within an expressed sequence tag (accession number AA388833) that maps to chromosome 22 just 5’ of exon 2 and that we have designated exon 1. To identify mRNA species utilizing exon 1 or 2 in association with other bid exons, 5’ RACE was performed. Neutrophil mRNA was chosen as the source of transcripts, because of our interest in death receptor signaling in these cells (25, 26) and because transcripts of alternatively spliced products were abundant in myeloid cell lines in preliminary experiments (data not shown). Using 5’ RACE, a number of alternatively spliced transcripts were isolated, including transcripts utilizing two further previously undescribed exons. The third of these novel exons we refer to as exon 3, the genomic locus of which is located 3’ of exon 2. mRNA transcripts contained exon 3 as the most 5’ exon, and this was identified either in continuity with exons 4, 5, 7, 8, and 9, or in alternatively spliced isoforms “missing” exon 4 or exons 4 and 5. The fourth novel exon, which we refer to as exon 6 was isolated between sequences corresponding to exons 2, 4, and 5 and exons 7, 8, and 9. Translation of these novel transcripts creates three potential novel proteins, which we have named BidL (accession number AF250233) for “short,” BidES (accession number AP250233) for “extra long,” and BidES for “extra short.” BidES is generated from a number of splice variants that share the common feature of loss of the first AUG together with the potential for translation from an internal AUG within exon 7 of the Bid gene. A summary of all the splice variants of bid isolated by PCR, RACE, and searching the GenBank™ data base is shown in Fig. 1A. For the sake of clarity, we will use “Bid,” to refer specifically to the previously described gene product of the Bid gene (8) and “Bid” where the reference is to a more general property of the gene and its products. Putative protein products of these splice variants are shown in Fig. 1A. BidKL is predicted to have an additional 45 amino acids at the N terminus that do not contain any recognized conserved domains. BidKL contains only the unopposed regulatory BH3-B domain, omitting the pro-apoptotic BH3 domain. There are an additional 63 amino acids at the C terminus of BidKL that are entirely novel and that contain no recognized conserved domains. BidES contains the final 99 amino acids of BidL, beginning with the terminal amino acids of the BH3 domain and including the sequence likely to be involved in mitochondrial targeting of Bid (27).

2 The abbreviations used are: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RACE, rapid amplification of cDNA ends; GFP, green fluorescent protein.
Novel Isoforms of Bid

**Differential Expression of Bid Isoforms—**Using specific primers, as described under “Experimental Procedures,” PCR was performed using a commercially available RNA panel (Premier RNA; Clontech) to screen a variety of tissues. Using primers in exons 2 and 9 of bid (BidF4, BidR2), a three-handed pattern was seen in all of the tissues studied (Fig. 2A). Sequencing of cDNA extracted from these bands confirmed that these correspond to cDNA for BidL and two transcripts for BidES (designated BidES (2) and BidES (3) in Fig. 1A). Using primers in exons 3 and 9 (BidF7 and BidR2), transcripts encoding BidEL were detected predominantly in tissues with large numbers of hemopoetic cells (e.g. spleen and bone marrow). BidL was also expressed at high levels in cerebral and cerebellar cortex, as might be expected given the importance of Bid in regulating neuronal cell death (28). Transcripts corresponding to cDNA for BidES (4) and BidES (5) were also detected. PCR using primers to exon 6 and 9 (BidF10 and BidR2) shows expression of cDNA for BidS in a similar distribution to cDNA for BidL. In addition, a range of transformed cell lines was screened for the presence of mRNA for Bid isoforms. mRNA for BidL, BidES (2), and BidES (3) were seen in Cos, HeLa, HL60, Jurkat, and 293T cells. mRNA for BidS was seen in all of the above cell lines, and mRNA for BidES was seen only in Jurkat cells (Fig. 2B). There is therefore evidence of endogenous mRNAs expressing all of the isoforms identified by RACE.

To demonstrate endogenous protein expression, we screened a panel of primary tissues by Western blotting. Immunoreactive bands of appropriate size were seen for BidL in spleen, placenta, and pancreas; for BidS in lung, pancreas, and spleen; and for BidES in lung and pancreas (Fig. 3A). Transformed cell lines with high levels of mRNA expression of various Bid isoforms were also screened for Bid protein expression. Both HeLa cells and 293T cells contain an immunoreactive band that migrates at the same size as in vitro translated BidL (Fig. 3B). Jurkat cells contain a low intensity protein band of ~27 kDa on Western blotting with an anti-Bid antibody that migrates at an identical size to BidES overexpressed in the 293T cell line (Fig. 3B) and to in vitro translated BidEL (data not shown). We were thus able to identify protein bands on Western blotting that correspond to either in vitro translated or transfected BidEL and BidS, confirming endogenous expression of these proteins.

Recently, a role for Bid in regulation of myeloid hemoestasis, including peripheral blood neutrophil numbers, has been demonstrated using Bid−/− mice (29). We therefore examined expression of Bid isoforms in two models of neutrophil maturation. NB4 cells are members of a myeloid leukemia cell line that, following treatment with retinoic acid, undergo biochemical and functional differentiation along the neutrophil lineage, such that by 24 h these cells share many characteristics with mature neutrophils (18, 30). With progressive maturation, NB4 cells expressed higher levels of BidES (4). BidS levels did not consistently change in these experiments. In addition, we studied BidL, BidEL, and BidS expression in myeloid precursors extracted from bone marrow by density gradient centrifugation (19, 31). Cells extracted in this way represent progressively more mature neutrophil precursors and are here compared with circulating neutrophils from the same donor. Mature neutrophils express more of both BidEL and BidS (Fig. 4), in part paralleling the data from NB4 cells. Thus, not only are transcripts for BidES and BidS expressed in a variety of primary

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**Fig. 1. Schematic representation of the Bid gene structure and protein isoforms.** A, the genomic organization of the Bid gene and mRNA species generated by alternative splicing are shown to scale. Introns are shown at 1/5 of the exon scale. The intron following exon 3 is 23,434 bp and has been abbreviated in this figure. Putative translation start (→) and stop (↑) codons are indicated, and the predicted protein products are named to the right of the illustration. None of the mRNA structures shown has been previously described, with the exception of BidL and BidES (1) (accession number AA358833). All splice variants are capable of encoding the putative 10-kDa protein BidES, by translation from an internal AUG in exon 5. The 5’ extent of all the mRNAs shown has not been unequivocally determined. B, potential protein species generated by the mRNA species shown in A. The location of the pro-apoptotic BH3 (black box) and inhibitory BH3B (gray box) domains are shown, along with the cleavage site for caspase-8 (vertical line). Novel protein sequence is shown as a shaded area. The number of amino acids and predicted molecular masses are indicated.
tissues, they are differentially regulated between tissue types and, during maturation of a single cell type, the neutrophil granulocyte.

Effects of Bid Isoforms on Cellular Apoptosis—Based on the predicted protein structure determined for the Bid isoforms (Fig. 1B), each of the three novel isoforms would be predicted to behave in different ways to modulate the activity of Bid following cleavage and activation. We hypothesized that BidS, containing only the inhibitory BH3B domain, could act as a naturally occurring inhibitor of tcBid-induced apoptosis. Bid EL is identical to BidL at the C terminus, contains the BH3 domain, and differs only in the N-terminal fragment. Bid EL might therefore induce apoptosis in a similar way to Bid L, but the additional N-terminal sequence might influence the subcellular localization of Bid EL or perhaps alter the cleavage and activation of Bid EL. Because Bid ES, which again lacks a BH3 domain, corresponds to the portion of Bid that localizes to the mitochondrial surface, we hypothesized that Bid ES might interrupt the action of cleaved Bid L at the mitochondria. To test these hypotheses we first assessed the potential of BidS, Bid EL, and Bid ES to modulate apoptosis in an overexpression assay.

Apoptotic morphology was assessed in A549 cells transfected with the following constructs: pCR3.1.empty vector, pCR3.1.Bid EL, pCR3.1.Bid S, or pCR3.1.Bid ES as described under “Experimental Procedures” (16). In keeping with our predictions, transfection with constructs expressing Bid L or Bid EL induced high levels of apoptotic morphology, whereas Bid S did not (Fig. 5A). Bid S did not protect against cell death caused by the transfection process. In contrast to the hypothesis based on predicted structure, Bid ES, which contains no functional BH3 homology domains, appeared to induce apoptosis, albeit to a lesser degree than Bid L and Bid EL.

We then tested the ability of the novel isoforms to interfere with Bid L signaling. To simulate the specific effects of Bid activation, the cells were transfected with a construct expressing tcBid L (pCR3.1.tcBid L). This mimics the effects of isolated Bid L cleavage, without direct activation of caspase pathways, as might occur with death receptor ligation. The ability of BidS, Bid ES, and Bid EL to inhibit tcBid L-induced cell death was tested in...
that the additional sequence present in BidS does not encode an additional functional localization motif. Cells expressing GFP-BidES showed co-localization of fluorescence with mitochondrial stains, in keeping with the predicted function of this region of the Bid molecule as a mitochondrial-targeting region.

Following transfection, GFP-BidKL localized to distinct regions within the cell (Fig. 6, c–f, middle panels). Further staining of organelles demonstrated co-localization with Golgi staining (BODIPY TR ceramide analogue; Fig. 6d) but not with nuclear structures (propidium iodide staining; Fig. 6e), mitochondrial structures (Mitotracker Red staining; Fig. 6e), or endoplasmic reticulum staining (rhodamine; Fig. 6f). This localization is confined to GFP fusion proteins to the N terminus of BidKL. When constructs expressing BidEL-GFP (i.e. a fusion of GFP to the C terminus of BidKL) were transfected, a significant part of the fluorescence is localized to the mitochondria (Fig. 6g), as is seen with GFP-BidES (Fig. 6h). In addition, Western blotting of lysates from transfected cells shows partial cleavage of GFP-BidKL but not GFP-BidL (Fig. 6). This suggested that BidKL is cleaved constitutively in transfected cells, with the N terminus localizing with the Golgi and the C terminus translocating to the mitochondria. This is supported by the observation that the broad spectrum caspase inhibitor, zVAD.fmk, is able to prevent both apoptosis and the localization of GFP-BidKL to Golgi, whereas an inhibitor of proteosomal degradation, M132, had no effect on the appearance of GFP-BidKL transfected cells.

The observed distribution of GFP-BidES was not solely due to the onset of apoptosis because apoptotic cells from transfections with the other GFP fusion vectors did not show the same pattern of distribution as was seen consistently with GFP-BidKL (data not shown). Transfection of these cells with pCR3.1BidKL and a vector expressing GFP alone (pEGFP-C1) does not have the same distribution (data not shown), suggesting that the observed subcellular distribution was not an artefact of GFP degradation in apoptotic cells. However, GFP constructs containing only the N-terminal cleavage fragment of BidKL (tBidKL) did not localize to the Golgi (Fig. 6i), which may indicate that further modification of BidKL during apoptosis is required for this observed localization.

Functional Interaction of Bid Isoforms with Other Bcl-2 Family Members—Bid was initially identified by its ability to interact with both pro- and anti-apoptotic Bcl-2 family members (8), and this interaction is thought to be dependent upon the BH3 domain and to be important in the function of Bcl-2 proteins. The ability of the Bid isoforms to interact with pro- and anti-apoptotic members of the Bcl-2 family was therefore assessed. It has been well documented that there is considerable potential for unphysiological heterodimerization of Bcl-2 family proteins in buffer systems (33–35), and moreover, because BidKL and BidS lack a BH3 domain, they would not be anticipated to heterodimerize with other Bcl-2 proteins. To establish whether the novel Bid isoforms would functionally interact with other pro- or anti-apoptotic Bcl-2 proteins, functional assays were devised. In these assays we assessed both the ability of Bid isoform-induced apoptosis to be inhibited by Bcl-2 and the ability of Bax induced apoptosis to be inhibited by anti-apoptotic Bid isoforms. Apoptosis induced by BidL, BidKL, but not BidES was inhibited by Bcl-2 overexpression (Fig. 7A). This is consistent with the predicted BH3-dependent (BidL and BidKL) and -independent (BidES) mechanisms of cell death. Bax-induced cell death was inhibited by Bcl-2 but not by BidL, and BidES was also without effect upon Bax-induced apoptosis (Fig. 7B).
DISCUSSION

Bcl-2 family proteins are important regulators of cellular apoptosis. As such, their activity is highly regulated. In addition to regulation at the level of transcription and translation, their activity is regulated by post-translational modification. For example, Bad is regulated by phosphorylation/dephosphorylation (36); Bid, Bcl-2, and Bcl-XL are regulated by caspase-mediated proteolysis (12, 13, 37–39), which in turn may be mediated by phosphorylation of Bid (40); Bax is cleaved by calpains (41), and tBid undergoes N-myristoylation (42). Bcl-2 proteins are also regulated by homodimerization and heterodimerization (43) and differential subcellular localization (44). In addition, many are known to be regulated by alternative splicing (15), which can regulate the apoptogenic potential (e.g. Bim (45)) or alter its function entirely (e.g. Bcl-xL and Bcl-xS (14)).

Bid acts to convert proteolytic signals from Granzyme B, caspases (predominantly caspase-8), and lysosomal proteases into apoptotic signals. Cleavage of a protease-sensitive domain of Bid yields an active fragment (tcBid) and an inhibitory fragment (tBid). We have identified three novel isoforms of Bid that may modulate the function of cleaved Bid. These proteins have a restricted and distinct tissue distribution and also differ in their intracellular localization. Importantly, they show distinct differences in their ability to modulate cellular apoptosis. These isoforms may therefore exist to provide an additional layer of control of Bid function.

BidEL exists in many cell types at mRNA level, as we have shown, and other groups have recently also deposited sequence corresponding to BidEL in the GenBank™ database (e.g. AK0947952 BC036364 (47)). Cells transfected with expression vectors for BidEL readily express protein, and an endogenous protein of identical size is seen in a number of primary tissue types and in Jurkat cell lysates. Overexpression of BidEL in A549 cells leads to apoptosis at levels indistinguishable from those produced by BidL overexpression. There is therefore good evidence to suggest that this protein species exists in vivo and that it may have functional relevance to the control of apoptosis.

The subcellular localization of BidEL is complicated by the cleavage of this protein. The N-terminal fragment localizes to the Golgi apparatus following onset of apoptosis, whereas the C-terminal fragment, as predicted, traffics to mitochondria. The findings could be an artifact of overexpression but are not seen with overexpression of other closely related isoforms of Bid or with the same protein when labeled with GFP at the C-terminus. The localization of the N-terminal fragment to the Golgi apparatus may remove the inhibitory effect of this portion of BidEL, potentially regulating the activity of BidEL following cleavage in vivo. In the co-transfection studies shown in

![Fig. 4. Modulation of Bid isoform expression during myeloid differentiation.](image)

NB4 cells

Subject 1

Subject 2

| Hours | BidL | BidEL | BidS | BidES (1) | BidES (2) | BidES (3) |
|-------|------|-------|------|-----------|-----------|-----------|
| 0     | 0    | 0     | 0    | 0         | 0         | 0         |
| 24    | 0    | 0     | 0    | 0         | 0         | 0         |
| 48    | 0    | 0     | 0    | 0         | 0         | 0         |
| 72    | 0    | 0     | 0    | 0         | 0         | 0         |

Levels of GAPDH remained constant, and those for BidL, BidES (2), and BidES (3) were consistent across repeated experiments. The figure shown is representative of three independent experiments. mRNA extracted from bone marrow myeloid precursors that had been separated into fractions of progressively increasing maturation (fractions 3, 2, and 1) was used as a template for reverse transcription-PCR as described under “Experimental Procedures.” Mature peripheral blood neutrophils were extracted from the same subjects and are indicated by N. Levels of GAPDH consistently decline during neutrophil maturation, but levels of BidL, BidES (1), and BidES (2) remain constant. BidS and BidEL increase dramatically with progressive maturation to the levels seen in peripheral blood neutrophils. The experiment shown using two independent subjects is representative of three separate experiments.
Fig. 5. Modulation of apoptosis by Bid isoforms. A, A549 cells were transfected with a PCR3.1 vector containing the constructs shown, a GFP reporter, and carrier DNA to a total of 5 μg. 24 h following transfection, the plates were examined, and the cells expressing the GFP reporter were assessed for apoptotic morphology. The results are shown as the means ± S.E. of four independent experiments performed in duplicate. The rates of apoptosis seen with Bid, BidEL, and BidLS are significantly larger than control or BidS (p < 0.01). BidES rates of apoptosis are significantly lower than those seen with either Bid or BidEL (p < 0.01). B, A549 cells were transfected as above with pCR3.1.tBid (50 ng), pEGFPc1 (1 μg), test vector (500 ng), and carrier DNA to a total of 5 μg. Compared with Bcl-2 and Bcl-xL, nBidL, BidS, and BidEL were less efficient inhibitors of apoptosis. In this series of experiments, inhibition of tBid by nBidL was not statistically significant. *, p < 0.05; **, p < 0.01 compared with tBid transfected cells alone. C. HepG2 cells were transfected with pCR3.1 empty vector control or pCR3.1.tBid (50 ng), with pEGFPc1 (1 μg) as a marker of transfection. After 24 h, CH-11 (an agonistic anti-Fas antibody) was added at a concentration of 500 ng/ml. A further 24 h later, transfected cells were assessed for apoptotic morphology, as above. BidLS was able to completely inhibit CH-11-induced apoptosis (p < 0.05).

Fig. 5B, rates of apoptosis in cells treated with tBidL and tBidEL were not significantly different from those seen with tBidL alone. These results may indicate that tBidEL differs from tBidL in its ability to act as an apoptosis inhibitor, perhaps as a consequence of its sequestration within the Golgi. However, BidEL is indistinguishable from BidL with regard to its pro-apoptotic effect, which is inhibited by Bcl-2.

In the case of BidEL, regulation of activity may be at the level of mRNA. We have not isolated RNAs containing both exon 3 and exon 2, which may suggest different promoter usage. If reliant on the same promoter, the differing 5′ sequence of mRNA species for BidL and BidEL may well alter relative stability or translation efficiency, leading to altered levels of protein present (48). In addition, the additional upstream reading frame seen in the mRNA for BidEL may function to regulate expression of BidL transcribed from this mRNA. It is recognized that transcripts with additional upstream reading frames (such as BidEL) may exist to tightly regulate the expression of translations beginning at the intended downstream AUG (49).

BidLS is expressed in a number of cell types at mRNA and protein level, although no expressed sequence tags are present in the GenBank™ data base that code for BidLS. In vitro translated BidLS migrates at the same size as endogenous immunoreactive bands seen in a variety of human tissues and in HeLa and 293T cells. In addition, cells transfected with expression vectors for BidLS express a protein band of appropriate size on Western blotting (data not shown). BidLS remains cytoplasmic when transfected as a GFP fusion protein, as does BidL. Transfection of A549 cells with GFP-BidLS does not induce apoptosis, as suggested by the predicted presence of a BH3B but not a BH3 domain. BidLS transfection is, however, able to inhibit apoptosis caused by truncated BidL and to prevent CH-11/Fas-induced cell death. BidLS does not, however, appear to directly interact with Bcl-2 or Bax in functional assays. Thus, BidLS has the potential to act as a naturally occurring inhibitor of tBidL-induced cell death and to modulate the response of a cell to a variety of death-inducing stimuli, including death receptor ligation. There is therefore evidence to suggest that BidLS exists in vivo and that it has functional relevance to the control of apoptosis.

The recent publication of data suggesting that Bid is essential for normal terminal differentiation of myeloid cells (29), together with the original derivation of BidLS and BidEL from human neutrophils, suggested to us that expression of Bid isoforms may be of interest in neutrophil precursors. Using parallel strategies we identified increases in levels of mRNA for BidEL and BidLS during myeloid differentiation. No changes in the levels of BidLS were seen in these experiments. This suggests that BidLS and BidEL may be involved differentially in the regulation of Bid-induced death in developing myeloid cells. In particular, they may influence the sensitivity of these cells to death receptor-induced apoptosis. Neutrophils contain high levels of lysosomal proteases, and BidLS may be present to prevent inadvertent cell death following proteolytic cleavage of Bid (50). The parallel increases in BidLS and BidEL, which initially appear conflicting in effect, may suggest a degree of subtlety in the regulation of Bid-induced apoptosis that is not revealed in these in vitro experiments.

A 10-kDa protein, BidES, is potentially generated from all of the Bid transcripts identified and corresponds to the C-terminal fragment of Bid, downstream of the BH3 domain. This protein would be the only product derived from a number of alternatively spliced mRNA species whose expression is detected during PCR analysis of the Bid gene (indicated in Fig. 1A), and this may explain the existence of these mRNAs, whose purpose is otherwise unknown. A protein band of appropriate size was identified on Western blotting, although this has not been completely characterized. The apparent actions of BidES as both a pro-apoptotic agent and as an inhibitor of tBid-
induced apoptosis are of interest. tBid has previously been shown to target mitochondria in a BH3-independent manner (27) and does not rely on the BH3 domain for trimerization and cytochrome c release (51). The same mechanisms that underly these processes could also account for the action of BidES in inducing apoptosis. The absence of a BH3 domain in BidES presumably accounts for its lack of direct interaction with either Bcl-2 or Bax in functional assays. The effects of BidES on

![Subcellular localization of GFP-Bid isoform fusion constructs](image)
apoptosis must therefore relate to effects other than the ability to interact with other BH3 domain-containing proteins. Targeting of tBid to mitochondria requires binding to cardiolipin (27) or related sites on the mitochondrial surface (46, 52). The effect of BidE52 in inhibiting tBid-induced apoptosis may relate to its ability to inhibit binding of tBid to the mitochondria. Thus, BidE52 might act in different ways to modulate apoptosis depending on the relative levels of pro-apoptotic and anti-apoptotic Bid moieties in the cell. In this way BidE52 could provide flexible and dynamic regulation of Bid-mediated apoptosis.

The important role of BH3 proteins in initiating apoptosis in response to a varied range of stimuli is becoming clear. The existence of a family of Bid proteins suggests that the fate of a cell following Bid cleavage may not be as simple as previously thought and may be influenced by the relative expression of the different Bid isoforms. The novel isoforms described here are endogenously expressed and perform distinct functions, as evidenced by differences in expression, cellular localization, and functional effects upon cellular apoptosis. This suggests that they have an important place in the complex network of pathways used by cells to determine their fate following a pro-apoptotic stimulus.

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