Interrelated Roles for Mcl-1 and BIM in Regulation of TRAIL-mediated Mitochondrial Apoptosis*

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The current study demonstrates a novel cross-talk mechanism between the TRAIL receptor death signaling pathway and the mitochondria. This newly identified pathway is regulated at the mitochondrial outer membrane by a complex between the prosurvival Bcl-2 member, Mcl-1 and the BH3-only protein, Bim. Under non-apoptotic conditions, Bim is sequestered by Mcl-1. Direct degradation of Mcl-1 by TRAIL-activated caspase-8 or caspase-3 produces Mcl-1-free Bim that mediates a Bax-dependent apoptotic cascade. Using Mcl-1 or Bim RNAi, we demonstrate that a loss in Mcl-1 expression significantly enhances the mitochondrial apoptotic response to TRAIL that is now mediated by freed Bim. Whereas overexpression of Mcl-1 contributes to the preservation of the mitochondrial membrane potential, Mcl-1 knockdown facilitates the Bim-mediated dissipation of this potential. Loss of Mcl-1 contributes to an increased level of caspase activity downstream of the mitochondrial response to TRAIL. Furthermore, the Mcl-1 expression level at the mitochondrial outer membrane determines the release efficiency for the apoptogenic proteins cytochrome c, Smac, and HtrA2 in response to Bim. These are the first findings to demonstrate the involvement of Bim in the TRAIL-mediated mitochondrial cascade. They also suggest that Mcl-1 may serve as a direct substrate for TRAIL-activated caspases implying the existence of a novel TRAIL/caspase-8/Mcl-1/Bim communication mechanism between the extrinsic and the intrinsic apoptotic pathways.

TRAIL-mediated apoptosis is mainly dependent on the extrinsic cell signaling pathway, which involves death receptor engagement, formation of the death-inducing signaling complex, activation of apical caspases, and consequently, the activation of effector caspases such as caspase-3 and caspase-7 (1). In certain types of cells, referred to as type II, effector caspase activation requires amplification of the apoptotic signal mediated by the death-inducing signaling complex through engagement of the intrinsic cell (mitochondrial) signaling pathway (2, 3). The cross-talk between these two prototypical pathways is partly mediated by caspase-8-truncated Bid (tBid) (4–6). The proapoptotic fragment of Bid translocates to the mitochondria where it activates Bax and thereby induces the release of the apoptogenic proteins, cytochrome c, AIF, endonuclease G, Smac, and HtrA2 (7, 8). Once released into the cytoplasm, cytochrome c associates with Apaf-1 and caspase-9 to form the “apoptosome” that serves to further activate caspase-9, which in turn activates caspase-3 and -7 downstream of mitochondrial apoptotic events. Released Smac and HtrA2 bind to members of the inhibitor of apoptosis (IAP) protein family, and thereby prevent IAP inhibition of caspase-3, -7, and -9 (9–11).

The requirement for Bax in TRAIL-induced apoptosis of type II cells has been mapped to the mitochondrial amplification cycle (2, 12, 13). Early events triggered by TRAIL, including DISC formation, caspase-8 activation, and Bid cleavage are not dependent on Bax; however, mitochondrial depolarization, cytochrome c release, and activation of caspase-9 were prevented in Bax-deficient cells.

The BH3-only protein Bid is currently the only known link between the extrinsic and the intrinsic apoptotic pathways mediated by TRAIL. Recently, we identified a novel mitochondrial apoptotic cascade that is initiated by the degradation of Mcl-1 and proceeds through the disruption of the Mcl-1-Bim complex (14, 15). Thus, degradation of Mcl-1 by either granzyme B or caspase-3 relieves Bim, a high affinity Mcl-1 binding partner, from sequestration and neutralization of its activity.

In the current study, we investigated the possibility that the mitochondrial Mcl-1-Bim complex serves as an additional link between the extrinsic and the intrinsic apoptotic pathways activated by TRAIL. Our findings are the first to demonstrate that Bim is involved in the mitochondrial response to TRAIL and suggest that Mcl-1 and Bim are functionally linked in the cell response to TRAIL. Furthermore, we demonstrate that Mcl-1 serves to maintain the mitochondrial membrane potential that is disrupted by the presence of Mcl-1-free Bim, leading to the release of apoptogenic proteins. Thus, our studies imply the existence of a cross-talk mechanism between the TRAIL death receptor and the mitochondrial apoptotic cascade that has not been previously considered.

EXPERIMENTAL PROCEDURES

Reagents—Anti-human Mcl-1 Abs were from Oncogene (Boston, MA; mouse clone RC13 generated against recombinant Mcl-1) and from Santa Cruz Biotechnology (Santa Cruz, CA; a polyclonal Ab generated against a 20-amino acid residue synthetic peptide of human Mcl-1). Anti-β-actin mAb (clone AC-15) was purchased from Sigma; rabbit anti-Bim Ab was from ProSci (Poway, CA); and Bim-specific rat mAb was from Apoptech (San Diego, CA; clone 1A4A8); Abs to lamin A/C and β-tubulin were from Santa Cruz Biotechnology; anti-caspase-3 was from StressGen (Victoria BC, Canada); Abs to caspase-8 and cytochrome c were from BD Pharmingen (San Jose, CA). Anti-Smac Ab was from Cell Signaling (Beverly, MA), and anti-HtrA2 Ab was from Apoptech. Z-VAD-FMK was from ICN (Aurora, OH). [35S]Methionine, protein A-Sepharose beads, and protein G-Sepharose beads were from Amersham Biosciences. Flow cytometry reagents Annexin V, JC-1,
DiCl₃ (5), FAM-DEVDFMK, and FAM-LETDFMK were from Molecular Probes (Carlsbad, CA).

Cell Lines, Cell Lysates, and Cell Extracts—HeLa, breast carcinoma CAMA-1, and colon cancer Hct116 cells were grown in Dulbecco’s modified Eagle’s medium containing 15% fetal calf serum, 20 mM L-glutamine, and 100 units/ml each of penicillin and streptomycin. Cell lysates were prepared with 1% Nonidet P-40, 20 mM Tris-HCl pH 7.4, 137 mM NaCl, 10% glycerol, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. To prepare cell extracts for caspase-3 or caspase-8 reactions, cultured cells were washed twice with phosphate-buffered saline and then resuspended in ice-cold buffer (20 mM HEPS, pH 7.0, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 20% glycerol, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). After incubation on ice for 20 min, cells (2.5 × 10⁶/0.5 ml) were disrupted by Dounce homogenization. Nuclei were removed by centrifugation at 650 × g for 10 min at 4 °C. Cellular extracts were obtained as the supernatants resulting from centrifugation at 14,000 × g at 4 °C for 30 min.

In Vitro Cleavage Reaction with Caspase-3 or Caspase-8—In vitro cleavage reactions were performed in a total volume of 20 μl. The reaction buffer consisted of 20 mM HEPS (pH 7.4), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 20% glycerol, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Each reaction also contained 1 μl of reticulocyte lysate containing 35S-labeled Mcl-1 and also reticulocyte lysate system (Promega). The Mcl-1 siRNA sequence used above was included in the reaction buffer. After incubation at 30 °C for 90 min, the reaction products were cleavage reactions were performed in a total volume of 20 μl. The reaction buffer consisted of 20 mM HEPS (pH 7.4), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 20% glycerol, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Each reaction also contained 1 μl of reticulocyte lysate containing 35S-labeled Mcl-1 and also 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.

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 previously described (17). Following probing with a specific primary Ab and horseradish peroxidase-conjugated secondary Ab, the protein bands were detected by enhanced chemiluminescence (Pierce). Protein bands were quantified by Molecular Dynamics Densitometer SI and the ImageQuaNT software.

Mcl-1, Bim, and Caspase-3 RNAi—Short interfering RNAs for Mcl-1 and Bim were obtained as duplexes in purified and desalted form (Option C) from Dharmaco. The two siRNAs had the following sense strand sequences: Mcl-1-5′-GAAACCGGGGAAUUGCCACUdTdT-3′; Bim-5′-GACCCGAAGGUACUAAUUdIddTdT-3′. Human caspase-3 siRNA was obtained as the siGENOME SMARTpool reagent (M-0004307-01-0005, Dharmaco). The non-targeting siRNA control for Mcl-1, Bim, and caspase-3 siRNAs was also obtained from Dharmaco (siCONTROL Non-Targeting siRNA #1) with the sense strand sequence 5′-UAGGCCG- UAAACACACAAUU-3′. CAMA-1 cells (2.5 × 10⁵) were plated in a 6-well plate, and following 24 h (at ~30% confluency) were transfected with 200 nM siRNA in Opti-MEM medium (Invitrogen) without fetal calf serum using Oligofectamine reagent (Invitrogen) according to the manufacturer’s transfection protocol. After 4 h, fetal calf serum was added to a final concentration of 10%. At 40 h, the medium over the cells was adjusted to 1 ml before the addition of an apoptotic agent. Mcl-1 short hairpin RNA (shRNA) was generated using the siSTRIKE U6 Hairpin Cloning System (Promega). The Mcl-1 siRNA sequence used above was included in the following sense and antisense oligonucleotides: 5′-ACCGAGAACG- GTTAATCCGACCTTCTCTGCAAGTTACCGGTTTCTT- TTTTC-3′ and 5′-TGCGAGAAAAAGAACCGGTATCTGCC- TTGACAGAAGATCCGATTACCGGTTT-3′. Sense and antisense strands were annealed and ligated into the linearized psiSTRIKE NeoVector following the manufacturer’s directions. Sequence analysis of transformed randomly picked clones (University of Pittsburgh DNA Sequencing Core Facility) confirmed the sequence integrity of the Mcl-1 shRNA plasmids.

Stable Transfection—Hct116 or HeLa cells were washed in cold phosphate-buffered saline and resuspended in electroporation buffer (AMAXA) at a final concentration of 3 × 10⁶ cells/ml. 5 μg of linearized Mcl-1 plasmid, linearized pCR3.1 vector, or linearized Mcl-1 shRNA plasmid were mixed with 0.1 ml of cell suspension, transferred to a 2.0-mm electroporation cuvette, and nucleofected with an Amx200 Nucleofector apparatus (Amx200, Inc., Gaithersburg, MD) utilizing the appropriate program according to the manufacturer’s directions. Genetin-resistant cell lines were grown in the presence of G418 (1500 μg/ml). Genetin-resistant clonal cell lines expressing either Mcl-1 or Mcl-1 shRNA were generated by dakocytomation (1 cell/well) utilizing a MOFLO high speed cell sorter and Summit Software.

Flow Cytometry—Cytofluorometric analyses of apoptosis were obtained by staining with propidium iodide (PI) and fluorescein isothiocyanate-Annexin V conjugates (Becton Dickenson). Activity of caspase-3 and -8 was assessed with the caspase-specific Vybrant-FAM substrates and -8 was assessed with the caspase-specific Vybrant-FAM substrates from Molecular Probes. Mitochondrial depolarization was measured with the MitoProbe JC-1 and DiCl₃ (5) from Molecular Probes. The staining was performed according to the manufacturer’s procedures, assessed by a Beckman Coulter Epics XL-MCL and analyzed with the EXPO32 software.

Mitochondria Purification—To obtain an enriched mitochondrial fraction, HeLa cells were suspended in mitochondrial buffer (MIB) composed of 0.3 M sucrose, 10 mM MOPS, 1 mM EDTA, and 4 mM KH₂PO₄, pH 7.4, and lysed by Dounce homogenization as previously described (14). Briefly, nuclei and debris were removed by a 10-min centrifugation at 650 × g, and a pellet containing mitochondria was obtained by two successive spins at 10,000 × g for 12 min. To obtain the S-100 fraction, the postnuclear supernatant was further centrifuged at 100,000 × g for 1 h at 4 °C. To obtain the enriched mitochondrial frac-
tion, 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 × 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 × g and used immediately. Purity was assessed by electron microscopy and by enzyme marker analysis (18). For enzyme analysis, the following enzymes were assayed: aryl sulfatase (lysosomes/ granules); N-acetyl-β-D-glucosaminidase, α-L-fucosidase, and β-glucoronidase (lysosome); lactate dehydrogenase (cytosol); cytochrome oxidase; or monoamine oxidase (mitochondria); thiamine pyrophosphatase (Golgi); NADH oxidase (endoplasmic reticulum); dipeptidyl peptidase IV (plasma mem-
brane). The purity was assessed at 95%, with ~5% or less contamination from the microsomal fraction.

Release of Mitochondrial Apoptogenic Proteins—Purified mitochondria (50 μg of protein) were incubated with various doses of His-BimL in 25 μl of MIB at 37 °C for 30 min. Generation of His-BimL has been previously described (14). 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 apoptogenic proteins, cytochrome c, Smac, and HtrA2.

RESULTS

Involvement of Caspase-8 and Caspase-3 Activity in the Down-regulation of Mcl-1 during TRAIL-mediated Apoptosis—To investigate a potential role for Mcl-1 in the mitochondrial response to TRAIL, we assessed its expression in lysates of tumor cell lines treated with TRAIL.

FIGURE 3. TRAIL-mediated cleavage of Mcl-1 in caspase-3 knockdown or Bax knock-out cells. A and B, cleavage of Mcl-1 in caspase-3 knockdown cells. Hct116 (A) and CAMA-1 (B) cells were treated with caspase-3 siRNA for 42 h and subsequently treated with TRAIL (150 ng/ml) for 6 h. The cells were lysed, and the postlysis products were assessed by immunoblotting for the expression of caspase-3, Mcl-1, and caspase-8. β-Tubulin serves as an equal loading control. The asterisks indicate unidentified protein bands and the arrowheads indicate cleavage products. C, TRAIL resistance of Hct116 Bax−/− cells. Hct116 Bax−/− or Bax+/− cells were treated with the indicated doses of TRAIL for 16 h, and assessed by flow cytometry for staining with Annexin V-FITC and PI. One experiment of at least five performed is shown. D, cleavage of Mcl-1 in cells with disrupted mitochondrial apoptosis. Hct116 Bax−/− or Bax+/− cells were treated with TRAIL as described in C, and then were lysed and assessed by immunoblotting for the expression of the indicated proteins. In vitro translated Mcl-1ΔN127 was used as a migration marker for Mcl-1 cleavage product (top panel, right lane). The same membrane was stripped and reprobed for the various proteins.
Consistent with recent observations (19, 20), TRAIL induced a significant Mcl-1 degradation in multiple tumor cell lines, including HCT116 (Fig. 1A), CAMA-1, HeLa, and Jurkat. Mcl-1 degradation was accompanied by the processing of caspase-8 and caspase-3 that were blocked in the presence of the potent caspase inhibitor, Z-VAD-FMK. In the presence of this caspase inhibitor, neither a TRAIL-mediated loss in the level of Mcl-1 was detected nor was there cleavage of caspase-8. A certain level of p20 caspase-3 was detected in the presence of Z-VAD-FMK, but as the inhibitor binds to this fragment of caspase-3, no further processing of the p20 caspase-3 subunit to its p19 or p17 active forms was detected (Fig. 1A). Recent reports by us (14, 15) and others (21, 22) have demonstrated that Mcl-1 serves as a substrate for caspase-3, but its potential cleavage by caspase-8 has not yet been examined. In the TRAIL-mediated apoptotic cascade, activation of caspase-8 is mainly upstream of caspase-3. Because the potential cleavage of Mcl-1 by caspase-8 may serve as a new cross-talk mechanism between the extrinsic and the intrinsic apoptotic pathways, we examined the possibility that Mcl-1 also serves as a substrate for caspase-8. For this purpose, we added recombinant caspase-3 and recombinant caspase-8 into Hct116 cell extracts and assessed by immunoblotting changes in the levels of Mcl-1. In the presence of either caspase-3 or caspase-8 Mcl-1 was cleaved, generating fragments that exhibit similar migration patterns on SDS-PAGE (Fig. 1B). To confirm that Mcl-1 was cleaved directly by caspase-8, rather than by an unknown endogenous caspase-activated protease(s), we tested for the ability of recombinant caspase-3 or recombinant caspase-8 to directly cleave 35S-labeled in vitro translated Mcl-1 (Fig. 2A). As assessed by autoradiography, a similar pattern of cleavage was generated by the two recombinant caspases. We previously mapped the caspase-3 cleavage sites to aspartic acid residues 127 and 157 of Mcl-1 (15). To map the caspase-8 cleavage sites in Mcl-1, we utilized mutant Mcl-1 plasmids that possess single residue conversions of aspartic acid to alanine, including D72A, D127A, D157A, or a mutant for both residues 127 and 157. The mutated Mcl-1 plasmids were transcribed and translated by an in vitro system, and the products were treated with recombinant caspase-3 or caspase-8 (Fig. 2B). As determined by autoradiography, Mcl-1 cleavage by caspase-8 was mapped to the same aspartic acid residues, 127 and 157, as the cleavage by caspase-3. The double mutant for aspartic acid residues 127 and 157 was resistant to cleavage by each of these caspases. To further examine the possibility that Mcl-1 cleavage takes place in the absence of caspase-3, we used RNAi to knockdown the expression of caspase-3 in Hct116 and CAMA-1 tumor cell lines. Despite a significant knockdown of caspase-3 in each of these cell lines, treatment with TRAIL induced Mcl-1 cleavage in association with caspase-8 activation (Fig. 3, A and B). Interestingly, in Hct116 cells, increased TRAIL-induced activity of caspase-8 was detected in the absence of caspase-3 as compared with control siRNA cells.

To ascertain whether caspase activity involved in Mcl-1 cleavage can operate upstream of the mitochondria, and therefore is distinct from effector caspases like -3 and -7 that are activated downstream of mitochondrial involvement, we included in our studies the Hct116 Bax+/− clonal cell line and its counterpart the Hct116 Bax−/− clonal cell line. The Bax knock-out cells have been shown to be TRAIL-resistant because of Bax deficiency, despite caspase-8 activation in response to TRAIL (2, 12). In agreement with the previous reports, no Annexin V-positive cells were detected in Bax knock-out cells; these cells were independent of the reaction kinetics or the dose of TRAIL utilized (Fig. 3C). However, TRAIL treatment of these isogenic cell lines resulted in Mcl-1 cleavage in association with caspase-8 activation (Fig. 3D). As expected, caspase-3 and -7 were activated in Hct116 Bax−/−, but not in Bax+/− cells, where mitochondrial involvement is blocked (Fig. 3D). In Bax−/− cells, caspase-3 is processed down to the p20 subunit, but as reported previously, activation of this subunit requires mitochondrial involvement, particularly the release of Smac (12, 23). The more efficient processing of caspase-8 in Bax+/− as compared with Bax−/− Hct116 cells, may be produced by a secondary cleavage mediated by caspase-3 and -7 that are activated downstream of the mitochondrial involvement in Bax−/−, but not in Bax+/− Hct116 cells. Thus, utilizing conditions where caspase-3 and -7 are not activated, these results suggest that Mcl-1 is a substrate for caspase-8 upstream of the mitochondria.

Presence of Mcl-1-free Bim in Mcl-1 Knockdown Cells—We previously reported that a high affinity complex comprised of Mcl-1 and Bim is present at the outer mitochondrial membrane of non-apoptotic cells (14). Such a mechanism for sequestration of Bim in T lymphocytes has also been proposed by Korsmeyer’s studies (24). We have also reported that caspase-generated Mcl-1 fragments are subjected to further non-caspase-mediated degradation in the cell extracts, resulting in the eventual elimination of Mcl-1 and its cleaved fragments (15). To demonstrate that the degradation of Mcl-1 results in an excess of Mcl-1-free Bim, we mimicked the caspase-mediated down-regulation of Mcl-1 by knocking down Mcl-1 utilizing RNA interference. Cells treated with Mcl-1 siRNA were used to qualitatively assess the presence of Bim either in a complex with Mcl-1 or as a Mcl-1-free protein. Conversely, we utilized Bim knockdown cells to express Bim-free Mcl-1 (Fig. 4). Immunoprecipitation of Mcl-1 from either control or Bim-knockdown cells completely depleted the supernatant of Mcl-1 (Fig. 4A, lanes 4–6). Whereas Bim co-precipitated efficiently with Mcl-1 in control cells, in
A Role for Mcl-1·Bim Complex in TRAIL-mediated Apoptosis

The potential-sensitive color shift of JC-1 is caused by changes in the mitochondrial membrane potential. To investigate the functional linkage between Mcl-1 and Bim in TRAIL-mediated apoptosis, we assessed the apoptotic response of cells with a combined knockdown of both Mcl-1 and Bim (Fig. 5, A–C). Interestingly, the increased susceptibility to TRAIL that was gained by Mcl-1 knockdown was significantly, but not completely reversed by the presence of Bim siRNA in the same cells (Fig. 5, B and C). The incomplete reversal of the Mcl-1 knockdown-mediated TRAIL susceptibility may relate to the presence of Bim-free Mcl-1 as a part of the Mcl-1 cellular pool (Fig. 4E).

To further investigate the ability of Mcl-1 to protect the mitochondria from TRAIL-mediated disruption of the mitochondrial membrane potential, we utilized Hct116 colon carcinoma cells that were stably transfected with Mcl-1 (Fig. 6A). As assessed by two measures for mitochondrial membrane potential (JC-1 and DiIC1, Ref. 5), overexpression of Mcl-1 significantly inhibited the TRAIL-mediated dissipation of the mitochondrial membrane potential, even at a dose of TRAIL as high as 100 ng/ml (Fig. 6, B and C). Similarly, the Mcl-1 double mutant (D127A/D157A) transfected into Hct116 cells had an inhibitory effect on TRAIL-induced disruption of mitochondrial membrane potential, suggesting that the cleavage of Mcl-1 facilitates the apoptotic process (data not shown).

As our results suggest that the knockdown of Mcl-1 enhances the mitochondrial apoptotic response to TRAIL, we investigated whether it
will overcome the mitochondrial resistance that is mediated by Bax deficiency. To this end, we knocked down Mcl-1 expression in the two isogenic Hct116 clonal cell lines, Bax^+/−/Bax^−/− and Bax^+/−/Bax^+/+, using RNAi and assessed their apoptotic response to TRAIL. Knockdown of Mcl-1 did not compensate for the deficiency in Bax, and the majority of the cells maintained their TRAIL resistance (Fig. 6D). The mild increase (~10%) in Bax^+/− cell response to TRAIL following Mcl-1 siRNA treatment may relate to the ability of freed Bim to activate Bak (25). These findings suggest that the regulatory activity of the Mcl-1-Bim complex at the mitochondrial outer membrane is upstream of Bax, and that the effector function of Bax is required for the Mcl-1-Bim complex to mediate TRAIL susceptibility.

**Knockdown of Mcl-1 Enhances Caspase Activity Downstream of the Mitochondria**—The effects of Mcl-1 and Bim on the dissipation of mitochondrial membrane potential in response to TRAIL may contribute to the subsequent apoptotic events that take place either at the mitochondrial level, like the release of apoptogenic factors, or downstream of the mitochondria, as in the generation of the apoptosome and subsequent caspase activation. Indeed, immunoblot analysis of caspase-3 and caspase-8 demonstrated enhanced TRAIL-mediated processing of these caspases in Mcl-1-knockdown cells, compared with cells treated with control siRNA (Fig. 7, A and C). Enhanced caspase-3 activation in Mcl-1-knockdown cells is demonstrated by the increased processing of caspase-3 p20 into the p19 and p17 subunits (Fig. 7A). Interestingly, in Bim knockdown cells, the p20 subunit of caspase-3 did not undergo further autoprocessing, most likely because of a reduced release of mitochondrial IAP inhibitors, SMAC and HtrA2. Likewise, increased TRAIL-mediated loss of caspase-8 prodomain and production of its subunits was detected in Mcl-1 knockdown cells as compared with control CAMA-1 cells (Fig. 7D). To confirm the role of Mcl-1 in protection against caspase activation downstream of the mitochondria, we assessed the activity of caspase-3 and caspase-8 by flow cytometry, using fluorescent substrates that bind either active caspase-3 and -7 (DEVD-FMK), or active caspase-8 (LETD-FMK). Although immunoblotting did not detect any caspase-3 processing in cells treated with control siRNA alone (Fig. 7A), a low level of binding of fluorescent DEVD substrate was detected in these cells by flow cytometry (Fig. 7B). Such differential detection may relate to the higher sensitivity of the flow cytometry approach. As expected from the immunoblotting results, knockdown of Mcl-1 enhanced significantly the ability of TRAIL to activate caspase-3 and/or -7, whereas Bim knockdown reduced the TRAIL-induced activation of these caspases (Fig. 7B). Knockdown of these two Bcl-2 family members also affected the activity of caspase-8, albeit, to a lesser extent (Fig. 7D).
The Balance between Mcl-1 and Bim Regulates the Release of Mitochondrial Apoptogenic Proteins—To investigate the interactive regulatory roles of Mcl-1 and Bim in the release of apoptogenic proteins, we utilized a cell-free system composed of recombinant Bim and mitochondria purified from HeLa clonal cell lines that differed in the levels of Mcl-1 expression. Such HeLa clonal cell lines were obtained by stable transfection of the WT clonal cells with either a plasmid encoding Mcl-1 or a plasmid encoding Mcl-1 shRNA (Fig. 8A). The mitochondria were incubated with four escalating doses of His-Bim at 37 °C and controls were incubated under similar conditions or on ice. The release of mitochondrial apoptogenic proteins was assessed by immunoblotting (Fig. 8, B1, C1, and D1), and each of the protein bands (for a specific apoptogenic factor) was quantified relative to the signal generated by immunoblotting of that factor in the mitochondrial pellet utilized in the release reaction (not shown). The calculated ratios between the released protein and its input in the mitochondrial pellet are illustrated in Fig. 8, B2, C2, and D2. The Bim-mediated release of cytochrome c, Smac, and HtrA2 correlated to the level of Mcl-1 expression. Thus, mitochondria purified from Mcl-1 shRNA-transfected HeLa cells demonstrated increased release of each of the tested apoptogenic proteins as co-

![Image of a figure showing immunoblots and flow cytometry graphs.]
pared with mitochondria from cells transfected with Mcl-1 (Fig. 8, B2, C2, and D2). Interestingly, the spontaneous release of cytochrome c followed a different pattern than that of Smac and HtrA2. Whereas no release of cytochrome c was detected in control mitochondria incubated at 37 °C in the absence of Bim (Fig. 8B1), a certain level of either Smac or HtrA2 was detected (Fig. 8, C1 and D1). Furthermore, Mcl-1 knockdown rendered the mitochondria somewhat more permeable to Smac and HtrA2 even in the absence of exogenous Bim. Because of this increased spontaneous release from Mcl-1 shRNA mitochondria, only slight differences in the release levels of Smac and HtrA2 were detected between WT mitochondria and Mcl-1 shRNA mitochondria treated with the highest dose of Bim. However, at the same high dose of Bim (Fig. 8, B2, C2, D2, lane 6) a significantly higher release of cytochrome c was detected from the Mcl-1 shRNA mitochondria, compared with mitochondria from WT HeLa cells. These differences between the patterns of release of cytochrome c and Smac or HtrA2 may relate to differential electrostatic interactions of these proteins with the mitochondrial membrane as recently reported (28). The consistent differences between cells overexpressing Mcl-1 and Mcl-1 knockdown cells in the Bim-mediated release levels of cytochrome c, Smac and HtrA2 demonstrate that the ratio between Mcl-1 and Bim determines the release levels of these mitochondrial apoptogenic proteins.

DISCUSSION

The current study identified a novel regulatory mechanism for the mitochondrial cascade induced by TRAIL. Previous studies involving Bax-deficient tumor cells have established the significance of a mitochondrial amplification cascade for TRAIL-mediated apoptosis in certain cell types. Thus, Bax deficiency endows certain cell types with a significant TRAIL resistance, indicating the essential role of this apoptotic executioner in the extrinsic apoptotic cascade initiated via TRAIL-Rs (2, 12). Until now, Bid was the sole BH3-only protein considered as a Bax activator in the TRAIL-mediated cascade. In this communication pathway, caspase-8-cleaved Bid translocates to the mitochondrial outer membrane, where it activates Bax. Our studies identified a cross-talk pathway between the TRAIL death receptor and the mitochondrial executioner Bax that has not been previously considered. This cell membrane to mitochondrial membrane communication is delivered by the caspase-mediated disruption of the Mcl-1-Bim complex at the mitochondrial outer membrane. We demonstrate that Mcl-1 is a direct substrate for caspase-8, and that loss of Mcl-1 generates Mcl-1-free Bim that is involved in the ensuing apoptotic events. Although the involvement of Mcl-1 in TRAIL-mediated apoptosis has been documented previously (20), the exact mechanism(s) of Mcl-1 function remained unknown. The current study has elucidated the caspase-8/3-released mitochondrial apoptogenic proteins. 

FIGURE 8. The level of Mcl-1 expression regulates the mitochondrial permeability for apoptotic factors cytochrome c, Smac, and HtrA2. A, expression of Mcl-1 in HeLa cells stably transfected with plasmids encoding Mcl-1 or Mcl-1 shRNA. HeLa clonal cell lines transfected with plasmids encoding Mcl-1 or Mcl-1 shRNA were assessed by immunoblotting for the expression of Mcl-1. Controls including WT HeLa cells (shown), or HeLa cells stably transfected with vector controls for Mcl-1 or Mcl-1 shRNA (not shown) had comparable levels of Mcl-1 expression. The membrane was reprobed with β-actin to demonstrate equal loading. B, C, and D, Bim-mediated release of mitochondrial apoptogenic proteins is regulated by the level of expression of Mcl-1. Mitochondria were purified from WT HeLa cells or clones that were stably transfected with plasmids for Mcl-1 or Mcl-1 shRNA. The mitochondria were incubated with increasing doses of His-Bim (2–20 μM, lanes 3–6) at 37 °C for 30 min. Control mitochondria were incubated for a similar time either on ice (lane 1) or at 37 °C (lane 2) to assess spontaneous release. After a 30-min incubation, the mitochondrial supernatant (Mit-Sup) was assessed for the release of cytochrome c (B1), Smac (C1), or HtrA2 (D1). The released protein gel bands as well as the gel bands that represent total protein input (for a specific apoptogenic factor) in the respective mitochondrial pellet used in the release reaction (not shown) were quantified by a Molecular Dynamics Densitometer 51 and ImageQuaNT software. The ratios between the released protein and its original level in the mitochondrial pellet are plotted in B2 (cytochrome c), C2 (Smac), and D2 (HtrA2). The numbers indicated by the x-axis on B2, C2, and D2 correspond to the lane numbers indicated on B1, C1, and D1.
A Role for Mcl-1·Bim Complex in TRAIL-mediated Apoptosis

mediated cleavage of Mcl-1 as an initiating signal for the TRAIL-mediated mitochondrial cascade, and that unsequestered Bim is involved in the execution mechanism at the mitochondrial outer membrane.

Our previous studies have identified the disruption of Mcl-1·Bim complex as a mechanism utilized by granzyme B to induce a mitochondrial apoptotic cascade (14, 15). Mcl-1 is cleaved directly by granzyme B and indirectly by granzyme B-activated caspase-3. We have mapped the granzyme B cleavage sites to aspartic acid residues 117, 127, and 157, and the cleavage sites for caspase-3 to aspartic acid residues 127 and 157 (15). We also demonstrated that ectopic expression of the C-terminal cleavage products did not induce apoptosis, and did not enhance the apoptotic process induced by granzyme B or cytotoxic drugs. Rather, following the Mcl-1 initial cleavage, the resultant fragments underwent further degradation by other proteolytic activities present in the cytoplasm. In the present study, we demonstrate that Mcl-1 is a substrate for caspase-8, and that caspase-8, like caspase-3, cleaves Mcl-1 at aspartic acid residues 127 and 157. The cleavage of Mcl-1 by caspase-8 is significant, as it implies the potential existence of a novel cross-talk mechanism between the extrinsic and the intrinsic apoptotic pathways that may not require caspase-3 activity to initiate the intrinsic pathway. In support of such a possibility, we demonstrated that TRAIL-induced cleavage of Mcl-1 occurs in cells with a significant knockdown level of caspase-3. Utilizing the two isogenic clonal cell lines, Hct116 Bax<sup>−/−</sup> and Bax<sup>+/+</sup>, we further demonstrated that Mcl-1 cleavage occurs upstream of mitochondrial apoptotic events in association with the activation of caspase-8, but not caspase-3 or caspase-7. Our cumulative results suggest that TRAIL-induced caspase activity that targets the Mcl-1·Bim complex at the mitochondrial outer membrane represents a novel cross-talk mechanism between the TRAIL death receptor and the mitochondria.

A role for Mcl-1 in TRAIL-mediated apoptosis has been identified in cholangiocarcinoma cell lines whose TRAIL resistance was reversed by Mcl-1 RNAi (20). Our current study is the first to demonstrate the involvement of Bim in TRAIL-mediated apoptosis that is associated with loss of Mcl-1 function. Thus, we show that knockdown of Mcl-1 produces excess Mcl-1-free Bim that mediates its effect upstream of Bax. Although either Bax or Bak may mediate the mitochondrial porosity for apoptogenic proteins, our previous studies demonstrated that TRAIL preferentially utilizes Bax over Bak for the induction of mitochondrial apoptotic events (23). These findings are in line with the reported resistance to TRAIL-mediated apoptosis in Bax-deficient Hct116 cells (2, 12). The involvement of the Mcl-1·Bim complex in TRAIL-mediated apoptosis is upstream of Bax, since Mcl-1 RNAi did not reverse the resistance of Bax-deficient Hct116 cells to TRAIL. The function of Bim through either Bax or Bak has recently been demonstrated to be mediated by thymocytes from Bim and Bax or Bim and Bak double knockout mice (25, 29). Thus, thymocytes lacking Bim and Bak or Bak displayed a marked reduction in apoptosis, although not as complete as that observed in thymocytes lacking both Bax and Bak. Additional support for the possibility that Bim acts directly on Bax/Bak was provided in a recent report demonstrating that the Bim BH3 peptide, but not the PUMA BH3 peptide, was sufficient to induce conformational changes, oligomerization, and activation of Bax and Bak to permeabilize the mitochondrial membrane (29).

Our studies demonstrate that sequestration of Bim by Mcl-1 plays a role in preserving the mitochondrial membrane potential. The mitochondria of healthy cells maintain an electrochemical gradient across the mitochondrial inner membrane that is created by pumping protons from the matrix to the intermembrane space in conjunction with electron transport through the respiratory chain. The proton gradient and membrane potential are the proton-motive force that is used to drive ATP synthesis. Coupling of electron transport through the respiratory chain and ATP generation can be disrupted by some acidic aromatic substances, such as CCCP and 2,4-dinitrophenol. Such uncouplers of oxidative phosphorylation carry protons across the inner mitochondrial membrane, leading to a reduction in the mitochondrial membrane potential and a collapse of the pH gradient. TRAIL-mediated apoptosis is associated with similar alterations in the mitochondrial membrane potential.

Our studies suggest that in CAMA-1 cells, Bim is a major player in mediating these alterations, since they are significantly blocked in Bim knockdown cells. The functional linkage between Mcl-1 and Bim is demonstrated by the ability of Bim knockdown to reverse TRAIL susceptibility that is gained through Mcl-1 knockdown. Additional support for the interactive regulatory roles that Mcl-1 and Bim play in the development of a mitochondrial apoptotic cascade is provided by the cell-free system for purified mitochondria that we employed. Thus, purified mitochondria from HeLa cells stably transfected with plasmids encoding either Mcl-1 or Mcl-1 shRNA were assessed for the release levels of apoptogenic proteins in response to recombinant BimL. In this in vitro system, the ratio between endogenous Mcl-1 and exogenous Bim determined the release levels for the apoptogenic proteins cytochrome c, Smac, or HtrA2. Although in vivo, Bim is also sequestered by Bcl-2 and Bcl-XL, the majority of Bim is bound by Mcl-1 (14, 27). Therefore, Mcl-1 plays a dominant role in the mitochondrial cascade executed by Bim.

In summary, the current findings are significant on two levels: they elucidate a new communication mechanism between the extrinsic and the intrinsic apoptotic pathways, and they identify Bim as a prominent player for TRAIL-mediated apoptosis in cells that require a mitochondrial amplification cascade for their subsequent demise.

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