

Endogenous Bak inhibitors Mcl-1 and Bcl-x_L: differential impact on TRAIL resistance in Bax-deficient carcinoma

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Tumor necrosis factor (α)-related apoptosis-inducing ligand (TRAIL) is a promising anticancer agent that preferentially kills tumor cells with limited cytotoxicity to nonmalignant cells. However, signaling from death receptors requires amplification via the mitochondrial apoptosis pathway (type II) in the majority of tumor cells. Thus, TRAIL-induced cell death entirely depends on the proapoptotic Bcl-2 family member Bax, which is often lost as a result of epigenetic inactivation or mutations. Consequently, Bax deficiency confers resistance against TRAIL-induced apoptosis. Despite expression of Bak, Bax-deficient

cells are resistant to TRAIL-induced apoptosis. In this study, we show that the Bax dependency of TRAIL-induced apoptosis is determined by Mcl-1 but not Bcl-x_L. Both are antiapoptotic Bcl-2 family proteins that keep Bak in check. Nevertheless, knockdown of Mcl-1 but not Bcl-x_L overcame resistance to TRAIL, CD95/FasL and tumor necrosis factor (α) death receptor ligation in Bax-deficient cells, and enabled TRAIL to activate Bak, indicating that Mcl-1 rather than Bcl-x_L is a major target for sensitization of Bax-deficient tumors for death receptor-induced apoptosis via the Bak pathway.

Introduction

TNF-related apoptosis-inducing ligand (TRAIL), a cytotoxic ligand of the TNF family, is a promising anticancer agent (Huang and Sheikh, 2007; Mérimo et al., 2007; Fulda, 2009). TRAIL induces cell death in a wide range of human cancers independently of their p53 status without apparent toxic side effects in normal tissues. Furthermore, recent studies demonstrated that TRAIL and other death ligands, including CD95/FasL and TNF, could sensitize tumor cells for ionizing radiation- and drug-induced apoptosis. Such a combined modality may circumvent the resistance of tumors against chemo- and radiotherapy (Marini and Belka, 2003; Schmelz et al., 2004; Marini et al., 2005; Fulda, 2009; Newsom-Davis et al., 2009; for review see Daniel et al., 2001). Ligands of the TNF family initiate the extrinsic apoptotic pathway through binding to cell surface death receptors of the TNF receptor superfamily. Engagement of the death receptors leads to receptor oligomerization and the

formation of the death-inducing signaling complex followed by activation of the initiator caspase-8 (Dhein et al., 1992; Muzio et al., 1996). In so-called type I cells, active caspase-8 initiates sufficient processing and concomitant activation of the downstream effector caspase-3 that ultimately leads to execution of apoptosis (Scaffidi et al., 1998).

In contrast, in type II cells, the amount of caspase-3 activated via caspase-8 is not sufficient to trigger apoptosis. In such cells, death receptor-mediated apoptosis requires amplification of the death signal through activation of the intrinsic (mitochondrial) cell death pathway (for reviews see Daniel et al., 2001; Kaufmann and Steensma, 2005). Proteins of the Bcl-2 family, which comprises anti- and proapoptotic proteins, tightly regulate this mitochondrial cell death pathway. The antiapoptotic proteins of this family (Bcl-2, Bcl-x_L, Mcl-1, Bfl-1/A1, and Bcl-w) are characterized by the presence of all four Bcl-2

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Abbreviations used in this paper: BH, Bcl-2 homology; PI, propidium iodide; tBid, truncated Bid; TRAIL, TNF-related apoptosis-inducing ligand; wt, wild type.

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homology (BH) domains. Proapoptotic homologues lack a BH4 domain and can be further subdivided into two subfamilies. The multidomain Bax homologues including Bax, Bak, and Bok/Mtd contain BH1–3, whereas the proteins of the BH3-only subfamily, which comprises Bad, Bid, Bim, Puma, Noxa, Nbk/Bik, Bmf, and Hrk, only share the BH3 interaction domain (Daniel et al., 2003; van Delft and Huang, 2006). The BH3-only proteins are essential initiators of apoptosis, which, upon a specific stimulus, activate a conformational switch and induce oligomerization of the executioner proteins Bax and Bak. Activated, Bax, and Bak initiate mitochondrial membrane permeabilization and thereby induce the release of cytochrome *c* from the mitochondria into the cytoplasm. There, cytochrome *c* associates with Apaf-1 and caspase-9 to form the apoptosome that serves to facilitate autocatalysis and activation of caspase-9, which in turn triggers the downstream executioner caspases. Mitochondrial amplification of the death receptor signal in type II cells is achieved by caspase-8–mediated cleavage of the BH3-only protein Bid. The resulting active, truncated Bid (tBid) activates Bax, thereby inducing apoptosome and Smac/Diablo-mediated caspase activation. Thus, Bcl-2 family members play a critical role in modulating TRAIL-mediated cell death in tumor cells.

Interestingly, it has been shown that overexpression of Bcl-2, Bcl-x_L, or Mcl-1 inhibits TRAIL-induced apoptosis (Henson et al., 2003; Taniai et al., 2004; Zhang and Fang, 2005). Likewise, Bax deficiency confers TRAIL resistance of cancer cells (Deng et al., 2002; LeBlanc et al., 2002; Ravi and Bedi, 2002; Theodorakis et al., 2002). However, Bax was dispensable for TRAIL-induced caspase-8 activation and subsequent cleavage of Bid but crucial for the release of cytochrome *c* and Smac/Diablo from mitochondria and downstream activation of caspases. In this line, we have shown that the synergistic induction of apoptosis by TRAIL and anticancer drugs or ionizing irradiation depends on Bax but not on the homologous Bak (von Haefen et al., 2004; Wendt et al., 2005).

In this study, we show that the requirement for Bak in TRAIL-induced apoptosis is determined by the antiapoptotic Bcl-2 homologue Mcl-1 rather than Bcl-x_L, the second major endogenous inhibitor of Bak (Willis et al., 2005). We found that Bax-deficient, Bak-expressing carcinoma cells were resistant to TRAIL-induced apoptosis, and reconstitution of Bax sensitized these cells for TRAIL-induced apoptosis. However, down-regulation of Mcl-1 sensitized Bak-proficient but not Bak-deficient cancer cells for TRAIL-induced apoptosis regardless of their Bax expression status. In contrast, knockdown of Bcl-x_L failed to overcome TRAIL resistance in Bax-deficient cells. As Bax is often lost in tumors, inhibition of Mcl-1 rather than Bcl-x_L represents an opportunity to open the Bak pathway and overcome TRAIL resistance.

Results

In type II cells, death receptor–mediated apoptosis requires amplification of the death signal through activation of the intrinsic (mitochondrial) cell death pathway. Several studies indicate that Bax is crucial for TRAIL-mediated apoptosis in these cells and that loss of Bax protects cancer cells from apoptosis induced

by TRAIL (Deng et al., 2002; LeBlanc et al., 2002; Ravi and Bedi, 2002; Theodorakis et al., 2002; von Haefen et al., 2004; Wendt et al., 2005). However, the role of Bak in TRAIL-induced apoptosis is barely defined. To elucidate the specific role of Bax and Bak, we investigated TRAIL-induced cell death in HCT116 cells deficient of Bax, Bak, or both. First, to clarify that HCT116 cells are type II cells, we analyzed how knockdown of caspase-8 or Bid by siRNAs affected apoptosis induced by TRAIL. Induction of apoptosis was analyzed by flow cytometric measurement of genomic DNA fragmentation, and apoptotic cells were identified as cells with a hypodiploid, i.e., sub-G1, DNA content. Caspase-8 and Bid expression were specifically down-regulated by the respective siRNA but not by a control siRNA (Fig. 1 A, left). This down-regulation resulted in strong inhibition of TRAIL-induced apoptosis. Despite TRAIL treatment, only 8% of caspase-8 knockdown cells and 13% of Bid knockdown cells showed a hypodiploid DNA content compared with 33% of control cells (Fig. 1 A, right). This indicates that in the absence of Bid, caspase-8 is not sufficient to initiate execution of the death signal by TRAIL, and this Bid dependency clearly identifies HCT116 cells as type II cells.

Next, we determined how the specific loss of Bax or Bak affected TRAIL-induced apoptosis in HCT116 cells. Specific loss of protein expression was achieved by knockout of the Bax gene (Zhang et al., 2000) and Bak knockdown by short hairpin RNA (Theodorakis et al., 2002). The HCT116 wild-type (wt) cells and isogenic cell lines devoid of either Bax or Bak expression alone (termed Bax⁻ and Bak⁻, respectively) or both (termed Bax⁻/Bak⁻) were treated with 50 ng/ml TRAIL for 24 h (Fig. 1 B). In wt cells, TRAIL treatment resulted in DNA fragmentation in ~36% of the cells, whereas Bax-deficient cells were resistant to TRAIL-induced DNA fragmentation. Less than 6% of Bax⁻ or Bax⁻/Bak⁻ cells showed a hypodiploid, i.e., sub-G1, DNA content. In sharp contrast to the loss of Bax, Bak deficiency did not confer resistance to TRAIL-induced apoptosis. Upon TRAIL treatment, 33% of the Bak⁻ cells showed DNA fragmentation, which is comparable with TRAIL-induced cell death in wt cells (Fig. 1 B).

Western blot analysis showed that TRAIL treatment is accompanied by cleavage of the executioner caspase-3 in HCT116. However, in contrast to Bax-proficient cells, cleaved caspase-3 is barely detectable in Bax-deficient HCT116 cells regardless of whether or not they expressed Bak (Fig. 1 C).

To further address the role of Bax and Bak and of mitochondria in TRAIL-induced apoptosis and to confirm that loss of Bak does not affect TRAIL-induced mitochondrial apoptosis, we determined the release of cytochrome *c* upon TRAIL treatment. Western blot analysis of cytosolic extracts obtained at 24 h after treatment showed that TRAIL induces the release of cytochrome *c* in both HCT116 wt and HCT116 Bak⁻ cells but not in Bax-deficient cell lines (Fig. 1 C, bottom).

Altogether, these results establish that at least in TRAIL-induced apoptosis, Bax and Bak do not exert redundant functions. Although Bak knockdown does not affect TRAIL-induced apoptosis, loss of Bax efficiently protects HCT116 cells from cell death induced by TRAIL. Furthermore, the presence of endogenous Bak does not influence the proportion of cells

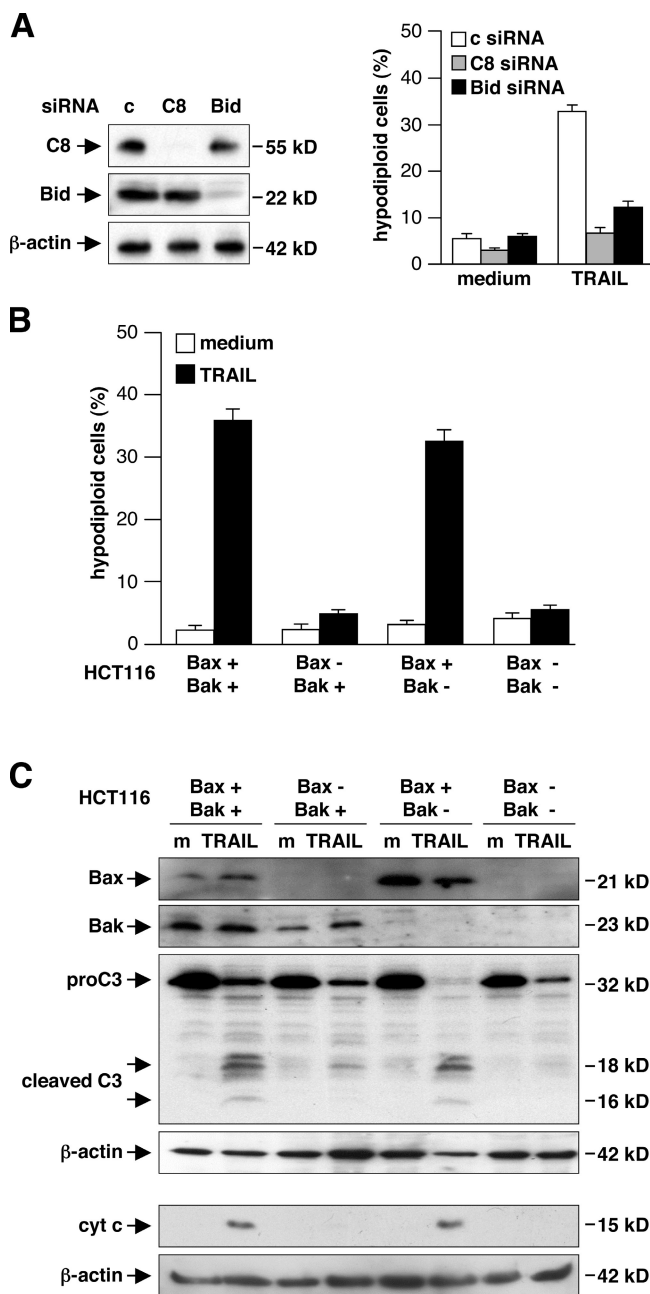


Figure 1. Loss of Bax but not Bak impairs TRAIL-induced apoptosis in the type II cell line HCT116. (A) Impaired induction of TRAIL-induced apoptosis after down-regulation of caspase-8 or Bid revealed that HCT116 cells are of type II. (left) To analyze siRNA-mediated down-regulation of Bid and caspase-8, HCT116 wt cells were transfected with the respective siRNA and cultured for 24 h followed by Western blot analysis for the presence of the respective proteins. (right) 24 h after transfection, HCT116 cells were treated with 50 ng/ml TRAIL and cultured for an additional 24 h until apoptotic DNA fragmentation was determined on a single-cell level by flow cytometric measurement of the cellular DNA content. Cells displaying a sub-G1, hypodiploid DNA content are considered apoptotic. c, control. (B) TRAIL-induced apoptosis is impaired in Bax-deficient cells but not in Bak-deficient HCT116 cells. HCT116 wt, Bax⁻, Bak⁻, and Bax⁻/Bak⁻ cells were treated with 50 ng/ml TRAIL and cultured for 48 h. Control cells were grown in the absence of TRAIL. Apoptotic DNA fragmentation was determined as described in A. (C) Analysis of Bax and Bak expression, processing of procaspase-3, and cytochrome c (cyt c) release are shown. Cells were treated with TRAIL and cultured until whole cell extracts were analyzed. For the detection of cytochrome c release, cytosolic extracts were prepared and subjected to Western blot analysis. m, medium. Error bars indicate means \pm SD from three experiments.

undergoing TRAIL-induced apoptosis, regardless of whether or not they express Bax.

This indicates that, in contrast to Bax, Bak is not activated during TRAIL-induced apoptosis and fails to mediate the death signal by TRAIL. Bak is mainly inhibited by the antiapoptotic Bcl-2 family members Bcl-x_L and Mcl-1 (Willis et al., 2005). To functionally address the contribution of Mcl-1 in Bak inhibition, we down-regulated Mcl-1 by RNA interference in HCT116 wt, Bax⁻, and Bak⁻ cells. In all three cell lines, Mcl-1 expression levels were specifically decreased by Mcl-1 siRNA but not by a control siRNA (Fig. 2, A–C, left). This knockdown of Mcl-1 slightly sensitized HCT116 wt cells for TRAIL-induced apoptosis. In HCT116 wt cells treated with a random siRNA, TRAIL induced apoptosis in \sim 30% of the cells, whereas Mcl-1 down-regulation resulted in 38% of the cells undergoing apoptosis (Fig. 2 A, right). More importantly, the down-regulation of Mcl-1 rendered Bax-deficient HCT116 Bax⁻ cells, which are resistant to TRAIL-induced apoptosis, susceptible for TRAIL-induced cell death. Compared with 5% of control cells, TRAIL induced apoptosis in \sim 28% of the cells after knockdown of Mcl-1 (Fig. 2 B, right). In contrast, Mcl-1 down-regulation in Bak-negative, but Bax-proficient, HCT116 cells failed to enhance induction of apoptosis by TRAIL (Fig. 2 C, right).

To corroborate that all death occurs by apoptosis and to control viability, we analyzed TRAIL-treated cells after down-regulation of Mcl-1 by propidium iodide (PI)/annexin V FITC staining. As in the case of DNA fragmentation analysis, TRAIL induced apoptosis in HCT116 wt and Bak-deficient HCT116 cells, indicated by the detection of cells positive for phosphatidylserine exposure and negative for PI staining (early apoptotic) and detection of annexin V/PI positivity (late apoptotic) cells (Fig. S1). In contrast, annexin V-positive cells were hardly detectable in the TRAIL-treated HCT116 Bax⁻ cell line, confirming the resistance of this cell line. Also in line with the DNA fragmentation analysis, down-regulation of Mcl-1 slightly sensitizes HCT116 wt but not Bak-deficient HCT116 cells for TRAIL-induced apoptosis. In contrast, knockdown of Mcl-1 in the resistant cell line HCT116 Bax⁻ strongly increases the number of early apoptotic and late apoptotic cells after TRAIL treatment, up to 25% each (Fig. S1). The result that Bak-expressing HCT116 cells but not Bak-deficient cells were sensitized indicates that Mcl-1 knockdown facilitates TRAIL to induce apoptosis via a specific, Bak-dependent pathway.

To further investigate the mechanism of TRAIL resistance in Bax-deficient cells, we analyzed the influence of combined Mcl-1 down-regulation and TRAIL treatment on the activation of Bak. During apoptosis, Bak undergoes a conformational change, leading to the exposure of its N terminus that is inaccessible in vital cells. To study Bak activation, we performed a flow cytometric immunofluorescence analysis by use of a conformation-specific antibody directed against the Bak N terminus in Bax-deficient HCT116 cells. In HCT116 Bax⁻ cells treated with a control siRNA, TRAIL failed to activate Bak. In contrast, knockdown of Mcl-1 led to exposure of the Bak N terminus, i.e., Bak activation, in 33% of the HCT116 Bax⁻ cells (Fig. 2 D).

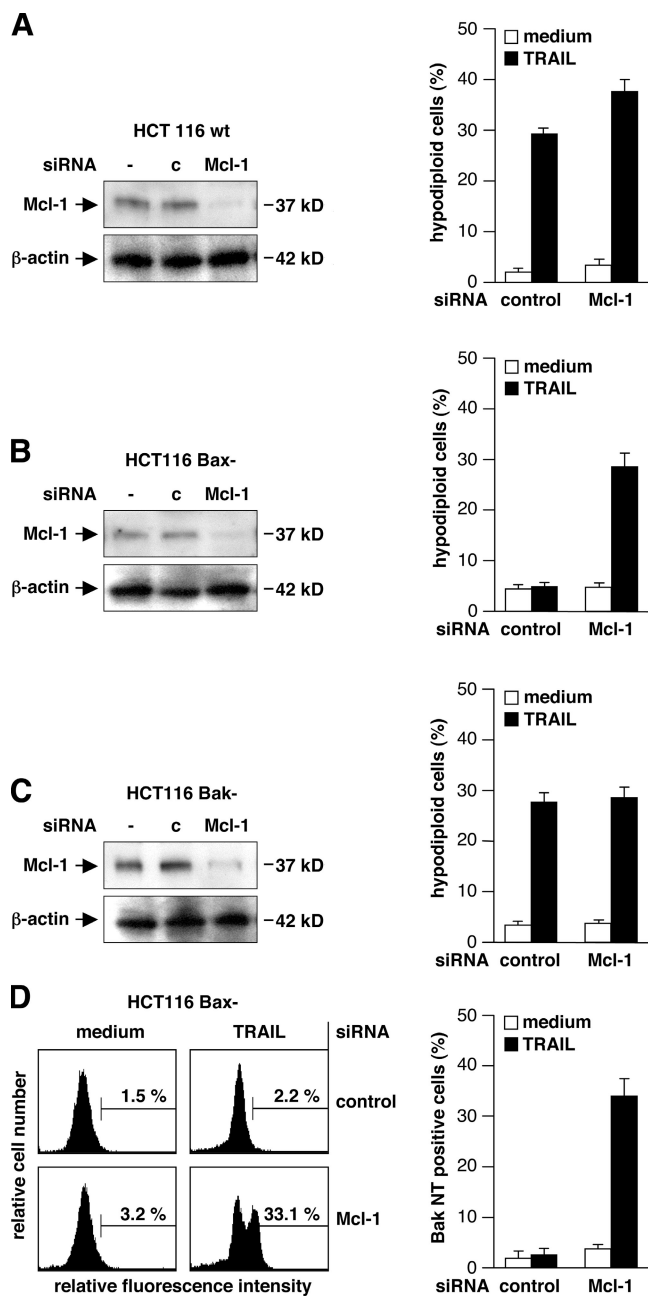


Figure 2. Silencing of Mcl-1 by RNA interference sensitizes Bak-proficient HCT116 cells for TRAIL-induced apoptosis and enables TRAIL to activate Bak. HCT116 wt, Bax^{-/-}, and Bak^{-/-} cells were either left untreated or were transfected with control (c) or Mcl-1 siRNAs. (A–C, left) After 24 h, total protein cell extracts were prepared, and down-regulation of Mcl-1 was confirmed by Western blot analysis. Mcl-1 down-regulation for HCT116 wt, Bax^{-/-}, and Bak^{-/-} cells is shown in A, B, and C, respectively. (right) Cells were treated with 50 ng/ml TRAIL, cultured for an additional 24 h, and harvested, and apoptotic cells were determined by flow cytometric measurement of cellular DNA content. Means ± SD from three experiments for the respective cell lines as shown in A–C. (D) To analyze Bak activation upon TRAIL treatment and Mcl-1 down-regulation, Bax-deficient HCT116 cells were stained with a conformation-specific antibody against the Bak N terminus and analyzed by flow cytometry. The percentage of immunostained cells, indicating Bak activation, is shown between the markers (representative histograms). (right) Means from triplicates ± SD are shown.

To support the specific role of Bak inhibition by Mcl-1 in TRAIL-induced apoptosis, we overexpressed the BH3-only proteins Nbkl and Noxa in HCT116 Bax^{-/-} cells. Nbkl preferentially binds to Bcl-2 and Bcl-x_L but not to Mcl-1. In contrast, Noxa shows specificity for Mcl-1 binding. To induce expression of Nbkl or Noxa, we used a regulable adenoviral vector system (Gillissen et al., 2003), and expression of the respective protein under “on” condition was confirmed by Western blot analysis (Fig. 3 A). After expression of Nbkl or Noxa for 14 h, cells were treated with TRAIL. Measurement of hypodiploid cells revealed that Noxa but not Nbkl sensitized for TRAIL-induced apoptosis. Upon Nbkl expression and TRAIL treatment, <10% of the cells became hypodiploid, whereas Noxa expression combined with TRAIL treatment induced DNA fragmentation in 25% of the cells (Fig. 3 B). This was confirmed by annexin V FITC/PI staining. Annexin V-positive cells were detected at background levels in the TRAIL-treated HCT116 Bax^{-/-} cell upon Nbkl expression. In contrast, Noxa expression strongly increased the number of early apoptotic and late apoptotic cells after TRAIL treatment up to 15% and 21%, respectively (Fig. 3 C).

To confirm the impact of Mcl-1 on Bak in TRAIL-induced apoptosis, we investigated the prostate carcinoma cell line DU145, which carries a frameshift mutation in the *bax* gene. Consequently, DU145 cells completely lack Bax expression, whereas *bak* is not mutated, and Bak is endogenously expressed to a moderate extent (Gillissen et al., 2003). We previously established DU145 cells stably reexpressing the Bax-α cDNA under the control of a cytomegalovirus promoter (von Haefen et al., 2002). Despite expression of Bak, Bax-deficient DU145 mock cells were resistant to TRAIL-induced apoptosis. However, reconstitution of Bax sensitized these DU145-Bax cells for TRAIL-induced apoptosis. After treatment with 100 ng/ml TRAIL, only 13% of the Bax-deficient DU145 cells showed DNA fragmentation, whereas ~40% of the Bax-reexpressing DU145 cells were apoptotic (Fig. 4 A, left). To verify whether TRAIL-mediated apoptosis requires amplification via the mitochondrial pathway in DU145 cells, we down-regulated caspase-8 and Bid by siRNA and examined induction of apoptosis in DU145-Bax cells. Although down-regulation of caspase-8 was not complete, reduced caspase-8 expression diminished TRAIL-induced apoptosis (Fig. 4 A, right). More importantly, knockdown of Bid impeded TRAIL-induced cell death in DU145-Bax cells. Only 12% of the cells transfected with Bid siRNA and treated with TRAIL were detected as apoptotic compared with 42% of the cells transfected with control siRNA (Fig. 4 A, right). Together with the Bax dependency, these results clearly establish that DU145 cells are type II cells and require the mitochondrial cell death pathway to undergo death ligand-triggered apoptosis. Furthermore, these data indicate that Bak fails to transmit the cell death signal triggered by TRAIL in the DU145 system.

To address the question of whether Bak is kept in check by Mcl-1 during TRAIL-induced cell death, we down-regulated Mcl-1 by RNA interference in DU145 mock and Bax-reexpressing DU145-Bax cells. Mcl-1 expression was specifically decreased in both cell lines by Mcl-1 siRNA but not by control siRNA (Fig. 4, B and C, left). The transfection with Mcl-1 siRNA

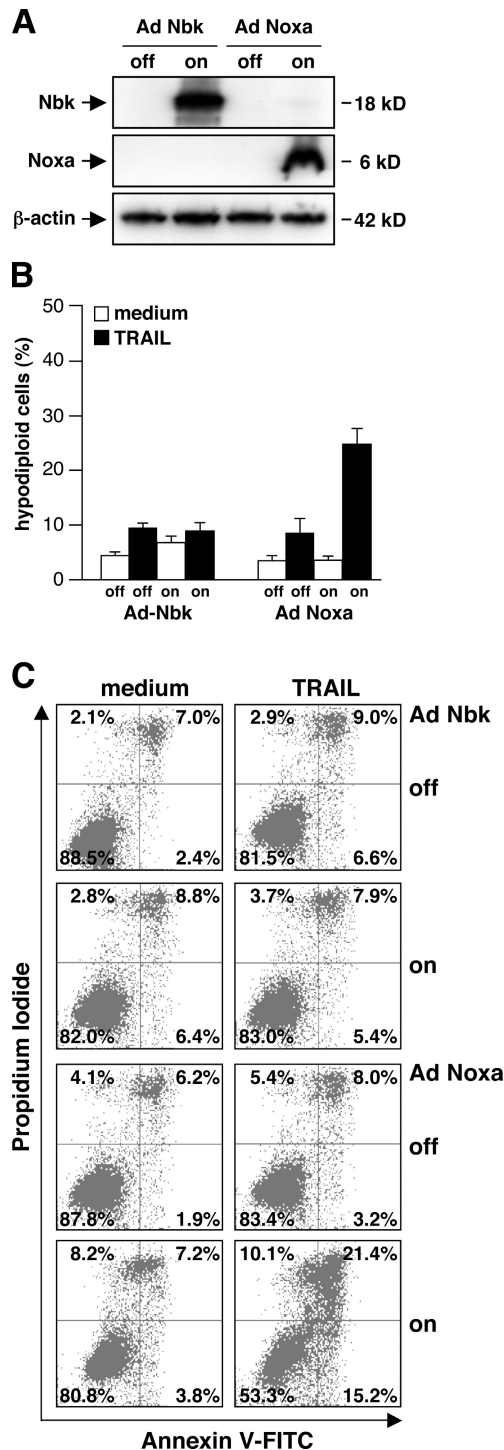


Figure 3. Expression of Noxa but not Nbk overcomes TRAIL resistance in Bax-deficient HCT116 cells. HCT116 Bax^{-/-} cells were transduced with Ad-mycNbk-tTA or Ad-mycNoxa-tTA and cultured under “off” or “on” conditions. (A) Expression of Nbk and Noxa was confirmed by immunoblotting. (B and C) 14 h after induction of expression, cells were treated for an additional 24 h with TRAIL. Apoptotic cells were determined by flow cytometric measurement of cellular DNA content (B) and annexin V/PI staining (C). Means \pm SD from triplicates are shown in B.

sensitized DU145-Bax cells for TRAIL-induced apoptosis as compared with the control siRNA. In detail, DU145-Bax cells transfected with control siRNA and treated with 50 ng/ml TRAIL showed apoptotic DNA fragmentation in 32% of the

cells, whereas apoptosis was increased in cells with Mcl-1 down-regulation to 60% (Fig. 4 B, right). More importantly, knockdown of Mcl-1 overcame the TRAIL resistance of Bax-deficient DU145 mock cells. Treatment with 50 ng/ml TRAIL induced apoptotic DNA fragmentation in only 8% of the DU145 mock cells transfected with control siRNA, whereas Mcl-1 knockdown sensitized for cell death induction by TRAIL. Despite Bax deficiency, 39% of the cells became apoptotic (Fig. 4 C, right). This indicates that TRAIL induces apoptosis via a Bak-dependent pathway upon inactivation of Mcl-1.

To analyze the impact of Bcl-x_L (the second major endogenous inhibitor of Bak) on TRAIL resistance, we down-regulated Bcl-x_L by siRNA (Willis et al., 2005). The knockdown of Bcl-x_L was specific and considerable (Fig. 5, A and B, top). In analogy to Mcl-1 knockdown, down-regulation of Bcl-x_L enhanced apoptosis by TRAIL from 27% in control cells to 48% in DU145-Bax cells (Fig. 5 A, right). However, in sharp contrast to Mcl-1 knockdown, the Bcl-x_L down-regulation failed to overcome TRAIL resistance in Bax-deficient DU145 cells. Although Bcl-x_L expression was efficiently blocked, only 9% of the cells showed an apoptotic phenotype in response to TRAIL treatment (Fig. 5 B, right) compared with 39% after Mcl-1 down-regulation (Fig. 4 C, right). Thus, Bcl-x_L appears to act in the Bax but not the Bak pathway in TRAIL-induced apoptosis. This also indicates that Mcl-1, rather than Bcl-x_L, is the major factor mediating TRAIL resistance in a Bax-deficient setting. Moreover, sensitization by Mcl-1 knockdown is specific and not caused by a generally reduced amount of anti-apoptotic Bcl-2 proteins.

Analysis of Bak activation by use of a conformation-specific antibody directed against an activation-induced epitope in the Bak N terminus revealed that Bax-deficient DU145 cells treated with a control siRNA are resistant to TRAIL. In contrast, knockdown of Mcl-1 in addition to TRAIL treatment led to exposure of the Bak N terminus, i.e., Bak activation, in 44% of the DU145 cells (Fig. 6 A). In agreement with the conformational switch of Bak, TRAIL treatment induced a strong clustering of EGFP-Bak after Mcl-1 knockdown in DU145-EGFP-Bak cells (Fig. 6 B). In contrast, cells treated with a control siRNA showed no EGFP-Bak clustering after TRAIL treatment.

Given the therapeutic impact of our finding on resistance to TRAIL receptor ligation, we next asked whether small molecules, known to down-regulate Mcl-1 expression, sensitize Bax-deficient DU145 cells for TRAIL-induced apoptosis. The multikinase inhibitor sorafenib (Nexavar; BAY43-9006) has been shown to induce apoptosis and sensitizes tumor cells for chemotherapy coinciding with down-regulation of Mcl-1 (Dasmahapatra et al., 2007; Ding et al., 2008; Yang et al., 2008). Therefore, we treated DU145 cells with different concentrations of sorafenib. As expected, treatment of DU145 cells with sorafenib resulted in a dose-dependent reduction of Mcl-1 protein expression (Fig. 7 A). When Bax-deficient DU145 mock cells were exposed to 50 ng/ml TRAIL, apoptotic DNA fragmentation was detectable in only 6% of the cells. The addition of 10 μ M sorafenib alone did marginally increase background apoptosis. However, 10 μ M sorafenib overcame TRAIL

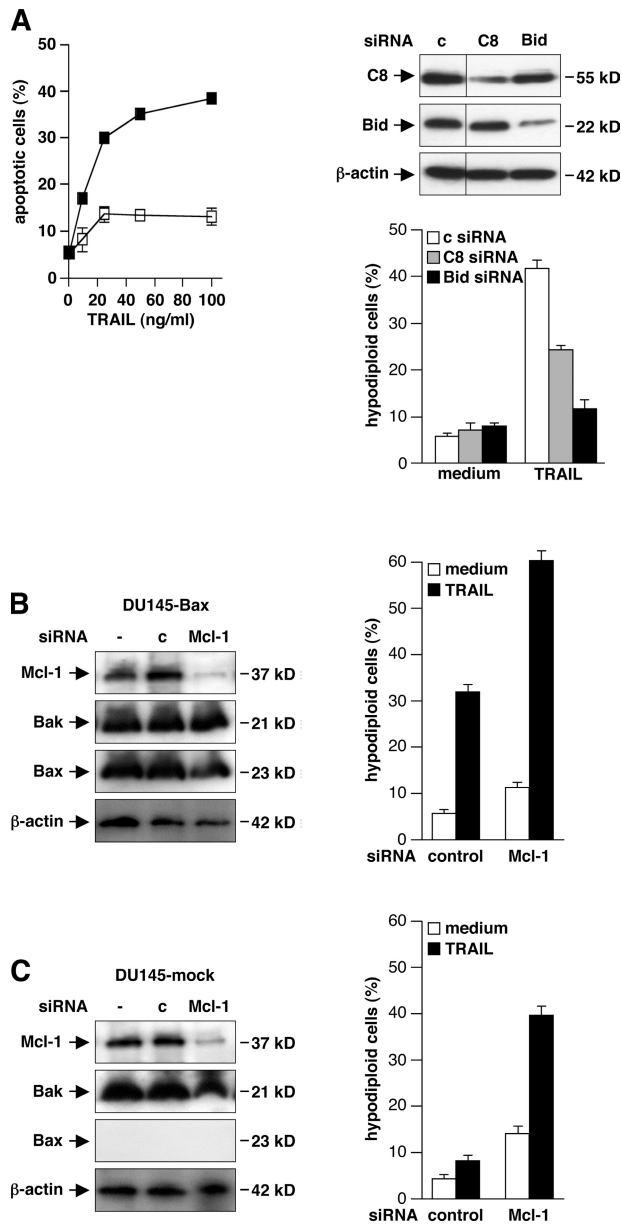


Figure 4. Mcl-1 knockdown sensitizes type II DU145-Bax cells for TRAIL-induced apoptosis and overcomes TRAIL resistance in Bax-deficient DU145 cells. (A, left) DU145 mock or DU145-Bax cells were treated with increasing concentrations of TRAIL and cultured for 24 h. Measurement of apoptotic DNA fragmentation revealed that reexpression of Bax overcomes TRAIL resistance of DU145 cells. Open squares, DU145 mock cells; closed squares, DU145-Bax cells. (right) To confirm that DU145 cells are type II, cells were transfected with control (c), caspase-8, or Bid siRNA and cultured for 24 h. (top) Total cell extracts were prepared, and down-regulation of respective proteins was confirmed by Western blot analysis. Black lines indicate that intervening lanes have been spliced out. (bottom) 24 h after siRNA-mediated knockdown of caspase-8 or Bid, cells were treated with 50 ng/ml TRAIL and cultured for an additional 24 h. (right) Cells were harvested, and apoptotic cells were determined by flow cytometric measurement of cellular DNA content. (B and C) DU145-Bax (B) and DU145 mock cells (C) were transfected with control or Mcl-1 siRNA and cultured for 24 h. Mcl-1 down-regulation and expression status of Bax and Bak were confirmed by Western blot analysis. After knockdown of Mcl-1, cells were treated with TRAIL at 50 ng/ml and cultured for an additional 24 h. Measurement of apoptotic DNA fragmentation revealed that Mcl-1 down-regulation sensitized DU145-Bax cells for TRAIL-induced apoptosis and overcame TRAIL resistance in DU145 mock cells. Error bars indicate means \pm SD from three experiments.

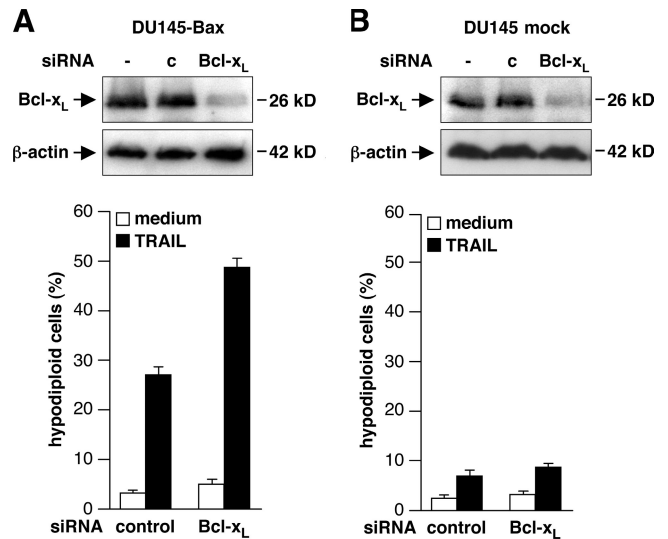


Figure 5. Down-regulation of Bcl-x_L fails to circumvent TRAIL resistance of Bax-deficient DU145 cells. (A and B) DU145-Bax (A) and DU145 mock cells (B) were transfected with control (c) siRNA or Bcl-x_L siRNA and cultured for 24 h. Down-regulation of Bcl-x_L was confirmed by Western blot analysis. After knockdown of Bcl-x_L, cells were treated with TRAIL at 50 ng/ml and cultured for an additional 24 h. Measurement of apoptotic DNA fragmentation revealed that Bcl-x_L down-regulation sensitized DU145-Bax cells for TRAIL-induced apoptosis but failed to circumvent TRAIL resistance of DU145 mock. Error bars indicate means \pm SD from three experiments.

resistance and facilitated apoptosis in 33% of the cells despite Bax deficiency (Fig. 7 B). Similar results were obtained with roscovitine (CYC202 or seliciclib), a small molecule, cyclin-dependent kinase inhibitor that was also shown to down-regulate Mcl-1 (MacCallum et al., 2005; Raje et al., 2005; Ortiz-Ferrón et al., 2008). Preincubation of DU145 mock cells with nontoxic concentrations of roscovitine strongly sensitized and efficiently overcame TRAIL resistance of these Bax-deficient cells (Fig. 7 C). Down-regulation of Mcl-1 by Sorafenib or roscovitine also enhanced cell death induction in Bax-expressing DU145-Bax cells upon TRAIL treatment in a dose-dependent manner (Fig. S2).

So far, we have shown that knockdown of Mcl-1 but not Bcl-x_L can sensitize cells for TRAIL-induced apoptosis and overcomes TRAIL resistance of Bax deficient cells. To analyze the role of Mcl-1 and Bcl-x_L in the regulation of cell death induced by other death receptors, we treated DU145 wt and DU145-Bax cells with TNF or CD95/FasL. As shown in Fig. 8 A (left), DU145-Bax cells are sensitive to TNF-induced apoptosis, and sensitivity is increased after down-regulation of Mcl-1 or Bcl-x_L. DU145-Bax cells are also susceptible for CD95/FasL-induced cell death, which is also enhanced after Mcl-1 or Bcl-x_L knockdown (Fig. 8 A, right). In contrast, neither TNF nor CD95/FasL induced an appreciable number of apoptosis in Bax-deficient cells. However, this resistance to TNF- and CD95/FasL-induced cell death can be overcome by down-regulation of Mcl-1 but not Bcl-x_L (Fig. 8 B). Collectively, these data show a differential impact of the endogenous Bak inhibitors Mcl-1 and Bcl-x_L on death receptor-induced apoptosis in Bax-deficient cells.

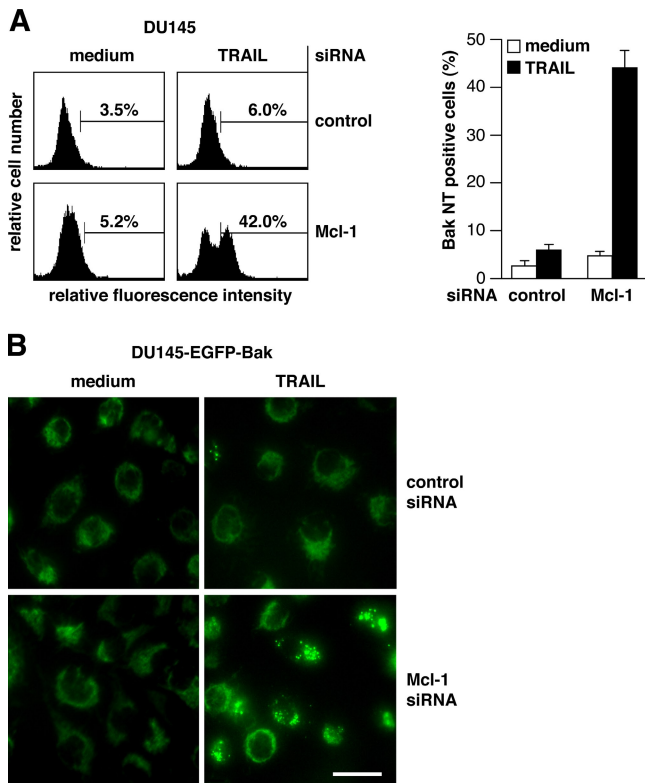


Figure 6. Mcl-1 silencing enables TRAIL to activate Bak. 24 h after Mcl-1 down-regulation, Bax-deficient DU145 cells were treated with 50 ng/ml TRAIL and cultured for an additional 24 h. (A) Cells were stained with a conformation-specific antibody against the Bak N terminus (NT) and analyzed by flow cytometry. The percentage of immunostained cells, indicating Bak activation, is shown between the markers (representative histograms). Means from triplicates \pm SD are shown on the right. (B) Clustering of EGFP-Bak after siRNA repression of Mcl-1 and TRAIL treatment. The distribution of EGFP-Bak was determined by fluorescence microscopy. Bar, 50 μ m.

Apart from the HCT116 and DU145 systems, we analyzed the impact of Mcl-1 and Bcl-x_L on TRAIL-induced apoptosis in three additional cell lines: the osteosarcoma cell line U2OS, the cervical cancer cell line HeLa, and the colon carcinoma cell line LoVo. It is known that U2OS and HeLa cells are resistant to TRAIL-induced apoptosis. Western blot analysis revealed that both cell lines are positive for Bax and Bak expression (Fig. S3). Thus, other mechanisms than loss of Bax have to account for the insensitivity to TRAIL-induced apoptosis. However, down-regulation of either Mcl-1 or Bcl-x_L sensitizes these cell lines for TRAIL-induced apoptosis (Fig. 9, A and B). These data support our notion that Mcl-1 and Bcl-x_L are involved in the regulation of TRAIL-induced cell death in Bax/Bak-proficient cells. In contrast, and despite loss of Bax as a result of biallelic frame shift mutation (Sturm et al., 2001), LoVo cells are not entirely resistant. This might be because of the high expression level of Bak (Fig. S3). Nevertheless, sensitivity of LoVo cells toward TRAIL-induced apoptosis can be increased by the down-regulation of Mcl-1 but not Bcl-x_L (Fig. 9 C). Collectively, these data support our finding that the endogenous inhibitors of Bak, Mcl-1 and Bcl-x_L, have a differential impact on the Bak pathway in TRAIL resistance of Bax-deficient carcinomas.

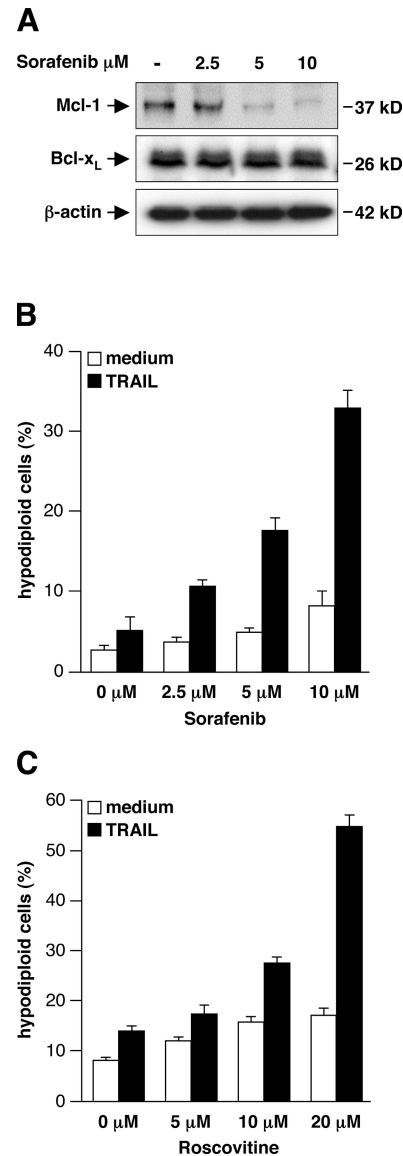


Figure 7. Sorafenib and roscovitine overcome TRAIL resistance in Bax-negative DU145 cells. (A) Bax-deficient DU145 mock cells were treated with different concentrations of sorafenib, and down-regulation of Mcl-1 but not Bcl-x_L was observed upon Western blot analysis. (B and C) DU145 cells were preincubated with or without the indicated concentrations of sorafenib (B) or roscovitine (C), treated with 50 ng/ml TRAIL, and cultured for 24 h. Apoptotic cells were detected by flow cytometric measurement of cellular DNA content. Means \pm SD from three experiments are shown.

Discussion

Despite the fact that TRAIL preferentially induces apoptosis in tumor cells, resistance of many cancers toward TRAIL-induced apoptosis limits the therapeutic application of this death ligand. Apart from loss of TRAIL receptor expression, resistance can be acquired by different mechanisms, including up-regulation of TRAIL decoy receptors (Mérino et al., 2007), increased expression of the caspase-8 inhibitor cFLIP (Krueger et al., 2001; Micheau, 2003), loss of caspase-8 (Grotzer et al., 2000), or enhanced AKT kinase activity caused by PTEN phosphatase deletion (Nesterov et al., 2001). In type II cells, which represent the majority of malignancies, TRAIL-mediated

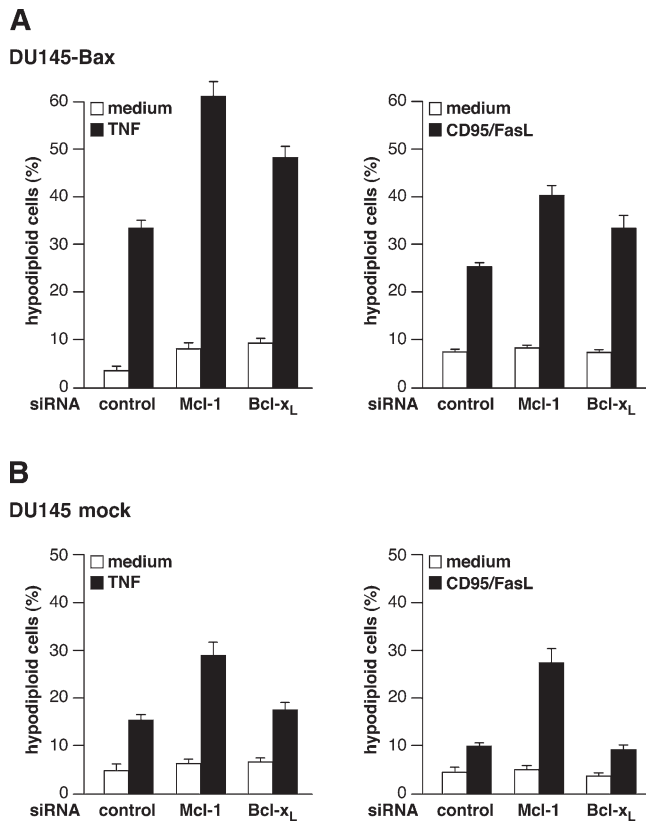


Figure 8. Mcl-1 but not Bcl-x_L mediates resistance to TNF and CD95/FasL-induced apoptosis in Bax-deficient DU145 cells. (A and B) DU145-Bax (A) and DU145 mock cells (B) were transfected with control, Mcl-1, or Bcl-x_L siRNA and cultured for 24 h. Down-regulation of Mcl-1 and Bcl-x_L was confirmed by Western blot analysis. After knockdown of Mcl-1 or Bcl-x_L, cells were treated with TNF (left) or CD95/FasL (right) and cultured for an additional 24 h. Measurement of apoptotic DNA fragmentation revealed that Mcl-1 or Bcl-x_L down-regulation sensitized DU145-Bax cells for TRAIL-induced apoptosis. However, only Mcl-1 knockdown overcomes resistance of Bax-deficient DU145 cells, whereas Bcl-x_L knockdown failed to circumvent resistance. Means \pm SD from triplicates are shown.

apoptosis depends on the mitochondrial death pathway. Consequently, deregulation of Bcl-2 family members may also contribute to TRAIL resistance (Rudner et al., 2005). Several studies have shown that loss of the proapoptotic Bcl-2 family member Bax protects cancer cells from apoptosis induced by TRAIL (Deng et al., 2002; LeBlanc et al., 2002; Ravi and Bedi, 2002; Theodorakis et al., 2002; von Haefen et al., 2004; Wendt et al., 2005). This is in line with our results that, despite expression of functional Bak (Gillissen et al., 2003, 2007), Bax-mutated DU145 cells are resistant to TRAIL-induced apoptosis and reexpression of Bax overcomes this resistance. Likewise, knockout of Bax renders TRAIL-sensitive HCT116 tumor cells resistant to TRAIL. Nevertheless, regarding a rheostat model of a balance between pro- and antiapoptotic Bcl-2 family proteins (Gallenne et al., 2009), it might be that loss of Bax protects cells just by decreasing the amount of proapoptotic multi-BH domain proteins under a critical threshold necessary for TRAIL to induce apoptosis. In this study, we show that in sharp contrast to Bax deficiency, loss of Bak expression fails to protect HCT116 colon cancer cells from TRAIL-induced apoptosis. This indicates that in human carcinoma

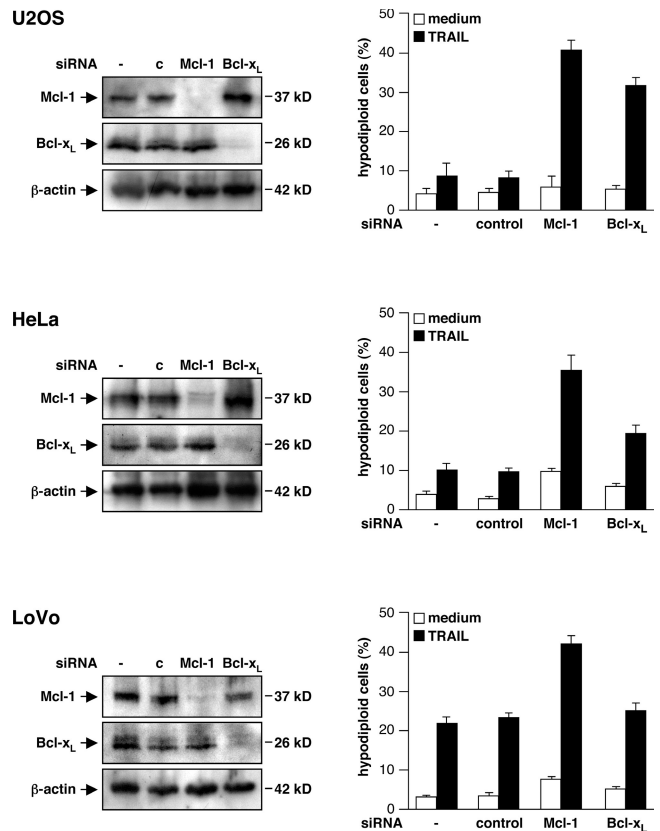


Figure 9. Different impact of Mcl-1 and Bcl-x_L silencing on TRAIL-induced apoptosis in Bax-proficient and -deficient cell lines. U2OS, HeLa, and LoVo cells were transfected with control (c), Mcl-1, or Bcl-x_L siRNA. (left) Down-regulation of respective proteins was confirmed by Western blot analysis. After knockdown of Mcl-1 or Bcl-x_L, cells were treated with TRAIL and cultured for an additional 24 h. (right) Measurement of hypodiploid cells revealed that Mcl-1 down-regulation sensitized U2OS, HeLa, and LoVo cells for TRAIL-induced apoptosis. In contrast, Bcl-x_L knockdown sensitized only U2OS and HeLa cells (both Bax proficient) but failed to sensitize Bax-deficient LoVo cells. Means \pm SD from triplicates are shown.

cells, induction of cell death by TRAIL is mediated specifically via an apparently Bak-independent pathway that relies in turn entirely on Bax. Evidence for a differential role of Bak versus Bax in cell death regulation also comes from analyses on BH3-only proteins or bacterial toxins (Cartron et al., 2003b; Gillissen et al., 2003, 2007; Lindenboim et al., 2005; Pardo et al., 2006).

Interestingly, recent studies have shown involvement of Mcl-1, an endogenous Bak inhibitor, in the regulation of TRAIL-induced apoptosis (Henson et al., 2003; Taniai et al., 2004; Wirth et al., 2005; Han et al., 2006; Meng et al., 2007). Similar data are available for Bcl-x_L, the second major endogenous inhibitor of Bak (Willis et al., 2005). Therefore, we asked whether down-regulation of Bcl-x_L or Mcl-1 overcomes the failure of TRAIL to trigger the Bak pathway and thereby sensitizes cancer cells for TRAIL-induced apoptosis to bypass the TRAIL resistance of Bax-deficient cancer cells. Mcl-1 down-regulation sensitized Bak-expressing cells for TRAIL-induced apoptosis regardless of their Bax status, whereas down-regulation of Bcl-x_L failed to overcome TRAIL resistance in Bax-deficient cells. In contrast to Mcl-1, down-regulation of Bcl-x_L failed to

sensitize Bak-positive/Bax-negative DU145 cancer cells, showing that sensitization for TRAIL is not caused by a decreased amount of antiapoptotic proteins in general but requires specific knockdown of Mcl-1 to allow for the activation of the Bak pathway. This is in agreement with the finding that down-regulation of Mcl-1 failed to sensitize Bak-deficient/Bax-positive HCT116 cells, indicating that in contrast to Bcl-x_L, Mcl-1 is not involved in the regulation of Bax activity during TRAIL-mediated apoptosis.

Insights into the mechanism by which TRAIL relies on Bax but fails to activate the Bak pathway came from recent publications, showing that apoptosis relies on selective interactions between particular subsets of Bcl-2 proteins. Although controversial models suggest (Cartron et al., 2003a, 2004) or object (Uren et al., 2007; Willis et al., 2007) binding of BH3-only proteins to Bax/Bak, interactions between BH3-only proteins or multidomain proteins with antiapoptotic Bcl-2 family members are well established. Bak binds to Mcl-1 and Bcl-x_L but not to Bcl-2. Thus, in healthy cells, Bak is specifically held in check by Bcl-x_L and Mcl-1 (Willis et al., 2005). In addition, Chen et al. (2005) suggests that Mcl-1 does not bind with appreciable affinity to tBid, the active form of Bid generated through caspase-8-mediated cleavage during TRAIL-induced apoptosis. These results are consistent with other studies showing that tBid is not associated with Mcl-1 after TRAIL treatment (Meng et al., 2007). This indicates that tBid is not sufficient to release Bak from its inhibitory binding to Mcl-1. In contrast, TRAIL caused increased binding of Bim and Puma to Mcl-1 (Meng et al., 2007). Nevertheless, binding of Bim, Puma, or Nbk has been shown to stabilize Mcl-1 (Mei et al., 2005; Czabotar et al., 2007; Gillissen et al., 2007). Inhibition of proapoptotic Bcl-2 family members by Mcl-1 might therefore be further enforced by increased expression of Mcl-1 induced by TRAIL through activation of the NF-κB pathway (Ricci et al., 2007) that would also facilitate expression of Bcl-x_L, an NF-κB target. Nevertheless, our functional data delineate that Mcl-1 but not Bcl-x_L mediates inhibition of the Bak pathway as an important mechanism of TRAIL resistance in Bax-deficient human carcinoma. Of note, this also proves true for TNF- and CD95/FasL-induced apoptosis. Bax-deficient DU145 and HCT116 cells are resistant to TNF- and CD95/FasL-induced apoptosis, which can be overcome by down-regulation of Mcl-1 but not Bcl-x_L. Supporting evidence for a differential impact of Mcl-1 and Bcl-x_L comes from our recent observation that Mcl-1 but not Bcl-x_L mediates inhibition of the Bak pathway upon the expression of the BH3-only protein Nbk (Gillissen et al., 2007). Furthermore, Bax-deficient DU145 cells are more susceptible for anticancer drug-induced apoptosis upon Mcl-1 down-regulation (unpublished data). Collectively, these data emphasize the central role of Mcl-1 in the regulation of Bak activity upon different death stimuli. Finally, inhibition of Mcl-1 upon expression of the BH3-only protein Noxa sensitizes Bax-deficient cells for TRAIL-induced apoptosis. Noxa shows specificity for Mcl-1 binding, thereby enabling TRAIL to induce killing via Bak. In contrast, the BH3-only protein Nbk does not bind to Mcl-1 and fails to disrupt the Mcl-1–Bak binding and therefore fails to facilitate TRAIL killing via the Bak pathway.

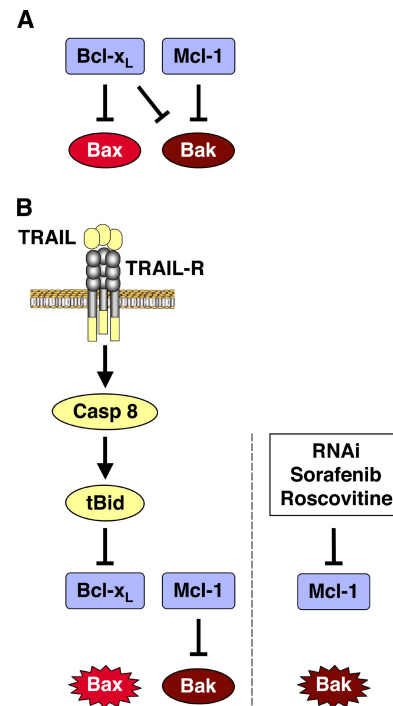


Figure 10. **Model for the regulation of TRAIL-induced apoptosis by Bcl-2 family members.** (A) In healthy cells, Bax is inactivated by Bcl-x_L (and Bcl-2), whereas Bak is sequestered by Bcl-x_L and Mcl-1. (B) Via the caspase-8/tBid signaling pathway, TRAIL activates the proapoptotic Bax through neutralization of the antiapoptotic Bcl-x_L. In contrast, the antiapoptotic Mcl-1, which is not targeted by tBid, keeps Bak in check. Down-regulation of Mcl-1 by RNA interference or targeted therapeutics, like sorafenib or roscovitine, enables TRAIL to activate Bak.

Therefore, small molecules known to down-regulate Mcl-1 might represent suitable means to overcome TRAIL resistance. The kinase inhibitor sorafenib blocks, e.g., NF-κB, and thereby increases TRAIL-induced apoptosis in a variety of neoplastic human cell lines including human leukemia cells (Meng et al., 2007; Rosato et al., 2007). Roscovitine induces cell death in multiple myeloma cells by down-regulation of Mcl-1 (MacCallum et al., 2005; Raje et al., 2005) and sensitizes glioma cells to TRAIL-mediated apoptosis. In this study, sorafenib and roscovitine circumvent TRAIL resistance in Bax-negative carcinoma cell lines and enable TRAIL to activate Bak. However, sensitization of DU145 cells by roscovitine is even more powerful than Mcl-1 down-regulation by siRNA. Thus, other mechanisms might be involved in sensitization by roscovitine. In this vein, roscovitine was shown to sensitize cancer cells to TRAIL-mediated apoptosis by down-regulation of survivin and XIAP (Kim et al., 2004).

In summary, resistance against TRAIL occurring in consequence of Bax deficiency can be overcome by down-regulation of Mcl-1, which enables TRAIL to activate a Bak-dependent apoptotic pathway (Fig. 10). The model of Bak activation upon TRAIL treatment shown in Fig. 10 is based on the derepression mechanism. However, inactivation of Mcl-1 is a prerequisite for Bak activation irrespective of the derepression or the direct activator model. The result that inactivation of Mcl-1 enables TRAIL to activate Bak is an important finding because Bax is often mutated in tumors having a microsatellite mutator phenotype, and loss of Bax expression has been associated

with tumor progression and a bad prognosis in colon, esophageal, and gastric carcinoma (Sturm et al., 1999, 2001; Ionov et al., 2000; Mrózek et al., 2003). Other studies suggest that mismatch repair defects in hematopoietic tumors are linked to Bax tumor suppressor frameshift mutations and that the resulting resistance to apoptosis may be a key feature of some lymphomas and leukemias (Brimmell et al., 1998; Meijerink et al., 1998). In fact, loss of Bax is a frequent event in human cancer, whereas Bak expression persists in most cancers. Thus, down-regulation or repression of endogenous Bak inhibitors like Mcl-1 appears to be a promising strategy to overcome restraints in apoptosis signaling as a result of Bax deficiency and to sensitize these tumors for TRAIL-induced apoptosis via a Bak-dependent pathway. Similar mechanisms are involved in resistance to CD95/FasL and TNF, indicating that control of the Bak pathway by Mcl-1 rather than Bcl-x_L is a common feature in death receptor-mediated apoptosis.

Materials and methods

Cell culture

HCT116 wt cells and their isogenic knockout sublines HCT116-Bax^{-/-} were provided by B. Vogelstein (Johns Hopkins Cancer Center, Baltimore, MD). The stable knockdown of Bak was performed in either HCT116 wt or HCT116-Bax knockout cells yielding Bax⁺/Bak⁻ and Bax⁻/Bak⁻ cells. Transfectants were generated, and cells were cultured as described previously (Gillissen et al., 2003; von Haefen et al., 2004; Hemmati et al., 2006). DU145 were obtained from the German Collection of Microorganisms and Cell Cultures. Control retroviral vector (HyTK mock transfectant) and HyTK-Bax-transfected DU145 prostate carcinoma cells (von Haefen et al., 2002; Gillissen et al., 2003) were grown in DME supplemented with 10% FCS, 100,000 U/liter penicillin, and 0.1 g/liter streptomycin at 37°C with 5% CO₂ in a fully humidified atmosphere. EGFP-Bak transfectants were generated, and cells were cultured as described previously (von Haefen et al., 2004). Media and culture reagents were obtained from Invitrogen.

Antibodies and reagents

Monoclonal mouse anti-Bax antibody (clone YTH-2D2; raised against a peptide corresponding to aa 3–16) was purchased from Trevigen, and rabbit anti-Mcl-1 (H-260; epitope corresponding to aa 1–260, mapping at the N terminus of Mcl-1 of human origin) was obtained from Santa Cruz Biotechnology, Inc. Monoclonal mouse anti-human cytochrome c (clone 7H8.2C12) was obtained from BD, monoclonal mouse anti-caspase-8 (clone 12F5) was obtained from Enzo Life Sciences, Inc., and polyclonal rabbit anti-Bcl-x_L antibody (raised against aa 18–233 of rat Bcl-x_L) was obtained from BD. Goat anti-caspase-9 antibody (raised against human caspase-9 aa 139–330) and polyclonal anti-human caspase-3 produced in goat and rabbit anti-Bid were obtained from R&D Systems. The monoclonal mouse anti-Bak N terminus antibody (clone TC102) was obtained from EMD. Polyclonal anti-human actin antibody produced in rabbits was obtained from Sigma-Aldrich. Secondary anti-rabbit, anti-goat, and anti-mouse HRP-conjugated antibodies were obtained from Promega or SouthernBiotech. RNase A was obtained from Roth, roscovitine was obtained from EMD, and the recombinant human TRAIL was obtained from R&D Systems.

Immunoblotting

After cell trypsinization and washing, protein expression was detected by ECL-based Western blot analysis as described previously (Wendt et al., 2005). For analysis of cytochrome c release, cells were lysed in a hypotonic digitonin buffer as described previously (von Haefen et al., 2003).

RNA interference

On-target plus Mcl-1 siRNA, caspase-8 siRNA, Bid siRNA, and control siRNA were purchased from Thermo Fisher Scientific. Transfection of the cells was performed by use of transfection reagent (DharmaFECT; Thermo Fisher Scientific) according to the manufacturer's instructions. After 24 h, cells were treated with TRAIL.

Measurement of apoptotic cell death by flow cytometry

Apoptosis was determined on the single cell level by measuring the DNA content of individual cells by flow cytometry as described previously (Gillissen et al., 2007). Cellular DNA content was measured with a logarithmic amplification in the FL-3 channel of a flow cytometer (FACScan; BD) equipped with the CELLQuest software. Data are given in percent hypodiploidy (i.e., the percentage of cells with a sub-G1 DNA content), which reflects the percentage of apoptotic cells with fragmented genomic DNA. Alternatively, apoptosis was determined by measuring binding of annexin-V-FITC upon exposure of phosphatidyl serines on the cell surface and PI counterstaining (Gillissen et al., 2003).

Analysis of Bak clustering

EGFP-Bak oligomerization was determined by the use of fluorescence microscopy in DU145 cells stably expressing EGFP-Bak. Cells were transfected with Mcl-1 siRNA or control siRNA and after 24 h treated with or without 50 ng/ml TRAIL for a further 24 h. Cells were fixed in PBS containing 4% paraformaldehyde for 15 min, washed twice in PBS, and mounted in Aquamount. Mounted cells were inspected at room temperature using a microscope (Axiovert 200; Carl Zeiss, Inc.) equipped with a Plan Aplanachromat 63×/1.4 NA objective lens (Carl Zeiss, Inc.) and a digital camera (ORCA ER; Hamamatsu Photonics). Images were acquired using Openlab software (PerkinElmer) on a Mac computer (OS X 10.5; Apple, Inc.).

Construction of recombinant adenovirus

The recombinant adenovirus for Noxa expression was constructed as described previously for Nbk (Gillissen et al., 2003). In brief, the E3 region of Ad5 was replaced by tTA under the control of a cytomegalovirus promoter and an SV40 poly(A) tail. Noxa cDNA was introduced into the E1 region under the control of the Tet-off system to achieve conditional expression in the absence of doxycycline. The resulting DNA construct was transfected in HEK293-packaging cells to produce vector stocks. Adenoviral stocks were propagated according to standard procedures described previously in Hemmati et al. (2002).

Online supplemental material

Fig. S1 shows sensitization of Bak-proficient HCT116 cells to TRAIL-induced phosphatidyl serine exposure upon down-regulation of Mcl-1. Fig. S2 shows sensitization of Bax-proficient DU145 cells to TRAIL-induced apoptosis upon sorafenib and roscovitine treatment. Fig. S3 shows Bcl-x_L, Mcl-1, Bax, and Bak protein expression levels of the cell lines used in this study. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200912070/DC1>.

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