Disruption of Mcl-1-Bim Complex in Granzyme B-mediated Mitochondrial Apoptosis*

Recently, we reported the identification of a novel mitochondrial apoptotic pathway for granzyme B (GrB) (Han, J., Goldstein, L. A., Gastman, B. R., Froelich, C. J., Yin, X. M., and Rabinowich, H. (2004) J. Biol. Chem. 279, 22020–22029). The newly identified GrB-mediated mitochondrial cascade was initiated by the cleavage and subsequent degradation of Mcl-1, resulting in the release of mitochondrial Bim from Mcl-1 sequestration. To investigate the biological significance of Mcl-1 cleavage by GrB, we mapped the major GrB cleavage sites and evaluated the apoptotic potential of the cleavage products. GrB cleaves Mcl-1 after aspartic acid residues 117, 127, and 157, generating C-terminal fragments that all contain BH-1, BH-2, BH-3, and transmembrane domains. These fragments accumulate at an early apoptotic phase but are eliminated by further degradation during the apoptotic process. The major Mcl-1 C-terminal fragment generated by GrB (residues 118–350) was unable to induce or enhance apoptosis when transfected into tumor cells. Instead, this Mcl-1 C-terminal fragment maintained a partial protective capability against GrB-mediated apoptosis via its lower affinity to Bim. In comparison with ectopically expressed full-length Mcl-1, the stably transfected C-terminal fragments of Mcl-1 were less efficiently localized to the mitochondria. Knockdown of Mcl-1, as achieved by transfection with Mcl-1-specific short interfering RNA, resulted in a significant level of apoptosis in the absence of external apoptotic stimulation and, in addition, enhanced the susceptibility of breast carcinoma cells to GrB cytotoxicity. The significance of Bim in this GrB apoptotic cascade was indicated by the marked protection against GrB-mediated apoptosis endowed on these cells through Bim knockdown. Our studies suggest that the disruption of the Mcl-1-Bim complex by GrB initiates a major Bim-mediated cellular cytotoxic mechanism that requires the elimination of Mcl-1 following its initial cleavage.

Mcl-1 was originally identified as an “early induction” gene during myeloblastic leukemic cell differentiation (1). It has since been established as an essential protein in development (2, 3) and in the survival and homeostasis of mature lymphoid cells (4, 5). Mcl-1 is an anti-apoptotic Bcl-2 family member with three putative Bcl-2 homology domains (BH1–3) (1, 4). Other pro-survival Bcl-2 family members, including Bcl-2, Bcl-XL, and Bcl-w, also possess a BH4 domain, which is absent in Mcl-1 and A1/Bfl-1 (6). Because the BH4 domain is required for molecular interactions with other proteins (7–9), its absence in Mcl-1 suggests that this Bcl-2 member interacts with a different set of proteins as compared with Bcl-2 and Bcl-XL. We and others (5, 10) have recently identified a Mcl-1 high affinity binding capacity for Bim, whereas its affinity for Bid, Bad, Bak, and Bak was low. By contrast, Bcl-2 displayed comparable binding to Bim and Bad (5). These findings suggest that the anti-apoptotic activity of different Bcl-2 family members depends on their selective interactions with other proteins, including various pro-death Bcl-2 members. Mcl-1 has a C-terminal hydrophobic domain that mediates its localization to membranous organelles (11, 12). Because both Mcl-1 and Bim are mainly localized at the mitochondrial membrane (13), sequestration of Bim by Mcl-1 is expected to block the Bim-mediated mitochondrial apoptotic cascade (10). Mcl-1 has a fast turnover rate and the shortest half-life among anti-apoptotic Bcl-2 family members (4). This rapid turnover may serve the apoptotic process, particularly in light of the finding that early elimination of Mcl-1 is required for a UV-mediated mitochondrial cascade in HeLa cells (14). Mcl-1 expression is highly regulated at both transcriptional and post-transcriptional levels. Its expression is dependent on environmental survival stimuli, mediated by various growth factors, such as IL-2,1 IL-3, IL-4, IL-7, IL-13, and granulocyte-macrophage colony-stimulating factor (5, 15–18). Several post-transcriptional mechanisms have been implicated in its down-regulation, including decreased protein synthesis and various proteolytic activities that are mediated by proteasomes or caspases (5, 19, 20).

We reported that Mcl-1 is a direct substrate for GrB (10). Based on this observation and the identification of the high affinity of Mcl-1 for Bim, we proposed that the GrB-mediated degradation of Mcl-1 may free sequestered Bim and therefore allows for the execution of a potent Bim-mediated mitochondrial apoptotic cascade. However, other Mcl-1-related pro-survival Bcl-2 members, Bcl-2 and Bcl-XL, are converted into pro-apoptotic effectors upon their cleavage by caspase activity.

---

* This work was supported by grants from National Institutes of Health Grant RO1 CA 109285 (to H. R.) and Department of Defense Health Grant RO1 CA 109285 (to H. R.). 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.

§ These authors contributed equally to this work.

** To whom correspondence should be addressed: University of Pittsburgh Cancer Institute, The Hillman Cancer Center, Research Pavilion, Rm. G17c, 5117 Centre Ave., Pittsburgh, PA 15213. Tel.: 412-623-3212; Fax: 412-623-1119; E-mail: rabinow@pitt.edu.
Biologic Significance of Mcl-1 and Bim in GrB Function

(21, 22). In the current study, we investigated the apoptotic nature of GrB-cleaved Mcl-1 products, as well as the significance of a complex between Bim and Mcl-1 or its GrB-generated C-terminal cleavage products for cellular survival.

EXPERIMENTAL PROCEDURES

Reagents—Anti-human Mcl-1 Abs were from Oncogene (Boston, MA; mouse clone RC13 generated against recombinant Mcl-1, Ab-2) and from Santa Cruz Inc. (Santa Cruz, CA; mAb generated against recombinant Mcl-1, Ab-1), and a polyclonal Ab generated against a 20 amino acid residue synthetic peptide of human Mcl-1, Ab-3). Anti-β-actin mAb (clone AC-15) was purchased from Sigma; anti-Cox IV mAb was from Molecular Probes (Eugene, OR); rabbit anti-Bim Ab was from ProSci (Pullman, WA); Bim-specific rat mAb was from Apoptech (San Diego, CA; Clone 14A8); Abs to AIF, Lamin A/C, Bcl-2, and Bcl-XL were from Clontech (Palo Alto, CA); Abs to AIF, Lamin A/C, Bcl-2, and Bcl-XL were from Santa Cruz; Z-VAD-FMK, and purified GrB were from ICN (Aurora, OH; [35]Methionine, protein G-Sepharose beads, and protein G-Sepharose beads were from Amersham Biosciences.

Cell Lines, Cell Lysates, and Cell Extracts—Jurkat T leukemic cells were grown in RPMI 1640 medium containing 10% fetal calf serum, 20 mM HEPES, 2 mM t-glutamine, and 100 units/ml each of penicillin and streptomycin. HeLa, breast carcinoma CAMA-1, and colon cancer HCT-116 cells were grown in Dulbecco’s modified Eagle medium containing 15% fetal calf serum, 20 mM t-glutamine, and 100 units/ml each of penicillin and streptomycin. The cell lysates were prepared with 1% Nonidet P-40, 20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. To prepare cell extracts for GrB or caspase-3 reactions, cultured cells were washed twice with phosphate-buffered saline and then resuspended in ice-cold buffer (20 mM HEPES, pH 7.0, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 250 mM sucrose, and protease inhibitors). After incubation on ice for 20 min, the cells (2.5 × 10⁶/0.5 ml) were disrupted by Dounce homogenization. The nuclei were removed by centrifugation at 650 x g for 10 min at 4 °C. Cellular extracts were obtained as the supernatants resulting from centrifugation at 14,000 x g for 30 min.

Clones Encoding GST-Mcl-1 Fusion Proteins—E. coli BL21 cells (Novagen) were grown and transformed with recombinant pGEX-4T-1 Mcl-1 plasmids (see above) and the EGFP control plasmid pEGFP-C2 (BD Biosciences) using GenePorter transfection reagent following the manufacturer’s protocol.

Production of Mcl-1, GrB, and Caspase-3 Mcl-1 C-terminal Fragments—Production of GST-Mcl-1 Fusion Proteins—Construction of recombinant cDNA clones capable of expressing WT Mcl-1 and Mcl-1 C-terminal fragments was performed as described previously (10). All Asp to Ala conversions were confirmed by automated DNA sequence analysis (University of Pittsburgh DNA Sequencing Core Facility) of randomly selected clones.

Molecular Cloning of Recombinant Plasmids Encoding the Mcl-1 GrB and Caspase-3 C-terminal Fragments—cDNA clones that encode the C-terminal fragments of Mcl-1 generated by GrB cleavage at Asp₁₁₇, Mcl-1Δ₁₂₂–₁₁₇, and by caspase-3 digestion at Asp₁₂₇, Mcl-1Δ₁₂₂–₁₂₇, will be referred to as Mcl-Δ₁₂₂ and MclΔ₁₂₂Δ₁₂₂, respectively. Both were produced by PCR (see above) of a WT Mcl-1 cDNA clone with the primer pairs (forward and reverse): GrB, 5'-GGCCGATATCCGCGAAGGAGCACCCCTGAGTTACGTAC -3' and 5'-GGCCGATATCCGCGAAGGAGCACCCCTGAGTTACGTACCTTCTCCTGACGTTCTC-3' (corresponds to Mcl-1 amino acid residues Gly₁-twelve through Gly₂-three) and 5'-GGCCGATATCCGCGAAGGAGCACCCCTGAGTTACGTACCTTCTCCTGACGTTCTC-3' (corresponds to Mcl-1 amino acid residues Gly₂-three through Gly₂-three); the reverse primer is the same as for GrB and caspase-3 Mcl-1 ampiclons were purified and subcloned as described previously (10) except that they were digested with the restriction enzymes BamHI and SalI and subsequently ligated into the BamHI and Xhol sites of the plasmid pEGFP-C2 (BD Biosciences). Transient transfection of CAMA-1 cells was carried out with Mcl-1 recombinant plasmids (see above) and the EGF fusion control plasmid pEGFP-C2 (BD Biosciences) using GenePorter transfection reagent following the manufacturer’s protocol.

Production of Mcl-1, GrB, and Caspase-3 Mcl-1 C-terminal Fragments—Production of Mcl-1, GrB, and Caspase-3 Mcl-1 C-terminal fragments was performed as described previously (10). All Asp to Ala conversions were confirmed by automated DNA sequence analysis (University of Pittsburgh DNA Sequencing Core Facility) of randomly selected clones.

Molecular Cloning of Human BimEL—Production of human BimEL cDNA clones was as described previously (10).

Transfection—HeT₁₁6 cells were washed in cold phosphate-buffered saline and resuspended in electroporation buffer (Amaxa) at a final concentration of 3 × 10⁶ cells/ml. Five μg of linearized plasmid DNA was added with 0.1 ml of suspension, transfected with a 400 μl electroporation cuvette, and nuleofected with an Amaxa Nucleofector apparatus (Amaxa, www.amaxa.com) utilizing program T20, according to the manufacturer’s directions. CAMA-1 cells were transfected by the GenePorter Transfection Reagent (Gene Therapy Systems Inc., San Diego, CA) according to the manufacturer’s directions. Geneticin-resistant cell lines were grown in the presence of G418 (1500 μg/ml). GeneConter was generated by cDNA microinjection (1 cell/well) utilizing a MOFLO high speed cell sorter and Summit Software. Transient transfection of CAMA-1 cells was carried out with Mcl-1 recombinant plasmids (see above) and the EGF fusion control plasmid pEGFP-C2 (BD Biosciences) using GenePorter transfection reagent following the manufacturer’s protocol.

Production of Mcl-1, GrB, and Caspase-3 Mcl-1 C-terminal Fragments—Production of GST-Mcl-1 Fusion Proteins—Construction of recombinant cDNA clones capable of expressing WT Mcl-1 and Mcl-1 C-terminal fragments was performed as described previously (10). All Asp to Ala conversions were confirmed by automated DNA sequence analysis (University of Pittsburgh DNA Sequencing Core Facility) of randomly selected clones.

Molecular Cloning of Recombinant Plasmids Encoding the Mcl-1 GrB and Caspase-3 C-terminal Fragments—cDNA clones that encode the C-terminal fragments of Mcl-1 generated by GrB cleavage at Asp₁₁₇, Mcl-1Δ₁₂₂–₁₁₇, and by caspase-3 digestion at Asp₁₂₇, Mcl-1Δ₁₂₂–₁₂₇, will be referred to as Mcl-Δ₁₂₂ and MclΔ₁₂₂Δ₁₂₂, respectively. All Asp to Ala conversions were confirmed by automated DNA sequence analysis (University of Pittsburgh DNA Sequencing Core Facility) of randomly selected clones.

Molecular Cloning of Recombinant Plasmids Encoding the Mcl-1 GrB and Caspase-3 C-terminal Fragments—cDNA clones that encode the C-terminal fragments of Mcl-1 generated by GrB cleavage at Asp₁₁₇, Mcl-1Δ₁₂₂–₁₁₇, and by caspase-3 digestion at Asp₁₂₇, Mcl-1Δ₁₂₂–₁₂₇, will be referred to as Mcl-Δ₁₂₂ and MclΔ₁₂₂Δ₁₂₂, respectively. All Asp to Ala conversions were confirmed by automated DNA sequence analysis (University of Pittsburgh DNA Sequencing Core Facility) of randomly selected clones.
added to the pellet extraction buffer. GST-Mcl-1 fusion proteins eluted from the microspin columns were concentrated and underwent buffer exchange using YM-10 Microcon centrifugal filter devices (Millipore). RNAs were obtained as duplexes in purified and desalted form (Option C) from Pharmacia. The three siRNAs had the following sense strand sequences: Mcl-1, 5′-GAAACGCCGUAUCCGACUdTtdT-3′; Bim, 5′-GGCGAGAAUGUGACAAUUGdTtdT-3′; and Lamin, 5′-CUGGACU-UCGAGAAGAAdTtdT-3′. CAMA-1 cells (2.5 × 10⁵) were plated in a 6-well plate and following 24 h (at ~30% confluence) they were transfected with 200 nM siRNA in Opti-MEM medium (Invitrogen) without fetal calf serum using Oligofectamine reagent (Invitrogen) according to the manufacturer’s transaction protocol. After 4 h, fetal calf serum was added to a final concentration of 10%. At 48 h, the medium over the samples was adjusted to 1 ml before the addition of an apoptotic agent.

Release of Mitochondrial Apoptotic Proteins—Purified mitochondria (50 μg of protein) were incubated with various doses of recombinant Mcl-1 (GST-Mcl-1), caspase-3-cleaved C-terminal fragment of Mcl-1 (GST-Mcl-1ΔN127), and the GrB-cleaved C-terminal fragment of Mcl-1 (GST-Mcl-1ΔN117) in 25 μl of MIB at room temperature for 10 min. His-BimL was then added for a co-incubation at 37 °C for 30 min. Mitochondria were pelleted by centrifugation at 10,000 × g for 10 min. The resulting supernatants or mitochondria were mixed with SDS sample buffer, resolved by SDS-PAGE, and analyzed by immunoblotting for the presence of mitochondrial apoptotic proteins, cytochrome c, and AIF.

In Vitro Transcription-Translation—Mcl-1, Mcl-1ΔN127, Mcl-1ΔN117, and Bim-EL cDNAs 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 phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Each reaction also contained 1 μl of reticulocyte lysate containing 35S-labeled Mcl-1, or Bim-EL, and also 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 boiling for 5 min.

Immunoprecipitation—For Mcl-1 and Bim immunoprecipitation experiments, the 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 phenylmethylsulfonyl fluoride, and 1% CHAPS). The lysates were preclreated with protein A- or G-Sepharose beads, and incubated with anti-Mcl-1 or anti-Bim Abs at 4 °C for 4 h. The immune complexes were then precipitated with protein A- or G-Sepharose beads, and incubated with anti-Mcl-1 or anti-Bim 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, mitochondria, or S-100 were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes, as described previously (25). Following probing with a specific primary Ab and horseradish peroxidase-conjugated secondary Ab, the protein bands were detected by enhanced chemiluminescence (Pierce).

Flow Cytometry—Cytofluorometric analyses of apoptosis were performed by co-staining with propidium iodide and fluorescein isothiocyanate-annexin V conjugates (Becton-Dickenson). The staining was performed according to the manufacturer’s procedures, assessed by a Beckman Coulter Epics XL-MCL, and analyzed with the EXPO32 software.

RESULTS

Elimination of Mcl-1 during Caspase-3 and GrB-mediated Apoptosis—We recently reported the identification of a novel mitochondrial cascade for GrB-mediated apoptosis that encompasses the release of Bim from sequestration by mitochondrial Mcl-1 (10). Our reported studies suggest that the disruption of the Mcl-1-Bim complex is mediated by cleavage of Mcl-1 by GrB or caspase-3. Examples for the observed down-regulation in Mcl-1 expression are depicted in Fig. 1. Thus, in breast carci-

FIG. 1. Degradation of endogenous Mcl-1 during apoptosis mediated by GrB/Ad or VP-16. A, elimination of Mcl-1 in GrB/Ad-treated cells. CAMA-1 cells were treated with GrB/Ad (33 nm/20 plaque-forming units/ml) for 2, 4, or 8 h at 37 °C. The cells were then lysed in the presence of Ac-IETD-CHO (500 μM), a GrB inhibitor, to block potential GrB activity during the lysis procedure. Expression of Mcl-1 was assessed by immunoblotting utilizing rabbit anti-Mcl-1 Ab-3 that detects both full-length Mcl-1 and one of its cleavage products. B, etoposide-mediated elimination of Mcl-1. Jurkat cells were treated with VP-16 (20–80 μM, for 16 h) in the presence or absence of Z-VAD-FMK (100 μM). Expression of Mcl-1 was determined by immunoblotting with mAb (Ab-1) that does not detect Mcl-1 cleavage products. VP-16-mediated caspase-3 activity is demonstrated by the presence of p20 caspase-3 subunit that is blocked by Z-VAD-FMK from further auto-processing into p19 and p17. C, degradation of endogenous Mcl-1 in tumor cell extracts treated with recombinant caspase-3 or purified GrB. Extracts of CAMA-1 (top panels) or HeLa cells (bottom panels) were treated with various doses of caspase-3 (25–100 μM) or GrB (33–330 mM) for 30 min at 37 °C. The reactions were stopped by boiling in sample buffer, and the presence of Mcl-1 was assessed by immunoblotting with Mcl-1-specific Ab-1. The membranes were stripped and reprobed with anti-β-actin mAb to demonstrate equal loading.
ination of Mcl-1 (14, 28). When added directly into cell extracts, recombinant caspase-3 or purified GrB efficiently reduces the expression level of Mcl-1 (Fig. 1C). To further investigate the apoptotic mechanisms involved in GrB-mediated cytotoxicity, we investigated the biological significance of the GrB-generated Mcl-1 cleavage products.

Mapping GrB and Caspase-3 Cleavage Sites in Mcl-1—To map the cleavage sites mediated by GrB or caspase-3, we generated mutant Mcl-1 cDNAs that involved Asp (GAC) to Ala (GCC) conversions for amino acid residue positions 72, 88, 117, 127, and 157 from WT Mcl-1 cDNA by introducing A→C point mutations utilizing extension overlap mutagenesis by PCR (24). Thus, mutant Mcl-1 cDNAs that encode single residue conversions (Asp→Ala) at one of the five sites as well as a mutant for both residues 127 and 157 were produced. All Mcl-1 cDNAs were ligated into the mammalian expression vector pCR3.1. The mutated Mcl-1 plasmids were transcribed and translated by an in vitro system, and the products were treated with recombinant caspase-3 or GrB. As determined by autoradiography (Fig. 2A, top panel) or by immunoblotting (bottom panel), Mcl-1 cleavage by caspase-3 was mapped to aspartic acid residues 127 and 157. Mcl-1 cleavage by caspase-3 was completely blocked in the Mcl-1 mutant for both the 127 and 157 aspartic acid residues. These cleavage sites for caspase-3 were recently reported by two independent groups (19, 20). GrB cleavage sites were mapped to aspartic acid residues 117, 127, and 157 (Fig. 2B). As demonstrated by immunoblotting (Fig. 2B, bottom panel), the major cleavage site for GrB is aspartic acid 117, because the D117A mutation blocked significantly the processing of Mcl-1, whereas D127A, D157A, and the double mutant of D127A/D157A were significantly less efficient in blocking the loss in full-length Mcl-1.

Kinetics of Elimination of Mcl-1 Cleavage Products—Cleavage of other anti-apoptotic Bcl-2 members, such as Bcl-2 and Bcl-XL, results in the conversion to pro-apoptotic proteins (21, 22). Caspase cleavage of either Bcl-2 or Bcl-XL removes from each of these proteins a BH4 domain that may be required for their antiapoptotic activity. Mcl-1 does not possess a BH4 domain, and cleavage by GrB or caspase-3 removes a 117- (GrB-only), 127-, or 157-residue N-terminal fragment, producing a C-terminal Mcl-1 fragment(s) that contains the transmembrane and BH1–3 domains (Fig. 2C). To gain a better insight into the apoptotic significance of the cleavage products, we determined their fate during the reaction of Mcl-1 with caspase-3 or GrB. The C-terminal cleavage products of Mcl-1 represented by residues 158–350 and 118–350 that are generated by caspase-3 and GrB, respectively, appear to accumulate during the initial phase of the reaction but start to decline within 2 h of enzymatic exposure (Fig. 3A). To generate a reaction setting that mimics the intracellular condi-
products in the presence of cell extracts alone or cell extracts and GrB. 

Elimination during the later phase of this process. We mixed 35S-labeled in vitro translated Mcl-1 and GrB (Mcl-1ΔN127 and Mcl-1ΔN117, respectively) with cell extract and assessed the stability of the radioactively labeled proteins following a 1–8-h incubation in the presence or absence of GrB. Significant loss of these proteins was detected at 8 h in the absence of GrB and at 4 h in the presence of GrB (Fig. 3B). In addition to its effect on the degradation of full-length Mcl-1, GrB also accelerated the degradation of the Mcl-1 deletion mutants, because each contains at least one GrB cleavage site at residue Asp157. These observations also suggest that the major cleavage products of Mcl-1 generated by either caspase-3 or GrB are being further degraded by proteolytic activity present in the cell extract during an advanced apoptotic process.

The Apoptotic Response to GrB Is Significantly Attenuated by Stable Transfection of Full-length Mcl-1—To assess the significance of the expression of Mcl-1 and its cleavage products on survival, we transiently co-transfected the breast carcinoma CAMA-1 cell line with plasmids that express full-length Mcl-1, Mcl-1ΔN127 (GrB-cleaved C-terminal product), and a control plasmid encoding EGFP at a 10:1 ratio, respectively. As judged by EGFP/annexin V flow cytometry, we did not observe induction of apoptotic death in successfully transfected cells (EGFP-positive) (results not shown). To analyze the significance of the presence of Mcl-1 and its cleaved forms for survival under better defined conditions, we stably transfected colon carcinoma Hct116 and breast carcinoma CAMA-1 cell lines with the plasmids encoding these different Mcl-1 forms. We obtained a comparable number of geneticin-resistant clones from each of the cell lines (~40 clones for each plasmid) and assessed the levels of Mcl-1 expression by Western blotting (Fig. 4A). The majority of the transfected clones demonstrated equal levels of expression of the transfected proteins. Geneticin-resistant Hct116 or CAMA-1 clonal cell lines that were confirmed by immunoblotting to overexpress the aforementioned Mcl-1-related proteins at equal levels were assessed for susceptibility to death mediated by GrB/Ad. A significant reduction in the apoptotic response to GrB/Ad as assessed by annexin V/propidium iodide was detected in each of the tumor cell lines transfected with full-length Mcl-1 (Fig. 4, B and C). However, overexpression of the Mcl-1 fragments that correspond to the caspase-3 and GrB C-terminal cleavage products provided a reduced protective effect from GrB-mediated cytotoxicity (Fig. 4, B and C). The two cell lines included in this analysis (CAMA-1 or Hct116) were sensitive to GrB-mediated apoptosis, but demonstrated different levels of phosphatidylserine externalization (Fig. 4, B and C). Differential detection of phosphatidylserine exposure has been reported to be cell type-specific and in various cells dependent on caspase activity or intracellular ATP levels (29–31). Thus, in CAMA-1 cells, where only few cells were stained by annexin V, GrB may not directly activate intracellular molecules that are involved in phosphatidylserine externalization. Similar to the observations in transient transfected cells, overexpression of the C-terminal Mcl-1 fragments in either the Hct116 or CAMA-1 cell line was not associated with induction of apoptosis.

As we have previously reported, endogenous Mcl-1 localizes mainly to the outer mitochondrial membrane (10). Following subcellular fractionation to cytosolic S-100 and purified mitochondrial fractions, we assessed the localization of the stably transfected full-length and C-terminal fragments...
FIG. 4. Reduced susceptibility to GrB/Ad of clonal tumor cell lines stably transfected with full-length Mcl-1 or plasmids encoding Mcl-1 C-terminal fragments corresponding to those generated by GrB or caspase-3. A, expression of stably transfected Mcl-1-related proteins in Hct116 and CAMA-1 clones. Geneticin-resistant clonal Hct116 (top panels) and CAMA-1 (bottom panels) cell lines that were transfected with the indicated plasmids were assessed by Western blotting to demonstrate the expression of the transfected proteins (lanes 1–4). In vitro translated Mcl-1 (lane 5), Mcl-1ΔN117 (lane 6), and Mcl-1ΔN127 (lane 7) were used as migration markers. The asterisks indicate unidentified protein bands. The membrane was reprobed with anti-β-actin mAb to demonstrate equal loading. To demonstrate equal levels of expression, the Mcl-1 protein bands were quantitated using a Molecular Dynamics Densitometer SI and the ImageQuant software. The ratios of the Mcl-1 proteins to their corresponding β-actin proteins for Hct116 cells are as follows: 3.0 (endogenous Mcl-1 control, lane 1), 6.7 (endogenous + full-length Mcl-1, lane 2), 7.2 (endogenous + Mcl-1ΔN127, lane 3), and 7.6 (endogenous + Mcl-1ΔN117, lane 4). The ratios of Mcl-1 to β-actin for CAMA-1 cells are: 2.6 (endogenous control, lane 1); 5.1 (endogenous + full-length Mcl-1, lane 2), 4.6 (endogenous + Mcl-1ΔN127, lane 3), and 4.8 (endogenous + Mcl-1ΔN117, lane 4). These Mcl-1/β-actin ratios suggest that for the different Mcl-1 transfected cell lines, the combined level of endogenous and transfected full-length Mcl-1 is approximately equal to the combined levels of endogenous Mcl-1 and the transfected Mcl-1 C-terminal fragments. B and C, flow cytometry analysis for the determination of susceptibility to GrB/Ad of Hct116 and CAMA-1 clones stably transfected with Mcl-1, Mcl-1ΔN127 or Mcl-1ΔN117 plasmids. The Hct116 cells (B) were treated with 34.25 µM/20 plaque-forming units/ml GrB/Ad for 12 h, and the CAMA-1 cells (C) were treated with 8 nM/20 plaque-forming units/ml GrB/Ad for 6 h. Following the GrB/Ad treatment, the cells were assessed by flow cytometry for annexin V/propidium iodide staining. The percentage of apoptotic cells is indicated.
achieve a similar effect. These findings suggest that the Mcl-1 C-terminal fragments generated by either GrB or caspase-3 can at least partially inhibit Bim-mediated cytochrome c release from purified mitochondria.

GrB Susceptibility of Breast Carcinoma Cells Is Enhanced by siRNA Silencing of Mcl-1 and Inhibited by Bim Silencing—Our results (Fig. 3) suggest that cleavage of Mcl-1 by either GrB or caspase activity leads to an eventual elimination of Mcl-1. To investigate the effects of Mcl-1 elimination on breast carcinoma cell viability and their susceptibility to GrB-mediated apoptosis, we subjected CAMA-1 cells to RNAi for Mcl-1, Bim, or Lamin A/C. Successful knockdown of these genes was confirmed by immunoblotting at 40–48 h post-transfection with the specific siRNA (Fig. 8A). At 40 h post-transfection, the cells were treated with GrB/Ad and assessed 6 h later for viability by flow cytometry of annexin V and propidium iodide staining (Fig. 8B). Silencing of Mcl-1 alone, but not of Bim or Lamin A/C, reduced the viability of CAMA-1 cells even in the absence of external apoptotic stimulation. This increase in background apoptosis may relate to the ability of Mcl-1 to sequester and/or neutralize Bim and probably other mediators of apoptosis. Furthermore, elimination of Mcl-1 enhanced the susceptibility of CAMA-1 cells to GrB/Ad-mediated apoptosis. Such an increased susceptibility in the absence of Mcl-1 has been reported for other apoptotic inducers, including UV and TRAIL, and therefore is not GrB-specific (14, 32). However, apoptotic activity of GrB in CAMA-1 cells was predominantly mediated by the Mcl-1 high affinity binding partner, Bim, because it was significantly blocked in cells transfected with Bim-specific siRNA (Fig. 8B). Control silencing of Lamin A/C did not have any effect on the response of these cells to GrB/Ad. These results underscore the important contribution of the Mcl-1-Bim cascade in CAMA-1 cell susceptibility to GrB-mediated apoptosis and further emphasize the importance of Mcl-1 elimination in the execution of this apoptotic response.

DISCUSSION

Mitochondrial disruption has been established as a key event during GrB-mediated apoptosis, but the exact mechanism for GrB function has remained unclear (33, 34). Recent studies have identified Bid as a direct substrate for GrB and therefore as a direct link to the mitochondrial apoptotic cascade mediated by Bax or Bak (35–37). Although in a cell-free system GrB-cleaved Bid is a potent inducer for the release of mitochondrial apoptotic proteins (38), a recent study questions whether a direct cleavage of Bid by GrB occurs under physiologic conditions (34, 39). Furthermore, in response to GrB treatment, embryonic fibroblasts from Bid–/– mice display disrupted mitochondrial transmembrane potentials (40). These studies implied that cytosolic mediators other than Bid may act as a link between GrB and the mitochondria. Our recent studies (10) have identified Mcl-1 as a direct substrate for GrB and as a
Control GST protein had no inhibitory effect on Bim-mediated release of cytochrome c. Immunoblotting for the presence of AIF and cytochrome c revealed that caspase-3 or GrB cleavage products of Mcl-1. H. Rabinowich, unpublished data.

Mcl-1 is a high affinity binding partner for Bim. We proposed that the Mcl-1-Bim cooperation may constitute an alternative mitochondrial apoptotic pathway that is activated directly by GrB, independent of Bid. In the current study, we investigated the functional mechanism and biological significance of this novel GrB-mediated mitochondrial apoptotic cascade.

Caspase cleavage of the pro-survival Bcl-2 members, Bcl-2 and Bcl-XL, converts them into death effector proteins that further amplify the apoptotic cascade (21, 22). In contrast to Mcl-1, Bcl-2 and Bcl-XL are not susceptible to GrB activity. The current study has mapped the GrB cleavage sites of Mcl-1 to aspartic acid residues 117, 127, and 157 and confirmed the recently reported caspase-3 cleavage sites of Mcl-1 at aspartic acid residues 127 and 157 (19, 20). Interestingly, cleavage of Mcl-1 by either caspase-3 or GrB activities resembles the caspase-3 cleavage of Bcl-2 and Bcl-XL in its removal of an N-terminal fragment while producing a C-terminal protein that contains the BH1–3 domains. Therefore, we investigated the apoptotic nature of the Mcl-1 C-terminal fragments generated by either caspase-3 or GrB activity. Whereas stable transfection of full-length Mcl-1 endowed significant protection from apoptosis on GrB-susceptible tumor cells, only mild (CAMA-1) and Bim cooperation may constitute an alternative mitochondrial apoptotic pathway.

Because various apoptotic agents are targeting the same mitochondrial events, the regulation of mitochondrial events by sequestering Bim, therefore preventing the activation of the aforementioned mitochondrial apoptotic events. Because cleavage of Mcl-1 by GrB does not completely abolish the sequestration of Bim, we reasoned that a proteolytic mechanism(s) other than via GrB and caspases may be involved in the further degradation of the Mcl-1 cleavage products. Indeed, we obtained evidence that the Mcl-1 cleavage products are further degraded by proteolytic activity present in the cell extract. Such activity may be mediated by the proteasome, because several studies have documented increased stability of Mcl-1 in the presence of proteasome inhibitors (14, 41, 42). To investigate the significance of decreased Mcl-1 expression for GrB function, we utilized the RNAi approach for Mcl-1. Knockdown of Mcl-1 was associated with a marked increase in the level of susceptibility of tumor cells to GrB-mediated apoptosis, highlighting the significance of Mcl-1 in the protection of cells from an immune attack.
apoptotic cascades, knockdown of Mcl-1 is very likely to increase the sensitivity of the cells to an array of apoptotic agents, including GrB. Indeed, it has recently been reported that Mcl-1 knockdown was associated with an increased sensitivity to UV or TRAIL (14, 32). In the context of the current study, Mcl-1 knockdown may be viewed as a model for cells that undergo Mcl-1 elimination. Thus, in addition to increased susceptibility to apoptotic agents, knockdown of Mcl-1 may also free Bim and other apoptotic Bcl-2 proteins from being sequestered by Mcl-1. Such a scenario may explain the significant level of apoptosis (24%) detected following Mcl-1 siRNA transfection in the absence of an external apoptotic signal. Recently, an example of apoptosis induction by Bcl-2 knockdown was reported (43). Silencing of Bcl-2 induced p53-mediated apoptosis that occurred without stimulation by genotoxic drugs, and that was not seen in the controls or in isogenic clonal cells deficient in p53. The increased level of background apoptosis we observed in cells treated with Mcl-1 siRNA suggests a role for Mcl-1 in the regulation of cell survival under homeostatic conditions. The biological importance of the Mcl-1-Bim cascade in GrB function was further underscored by the significant block in the response of CAMA-1 cells to GrB/Ad in the absence of Bim. Because GrB has multiple potential entry points for initiation of a caspase-dependent apoptotic cascade (34, 44), it was surprising that the absence of Bim arrested, at least within the time frame of the first 6 h of exposure to GrB/Ad, the apoptotic response seen in controls.

In summary, our studies suggest that the disruption of Mcl-1-Bim complex is initiated by GrB cleavage of Mcl-1. The GrB-generated Mcl-1 C-terminal fragment(s) exerts a variable but
partial protective effect against GrB-mediated apoptosis by maintaining limited ability to bind and sequester Bim. The inefficient protective effect of this GrB-cleaved Mcl-1 fragment is most likely due to the combination of its altered subcellular localization to the mitochondria and reduced binding to Bim. Based on these findings and the studies involving Mcl-1 or Bim knockdown, we propose that the disruption of the Mcl-1-Bim complex by GrB initiates a major Bim-mediated cellular cytotoxic mechanism that requires the elimination of Mcl-1 following its initial cleavage.

REFERENCES

1. Kozopas, K. M., Yang, T., Buchan, H. L., Zhou, P., and Craig, R. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3516–3520
2. Rinkenberger, J. L., Horning, S., Klocke, B., Roth, K., and Korsmeyer, S. J. (2000) Genes Dev. 14, 23–27
3. Okita, H., Umezawa, A., Suzuki, A., and Hata, J. (1998) Biochim. Biophys. Acta 1398, 335–341
4. Craig, R. W. (2002) Leukemia 16, 444–454
5. Opferman, J. T., Letsi, A., Beard, C., Sorcinelli, M. D., Ong, C. C., and Korsmeyer, S. J. (2003) Nature 426, 671–676
6. Droin, N. M., and Green, D. R. (2004) Biochim. Biophys. Acta 1644, 179–188
7. Sorensen, C. M. (2000) J. Biol. Chem. 275, 11368–11374
8. Denis, G. V., Yu, Q., Ma, P., Deeds, L., Fuller, D. V., and Chen, C. Y. (2003) J. Biol. Chem. 278, 5775–5785
9. Shimizu, S., Konishi, A., Kodama, T., and Tsujimoto, Y. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3100–3105
10. Han, J., Goldstein, L. A., Gastman, B. R., Froelich, C. J., Yin, X. M., and Rabinowich, H. (2004) J. Biol. Chem. 279, 22020–22029
11. Yang, T., Kozopas, K. M., and Craig, R. W. (1995) J. Cell Biol. 129, 1173–1184
12. Akgul, C., Mouling, D. A., White, M. R., and Edwards, S. W. (2000) FEBS Lett. 478, 72–76
13. Zhu, Y., Huang, H. M., Huang, C. J., and Yen, J. J. (2000) Br. J. Haematol. 113, 15–25
14. Cheng, E. H., Kirsch, D. G., Clem, R. J., Ravi, R., Kastan, M. B., Bedi, A., Ueno, K., and Hardwick, J. M. (1997) Science 278, 1866–1868
15. Clem, R. J., Cheng, R. H., Karp, C. L., Kirsch, D. G., Ueno, K., Takahashi, A., Kastan, M. B., Griffin, D. E., Earnshaw, W. C., Velivska, M. A., and Hardwick, J. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 554–559
16. Matsko, C. M., Hunter, O. C., Rahimovitch, H., Lotan, M. T., and Amoscato, A. A. (2001) Biochem. Biophys. Res. Commun. 287, 1112–1120
17. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 51–59
18. Johnson, D. E., Gastman, B. R., Wieckowski, E., Wang, G. Q., Amoscato, A., Delach, S. M., and Rabinowich, H. (2000) Cancer Res. 60, 1818–1823
19. Froelich, C. J., Orth, K., Turbov, J., Seth, P., Gottlieb, R., Bahir, B., Shah, G. M., Bleackley, R. C., Dixit, V. M., and Hanna, W. (1996) J. Biol. Chem. 271, 29073–29079
20. Browne, K. A., Blink, E., Sutton, V. R., Froelich, C. J., Jans, D. A., and Trapani, J. A. (1999) Mol. Cell. Biol. 19, 8604–8615
21. Zhang, B., Goja, I., and Fenton, R. G. (2002) Blood 99, 1885–1893
22. Gleiss, B., Gogvadze, V., Orrenius, S., and Fadool, B. (2002) FEBS Lett. 519, 153–158
23. Fadool, B., Gleiss, B., Hogstrand, K., Chandra, J., Wiedner, T., Sims, P. J., Henier, J. I., Orrenius, S., and Samali, A. (1999) Biochem. Biophys. Res. Commun. 266, 504–511
24. MacDonald, G., Shi, L., Vande Velde, C., Liebermann, J., and Greenberg, A. H. (1999) J. Exp. Med. 189, 131–144
25. Froelich, C. J., Metorsk, S. K., and Raja, S. M. (2004) Cell Death Differ. 11, 369–371
26. Sutton, V. R., Davis, J. E., Cancilla, M., Johnstone, R. W., Ruefli, A. A., Sedelies, K., Browne, K. A., and Trapani, J. A. (2000) J. Exp. Med. 192, 1405–1414
27. Alimonti, J. B., Shi, L., Baijal, P. K., and Greenberg, A. H. (2001) J. Biol. Chem. 276, 6974–6982
28. Heibine, J. A., Goping, I. S., Barry, M., Pinkoski, M. J., Shore, G. C., Green, D. R., and Bleackley, R. C. (2000) J. Exp. Med. 192, 1381–1402
29. Wang, G.-Q., Wieckowski, E., Goldstein, L. A., Gastman, B. R., Rahimovitz, A., Gambotto, A., Li, S., Fang, B., Yin, X.-M., and Rabinowich, H. (2001) J. Exp. Med. 194, 1325–1337
30. Metkra, S. S., Wang, B., Elshe, M. L., Kim, J. H., Lee, Y. J., Raja, S. M., and Froelich, C. J. (2003) J. Cell Biol. 160, 875–885
31. Thomas, D. A., Scorrano, L., Putcha, G. A., Korsmeyer, S. J., and Levy, T. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 14985–14990
32. Derouet, M., Thomas, L., Cross, A., Moots, R. J., and Edwards, S. W. (2004) J. Biol. Chem. 279, 26915–26921
33. Iglesias-Serret, D., Pique, M., Gil, J., Pons, G., and Lopez, J. M. (2003) Arch. Biochem. Biophys. 417, 141–152
34. Deb, A., and Milner, J. (2003) Genes Dev. 17, 832–837
35. Lord, S. J., Rajotte, R. V., Korbut, G. S., and Bleackley, R. C. (2003) Immuno- Rev. 193, 31–38