Mcl-1 interacts with truncated Bid and inhibits its induction of cytochrome c release and its role in receptor-mediated apoptosis

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Running title: Mcl-1 binds tBid and blocks cytochrome c release

SUMMARY

Engagement of death receptors such as TNF-R1 and Fas brings about the cleavage of cytosolic Bid to truncated Bid (tBid), which translocates to mitochondria to activate Bax/Bak, resulting in the release of cytochrome c. The mechanism underlying the activation, however, is not fully understood. Here, we have identified the anti-apoptotic Bcl-2 family member Mcl-1 as a potent tBid-binding partner. Site-directed mutagenesis reveals that the BH3 domain of tBid is essential for binding to Mcl-1, whereas all three BH domains (BH1, BH2, and BH3) of Mcl-1 are required for interaction with tBid. Site-directed mutagenesis reveals that the BH3 domain of tBid is essential for binding to Mcl-1, whereas all three BH domains (BH1, BH2, and BH3) of Mcl-1 are required for interaction with tBid. In vitro studies using isolated mitochondria and recombinant proteins demonstrate that Mcl-1 strongly inhibits tBid-induced cytochrome c release. In addition to its ability to interact directly with Bax and Bak, tBid also binds Mcl-1 and displaces Bak from the Mcl-1/Bak complex. Importantly, overexpression of Mcl-1 confirms resistance to the induction of apoptosis by both TRAIL and TNF-α in HeLa cells, whereas targeting Mcl-1 by RNA interference sensitises HeLa cells to TRAIL-induced apoptosis. Therefore, our study demonstrates a novel regulation of tBid by Mcl-1 through protein-protein interaction in apoptotic signalling from death receptors to mitochondria.

INTRODUCTION

The commitment of cells to apoptosis in response to diverse physiological cues and cytotoxic agents is primarily regulated by proteins of the Bcl-2 family that are evolutionarily conserved from nematodes to humans (1,2). Bcl-2 family proteins share one or more Bcl-2 homology (BH) domains and are divided into two main groups based on their pro- or anti-apoptotic activities. The anti-apoptotic members include Bcl-2, Bcl-xL, A1, Bcl-w and Mcl-1. The pro-apoptotic family members are further divided according to whether they contain multiple BH domains (such as Bax and Bak) or only the BH3 domain (such as Bid and Bim). The BH3 domain-only proteins require cooperation of other multi-domain family members to induce apoptosis (3-7).

In mammals two distinct apoptotic signalling pathways have been identified (8,9). In the extrinsic pathway, apoptosis is initiated through ligand binding to cell surface receptors of the tumour necrosis factor (TNF) family such as TNF-R1 and Fas. Upon ligation these receptors initiate the formation of a death-inducing signalling complex, which consists of adaptor molecules such as Fas-associated death domain protein
(FADD) and pro-caspase-8. Within the complex, caspase-8 undergoes auto-proteolytic activation. Once activated, caspase-8 can activate downstream caspases, for example, caspase-3 and -7, leading to orderly degradation of intracellular substrates and cell death. The cell-intrinsic pathway is initiated when the integrity of the outer mitochondrial membrane is lost in response to diverse apoptotic stimuli. This results in the release of cytochrome c and other apoptotic proteins into the cytoplasm, where cytochrome c binds to apoptotic protease activating factor 1 (Apaf-1). Apaf-1 in turn recruits pro-caspase-9 to form a multimeric complex, which leads to auto-proteolytic activation of caspase-9. The active caspase-9 then efficiently activates other downstream caspases, bringing about the morphological changes characteristic of apoptosis. This intrinsic pathway is thus mitochondria-dependent and tightly controlled by the Bcl-2 family proteins.

Although the two apoptotic pathways can function independently, an existing link between them is the BH3 domain-only protein Bid that is cleaved by active caspase-8 following engagement of death receptor Fas (10,11). Cleaved Bid, also known as truncated Bid (tBid), translocates to mitochondria to induce oligomerisation of Bax and/or Bak and cytochrome c release (4,12). In both Bax−/− and Bak−/− cells, tBid fails to induce cytochrome c release and apoptosis, suggesting that it requires Bax and/or Bak to exert its mitochondrial pro-apoptotic activity (5). However, the underlying mechanism and, in particular, the sequence of events that occurs after Bid cleavage and prior to cytochrome c release are not completely defined. In this study we investigated the mechanism of tBid-induced activation of the mitochondrial apoptotic pathway by searching for novel tBid interacting proteins using a yeast two-hybrid screen. We identified the anti-apoptotic Bcl-2 family protein Mcl-1 as a genuine tBid-binding partner. Further studies demonstrate that Mcl-1 effectively inhibits tBid-induced cytochrome c release from mitochondria and protects HeLa cells from apoptosis induced by both tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and TNF-α.

**EXPERIMENTAL PROCEDURES**

**Materials and Cell Culture** -- All media and cell culture reagents were purchased from Life Technologies (Paisley, UK). Other chemicals, unless otherwise stated, were obtained from Sigma (Poole, UK). Human cervical carcinoma HeLa cells were obtained from American Type Culture Collection (Rockville, MD), and cultured in DMEM, supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.5 U/ml penicillin and 0.5 mg/ml streptomycin.

**Cloning of cDNAs and Plasmids Construction** -- Mouse tBid cDNA was amplified by PCR from a murine pcDNA3-Bid template (gift from Dr. S. Korsmeyer, Harvard Medical School, Boston, MA) and inserted into EcoR I - BamHI sites downstream of the GAL4-DBD in the vector pGBK7T (Clontech, Palo Alto, CA). A cDNA encoding mouse Bax lacking the C terminal hydrophobic domain (BaxΔ21) was amplified by PCR from a pcDNA3-BaxΔ template (13) and cloned downstream of the GAL4-AD in pGADT7 vector (Clontech). A pUC18-bcl-2 construct (Clontexpress, Gaithersburg, MD) was used to clone the human Bcl-2 cDNA. This construct was digested with EcoR I and Hind III to release the Bcl-2 cDNA which was then ligated into the corresponding sites in pcDNA3.1/myc-HIS©(-) (Invitrogen, Groningen, The Netherlands). Human Bid and tBid cDNA were generated by RT-PCR using oligo-dT primer with total RNA obtained from human leukaemic Jurkat T cells. Both cDNAs were then cloned at the EcoR I - BamHI sites downstream of the HA-tag in pcDNA3.1-HA (Invitrogen) (14). The construct expressing HA-tagged human Mcl-1 was generated as previously described (14). GST fusion protein constructs were generated by PCR using pcDNA3.1- HA-Bid and -tBid as templates and cloned into the EcoR I - Xho I sites in the pGEX-6P-2 vector (Amersham, Little Chalfont, UK). Mutations in BH domains of tBid and Mcl-1 proteins were generated by site-directed mutagenesis using the GeneTailor™ Site-Directed Mutagenesis System according to manufacturer’s instruction (Invitrogen). The G94E mutation of BH3 domain of tBid was generated by PCR using the pGEX-tBid construct as a template. Using pcDNA3.1-HA-Mcl-1 as a template for PCR of all Mcl-1 mutants, we generated the G262E mutation at BH1 domain, the W305A and W312A double mutations for BH2...
domain, and the G217E and D218A double mutations of BH3 domain of Mcl-1. The cDNA fragment encoding human Mcl-1 generated by PCR was inserted into the Nde I and Sap I sites of the pTYB1 vector for the expression of recombinant human Mcl-1 protein (New England Biolabs, Beverly, MA). The accuracy of the molecular identity of all constructs was confirmed by sequencing. For details of the PCR primers used, see Supplemental data.

Yeast Two-hybrid Assay--All yeast two-hybrid procedures were carried out according to the manufacturer’s protocol (Clontech). The cDNA for mouse truncated Bid was cloned into the pGKT7 vector (Clontech) as described earlier. The cDNA library was generated from mouse primary thymocytes that had been treated with 5 Gy γ-irradiation and cultured for 5 hours to induce apoptosis. The RNA was then isolated from these cells and cDNA library was prepared by oligo dT priming and directionally cloned in the EcoRI – Xho I sites of the prey vector pAD-Gal4-2.1 (Stratagene, La Jolla, CA). The library was amplified once and found to have over 90% recombinants with an average insert size of 1.5 Kb. Screening was carried out by sequential transformation of the tBid construct followed by the cDNA library into yeast strain Y190. After transformation, the yeast were grown for 15 days on selection plates containing 17 mM 3-amino-1,2,4-triazole (3-AT). Colonies that grew on the plates were tested for activity of β-galactosidase reporter gene by filter-lift assay. Plasmids from the positive colonies were isolated and subject to PCR and sequencing to identify the prey cDNAs. Specificity of the interaction was confirmed by retransformation.

GST-fusion Protein Production and Binding Assay--GST-fusion proteins were produced in BL21 E. coli following the induction of expression by IPTG (Insight Biotechnology, Middlesex, UK), and purified using glutathione-sepharose beads (Amersham). In vitro translated proteins labelled with [35S]-methionine were prepared using the TNT® T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI), using pcDNA3.1-HA constructs as templates. The [35S]-labelled prey proteins were incubated with GST, GST-Bid or GST-tBid fusion proteins bound to glutathione-sepharose beads in bead-binding buffer (50 mM potassium phosphate, pH7.5, 150 mM KCl, 1mM MgCl₂, 10% glycerol, 1% Triton X-100) and protease inhibitors cocktail from Roche (Lewes, UK). The mixtures were incubated at 4°C for 2 hours with rotation. The beads were then pelleted and washed 5 times in ice-cold bead-binding buffer. Finally beads were re-suspended in SDS sample buffer and proteins resolved on SDS-PAGE gels, which were fixed, vacuum dried onto 3MM paper and then visualised using a phosphorimager (Typhoon 8600, Amersham).

Immunoprecipitation--For each immunoprecipitation experiment, HeLa cells were transfected with HA-tBid in pcDNA3.1 or empty vector in the presence of 75 μM Z-VAD.fmk (Enzyme System, Dublin, CA). Immunoprecipitation was essentially carried out as described (15). Briefly, 24 hours after transfection, cells are harvested and re-suspended in ice-cold lysis buffer containing 2% 3-[3-cholamidopropyl]-dimethyl-ammonio]-1-propanesulfonate (CHAPS), 20 mM Tris/HCl (pH7.4), 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100 and protease inhibitor cocktail (Roche). Lysates were precleared and then incubated with either mouse anti-HA monoclonal antibody (clone 12CA5, Roche) or rabbit anti-Mcl-1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 1 hour, and protein A sepharose beads (Pharmacia, Piscataway, NJ) was added to pull down the immuno-complexes. The beads were washed 5 times in washing buffer containing 0.2% Triton X-100, 20 mM Tris/HCl (pH7.4), 137 mM NaCl, 2 mM EDTA, 10% glycerol before being resuspended in SDS sample buffer and subject to SDS-PAGE. Immunoblotting was performed using, where appropriate, goat anti-Bid polyclonal antibody (R&D Systems, Minneapolis, MN), rabbit anti-Mcl-1 polyclonal antibody (Santa Cruz), rabbit anti-Bcl-2 polyclonal antibody (Santa Cruz), mouse anti-Mcl-1 monoclonal antibody (Chemicon International, Temecula, CA), rabbit anti-Bak (NT) polyclonal antibody (Upstate Biotechnology, Lake Placid, NY), or rabbit anti-Bax (NT) polyclonal antibody (Upstate). For quantification of Bak and Mcl-1 levels, bands representing respective Bak and Mcl-1 from membranes of three independent immunoprecipitation experiments were scanned and analysed using a GS-800 Calibrated...
Densitometer with Quantity One software (Bio-Rad Laboratories, Hercules, CA).

**Immunoblotting--SDS-PAGE** and immunoblotting were performed essentially as described (16). Briefly, cellular proteins were resolved on the polyacrylamide gels and transferred to nitrocellulose membrane (Amersham). The membranes were probed with, where appropriate, goat anti-Bid polyclonal antibody (R&D Systems, Minneapolis, MN), rabbit anti-Mcl-1 polyclonal antibody (Santa Cruz), mouse anti-cytochrome c monoclonal antibody (clone 7H8.2C12, Pharmingen, San Diego, CA), mouse anti-cytochrome oxidase subunit II monoclonal antibody (clone 12C4-F12, Molecular Probes, Eugene, OR), mouse anti-PARP monoclonal antibody (clone C2-10, R&D Systems), or rat anti-α-tubulin monoclonal antibody (Serotec, Oxford, UK). After incubating with respective secondary antibodies conjugated with HRP, the membranes were visualised by ECL Kit (Amersham).

**In vitro Assay for Mitochondrial Cytochrome c Release**--This assay was performed as described (17) with minimum modification. In brief, 20 × 10^6 HeLa cells were harvested and washed once in ice-cold PBS. The cell pellet was re-suspended in 5 x volume of buffer A (20 mM HEPES, pH7.4, 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl_2, 1mMEDTA, 1mM EGTA, 1mM DTT and protease inhibitor cocktail from Roche) and incubated on ice for 15 minutes. Cells were then disrupted by passing them through a 23-gauge needle 25 times before undergoing centrifugation in two sequential steps: 1000g and 10,000g. The 10,000g pellet was collected as mitochondrial fraction and re-suspended at a 5 µg/µl concentration in buffer A supplemented with 150 mM NaCl. Re-suspended mitochondria were incubated either alone or with caspase-8-cleaved recombinant human Bid (R&D Systems) at indicated concentrations at 37°C for 15 minutes. Following incubation, the mitochondria were centrifuged, with the resulting supernatant collected for examination of cytochrome c release by immunoblotting and the pellet cross-examined for loss of cytochrome c and cytochrome oxidase subunit II as sample loading control.

**Preparation of Recombinant Human Mcl-1**--The cDNAs of full-length human Mcl-1 and mutant Mcl-1mtBH3 were cloned into pTYB1 vector (New England Biolabs), which were used to transform BL21 cells, respectively. The recombinant proteins were induced with the addition of IPTG and purified according to manufacturer’s instruction (New England Biolabs). The proteins were further concentrated using centrifugal filter devices (Amicon Ultra-4 30,000 MWCO) (Millipore, Bedford, MA).

**Generation of Stably Transfected Cell Lines and Induction of Apoptosis**--HeLa cells were split to 40 – 50% confluence in 10 cm dishes the day prior to transfection and transfected with 10 µg of pcDNA3.1-HA or pcDNA3.1-HA-Mcl-1 using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturers instructions. 24 hour after transfection cells were split and cultured under selection with 1 mg/ml G418 (Life Technologies). Single cell clones were picked and expanded to establish the stable Mcl-1 over-expressing cells. Overexpression of the Mcl-1 protein was confirmed by immunoblotting. To induce apoptosis, wild type, vector only stable and HA-Mcl-1 over-expressing stable HeLa cells were all treated with soluble recombinant human TRAIL (Alexis Biochemicals, San Diego, CA) at indicated concentrations for 14 hours. Cells are also treated with TNF-α (15 ng/ml) in the presence of cycloheximide (CHX, 30 µg/ml) for 22 hours. Apoptosis was assessed by flow cytometry for cells with sub-G1 DNA content following propidium iodide (PI) staining and PARP cleavage as previous described (16).

**Lentivirus generation and expression of Mcl-1short hairpin RNA** -- A BLOCK-iT Lentiviral RNAi Expression System (Invitrogen) was used according to manufacturer’s instruction. Briefly, RNAi sequence for human Mcl-1, GGACTGGCTAGTTAAACAAAG, was identified using manufacturer’s RNAi Designer programme and corresponding oligonucleotides were cloned into pENTR™/U6 vector (Invitrogen). RNAi sequence for mouse eleven-nineteen leukaemia (ENL) gene, GCTGTGAGAAGCTCACCTTCA, was used to produce control shRNA and its oligonucleotides were also cloned into the vector. DH5α E. coli (Invitrogen) were transformed and clones were verified by sequencing. The correctly identified clones were transferred via a gateway reaction to a modified pLenti6/BLOCK-iT™-DEST vector (Invitrogen), a promoterless lentiviral destination.
vector in which the Blasticidin resistance marker is replaced with tailless human CD2 as a marker. 293 cells were transfected with the plasmids using Lipofectamine reagent (Invitrogen) to produce the virus. 48 h later, the lentivirus-containing supernatants were harvested to infect HeLa cells in the presence of polybrene. The infected HeLa cells were harvested 48 h later for the analysis of Mcl-1 expression by immunoblotting. Induction of apoptosis by TRAIL was performed essentially as described above.

RESULTS

Truncated Bid Interacts with Mcl-1--We initially performed a yeast two-hybrid screen using truncated Bid as the bait protein and identified a number of tBid-interacting proteins. These included two anti-apoptotic Bcl-2 family proteins Mcl-1 and Bcl-2. Only one positive clone representing a cDNA of Bcl-2 was found to interact with tBid, consistent with a previous report that Bid interacted with Bcl-2 (18). There were, however, three independent positive clones identified representing cDNAs encoding Mcl-1, and the in-frame amino acid sequences from each clones were aligned against the full length murine Mcl-1 (19) as shown in Figure 1A. One positive clone (clone 85) containing the shortest Mcl-1 cDNA lacked the 5' region encoding for the first 144 amino acids, suggesting that the C-terminus fragment of the Mcl-1 protein containing the BH1, BH2 and BH3 domains was responsible for interacting with tBid. To exclude the possibility of false interaction, the plasmids from the positive clones were isolated and re-transformed into yeast containing the plasmid of tBid cDNA, and the activity of the E-galactosidase reporter gene was examined using the filter-lift assay. As Bid has also been reported to interact with Bax (18), we used Bax as positive control with the mouse Baxα cDNA cloned in the pGADT7 vector during re-transformation. Filter-lift assay detected the E-galactosidase activity in the clone transformed with plasmid containing Bcl-2 cDNA in the presence of tBid (Fig. 1B, upper right panel, with middle right panel).

To see if the interaction of Mcl-1 with tBid could be confirmed by an independent method, we performed GST fusion protein pull-down experiments. GST-Bid and GST-tBid fusion proteins were immobilised on glutathione-sepharose beads, respectively, and incubated with either [35S]-methionine labelled Mcl-1 or Bcl-2 (Fig. 2A). This assay identified Mcl-1 as a protein specifically interacting with tBid rather than the full length Bid (Fig. 2A, comparing lane 4 with lane 3, upper panel). Bcl-2 was also seen to preferentially interact with the truncated form of Bid (Fig. 2A, comparing lane 4 with lane 3, lower panel). Similar to the result shown in Figure 1B, tBid fusion protein again showed a higher affinity for Mcl-1 than Bcl-2 (Fig. 2A).

Next, we carried out co-immunoprecipitation experiments to check if this interaction occurs within cells. A HA tagged tBid construct was generated and subsequently used to over-express tBid in HeLa cells in the presence of the pan caspase inhibitor Z-VAD.fmk to delay cell death. Cells were lysed 24 h after transfection and immunoprecipitation was done using an anti-HA antibody. The endogenous Mcl-1 was detected in complex with HA-tBid by immunoblotting (Fig. 2B, upper panel, left). In addition, endogenous Bcl-2 was also detected in complex with HA-tBid (Fig. 2B, lower panel, left), in agreement with an early report (18). Similarly, when an anti-Mcl-1 antibody was used to immunoprecipitate Mcl-1 complex from lysates of cells over-expressing HA-tBid, tBid was clearly observed to interact with Mcl-1 (Fig. 2B, right panel). It is important to mention that the endogenous full-length Bid was not immunoprecipitated by the anti-Mcl-1 antibody under the experimental conditions used (data not shown), further underlining the specificity of the interaction between Mcl-1 and the truncated form of the Bid protein.

BH3 Domain of tBid and All Three BH Domains of Mcl-1 Are Required for Interaction--We then investigated the binding sites responsible for the interaction between tBid and Mcl-1. Based on previous reports describing the amino acids critical for interactions between Bcl-2 family proteins (18,
and our observation from the yeast two-hybrid experiment that the C terminal fragment of Mcl-1 was required for interaction, we hypothesised that the interaction between Mcl-1 and tBid involved the BH domains of both proteins. To test this hypothesis we generated a number of constructs that contained cDNAs encoding for Mcl-1 and tBid but with their BH domains mutated. First, we compared the abilities of GST-tBid with mutant GST-tBid (mtBH3) (containing G94E mutation at BH3 domain) to interact with \[^{35}\text{S}]\text{-methionine labelled Mcl-1. GST-tBid, but not mutant GST-tBid (mtBH3), was shown to interact with Mcl-1 (Fig. 3A, compare lane 3 with lane 4). This indicated that the BH3 domain of tBid was required for interaction with Mcl-1. Next, we compared the binding ability of wild type Mcl-1 to GST-tBid with that of Mcl-1 proteins that had mutations at BH1 (G262E mutation), BH2 (W305A and W312A double mutations) or BH3 (G217E and D218A double mutations) domain, respectively. As shown in Figure 3B, apart from wild type Mcl-1, all three Mcl-1 proteins with their respective BH domain mutated failed to interact with GST-tBid. Mutations in BH1 and BH3 domains resulted in a complete loss of interaction whereas mutations in BH2 domains severely impaired the binding ability of Mcl-1 to GST-tBid. These results demonstrated that all three BH domains of Mcl-1 contribute to its ability to interact with tBid.

**Mcl-1 Prevents tBid-mediated Cytochrome c Release**—We then studied the functional significance of the interaction between tBid and Mcl-1. As tBid has been shown to possess potent cytochrome c release activity (10,11), an *in vitro* assay was set up to examine this activity. Mitochondria isolated from HeLa cells were treated with increasing amount of recombinant tBid, and as shown in Figure 4A (upper panel), tBid induced cytochrome c release into the supernatant in a dose-dependent manner. As a positive control, 30 \(\mu\text{g}\) of untreated mitochondria was used for the detection of cytochrome c (Fig. 4A, lane 1, upper panel). The same blot was re-probed for cytochrome oxidase (subunit II), a mitochondrial membrane protein and it confirmed that the supernatant samples were free from mitochondrial contamination (Fig. 4A, lanes 2-5, lower panel).

As Mcl-1 is an anti-apoptotic protein, we reasoned that its ability to interact with tBid would possibly interfere with the function of the tBid protein. To test this, isolated mitochondria from HeLa cells were pre-incubated with increasing amount of recombinant Mcl-1 protein before the addition of tBid. Recombinant Mcl-1 alone (5 ng/\(\mu\text{l}\)) had no effect on the release of cytochrome c (Fig. 4B, lane 2, *supernatant*). Treatment of mitochondria with tBid (1 ng/\(\mu\text{l}\)) resulted in the release of cytochrome c from mitochondria (Fig. 4B, lane 3, *supernatant*), which is further confirmed by the disappearance of cytochrome c in the mitochondrial fraction (Fig. 4B, lane 3, upper panel, *pellet*). At a concentration of 1 ng/\(\mu\text{l}\), recombinant Mcl-1 failed to block tBid-mediated release of cytochrome c (Fig. 4B, lane 4, *supernatant*). At 2 ng/\(\mu\text{l}\) concentration, Mcl-1 significantly inhibited the release (Fig. 4B, lane 5, *supernatant*), whereas at 5 ng/\(\mu\text{l}\) Mcl-1 completely prevented tBid-induced cytochrome c release (Fig. 4B, lane 6, *supernatant*). The complete protection by Mcl-1 (5 ng/\(\mu\text{l}\)) was also confirmed by the full retention of cytochrome c in the mitochondrial fraction (Fig. 4B, lane 6, upper panel, *pellet*). The same membrane from the mitochondrial fraction was also re-probed for tBid to confirm its presence (Figure 4B, middle panel, *pellet*) and for cytochrome oxidase as sample loading control (Fig. 4B, lower pane, *pellet*). Thus, recombinant Mcl-1 inhibited tBid-induced cytochrome c release from the mitochondria in a dose-dependent manner. To further confirm that Mcl-1 inhibits tBid function through its interaction with tBid, we generated a recombinant Mcl-1 protein containing G217E and D218A double mutations in its BH3 domain (Mcl-1mtBH3), which, we have previously shown, failed to interact with tBid (Fig. 3B, lower panel). Pre-incubating mitochondria with the recombinant Mcl-1mtBH3 protein itself (5 ng/\(\mu\text{l}\)) had no effect on the release of cytochrome c (Fig. 4C, lane 5, *supernatant*). However, it did not protect mitochondria from tBid (1 ng/\(\mu\text{l}\)) mediated cytochrome c release (Fig. 4C, lane 4, *supernatant*), whereas recombinant wild type Mcl-1 (5 ng/\(\mu\text{l}\)) effectively blocked this release (Fig. 4C, lane 3, *supernatant*). This is also confirmed by the observation that the loss of cytochrome c in the mitochondria occurred when
both recombinant Mcl-1mtBH3 protein and tBid were present (Fig. 4C, lane 4, upper panel, pellet), but was prevented by recombinant wild type Mcl-1 (Fig. 4C, lane 3, upper panel, pellet). Thus, our data provided compelling evidence for a functional effect of the interaction between Mcl-1 and tBid.

**Truncated Bid Displaces Bak from Mcl-1-Bak Complex**—Recently it has been shown that Mcl-1 forms a complex with Bak in healthy, unstressed cells (23,24), we therefore wanted to examine what happens to this complex when tBid was present. We used a HA tagged tBid construct to over-express tBid in HeLa cells in the presence of the pan caspase inhibitor Z-VAD.fmk as previously described. Immunoprecipitation using an anti-HA antibody showed that tBid interacted with both endogenous Bak and Bax (Fig. 5A, upper and lower panels, respectively), which was consistent with the published reports (3,4). Immunoprecipitation using anti-Mcl-1 antibody showed that Bak indeed interacted with Mcl-1 in cells transfected with empty vector, as detected by immunoblotting (Fig. 5B, lane 1, upper panel). However, when cells were transfected with the HA-tBid construct the level of Bak in complex with Mcl-1 was greatly reduced (Fig. 5B, lane 2, upper panel), whereas the total amount of Mcl-1 in both immunoprecipitated samples was similar (Fig. 5B, middle panel). Interaction of Mcl-1 with tBid was again confirmed by re-probing Bid on the same membrane (Fig. 5B, lane 2, lower panel). We also probed for Bax and could not detect Bax in the samples immunoprecipitated by anti-Mcl-1 antibody under the experimental conditions used (data not shown). To test the possibility that overexpression of HA-tBid may alter the expression levels of endogenous Bak and/or Mcl-1, resulting in the reduction of Bak in complex with Mcl-1, we also checked the levels of Bak, Mcl-1 and tBid in the total cell lysates prior to the immunoprecipitation. Immunoblotting analysis showed that the expression levels of both Bak and Mcl-1 remained unchanged (Fig. 5B, lanes 3 and 4, upper and middle panels), regardless of the presence of HA-tBid (Fig. 5B, lane 4, lower panel). Densitometric analysis of bands representing respective Bak and Mcl-1 on membranes from three independent immunoprecipitation experiments indicated that the relative level of Bak to Mcl-1 was about four-fold less in immunoprecipitated sample from cells over-expressing tBid than that from control cells (Fig. 5C). Statistical analysis by Student t-test showed that the difference was significant (p<0.05). Therefore, in addition to its ability to interact directly with Bak and Bax, tBid can also bind Mcl-1 and displace Bak from the Mcl-1/Bak complex.

**Mcl-1 Inhibits Apoptosis Induced by TRAIL and TNF-α in HeLa cells**—Since Bid has been shown to be cleaved early during the induction of apoptosis by TRAIL in HeLa cells (25,26), we wished to investigate whether cells overexpressing Mcl-1 would be resistant to apoptosis induced by TRAIL. HeLa cells were transfected with either pcDNA3.1 vector alone or human Mcl-1 cDNA expression construct and selected in G418. Single cell clones were picked and expanded to establish the stable Mcl-1 over-expressing cells. The level of Mcl-1 expression was evaluated by immunoblotting, which showed that its level is higher in cells transfected with the Mcl-1 construct than those with vector only or wild type HeLa cells (Fig. 6A). Induction of apoptosis was assessed by flow cytometry for sub-diploid DNA content (sub-G1) following propidium iodide staining and PARP cleavage, a biochemical marker of apoptosis. As shown in Figure 6B, treatment of wild type HeLa cells with increasing doses of TRAIL resulted in a dose-dependent induction of apoptosis. This treatment also caused a similar dose-dependent induction of apoptosis in cells transfected with vector alone (Fig. 6B). Cells stably over-expressing Mcl-1 were, however, consistently protected from cell death following treatment with TRAIL at all concentrations (Fig. 6B). Student t test analysis showed that the reduction in TRAIL (250 ng/ml)-induced apoptosis of cells over-expressing Mcl-1, when compared with cell death in wild type HeLa cells and in cells transfected with vector alone, was statistically significant (both p<0.01). Also, treatment with increasing amount of TRAIL resulted in a dose-dependent cleavage of PARP in wild type and vector-only transfected HeLa cells (Fig. 6B). PARP cleavage was less complete in Mcl-1 over-expressing cells than that seen in wild type and vector-only transfected HeLa cells following treatment of TRAIL at 250 ng/ml
concentration (Fig. 6B, comparing lane 15 with lanes 13 and 14, respectively, \textit{PARP}).

As TNF-\alpha has been shown to induce apoptosis in HeLa cells through a Bid-dependent pathway (15), we also treated the above cells with TNF-\alpha to see if cells over-expressing Mcl-1 would be resistant to apoptosis induced by TNF-\alpha. Treatment with DMSO or cycloheximide (30 \mu g/ml) alone did not cause significant increase in cell death in all three types of cells (data not shown). Treatment with TNF-\alpha (15 ng/ml) in the presence of cycloheximide for 22 h resulted in similar levels of apoptosis in wild type HeLa cells and cells stably transfected with vector alone (Fig. 6C, lanes 2 and 4, respectively). Cells stably over-expressing Mcl-1 were indeed partially resistant to TNF-\alpha-induced cell death (Fig. 6C, lane 6). The reduction in TNF-\alpha-induced apoptosis in cells over-expressing Mcl-1, when compared with cell death in wild type HeLa cells and cells transfected with vector alone, was statistically significant (both p<0.05). Again, PARP cleavage was not as complete in Mcl-1 over-expressing cells as that seen in wild type HeLa cells and cells transfected with vector alone (Fig. 6C, comparing lane 6 with lanes 2 and 4, respectively, \textit{PARP}).

\textbf{DISCUSSION}

Most of the studies on protein-protein interaction of Bid in apoptosis have, to date, focused on its interaction with the pro-apoptotic Bax and Bak proteins. It has been shown that tBid can directly interact with both Bax and Bak and induce their oligomerisation, resulting in cytochrome c release (3,4). This function of tBid is vital for the transmission of apoptotic signals from death receptors to mitochondria in certain tissues and, indeed, apoptosis in hepatocytes is dependent on tBid-mediated amplification of the apoptotic signal via the mitochondria after engagement of the death receptor Fas (27). However, there has been little information about the role of anti-apoptotic Bcl-2 proteins in regulating the function of tBid. Here we show that Mcl-1 can regulate tBid-mediated apoptosis through its ability to interact with tBid. Our yeast two-hybrid screen identified Mcl-1 as a potent interaction partner for tBid. The interaction was confirmed by both GST fusion protein pull-down experiments \textit{in vitro} and co-immunoprecipitation \textit{in vivo}. In addition, we have compared Mcl-1 with Bcl-2 for the ability to bind tBid in both the yeast two-hybrid and GST pull-down experiments, and tBid appears to have a much higher affinity for Mcl-1 than Bcl-2. Co-immunoprecipitation assay showed that tBid interacts with Mcl-1 as well as Bcl-2 in cells. Recently, it has been shown that C-terminal fragments of Mcl-1 can also interact with tBid (28). However, in that study the interaction was
observed on the basis of exogenously expressed Mcl-1 proteins, whereas in our study tBid was shown to interact with endogenous Mcl-1.

It has been proposed that the BH3 domain-only proteins can be further divided into two subgroups – activating or sensitising (29,30). Those proteins with activating BH3 domains (e.g. Bid and Bim) appear to have a higher affinity for Bak or Bax than Bcl-2, thus perhaps directly activating Bak and Bax. The BH3-only proteins with sensitising BH3 domains (e.g. Bad) appear to preferentially bind Bcl-2 allowing Bak and Bax to be de-repressed. In this study, we confirm that tBid can directly interact with Bak and Bax. More importantly, we have also shown that tBid can bind to Mcl-1 and disrupt the Mcl-1/Bak complex. As a result, Bak is displaced from the complex. Recently, it has been reported that another BH3-only proteins Noxa can also displace Bak from Mcl-1/Bak complex (31), suggesting that this displacement is indeed a common feature for BH3-only proteins to activate Bak and/or Bax. Therefore, our study demonstrates dual modes of action by tBid, which is capable of both activating and sensitising other pro-apoptotic Bcl-2 family proteins. On the other hand, Mcl-1 can act through direct binding to neutralise tBid, thus preventing it from activating Bak or Bax. In this respect Mcl-1 may be playing an active role in counter balancing activating BH3 domain-only proteins. This notion is supported by the recent studies that Bim, another activating BH3 domain-only protein, has a higher affinity for Mcl-1 than Bcl-2 (32) and Mcl-1 effectively inhibits Bim-mediated release of mitochondrial cytochrome c (33). Our study shows that Mcl-1 interacts with tBid and impairs the ability of tBid to induce cytochrome c release and apoptosis. Recently, two independent studies revealed differential targeting of anti-apoptotic Bcl-2 proteins by BH3-only proteins using individual BH3 peptides (34,35). A Bim BH3 peptide appears capable of interacting with most anti-apoptotic proteins including Mcl-1 with high affinity, whereas the Bid BH3 peptide does not bind Mcl-1. This is not unexpected, as we have shown that full length Bid is not able to interact with Mcl-1. Only the truncated Bid, which may undergo further modification by post-proteolytic N-myristoylation, is able to bind Mcl-1, suggesting that there is a complex structural basis underlying the interaction of Bid with other proteins, which is not discernable in binding studies using Bid BH3 peptide alone.

Our in vivo experiments in HeLa cells were prompted by the involvement of Bid in apoptosis mediated by TRAIL and TNF-α in HeLa cells (15,25,26). We have shown that Mcl-1 can indeed protect HeLa cells from apoptosis induced by TRAIL and TNF-α. Mcl-1 silencing by shRNA approach sensitises HeLa cells to TRAIL-induced apoptosis. Recently, it has been reported that Mcl-1 mediates resistance to TRAIL-induced apoptosis in human cholangiocarcinoma cells (36). In their study, resistance was specifically associated with overexpression of Mcl-1 and depletion of Mcl-1 by small interfering RNA method also sensitises cells to TRAIL-mediated apoptosis despite Bcl-2 expression. Furthermore, another study has also shown that hepatocyte growth factor (HGF)-mediated Mcl-1 induction inhibits apoptosis induced by Fas in human primary hepatocytes (37). As TRAIL and Fas induce apoptosis via a similar mechanism (38), it is conceivable that Mcl-1 could bind to tBid and interfere with its function in activating Bax and/or Bak, resulting in protection of these cells from death receptor-mediated apoptosis. Our observations of interaction between Mcl-1 and tBid and subsequent prevention of cytochrome c release therefore provide a potential biochemical mechanism to explain the anti-apoptotic effect of Mcl-1 in death receptor-mediated apoptosis through specific targeting of tBid.

It is worth noting that under the experimental conditions used, over-expression of Mcl-1 does not fully protect cells from both TRAIL- and TNF-α-induced apoptosis although it significantly reduces the level of cell death in HeLa cells. It is possible that the level of Mcl-1 expression in stably transfected cells is not high enough to prevent cell death completely. In addition, in the case of TNF-α-induced apoptosis, the presence of protein synthesis inhibitor cycloheximide inevitably blocks production of Mcl-1 protein and thus reduces the anti-apoptotic function of Mcl-1. However, there is emerging evidence to suggest that complete protection of apoptosis requires multiple lines of resistance conferred by anti-
apoptotic proteins and Mcl-1 may provide the first line of resistance to the induction of apoptosis by TRAIL and TNF-α. This is consistent with the observation that in adenoviral protein E1A-induced apoptosis in HeLa cells, loss of Mcl-1 is required to initiate the apoptotic pathway (23). The idea is further supported by a recent study demonstrating that Mcl-1 functions upstream of and together with Bcl-xL in preventing UV irradiation-induced cytochrome c release from mitochondria and apoptosis in HeLa cells (17). Mcl-1 alone may not be able to offer complete protection of cells from apoptosis. Conversely, depletion of Mcl-1 alone may be insufficient to render all the cells sensitive to death receptor-mediated apoptosis.

Mcl-1 was initially discovered as an early induction gene during differentiation of the myeloid cell line, ML-1 (39) and is widely expressed in a variety of human tissues and cells as well as many tumours (40,41). Deletion of Mcl-1 in mice led to embryonic lethality during the peri-implantation stage, suggesting it is essential for embryonic development (42). Genetic studies with conditional knockout approach also reveal that Mcl-1 is required both in early lymphoid development and in the maintenance of mature B and T lymphocytes, which are rapidly lost when Mcl-1 is deleted (32). Mcl-1 also plays physiologically important roles in regulating myeloid cell survival (43,44). Given that Mcl-1 can interact strongly with tBid and other BH3-only proteins such as Bim and inhibits their induction of cytochrome c release and activation of the mitochondrial apoptotic pathway, loss of Mcl-1 may render the cells sensitive to apoptosis induced by a variety of apoptotic stimuli including the activation of death receptors of TNF family. Here we demonstrate a biochemical link between Mcl-1, an essential regulator of lymphoid homeostasis, and receptor-mediated apoptosis, the pre-eminent pathway controlling survival of the cells of the immune system (45,46).

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Figure legends

Figure 1. Identification of interaction of tBid with Mcl-1 by yeast two-hybrid screening. (A) Alignment of in-frame amino acid sequences from three clones containing cDNA encoding Mcl-1 with full-length murine Mcl-1 protein (mMcl-1). (B) The activity of the β-galactosidase reporter gene was examined by filter-lift assay after re-transformation of yeast containing either vector alone or tBid cDNA with plasmids from positive clones representing cDNA encoding Mcl-1 (upper panel) and Bcl-2 (middle panel). As a positive control, mouse BaxΔcDNA was used in the re-transformation (lower panel).

Figure 2. Truncated Bid interacts with Mcl-1 in vitro and in vivo. (A) In vitro translated [35S]-methionine labelled Mcl-1 (upper panel) and Bcl-2 (lower panel) were incubated with GST alone, GST-Bid or GST-tBid immobilised on glutathione-sepharose beads. Bound proteins were visualised using a phosphorimager after protein separation by SDS-PAGE. (B) HeLa cells were transfected with either pcDNA3.1 vector alone or a HA-tBid construct in the presence of 75 μM Z-VAD.fmk. 24 h after transfection, cells were lysed and subject to immnoprecipitation using anti-HA antibody (upper panel) or anti-Mcl-1 antibody (lower panel). Precipitated immuno-complexes were analysed by SDS-PAGE and immunoblotting using anti-Mcl-1 antibody (upper panel), anti-Bcl-2 antibody (middle panel) or anti-Bid antibody (lower panel).

Figure 3. BH domains of both tBid and Mcl-1 are required for interaction. (A) Requirement of BH3 domain of tBid to bind Mcl-1. In vitro translated [35S]-methionine labelled Mcl-1 was incubated with GST alone, GST-tBid or mutant GST-tBid (mtBH3) (G94E) immobilised on glutathione-sepharose beads. (B) BH1, BH2 and BH3 domains of Mcl-1 contribute to interaction with tBid. GST alone and GST-tBid were immobilised on glutathione-sepharose beads and incubated with in vitro translated [35S]-methionine labelled Mcl-1 (upper panel) or Mcl-1mtBH1 (G262E mutation) (upper middle panel) or Mcl-1mtBH2 (W305A and W312A double mutations) (lower middle panel) or Mcl-1mtBH3 (G217E and D218A double mutations) (lower panel). All bound proteins were analysed using a phosphorimager.

Figure 4. Mcl-1 prevents tBid-mediated cytochrome c release from isolated mitochondria. (A) Mitochondria isolated from HeLa cells were incubated with recombinant tBid at the indicated concentrations. At the end of incubation, supernatant of the mitochondrial suspension was collected and subject to SDS-PAGE and immunoblotting for cytochrome c (Cyt. c., upper panel). Untreated mitochondria (Mito.) were used as positive control for the detection of cytochrome c (lane 1). The same membrane was re-probed for cytochrome oxidase (subunit II) to check if the supernatant contains mitochondrial contamination (Cyt. Oxid., lower panel). (B) Mitochondria were pre-incubated with recombinant Mcl-1 at the indicated concentrations before the addition of tBid. Both supernatant and pellet of the mitochondrial suspension were collected at the end of incubation and subject to SDS-PAGE and immunoblotting for cytochrome c (Cyt. c., supernatant, and upper panel, pellet). Membrane from the pellet samples was also probed for tBid for its presence (middle panel, pellet) and for cytochrome oxidase (subunit II) as sample loading control (Cyt. Oxid., lower panel, pellet). (C) Mitochondria were pre-incubated with recombinant Mcl-1 (5 ng/μl) or mutant Mcl-1mtBH3 (G217E and D218A double mutations) (5 ng/μl) before the addition of tBid. At the end of incubation, both supernatant and pellet of the mitochondrial suspension were collected and subject to SDS-PAGE and immunoblotting as described in (B).
Figure 5. tBid binds Mcl-1 and displaces Bak from Mcl-1-Bak complex. (A) HeLa cells were transfected with either pcDNA3.1 vector alone or a HA-tBid construct in the presence of 75 µM Z-VAD.fmk. Cells were lysed 24 h after transfection and subjected to immunoprecipitation using anti-HA antibody. Precipitated immuno-complexes were analysed by SDS-PAGE and immunoblotting using anti-Bak antibody (upper panel) or anti-Bax antibody (lower panel). Asterisks denote non-specific bands. (B) The above cell lysates were immunoprecipitated by anti-Mcl-1 antibody. The precipitates were subject to SDS-PAGE and immunoblotting using anti-Bak antibody (upper panel, left), anti-Mcl-1 antibody (middle panel, left) or anti-Bid antibody (lower panel, left). Prior to immunoprecipitation, 5% of total cell lysates (TCL) from cells transfected with either pcDNA3.1 vector alone or a HA-tBid construct were also analysed by SDS-PAGE and immunoblotting for the expression levels of Bak (upper panel, right), Mcl-1 (middle panel, right) or Bid (lower panel, right). (C) Relative level of Bak to Mcl-1 in immunoprecipitated samples by anti-Mcl-1 antibody was analysed by densitometry after scanning the bands representing respective Bak and Mcl-1 on membranes from three independent immunoprecipitation experiments.

Figure 6. Mcl-1 inhibits apoptosis induced by TRAIL and TNF-α in HeLa cells. (A) HeLa cells were stably transfected with either pcDNA3.1 vector alone or plasmid containing the cDNA encoding human Mcl-1. The expression of Mcl-1 in wild type (w/t), pcDNA3.1 vector and Mcl-1 over-expressing HeLa cells was assessed by immunoblotting for Mcl-1 (upper panel). The same membrane was also probed for α-tubulin as sample loading control (lower panel). (B) Apoptosis was induced by the treatment of the three HeLa cell lines with soluble human recombinant TRAIL at the indicated concentrations for 14 h and assessed by both flow cytometry for cells with sub-G1 DNA content and PARP cleavage by immunoblotting. α-Tubulin was also probed as sample loading control. (C) Apoptosis was also induced by the treatment of the three HeLa cell lines with TNF-α (15 ng/ml) in the presence of cycloheximide (CHX, 30 µg/ml) for 22 h and assessed as described in (B).

Figure 7. Mcl-1 silencing by RNA interference sensitises HeLa cells to TRAIL-induced apoptosis. (A) HeLa cells were infected with lentivirus containing vectors expressing either control shRNA or Mcl-1 shRNA. The expression of Mcl-1 in wild type (w/t) HeLa cells, and cells infected with virus expressing either control shRNA or Mcl-1 shRNA was assessed by immunoblotting for Mcl-1 (upper panel). The same membrane was also probed for α-tubulin as sample loading control (lower panel). (B) Apoptosis was induced by the treatment of the three HeLa cell lines with soluble human recombinant TRAIL (100 ng/ml) for 14 h and assessed by both flow cytometry for cells with sub-G1 DNA content and PARP cleavage by immunoblotting, α-Tubulin was also probed as sample loading control.
Fig. 3

A

Input (10%) GST GST-Bid GST-T-Bid (mtBH3)

1 2 3 4

Mcl-1

B

Input (10%) GST GST-Bid

1 2 3

Mcl-1 mtBH1 mtBH2 mtBH3
Fig. 4

A

| Mito. 30ng | tBid (ng/μl) |
|------------|--------------|
| -          | 1            |
|            | 5            |
| 1          | 2            |
| 3          | 4            |
| 5          | 10           |

Cyt. c

Cyt. Oxid.

B

| Mcl-1 (ng/μl) | -5 | - | 1 | 2 | 5 |
|---------------|----|---|---|---|---|
| tBid (1ng/μl) | -  | - | + | + | + |

Supernatant

Cyt. c

Pellet

Cyt. Oxid.

C

| Mcl-1        | - | - | + | - | - |
|---------------|---|---|---|---|---|
| Mcl-1mtBH3    | - | - | - | + | + |
| tBid         | - | + | + | + | - |

Supernatant

Cyt. c

Pellet

tBid

Cyt. Oxid.
Fig. 5

A

IP: α-HA

HA-tBid - +
IB: α-Bak

IB: α-Bax

B

IP: α-Mcl-1 5% Input of TCL

HA-tBid - + 3 4
IB: α-Bak

IB: α-Mcl-1

IB: α-Bid

1 2 3 4

C

Relative level of Bak to Mcl-1

Vector HA-tBid
Fig. 7

A

Mcl-1
α-Tubulin

1 2 3

B

% Apoptosis

0 10 20 30 40

Hela, w/t + + - - - -
Control shRNA - + + + - -
Mcl-1 shRNA - - - + + +
TRAIL - + - + - +

PARP

α-Tubulin

1 2 3 4 5 6

Intact
85 kDa fragment
Figure 1. Mcl-1 inhibits apoptosis induced by TNF-α and TRAIL in another stable HeLa cell line over-expressing Mcl-1. (A) The expression of Mcl-1 in wild type (w/t), pcDNA3.1 vector and Mcl-1 over-expressing HeLa cells was assessed by immunoblotting for Mcl-1 (upper panel). The same membrane was also probed for α-tubulin as sample loading control (lower panel). (B) Apoptosis was induced by the treatment of the three HeLa cell lines with TNF-α (15 ng/ml) in the presence of cycloheximide (CHX, 30 μg/ml) for 22 h and assessed by flow cytometry for cells with sub-G1 DNA content. (C) Apoptosis was also induced by the treatment of the three HeLa cell lines with soluble human recombinant TRAIL (250 ng/ml) for 14 h and assessed as described in (B). For details of generating stable HeLa cell lines, refer to Experimental Procedures of the text.
Mouse tBid cDNA was amplified by PCR from a murine pcDNA3-Bid template (gift from Dr. S. Korsmeyer, Harvard Medical School, Boston, MA) and inserted into the pGBK7 bait vector (Clontech). The primers used for PCR were as follows: 5’-CCGGAATTCCGGCACGGCAGGC-3’ (forward) and 5’-CCGGATCTCTAGAGTCTCCTATTTCTAACCAAG-3’ (reverse). A mouse Baxα cDNA lacking the C terminal hydrophobic domain (baxα21) was cloned by PCR from a pcDNA3-Bax template and cloned into the vector pGAD7 (Clontech). The primers used for PCR were 5’-CCGGAATTCCAGTCGACGCGGTCG-3’ (forward) and 5’-CCATCGAGTCCGATGTGGG-3’ (reverse). A pUC18-bcl-2 construct (Clonexpress) was used to clone the human bcl-2 cDNA. This construct was digested with EcoRI and Hind III to release the bcl-2 cDNA which was then ligated into the corresponding sites in pcDNA3.1/myc-HIS©(-)(Invitrogen). Human Bid and tBid cDNA were generated by RT-PCR using oligo-dT primer with total RNA obtained from human leukemic Jurkat T cells. The primers used to amplify bid were as follows: 5’-CCGGAATTCCATGGGCGCGTACGTCG-3’ (forward) and 5’-CCATCGAGTCCGATGTGGG-3’ (reverse). The both cDNAs were then cloned at the EcoRI - BamHI sites down stream of HA-tag in pcDNA3.1-HA (Invitrogen). The construct expressing HA-tagged human Mcl-1 was generated as previously described (14). GST fusion protein constructs were generated by PCR using pcDNA3.1-HA-Bid and -tBid as templates and cloned into the EcoRI - XhoI sites in the pGEX-6P-2 vector (Amersham, Buckinghamshire, UK). The primers used to amplify Bid were 5’-CCGGAATTCCATGGGCGCGTACGTCG-3’ (forward) and 5’-CCGCTCGAGTCAGTCCATTTCTGGC-3’ (reverse), while the primers 5’-CCGGAATTCCCGGCAACCGCAGGCACC-3’ (forward) and 5’-CCGCTCGAGTCAGTCCATTTCTGGC-3’ (reverse) were used to amplify tBid.

Mutations in BH domains of tBid and Mcl-1 proteins were generated by site-directed mutagenesis using the GeneTailor™ Site-Directed Mutagenesis System according to manufacturer’s instruction (Invitrogen). The G94E mutation of BH3 domain of tBid was generated with the primers: 5’-CAGGCACCTCGCCCAGGTCGAGGACAGCATGGACC-3’ (forward) and 5’-CCGCTCGAGTCAGTCCATTTCTGGC-3’ (reverse) using the pGEX-tBid construct as a template. Using pcDNA3.1-HA-Mcl-1 as a template for all Mcl-1 mutants, the G262E mutation at BH1 domain of Mcl-1 was generated with the primers: 5’-CAGACGGCGTAACAAACTGGGAAAGGATTGTGACTC-3’ (forward) and 5’-CCCAGTTTGGTACGCCGTCGCTGAAACATG-3’ (reverse). The G217E and D218A double mutations of BH3 domain were generated with the primers: 5’-GAGGACCTTACCAGCGGTTGAGGCTGGCAGACGC-3’ (forward) and 5’-CAACCGTGTAGGTCTCAGGGCCTTCC-3’ (reverse). The double mutations for BH2 domain of Mcl-1 were generated by two-step PCR with primers: 5’-GTAAGGACAAAAACGGACGGGCTGGTAAACAAAG-3’ (forward) and 5’-GTCCCGTTTTTGGGCTTACCAGAAGCTGTCG-3’ (reverse) for W305A mutation, and primers: 5’-GCTAGTTAACAAAAAGGACGGGATGGGATTTGGG-3’ (forward) and 5’-GCCTCTTTGTATTTCTAATACGACGCACTCGG-3’ (reverse) for W312A mutation. The primers used to generate recombinant human Mcl-1 proteins using HA-Mcl-1 in pcDNA3.1 as template
were as follows: 5′-GGTGGTCATATGTTTGGCCTCAAAAGAAACG-3′ (forward) and 5′-
GGTGGTTGCTCTTCCGCATTTTATTAGATATGC -3′ (reverse). The PCR products were
inserted into the Nde I and Sap I sites of the pTYB1 vector (New England Biolabs). The
accuracy of the molecular identity of all constructs was confirmed by sequencing.
Mcl-1 interacts with truncated Bid and inhibits its induction of cytochrome c release and its role in receptor-mediated apoptosis
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