Degradation of Mcl-1 by Granzyme B

IMPLICATIONS FOR Bim-MEDIATED MITOCHONDRIAL APOPTOTIC EVENTS*

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Recent studies have suggested that in the absence of Bid, granzyme B (GrB) can utilize an unknown alternative pathway to mediate mitochondrial apoptotic events. The current study has elucidated just such a pathway for GrB-mediated mitochondrial apoptotic alterations. Two Bcl-2 family members have been identified as interactive players in this newly discovered mitochondrial response to GrB: the pro-survival protein Mcl-1L and the pro-apoptotic protein, Bim. Expression of Mcl-1L, which localizes mainly to the outer mitochondrial membrane, decreases significantly in cells subjected to CTL-free cytotoxicity mediated by a combination of GrB and replication-deficient adenovirus. The data suggest that Mcl-1L is a substrate for GrB and for caspase-3, but the two enzymes appear to target different cleavage sites. The cleavage pattern of endogenous Mcl-1L resembles that of in vitro translated Mcl-1L subjected to similar proteolytic activity. Co-immunoprecipitation experiments performed with endogenous as well as with in vitro translated proteins suggest that Mcl-1L is a high affinity binding partner of the three isoforms of Bim (extra-long, long, and short). Bim, a BH3-only protein, is capable of mediating the release of mitochondrial cytochrome c, and this activity is inhibited by the presence of exogenous Mcl-1L. The findings presented herein imply that Mcl-1L degradation by either GrB or caspase-3 interferes with Bim sequestration by Mcl-1L.

Granzyme B (GrB),¹ the prototype member of the granzyme family of serine proteases, shares substrate specificity with caspases for cleaving after aspartate residues (1). GrB has been reported to cleave caspasess, including caspase-3, -6, -7, -8, and -10 in vitro. It has been assumed that GrB has multiple entry points for initiating the caspase-dependent apoptotic cascade. However, GrB also activates cell death and apoptotic morphology in the presence of short peptide fluoromethyl ketones, which are potent caspase inhibitors, but do not inactivate GrB (2). These observations led to the notion that GrB may be capable of initiating an alternate death pathway in the presence of viral or cellular inhibitors of caspases. This concept is supported by the identification of caspase substrates that are also processed by GrB, including the sensor for initiation of DNA damage repair, poly-(ADP)ribose polymerase (3); the nuclear mitotic apparatus protein (4); inhibitor of caspase-activated DNase to liberate the caspase-activated DNase from its complex with the inhibitor (5); T cell receptor-γ chain, which is essential for T cell signaling (6); the catalytic subunit of DNA-dependent protein kinase, which is involved in repairing double-stranded DNA breaks (4); and the nuclear-envelope intermediate-filament protein, lamin B (7).

A potential role for mitochondria in the response to GrB has been indicated by the protection conferred by overexpression of Bcl-2, a member of a family of apoptotic regulators that resides mainly on the cytoplasmic side of intracellular membranes (8). Recent studies have identified Bid as a direct substrate for GrB, and thereby as a direct link to a mitochondrial apoptotic cascade mediated by GrB (9–11). Bid, a cytosolic BH3-only Bcl-2 family member, is cleaved by caspase-8, lysosomal proteases, or GrB. Although cleaved at different sites, each of the resultant truncated Bids translocates to the mitochondrial outer membrane, where it triggers the release of the pro-apoptotic proteins cytochrome c, SMAC/DIABLO, HtrA2/Omi, endonuclease G, and AIF (12). Although in a cell-free system GrB-cleaved Bid is a potent inducer for the release of mitochondrial apoptotic proteins, a recent study questions whether cleavage of Bid by GrB occurs directly and independently of caspase activation under physiologic conditions (13, 14). Despite this controversy regarding the direct role of GrB-cleaved Bid, the need for the mitochondrial amplification of the caspase pathway in GrB-mediated apoptosis is well established, particularly in tumor or viral infected target cells with increased expression of cellular or viral inhibitors of apoptosis (1, 2, 8). Cellular inhibitors of apoptosis (IAP), such as XIAP or cIAP1/2 that are overexpressed in numerous types of tumors (15) directly block caspase-3, -7, and -9 (16). Consequently, mitochondrial secreted antagonists of IAP, SMAC/DIABLO and HtrA2/Omi are required to relieve the inhibited caspases (17–19).

The mitochondrial function of tBid is dependent on the expression of either Bax and/or Bak (20) and can be inhibited by overexpression of the anti-apoptotic proteins Bcl-2 and Bcl-XL (21). Although a role for GrB-cleaved Bid in the physiologic...

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¹ The abbreviations used are: GrB, granzyme B; IAP, inhibitor of apoptosis; XIAP, x-linked inhibitor of apoptosis; Mcl-1L, mitochondrial buffer; Ac-IETD-CHO, acetyl-Ile-Glu-Thr-Asp-aldheyde; VDAC, voltage dependent anion-selective channel.
induction of a mitochondrial apoptotic cascade is controversial, it has been demonstrated that a deficiency in both Bax and Bak attenuates the mitochondrial response to GrB (13, 22). Furthermore, embryonic fibroblasts (MEFs) from Bid−/− mice or Bax/Bak double knockout mice still have disrupted mitochondrial transmembrane potential in response to GrB (23). These findings imply that one or more cytosolic mediators other than Bid may act as a link between GrB and the mitochondria. Alternatively, GrB may act directly on mitochondrial outer membrane proteins providing an explanation for the ability of GrB to disrupt the mitochondrial transmembrane potential in a caspase- and Bid-independent manner (10, 13).

Mitochondrial response to apoptotic stimuli is determined by the balance between pro- and anti-apoptotic Bcl-2 family members. Anti-apoptotic Bcl-2 family members, including Bcl-2, Bcl-XL, and Mcl-1L (myeloid cell leukemia-1) protect against mitochondrial apoptotic events, whereas pro-apoptotic Bcl-2 family members, including Bax, Bak, and BH3-only proteins, promote the release of apoptogenic proteins from the mitochondria. Mcl-1 is an anti-apoptotic Bcl-2 family protein that was discovered as an early induction gene during myeloblastic leukemia cell differentiation (24). The biological significance of Mcl-1 has been elucidated by recent studies demonstrating that a Mcl-1 deficiency results in peri-implantation embryonic lethality (25). Increased expression of endogenous full-length Mcl-1 is associated with the maintenance of cell viability and decreased expression with cell death (24, 26, 27). A short splice variant of Mcl-1, Mcl-1S, has recently been identified as a BH3 domain only pro-apoptotic protein (28, 29). Despite numerous studies on the induction of Mcl-1, scarce information is available regarding the mechanisms involved in its down-regulation. Based on the effects of proteasome inhibitors, two recent studies proposed that Mcl-1L is degraded by proteasomes in response to UV or actinomycin D, in HeLa or multiple myeloma cell lines, respectively (30, 31).

Our studies, described below, are the first to report that Mcl-1L is a direct substrate for caspase-3 and GrB. We have also identified Bim, a BH3-only protein, as a high affinity binding partner for Mcl-1. The Mcl-1L/Bim cooperation may constitute an alternative mitochondrial apoptotic pathway that could be activated by the direct effect of GrB on the mitochondria, independent of Bid.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Anti-human Mcl-1 Abs were from Oncogene (Boston, MA; Ab-2, mouse clone RC13 generated against recombinant Mcl-1L), and from Santa Cruz Biotechnology (Santa Cruz, CA; Ab-1, mAb generated against recombinant Mcl-1L; and Ab-3, polyclonal Ab raised against recombinant Mcl-1L). Anti-Bim Ab was a generous gift from Dr. Xiaodong Wang (Southwestern Medical Center, Dallas, TX). Anti-caspase-3, 20-amino acid residue synthetic peptide of human Mcl-1L. Anti-mouse Mcl-1 Abs were from Santa Cruz Biotechnology; Z-VAD-FMK, clone 14A8); anti-SMAC mAb from Apoptech (clone 10G7); anti-Bim Ab were from ProSci (Poway, CA) and from Apoptech (San Diego, CA). Anti-caspase-3, 1779–1799 internal epitope (mAb) was generated against a rat Mcl-1 clone 10G7-UTR (the UTR of the Mcl-1L mRNA sequence and SuperScript III RNase H reverse transcriptase (Invitrogen). PCR was performed using the GC-Rich PCR System kit (Roche Applied Science). Amplions containing the entire open reading frame (ORF) of Mcl-1L were generated using the forward primer Mcl-F, 5′-CTGGCAATTTGTGCGCTCAA-3′, which corresponds to nt 888–888 and then extends by 6 nt into the 5′-UTR and the reverse primer Mcl-R (see above). The Mcl-1L amplion was size-selected and purified using a 1% agarose gel and the QIAquick gel extraction kit (Qiagen). Amplions were subcloned into the vector pCR3.1 by utilizing the Eukaryotic TA Expression kit (Invitrogen). Mcl-1L clones were confirmed by automated DNA sequence analysis (University of Pittsburgh DNA Sequencing Core Facility) of randomly picked colonies.

**Generation of Mcl-1S**—We produced a Mcl-1S splice variant cDNA clone based on the published sequence (GenBank™ accession number AF203373) utilizing deletion mutagenesis of Mcl-1L cDNA by overlap extension using PCR. To generate the deletion site, two primers were designed as follows: the forward primer Mcl-10, 5′-GGCCCTCGACCGATGGGTTTTGAGTTCTTCC-3′, which corresponds to nt 1340–1352 and 1601–1612, and the reverse primer Mcl-11, 5′-CCACACACCCATCTTTGGAGAGCGCTCCTGTTGG-3′, which is complementary to nt 1612–1612 and 1352–1331. These primers overlap the sequence region (nt 1353–1600) that is deleted from Mcl-1L to generate the deletion variant. PCR was performed out with Mcl-1L DNA and primer pairs Mcl-F (see above) and Mcl-11 and Mcl-R (see above) and Mcl-10, respectively, using the Expand Long Template PCR system (Roche Applied Science). The deletion mutant amplions (0.5 μl of each) were combined in a subsequent PCR reaction using primers Mcl-F and Mcl-R to produce the putative Mcl-1S amplion. This amplion was then gel-purified and subcloned as above for Mcl-1L. Sequence analysis (as above) of randomly picked clones confirmed the Mcl-1S sequence.

**Molecular Cloning of Human BimEL**—Total RNA was isolated from Jurkat T cells as described for Mcl-1L. First strand cDNA synthesis was carried out using SuperScript II RNase H reverse transcriptase and oligo(dT)12–18 primer (Invitrogen). PCR was performed with the Expand Long Template PCR System kit (Roche Applied Science) utilizing the forward and reverse primer pair specific for the amino acid and carboxyl-terminal ends, respectively, of BimEL, BimL, and BimS ORFs. The sequence of the forward Bim primers is 5′-GCCACCATGCGAAGCAACCTTCTGAT-3′, whereas the reverse primer is 5′-TCAATGCAT- TCTCCACCAAG-3′. Reaction products were separated on a 1% agarose gel, and a band corresponding in size (~600 bp) to the BimEL ORF was excised and DNA extracted as described for Mcl-1L. The putative BimEL amplion was subcloned into the vector pCR3.1 as above. Random clones were sequenced also as above to confirm the presence of the BimEL ORF.

**Preparation of His-tagged BimL**—Mouse amino-terminal histidine-terminating BimL was expressed from a recombinant plasmid produced by ligating an NdeI-XhoI-digested BimL amplicon produced with the Expand Long Template PCR system kit (Roche Applied Science) utilizing mouse BimL cDNA and the primers BimL-F, 5′-GGAATTCATAGTGCCACGCAACCTTCT-3′, and BimL-R, 5′-CGCGCTGACTGTTCCACCAAG-3′, into the NdeI-XhoI-digested bacterial expression vector pET-14b (Novagen). The resulting plasmid was transformed and cultured at 37 °C in Terrific Broth. The induction of expression was started at an A600 of 0.8–1.0 by the addition of 0.4 mM isopropyl β-d-thiogalactoside with continued incubation of the culture at 37 °C for 2–3 h. The bacterial pellets were resuspended and sonicated in a buffer containing 5 mM imidazole, 500 mM NaCl, and 20 mM sodium phosphate (pH 7.9). After centrifugation, the cleared supernatant was passed through a His-Bind nickel agarose affinity chromatographic column pre-charged with 50 mM NiSO4 (Novagen). The columns were washed with wash buffer containing 60 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9. The His-tagged Bim was eluted with elution
buffer containing 400 mM imidazole, 500 mM NaCl, and 20 mM Tris-Cl, pH 7.9, and were further purified using a Sephacryl G-50 column equilibrated with phosphate-buffered saline. A single major band was detected by SDS-PAGE stained with Coomassie Blue.

GrB-induced Apoptosis—CTL-free apoptosis was induced by incubation of target cells with GrB (33–330 nM) and replication-deficient adenovirus type V (Ad; 10–100 μM) for 6 h. The cells were then washed to remove excess exogenous GrB. To avoid the enzymatic activity of GrB during the lysis procedure, the GrB inhibitor, Ac-IETD-CHO (500 μM) was added to the lysis buffer.

Cellular Fractionation and Mitochondria Purification—To obtain an enriched mitochondrial fraction, Jurkat or HeLa cells were suspended in mitochondrial buffer (MIB) composed of 0.3 M sucrose, 10 mM MOPS, 1 mM KCl, 1 mM EDTA, and 4 mM KH2PO4, pH 7.4, and lysed by Dounce homogenization as previously described (32, 33). Briefly, nuclei and debris were removed by a 10-min centrifugation at 650 x g, and a pellet containing mitochondria was obtained by two successive spins at 10,000 x g for 12 min. To obtain the S-100 fraction, the postnuclear supernatant was further centrifuged at 100,000 x g for 1 h at 4°C. To obtain the enriched mitochondrial fraction, the mitochondria containing pellet was resuspended in MIB and layered on a Percoll gradient consisting of four layers of 10, 18, 30, and 70% Percoll in MIB. After centrifugation for 30 min at 15,000 x g, the mitochondrial fraction was collected at the 30/70 interface. Mitochondria were diluted in MIB containing 1 mg/ml bovine serum albumin (at least a 10-fold dilution required to remove Percoll). The mitochondrial pellet was obtained by a 40-min spin at 20,000 x g and used immediately. Purity was assessed by electron microscopy and by enzyme marker analysis (33). For enzyme analysis, the following enzymes were assayed: aryl sulfatase (lysosomes/granules); N-acetyl-β-D-glucosaminidase, α-, β-fucosidase, and β-glucuronidase (lysosome); lactate dehydrogenase (cytosol); cytochrome oxidase or monoamine oxidase (mitochondria); thiamine pyrophosphatase (Golg1); NDH oxidase (endoplasmic reticulum); and dipeptidyl peptidase IV (plasma membrane). The purity was assessed at 95%, with ~5% or less contamination from the microosomal fraction. When indicated, mitochondria were pelleted then incubated in 0.1 M Na2CO3, pH 11.5, for 20 min on ice to remove loosely attached proteins (33). Alkali treatment was not associated with the release of mitochondrial intermembrane proteins, such as SMAC or AIF. Following alkali treatment, supernatants and mitoplasts were separated by centrifugation and boiled in SDS sample buffer. The fractions were analyzed by immunoblotting. Release of Mitochondrial Apoptogenic Proteins—Purified mitochondria (50 μg of protein) were incubated with His-BimL at various doses as indicated, in 25 μl of MIB at 37°C for 30 min. Mitochondria were pelleted by centrifugation at 10,000 x g for 10 min. The resulting supernatants or mitochondria were mixed with SDS sample buffer and analyzed by SDS-PAGE and immunoblotting for the presence of mitochondrial apoptogenic proteins.

In Vitro Transcription-Translation—Mcl-1L, Mcl-1S, and BirNCLiDNAs were expressed in the TNT T7 transcription-translation reticulocyte lysate system (Promega). Each coupled transcription-translation reaction contained 1 μg of plasmid DNA in a final volume of 50 μl in a methionine-free reticulocyte lysate reaction mixture supplemented with [35S]-labeled methionine according to the manufacturer’s instructions. After incubation at 30°C for 90 min, the reaction products were immediately used or stored at −70°C.

In Vitro Cleavage Reaction with Caspase-3 or GrB—In vitro cleavage reactions were performed in total volume of 20 μl. The reaction buffer consisted of 20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Each reaction also contained 3 μl of reticulocyte lysate containing [35S]-labeled Mcl-1L, Mcl-1S, or BirNCL and reticulocyte lysate minus plasmid in the presence or the absence of recombinant caspase-3 (5–100 nM) or GrB (33–330 nM) for 20 min at 37°C. The reactions were terminated by addition of SDS loading buffer and boiled for 5 min.

Immunoprecipitation—For Mcl-1 and Bir immunoprecipitation experiments cells (5–10 × 106) and mitochondria (200 μg of protein) were lysed in 1% CHAPS buffer (20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF, and 1% CHAPS). The lysates were precleared with Protein A- or G-Sepharose beads and incubated with anti-Mcl-1 or anti-Bir Abs at 4°C for 4 h. The immune complexes were then precipitated with Protein A- or G-Sepharose beads at 4°C overnight. The pellets were washed four times with the appropriate lysis buffer and boiled for 5 min in SDS sample buffer.

Western Blot Analysis—Proteins in cell lysates, cell extracts, mito-

FIG. 1. Bid-dependent and Bid-independent release of mitochondrial apoptogenic proteins. A, release of mitochondrial intermembrane proteins in response to GrB. Purified mitochondria from Jurkat cells were treated with a dose-range of GrB (33–330 nM) for 40 min at 37°C. The proteins in the mitochondrial supernatants (Mit-Sup) or control pellets (Mit) were resolved by SDS-PAGE and detected by immunoblotting. Expression of Cox IV serves as a loading control. B, purified mitochondria were treated with GrB (330 nM) for 40 min at 37°C. The mitochondrial pellet and supernatant proteins were resolved by SDS-PAGE and immunoblotted for the detection of cytochrome c and Cox IV. C, GrB-mediated degradation of full-length Bid attached to the mitochondria. Purified mitochondria obtained from Jurkat cells were treated with a GrB dose range of 33–99 nM for 40 min at 37°C. The proteins were resolved by SDS-PAGE and detected by immunoblotting. Expression of Cox IV serves as a loading control. D, GrB-mediated release of cytochrome c from purified liver mitochondria of wild-type and Bid−/− mice. Purified mitochondria were treated with GrB (330 nM) for 40 min at 37°C. The mitochondrial pellet and supernatant proteins were resolved by SDS-PAGE and immunoblotted for the presence of cytochrome c and Cox IV.

RESULTS

Bid-dependent and Bid-independent Mechanisms of Mitochondrial Release of Apoptogenic Proteins in Response to Direct Application of GrB—GrB has been reported to have a direct effect on mitochondria that results in the release of cytochrome c (10). Such GrB dose-dependent release of apoptogenic proteins, including, cytochrome c, SMAC, and AIF (Fig. 1A) may be mediated by full-length Bid that in addition to its cytoplasmic localization, is also associated with purified mitochondria. However, full-length Bid is loosely attached to purified mitochondria, because it is entirely removed by treatment with alkali, and therefore it is probably not anchored to the mitochondrial outer membrane (Fig. 1B). Exposure of purified mitochondria to GrB results in the processing of mitochondria-associated Bid as indicated by its reduced level of detection (Fig. 1C). To investigate the significance of mitochondria-attached full-length Bid in the response to direct application of GrB, we utilized liver mitochondria purified from Bid−/− mice. Direct application of various doses of GrB (33–330 nM) to murine wild-type liver mitochondria resulted in a significant release of cytochrome c (Fig. 1D). We confirmed that some full-length Bid was associated with mito-

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...in response to GrB/Ad, although an increased dose of GrB...inhibited. WT Jurkat cells were treated with Z-VAD-Fmk (100 μM) to inhibit potential activity of excess GrB during the lysis procedure (13). To differentiate between direct GrB activity in situ and activity of caspases stimulated by GrB, wild-type Jurkat cells were treated with GrB/Ad in the presence of a potent caspase inhibitor, Z-VAD-Fmk (100 μM), which does not inhibit GrB. In the presence of this inhibitor, the processing of caspase-3 p20 to the p19 and p17 subunits as well as its enzymatic activity were blocked (Fig. 2B). However, the presence of Z-VAD-Fmk did not completely arrest the GrB-mediated loss of Mcl-1. These results suggest that Mcl-1 is a substrate for both caspase and GrB activities. Thus, in cells treated with GrB/Ad, Mcl-1 appears to be degraded directly by GrB and also indirectly by GrB-activated caspases. Furthermore, this GrB activity is not significantly inhibited by either a deficiency in Bax and Bak, or overexpression of the anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-XL.

Subcellular Localization of Mcl-1L—Currently, two splice isoforms of Mcl-1 have been identified: Mcl-1L, an anti-apoptotic Bcl-2 family member (39), and Mcl-1S, a pro-apoptotic protein (24, 28, 29). Mcl-1L, contains BH1, BH2, and BH3 domains and a C-terminal transmembrane domain (39). Mcl-1S is a BH3-only protein devoid of the C-terminal transmembrane domain (28). To determine the subcellular localization of Mcl-1 proteins, we performed Western blot analyses on whole cell extracts, enriched mitochondrial fraction, or S-100 obtained from wild-type Jurkat or HeLa cells utilizing two Mcl-1-specific clones of mouse Ab (Ab-1 and Ab-2) and one source of polyclonal Ab (Ab-3). In whole cell extract, we detected two cross-reactive, faint bands (27–29 kDa) in addition to the expected 37-kDa protein band of Mcl-1L (Figs. 2B and 3A). These cross-reactive proteins appear to be close in size to that reported for Mcl-1S (29 kDa). To compare the SDS-PAGE migration of the endogenous protein bands detected by the anti-Mcl-1 Abs with that of Mcl-1L- and Mcl-1S-encoding plasmids in an in vitro reticulocyte lysate coupled transcription-translation system to obtain Mcl-1L and Mcl-1S translation products. To determine whether any of the cross-reactive protein bands corresponds to Mcl-1S, we ran them on SDS-PAGE, side by side with in vitro translated Mcl-1L and Mcl-1S. The three anti-Mcl-1 Abs utilized detected the in vitro translated products of both Mcl-1L and Mcl-1S (Fig. 3A, lanes 4–6). In Jurkat cellular extract, Ab-1 detected only Mcl-1L, whereas both Ab-2 and Ab-3 detected also a very faint protein band that corresponds in its SDS-PAGE migration to Mcl-1S (Fig. 3A, lane 1). Additional cross-reactive protein bands with a slightly faster migration than that of Mcl-1S were detected by Ab-2 and Ab-3. To determine the sub-cellular localization of endogenous Mcl-1 proteins, lysates of mitochondria or S-100 fractions were included in the assessment for co-migration with the in vitro translated Mcl-1L and Mcl-1S (Fig. 3A, lanes 2 and 3). The results obtained with the three anti-Mcl-1 Abs suggest that Mcl-1L is associated mainly with the mitochondria (Fig. 3A). In the experiment shown in Fig. 3A, the mitochondria and S-100

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fractions were loaded on the SDS-PAGE in cell equivalents, i.e. each lane corresponds to the same number of cells. Thus, the detection level of β-actin in the S-100 fraction was similar to that of the cellular extract (lanes 1 and 3), and the Cox IV detection level in the mitochondrial fraction was comparable to the one detected in the cellular extract (lanes 1 and 2). Therefore, the low detection of Mcl-1L in the S-100 fraction is a true representation of the in situ situation. However, Mcl-1L is also detected in the cytosol (mainly by the polyclonal Ab-3) probably due to its partial membrane attachment (Fig. 3B). In contrast, Cox IV, a mitochondrial matrix protein, or VDAC, an integral outer membrane protein that resides on the contact sites between outer and internal membranes, which were not detected in the alkali supernatant, Mcl-1 was only partly alkali-resistant. Nonetheless, relative to the mitochondria, there is only a low level of Mcl-1L in the cytosol. Through reverse transcription-PCR and immunoblot analyses performed with HeLa or Jurkat cells, we confirmed that the expression of Mcl-1S is significantly reduced relative to that of Mcl-1L, and therefore it was not consistently detected in cell lysates or S-100 by Abs capable of detecting in vitro translated Mcl-1S. The cross-reactive protein bands detected by Ab-2 in the S-100 fraction and by Ab-3 in the cell extract migrate faster than Mcl-1S and currently remain unidentified.

Direct Degradation of Mitochondrial Mcl-1L by GrB—To investigate whether GrB has a direct effect on Mcl-1L, purified mitochondria from either Jurkat or HeLa tumor cell lines were treated with various doses of purified GrB (Fig. 4, A–C). A dose-dependent loss in the level of Mcl-1L was detected by three Mcl-1-specific Abs. No change was detected in the expression levels of Bcl-XL, Bcl-2 or the three isoforms of Bim. Similar GrB-mediated Mcl-1L loss was detected in HeLa cells (Fig. 4B) and in breast carcinoma cell lines (data not shown). Utilizing a polyclonal anti-Mcl-1 Ab (Ab-3), a p26 Mcl-1 cleavage product was detected in GrB-treated mitochondria (Fig. 4A). To ascertain that the observed loss in Mcl-1L expression was mediated by GrB rather than by GrB-stimulated caspases, the experiment was repeated in the presence of Z-VAD-Fmk (Fig. 4C). This inhibitor did not have any effect suggesting that the reduction in the detection level of mitochondrial Mcl-1L was mediated by GrB directly or by non-caspase enzymatic activity associated with the mitochondria and stimulated by GrB. GrB also reduced the detection level of Mcl-1L in the S-100 fraction (Fig. 4D). These results suggest that endogenous Mcl-1L present in the mitochondrial or cytosolic fractions is a substrate for GrB activity.

Degradation of Endogenous Mcl-1 by Recombinant Caspase-3—Exposure of Jurkat cells to GrB/Ad in the presence of Z-VAD-Fmk (Fig. 2) indicated that the loss in Mcl-1 expression was mediated by both GrB and caspase activity. To confirm this observation, Jurkat cell extracts were treated with recombinant caspase-3 (Fig. 5). Endogenous Mcl-1L and Mcl-1S were sensitive to caspase-3 activity as their expression was reduced at a dose of caspase-3 as low as 10 nM, whereas at a dose range of 50–100 nM neither a loss in the level of pro-caspase-8 was detected (not shown), nor was there a significant level of processing of XIAP (Fig. 5A). However, XIAP and pro-caspase-8 are susceptible to a higher dose range (100–1000 nM) of recombinant caspase-3 (Fig. 5B).

Susceptibility of in Vitro Translated Mcl-1L and Mcl-1S to Caspase-3 and GrB—To further confirm that Mcl-1L and Mcl-1S are substrates for caspase-3 and GrB, we examined the susceptibility of in vitro translated Mcl-1L and Mcl-1S to these enzymes. Exposure of the in vitro translated Mcl-1L (Fig. 6A) or Mcl-1S proteins (Fig. 6B) to either recombinant caspase-3 or GrB resulted in a significant loss of the 37- and 29-kDa protein bands, respectively. The identity of the in vitro translation products as Mcl-1L and Mcl-1S was confirmed by immunoblotting of the same membrane with anti-Mcl-1 mAb (Ab-2). Both autoradiography and immunoblot analyses suggested that the patterns of cleavage by GrB are different from that obtained with caspase-3 for both in vitro translated Mcl-1L and Mcl-1S. To compare the cleavage patterns of in vitro translated Mcl-1L with that of endogenous Mcl-1L, caspase-3, or GrB reaction products obtained from in vitro translated Mcl-1L, Mcl-1S, cell extract, and S-100 were run side by side on SDS-PAGE and examined by autoradiography and immunoblotting (Fig. 7). Immunoblotting with Mcl-1-specific Ab (Ab-3) revealed a similarity between cleavage products obtained from in vitro trans-
lated Mcl-1L, cell extract, or S-100 treated with caspase-3 (lanes 4, 10, and 13). Likewise, treatment with GrB also generated the same size products from in vitro translated Mcl-1L, cell extract, and S-100 fractions (lanes 5, 11, and 14). Endoge-

nous Mcl-1S cleavage products similar to those produced by protease digestion of in vitro translated Mcl-1S were not detected in the cellular fractions, potentially due to its low level of expression in situ. Although the Mcl-1 cleavage patterns mediated by GrB and caspase-3 appear to be different, the exact aspartic acid residues targeted by these enzymes remain to be identified.

Co-immunoprecipitation of Mcl-1 with Bim—Because Mcl-1L is an anti-apoptotic Bcl-2 family member, we reasoned that the biological significance of its degradation might be manifested by the release of a pro-apoptotic Bcl-2 family member from a physical interaction with Mcl-1L. To this end, we screened the immunoprecipitated pellet of endogenous Mcl-1 for the presence of BH3-only proteins. Immunoblot analysis of immunoprecipitated Mcl-1 detected the co-presence of three Bim isoforms (Extra-Long (EL), Long (L), and Short (S)) (Fig. 8, A and B) but no Bid or Bad (data not shown). The binding of Bim to Mcl-1L was specific as the immunoprecipitation of Mcl-1L co-immunoprecipitated Bim (Fig. 8A), and vice versa, the immunoprecipi-
tation of Bim co-immunoprecipitated Mcl-1L (Fig. 8B). Immunoprecipitation studies were performed with cell extract, lysate of purified mitochondria, or S-100 fractions. Because the anti-Mcl-1 Ab utilized for the immunoprecipitation and immunoblotting (Ab-2) can detect in the cell extract both a cross-reactive protein and Mcl-1S, the identity of the protein that migrates faster than Mcl-1L, remains unclear and designated as cross-reactive. Mcl-1L was efficiently detected by Ab-2 in the cell lysate and in mitochondria, and was also effectively immunoprecipitated from these cellular fractions (note depletion of Mcl-1L in the remaining supernatant). Because Ab-2 does not detect a significant level of Mcl-1L in the S-100 fraction, no immunoprecipitated Mcl-1L was detected (Fig. 8A, left panel). BimEL, -L, and -S were effectively co-immunoprecipitated with Mcl-1L (Fig. 8A, right panel). Interestingly, a reduced level of Bim was also co-immunoprecipitated with the Mcl-1 cross-reactive protein present in the S-100 fraction (Fig. 8A, right panel, bottom). Immunoprecipita-
tion of Bim from the cell extract or purified mitochondria, effectively co-immunoprecipitated Mcl-1L (Fig. 8B, right panel). However, immunoprecipitation of Bim from the S-100 fraction was not accompanied by precipitation of Mcl-1L.
rather, the cross-reactive protein was co-immunoprecipitated. Because the cross-reactive protein is detected by Mcl-1-specific mAb (Ab-2) and is also capable of binding Bim, it may represent a Mcl-1 isoform not yet identified. Bim has been reported to bind to anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-XL, in cells transfected with these proteins or in cells stimulated to undergo apoptosis (21, 40). However, immunoprecipitation of naturally expressed, endogenous Bim from non-apoptotic cells, was associated with Mcl-1L, but not with Bcl-2, Bcl-XL, or Bcl-w (data not shown), further indicating the selectivity and high affinity of Bim for Mcl-1L.

To further investigate the specificity and affinity of binding between Mcl-1 and Bim, we examined whether the corresponding in vitro translated products will be co-immunoprecipitated with each other. To this end, $^{35}$S-labeled in vitro translated BimEL was mixed with $^{35}$S-labeled in vitro translated Mcl-1L or Mcl-1S. Without prior co-incubation, the mixture was placed on ice and subjected to immunoprecipitation by Mcl-1-specific Ab (Fig. 9A) or Bim-specific Ab (Fig. 9B). As assessed by autoradiography and immunoblotting performed with the same membranes, BimEL was co-immunoprecipitated with either Mcl-1L or Mcl-1S. However, Mcl-1L or Mcl-1S were each associated with immunoprecipitated BimEL. In contrast to Mcl-1L and Mcl-1S, BimEL was not cleaved by a 20-min exposure to either GrB or caspase-3 (data not shown). However, the activation status of Bim might be indirectly affected by the proteolytic activity of GrB or caspase-3 on Mcl-1, because the degradation of Mcl-1L may interfere with its ability to sequester or otherwise repress the apoptotic activity of Bim.

Bim-mediated Release of Mitochondrial Cytochrome c Is Inhibited by Mcl-1L

To investigate the ability of Mcl-1L to repress the apoptotic activity of Bim, we employed a cell-free cytochrome c releasing assay. Initially, the ability of Bim to mediate the release of mitochondrial apoptogenic proteins, was assessed by applying various doses of recombinant His-tagged BimL to mitochondria purified from wild-type Jurkat cells. Recombinant BimL mediated the release of cytochrome c in a dose-dependent manner (Fig. 10A). The detection of cytochrome c in the mitochondrial supernatant coincided with its reduced presence in the mitochondrial pellet. However, the presence of exogenous Mcl-1L resulted in a dose-dependent inhibition of BimL-mediated cytochrome c release (Fig. 10B). These results suggest that the interaction of BimL with Mcl-1L interferes with the cytochrome c releasing capability of BimL.
Current understanding of GrB apoptotic activity does not fully explain the direct effect of GrB on mitochondrial permeability transition or the ability of GrB to cause mitochondrial depolarization in cells deficient in Bid, or Bax and Bak. The inability to satisfactorily elucidate the mechanisms underlying the function of GrB led to the assumption that one or more unknown intracellular factors are involved in the mitochondrial response to GrB. In the current study we identified two interactive Bcl-2 family members, Mcl-1L and Bim, as novel mediators of the mitochondrial response to GrB.

The interaction of Mcl-1L and Bim at the outer mitochondrial membrane fits into the current dogma for regulation of mitochondrial apoptosis by heterodimerization between pro-survival and pro-death Bcl-2 family members. The Bcl-2 gene family includes three groups of proteins that regulate stress-induced apoptosis. The pro-survival members include Bcl-2, Bcl-XL, Bcl-w, A1, and Mcl-1 that share three or four regions of homology (BH1–4) (41). The two pro-death sub-groups of the Bcl-2 family include Bax/Bak-like proteins, which are structurally similar to Bcl-2, as well as the more distantly related BH3-only proteins (41, 42). The BH3 domain is essential for the binding of the BH3-only proteins to the anti-apoptotic members of the family and for their ability to kill cells. Heterodimerization is mediated by the insertion of the BH3 domain of the pro-apoptotic molecules into a hydrophobic cleft formed by the

**Fig. 8. Association of endogenous Mcl-1 with Bim.** A, Bim is co-immunoprecipitated with Mcl-1. Cell extract, purified mitochondria and S-100 fractions from Jurkat cells were immunoprecipitated with mouse anti-Mcl-1 Ab (Ab-2). Control mouse Ig (lane 1) and the initial lysates (lane 2), depleted supernatants (lane 3), and immunoprecipitated pellets (lane 4) were resolved by SDS-PAGE and immunoblotted with mouse anti-Mcl-1 Ab (left panel). The membrane was stripped and re-probed with rabbit anti-Bim Ab (right panel). Please note, the control mouse Ig loaded on lane 1 is detected by the anti-mouse secondary Ab, but not by the anti-rabbit secondary Ab used to detect the anti-Bim Ab. B, Mcl-1 is co-immunoprecipitated with Bim. Cell extract, purified mitochondria, and S-100 fractions from Jurkat cells were immunoprecipitated with mouse anti-Bim Ab. Control mouse Ig (lane 1) and the initial lysates (lane 2), depleted supernatants (lane 3), and immunoprecipitated pellets (lane 4) were resolved by SDS-PAGE and immunoblotted with rabbit anti-Bim Ab (left panel). The membranes were then stripped and re-probed with mouse anti-Mcl-1 Ab (Ab-2; right panel).
duce apoptosis in HeLa cells (31), Mcl-1 antisense therapy
apoptosis. Although Mcl-1 small interference RNA did not in-
Mcl-1 by antisense oligonucleotides causes a rapid entry into
neutrophils (43), differentiating human myeloblastic leukemia
constitutively detected by immunoblotting performed with various
protein levels) in both Jurkat and HeLa cells it was not con-
Due to low expression of endogenous Mcl-1S (at the RNA and
and therefore is expected to localize mainly to the cytoplasm.
Mcl-1S does not possess a C-terminal transmembrane domain
plasmic side of intracellular membranes. In contrast to Mcl-1L,
transmembrane domain, which can target them to the cyto-
pox, Bim-L elicits a rapid entry into apoptosis, which starts
preventing its interaction with Mcl-1L. Indeed, Bim-L can
bind to Mcl-1L, but not Bcl-2, Bcl-XL, or Bcl-w. The binding between
three Bim isoforms, but not Bid or Bad, with endogenous
Mcl-1L from either Jurkat cell extracts or purified mitochondria.
Although under apoptotic conditions or when overex-
pressed, Bim is capable of interaction with various anti-apo-
potic Bcl-2 family members, upon immunoprecipitation from
extracts of non-apoptotic cells, it preferentially co-precipitated
Mcl-1L, but not Bcl-2, Bcl-XL, or Bcl-w. The binding between  
in vitro translated BimEL and Mcl-1 that occurs at 4 °C with no
need for prior co-incubation suggests the high affinity nature of
this interaction.

Alternative splicing generates at least three isoforms of Bim:
BimS, BimL, and BimEL (46). All three isoforms are potent
inducers of apoptosis but may be subjected to differential reg-
ulation. The additional regions present in the longer isoforms
attenuate their activity, by allowing Bim sequestration to the
microtubule-associated dynein motor complex through binding
to dynein light chain LCS/DLC1 (46, 47). Certain apoptotic
stimuli, such as taxol, induce the release of a Bim-L-DCL1
complex from the dynein motor complex. The BimL-DCL1 com-
plex then relocalizes to the membranes of intracellular org-
anelles, such as mitochondria, where it binds to and inhibits
the function of Bcl-2. According to our sub-cellular fraction-
ation experiments, the three Bim isoforms are present in both
S-100 and purified mitochondria from non-apoptotic Jurkat
and HeLa cells. Other studies also reported the constitutive
presence of BimEL in the mitochondria (48). In support of these
observations several studies have demonstrated the co-local-
ization of Bim and LC8 to heavy membrane fractions that are
enriched in mitochondria (47, 48). Currently, the role of LC8/
DCL1 in regulating mitochondrial attached Bim is unknown
(48). Also, several studies suggested that the apoptotic activity
of Bim is regulated by its phosphorylation status (48, 49). The
role of Bim phosphorylation in its interaction with Mcl-1 has

BH1, BH2, and BH3 domains on the surface of the anti-apo-
potic proteins. Such a mechanism of heterodimerization
and potential neutralization of Bim apoptotic activity may underlie
the binding observed between endogenous as well as  in vitro
translated products for Mcl-1L and Bim. In  in vitro translated BimEL has the ability to also interact with Mcl-1S, but it is not
clear whether such binding occurs  in situ.

Like many pro- and anti-apoptotic members of the Bcl-2
family, Mcl-1L and the three Bim isoforms have a C-terminal
transmembrane domain, which can target them to the cyto-
plasmic side of intracellular membranes. In contrast to Mcl-1L,
Mcl-1S does not possess a C-terminal transmembrane domain
and therefore is expected to localize mainly to the cytoplasm.
Due to low expression of endogenous Mcl-1S (at the RNA and
protein levels) in both Jurkat and HeLa cells it was not con-
sistently detected by immunoblotting performed with various
sub-cellular fractions.

Current literature suggests that down-regulation in expres-
sion of Mcl-1L would facilitate the apoptotic process. In human
neutrophils (43), differentiating human myeloblastic leukemia
(44), and multiple myeloma cell lines (30) down-regulation of
Mcl-1 by antisense oligonucleotides causes a rapid entry into
apoptosis. Although Mcl-1 small interference RNA did not in-
duce apoptosis in HeLa cells (31), Mcl-1 antisense therapy
chemosensitized human melanoma to subsequent treatment
with decarbazine (45). The outcome of Mcl-1 elimination in
different cells may vary because of the co-expression of redu-
ant anti-apoptotic Bcl-2 family members, including Bcl-2, Bcl-
XL, and Bcl-w. However, its significance for cell survival is
suggested by the apoptotic sensitization mediated through its
down-regulation (45). The specific apoptotic mechanisms
induced by Mcl-1 down-regulation are not clearly understood.
Release of Bim from Mcl-1 sequestration may lead to Bim
activation. Bim-Mcl-1 interaction appears to be both specific
and of a high affinity nature. The specificity in Mcl-1-Bim
binding was indicated by the co-immunoprecipitation of the
three Bim isoforms, but not Bid or Bad, with endogenous
Mcl-1L from either Jurkat cell extracts or purified mitochondria.
Although under apoptotic conditions or when overex-
pressed, Bim is capable of interaction with various anti-apo-
potic Bcl-2 family members, upon immunoprecipitation from
extracts of non-apoptotic cells, it preferentially co-precipitated
Mcl-1L, but not Bcl-2, Bcl-XL, or Bcl-w. The binding between  
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DCL1 in regulating mitochondrial attached Bim is unknown
(48). Also, several studies suggested that the apoptotic activity
of Bim is regulated by its phosphorylation status (48, 49). The
role of Bim phosphorylation in its interaction with Mcl-1 has
not yet been investigated. Noteworthy, we did not observe changes in the SDS-PAGE migration pattern of Bim following exposure of cells to GRb/Ad or treatment of mitochondria with GRb (data not shown).

The constitutive, rather than apoptosis-induced binding of Bim to a pro-survival protein suggests that Bim proteins are the target for Mcl-1L inhibition. The ability of exogenous Mcl-1 to inhibit recruitment of Bim from releasing mitochondrial cytochrome c, provides further support to a potential regulatory role that Mcl-1L plays in Bim function. Thus, the cleavage of Mcl-1L by either GRb or GRb-stimulated caspases is a mitochondrial apoptotic event that may alter the activation status of Bim. However, this hypothesis requires further investigation.

The mitochondrial mechanisms underlying the response to Bim are not yet resolved. Studies conducted with Bax–/– mice suggested that MEF cell death induced by a retrovirus expressing BimS or tBid requires the function of either Bax or Bak (20,50).

Another study conducted with purified liver mitochondria from wild-type or Bax–/– mice suggested that Bim, but not tBid, induces cytochrome c release in both Bak-dependent and Bak-independent manners (51). Moreover, Bim, but not tBid, was able to induce cytochrome c release from yeast mitochondria that do not possess Bcl-2 family members. Bim-mediated mitochondrial permeability transition and cytochrome c release are VDAC-dependent, because they are both inhibited by a VDAC-specific Ab (51). Regardless of the underlying functional mechanisms, the ability of Bim that is free from Mcl-1 sequestration to mediate mitochondrial permeability transition and release of apoptogenic proteins is predicted to be the basis of the novel pathway we identified for GRb-mediated mitochondrial apoptosis.

The cleavage of Mcl-1L by either GRb or caspase-3 eliminates the presence of full-length Mcl-1L while producing several cleavage products. One of the cleavage products generated by GRb and one of those generated by caspase-3 appear to be sustained in the presence of increased doses of either enzyme (Fig. 6A) or a prolonged reaction period (data not shown). Thus, GRb or caspase-3 proteolytic activities may abrogate the protective effect of Mcl-1L and at the same time produce apoptotic cleavage products. In an analogous scenario, Bcl-2 and Bcl-XL that are cleaved by caspase-3 are known to possess potent pro-apoptotic activity (52,53). However, the Mcl-1 cleavage sites targeted by GRb and caspase-3 need to be identified to further investigate the nature and the biologic significance of its cleaved products.

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