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Reduction of TRAIL-Induced Mcl-1 and cIAP2 by c-Myc or Sorafenib Sensitizes Resistant Human Cancer Cells to TRAIL-Induced Death

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SUMMARY

Cells expressing oncogenic c-Myc are sensitized to TNF superfamily proteins. c-Myc also is an important factor in determining whether a cell is sensitive to TRAIL-induced apoptosis, and it is well established that the mitochondrial pathway is essential for apoptosis induced by c-Myc. We investigated whether c-Myc action on the mitochondria is required for TRAIL sensitivity and found that Myc sensitized cells with defective intrinsic signaling to TRAIL. TRAIL induced expression of antiapoptotic Mcl-1 and cIAP2 through activation of NF-κB. Both Myc and the multikinase inhibitor sorafenib block NF-κB. Combining sorafenib with TRAIL in vivo showed dramatic efficacy in TRAIL-resistant tumor xenografts. We propose the combination of TRAIL with sorafenib holds promise for further development.

INTRODUCTION

TNF-α-related apoptosis-inducing ligand (TRAIL, also known as Apo2L) is a member of the TNF family of death receptor ligands and has significant potential for use in cancer therapy because of its potent ability to selectively kill cancer cells while leaving normal cells unharmed (Kelley and Ashkenazi, 2004). TRAIL-based therapies are now in Phase I and II clinical trials (www.clinicaltrials.gov). TRAIL activates the extrinsic pathway of apoptosis by

SIGNIFICANCE

Activation of TRAIL death receptor signaling provides an exciting approach for cancer therapy because of its potency and lack of significant toxicity. Unfortunately, certain defects in the apoptosis signaling pathway can confer resistance to TRAIL. One approach to optimize TRAIL therapy is to pharmacologically enhance sensitivity to TRAIL. We found that combining TRAIL with either the multikinase inhibitor sorafenib or with oncogenic c-Myc sensitizes *bax*$^{-/-}$ cells to TRAIL through a mechanism involving downregulation of TRAIL-induced Mcl-1 and cIAP2 expression. TRAIL activates NF-κB, which regulates Mcl-1 and cIAP2 expression, and sorafenib decreases NF-κB binding at these promoters. We propose use of sorafenib as a potent sensitizer of cancer cells, especially those harboring apoptotic defects to TRAIL-mediated death.

binding one of two transmembrane cell surface receptors, Death Receptor 4 (DR4) or Death Receptor 5 (DR5) (Jin and El-Deiry, 2005). After binding TRAIL, these receptors form homotrimers that signal through the adaptor protein FADD. FADD recruits caspases 8/10, which then self-activate and initiate downstream caspase cleavage events.

An unanswered question that has gained even more significance now that TRAIL therapy has entered the clinic is what biomarkers are available to identify patients likely to respond to TRAIL therapy? We showed c-Myc to be a key mediator of cancer cell sensitivity to apoptosis induced by TRAIL (Ricci et al., 2004). We found that levels of endogenous c-Myc significantly correlated with TRAIL sensitivity in a panel of cancer cell lines. We identified c-FLIP as a target of c-Myc-mediated transcriptional repression. c-FLIP, which is highly homologous to caspase 8 but is catalytically inactive, can bind caspase 8 and FADD and block caspase 8 activation (Irmler et al., 1997; Shu et al., 1997). Downregulation of FLIP expression results in sensitization to TRAIL-induced apoptosis. If Myc expression results in decreased FLIP, this can increase a cell's ability to undergo apoptosis following TRAIL activation of the extrinsic pathway. These results suggested that cells expressing oncogenic levels of Myc and having defects in their intrinsic pathway of apoptosis (e.g., Bax deficiency) might still be sensitive to TRAIL.

To test whether c-Myc can bypass the mitochondria and sensitize cells to TRAIL, we used *bax*$^{-/-}$ HCT116 human colon carcinoma cells that are resistant to TRAIL (Burns and El-Deiry, 2001). c-Myc overexpression results not only in cell proliferation but also in apoptotic cell death, and it appears critical that the intrinsic pathway of apoptosis is inactivated for c-Myc to promote tumorigenesis (Askew et al., 1991; Evan et al., 1992). Intrinsic pathway activation is predicated on destabilization of the outer mitochondrial membrane following oligomerization of proapoptotic members of the Bcl-2 superfamily, such as Bax and Bak. This destabilization results in the release of cytochrome c and other factors that initiate caspase-mediated apoptotic cell death. The exact mechanisms that explain c-Myc-induced alterations of the proteins involved in maintaining mitochondrial membrane integrity may not be consistent across cell and tissue types, but it is clear that c-Myc exerts a powerful effect on the mitochondria (Juin et al., 1999, 2002; Mitchell et al., 2000; Soucie et al., 2001).

We found that increasing c-Myc expression in *bax*$^{-/-}$ cells sensitizes them to TRAIL. We also found that TRAIL dramatically induces expression of two potent antiapoptotic molecules, cellular inhibitor of apoptosis protein 2 (cIAP2) and the Bcl2 family member, Mcl-1. Myc expression represses TRAIL-induction of both Mcl-1 and cIAP2. In an attempt to mimic Myc action in *bax*$^{-/-}$ cells, we combined the multikinase inhibitor sorafenib with TRAIL. Sorafenib targets both the RAF/MEK/ERK signaling pathway to inhibit cell proliferation and the VEGFR-2/PDGFR-β signaling cascade to inhibit tumor angiogenesis (Wilhelm et al., 2004). Sorafenib was recently FDA approved for treatment of renal cancer and is currently undergoing investigation in over 30 clinical trials for use against a wide range of human cancers, including melanoma, prostate, ovarian, pancreatic, lung cancer, and others (www.clinicaltrials.gov). We found sorafenib sensitizes *bax*$^{-/-}$ cells to TRAIL through a mechanism involving downregulating Mcl-1 and cIAP2 expression. This mechanism involves repression of TRAIL-induced NF-κB transcriptional activation of both the *mcl-1* and *ciap2* promoters. We propose that combining sorafenib with TRAIL can sensitize cancer cells harboring defects in their intrinsic apoptotic signaling pathway by diminishing TRAIL-induction of antiapoptotic proteins.

RESULTS

c-Myc Sensitizes HCT116 *bax*$^{-/-}$ Cells to TRAIL-Mediated Cell Death

To investigate the importance of the intrinsic pathway for mediating c-Myc-induced sensitization to TRAIL, we examined the effect of c-Myc expression in Bax-deficient HCT116 cells. Parental HCT116 cells are heterozygous for *bax* and are referred to herein as HCT116 *bax*$^{+/-}$ (Zhang et al., 2000). HCT116 *bax*$^{+/-}$ cells are very sensitive to TRAIL-induced cell death, but the deletion of the remaining *bax* allele renders these cells TRAIL-resistant (Burns and El-Deiry, 2001). Adding TRAIL to *bax*$^{-/-}$ cells expressing c-Myc for 6 hr resulted in modest cell death (8%) that increased to 27% after 24 hr (Figure 1A). A dose-response analysis showed increasing c-Myc expression sensitized *bax*$^{-/-}$ cells to TRAIL in a direct, linear manner (Figure 1B).

c-Myc-Mediated Sensitization to TRAIL in *bax*$^{-/-}$ Cells Requires Caspase-9 Activation

We analyzed loss of mitochondrial membrane potential after TRAIL treatment to investigate whether c-Myc acts upon the intrinsic apoptotic pathway in *bax*$^{-/-}$ cells. c-Myc altered membrane potential in *bax*$^{-/-}$ cells and this effect increased 2-fold after TRAIL treatment (Figure 1C). TRAIL induced cleavage of procaspase 3 in HCT116 *bax*$^{-/-}$ cells irrespective of c-Myc-expression, but TRAIL cleaved caspase 9 only in cells expressing c-Myc or Bax (Figure 1D). We tested whether caspase 9 is required for c-Myc sensitization to TRAIL by adding a specific inhibitor of activated caspase 9 (Z-LEHD-FMK). The caspase 9 inhibitor completely blocked c-Myc-mediated sensitization of the *bax*$^{-/-}$ cells to TRAIL (Figure 1E).

TRAIL Resistance in *bax*$^{-/-}$ Cells Involves TRAIL-Induced cIAP2 and Mcl-1 Expression

Because Bax deficiency does not block TRAIL-induced Bid cleavage (Figure 2A) (Ravi and Bedi, 2002), we reasoned that c-Myc might alter levels of other Bcl-2 family members. Unexpectedly, we observed that TRAIL induced a profound increase in Mcl-1 protein expression in the *bax*$^{-/-}$ cells (Figure 2A). Human Mcl-1 is an antiapoptotic member of the Bcl-2 protein family with similar BH-multidomain structures as Bcl-2 and Bcl-xL (Reed et al., 2004). Increased Mcl-1 expression likely confers additional protection against tBid in TRAIL-treated Bax-deficient cells. Examination of Bcl-2, Bcl-xL, and Bid

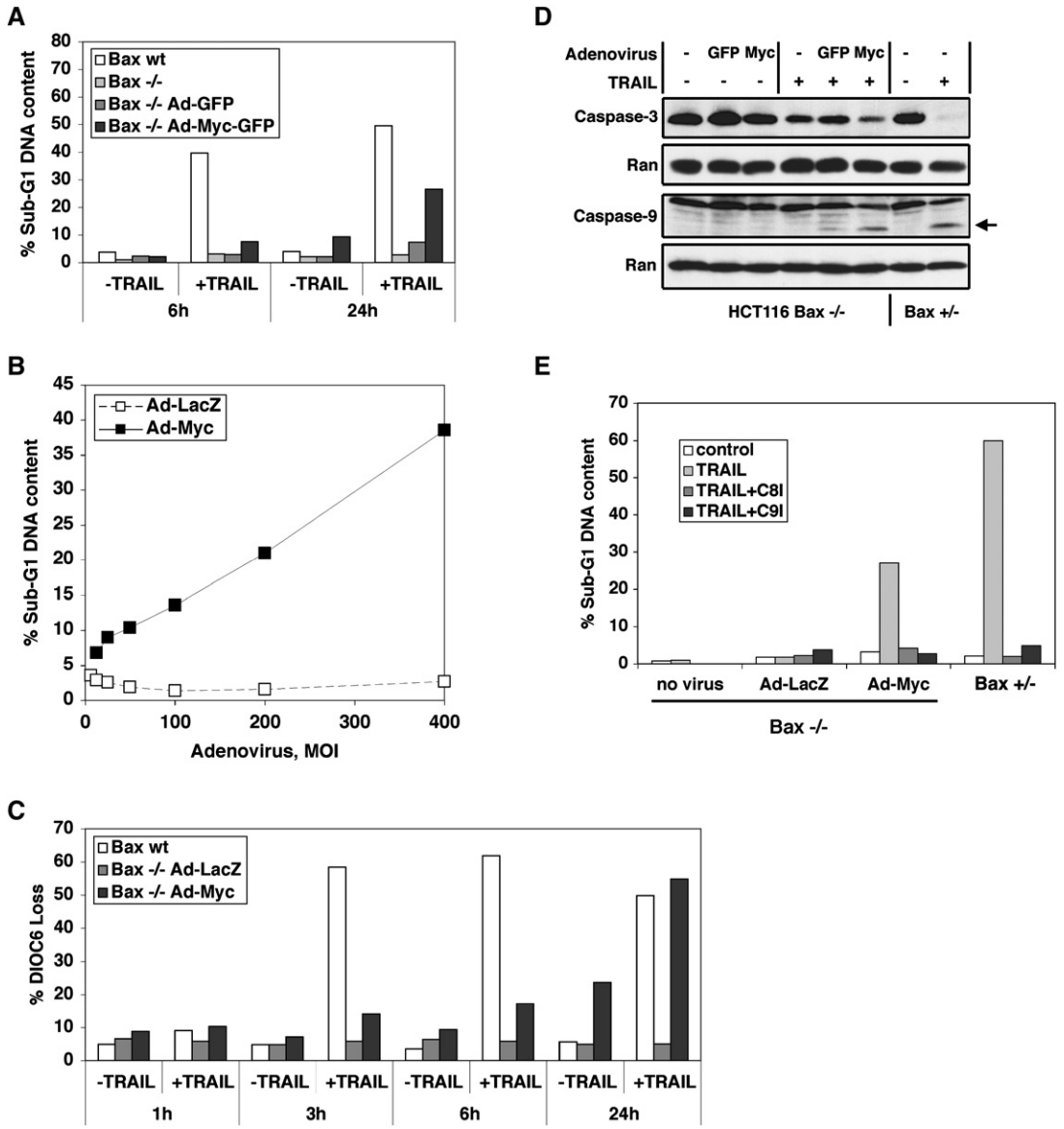


Figure 1. HCT116 *bax*$^{-/-}$ Cells Are Sensitized to TRAIL-Induced Apoptosis by c-Myc

(A) HCT116 *bax*$^{-/-}$ cells were infected with adenoviruses for 24 hr, then treated with TRAIL (50 ng/ml) for 6 hr or 24 hr. Percentages of cells with sub-G1 DNA content are shown.

(B) *bax*$^{-/-}$ cells were infected with adenoviruses for 24 hr, then treated with TRAIL (125 ng/ml) for 18 hr.

(C) Cells infected with non-GFP-expressing adenoviruses for 24 hr were treated with TRAIL (50 ng/ml) for the times indicated. Cells were collected and loss of mitochondria transmembrane potential was measured.

(D) Immunoblots using lysates from HCT116 *bax*$^{-/-}$ cells infected with adenovirus for 24 hr and treated with TRAIL for 6 hr.

(E) HCT116 *bax wt* cells and HCT116 *bax*$^{-/-}$ cells infected with adenovirus were treated with TRAIL, with or without an inhibitor to caspase 8 (C8I) or caspase 9 (C9I), for 24 hr.

levels in *bax*$^{-/-}$ cells did not show any meaningful changes in protein levels with Myc expression. Puma expression was diminished in TRAIL-treated Myc expressing cells, but Myc alone had no effect. Bim increased with TRAIL, and Myc expression reduced this induction. Given that all Bim isoforms are proapoptotic (O'Connor et al., 1998), these changes in Bim expression do not appear to impact the mechanism of Myc-mediated sensitization to TRAIL. TRAIL exposure also resulted in the loss of Bak expression in all treated cells. This observation is potentially in conflict with evidence showing that either Bax or Bak are required for apoptosis (Wei et al., 2001). It is possible that the initial presence of Bak permits the permeabilization of the outer mitochondrial membrane and tBid mediates cytochrome c release. A study from the Korsmeyer Laboratory described a BH3-independent function of tBid that supports this scenario (Scorrano et al., 2002). They found that tBid acts to mobilize

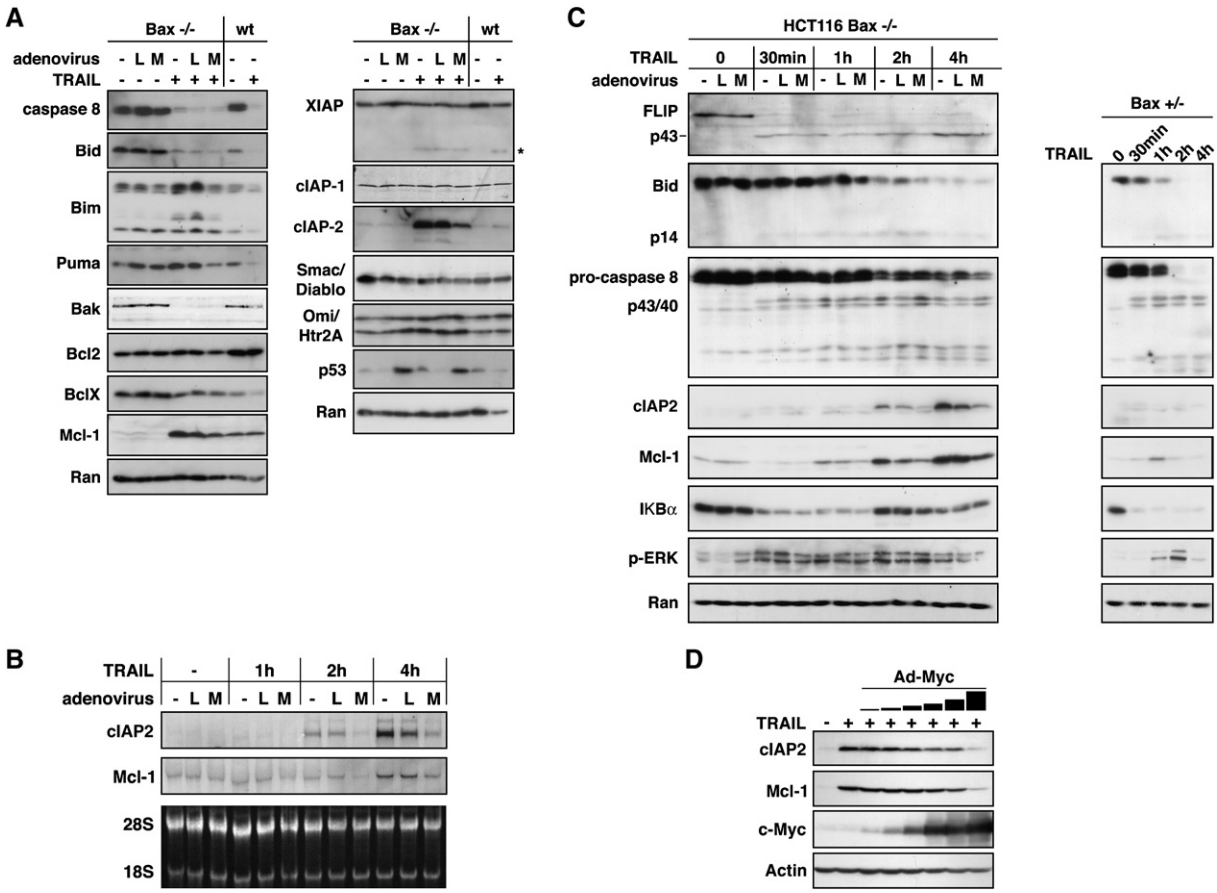


Figure 2. c-Myc Represses TRAIL-Induced cIAP2 and Mcl-1 Expression in *bax*$^{-/-}$ Cells

(A) *bax*$^{-/-}$ cells were infected with the indicated adenoviruses (L = Ad-LacZ; M = Ad-Myc) for 24 hr then treated with TRAIL for 24 hr. HCT116 *bax*$^{+/-}$ cells were treated with TRAIL for 6 hr and were not infected with adenoviruses.

(B) Cells infected with adenoviruses (24 hr) were treated with TRAIL for the times shown. Total RNA was isolated and northern blot analysis was performed.

(C) Immunoblots are shown for cells infected with adenoviruses for 24 hr and collected after TRAIL exposure for the times shown.

(D) Cells were infected with Ad-Myc as shown in Figure 1B for 24 hr then treated with TRAIL for 4 hr.

cytochrome c from isolated mitochondrial cristae into the intermembrane space and this property of tBid is independent of its BH3 domain. A small fraction of cytochrome c is present in the intermembrane space of mitochondria (~15%) that is quickly released after tBid activation. The remainder of cytochrome c sequestered in cristae (~85%) is subsequently mobilized to the intermembrane space by changes in mitochondrial structure initiated by tBid. It follows that in cells having elevated Mcl-1 expression, tBid is bound by Mcl-1 (Clohessy et al., 2006) and all of its proapoptotic activities are inhibited.

We continued our examination of factors that act downstream of Bid and are involved in blocking activated caspase 9. Inhibitor of Apoptosis proteins (IAPs) can block executioner caspases. Specifically, XIAP is a potent inhibitor of caspase 9, while cIAP1, cIAP2, and XIAP can inhibit caspases 3 and 7 (Roy et al., 1997; Shiozaki et al., 2003). When the mitochondrial membrane is disrupted during apoptosis, Smac/DIABLO and Omi/Htr2A are released and these proteins act to inhibit IAPs. c-Myc does not alter expression of the XIAP, cIAP1, or their controllers in *bax*$^{-/-}$ cells, but in a manner similar to Mcl-1, we found that TRAIL induced a profound increase in cIAP2 protein (Figure 2A).

Oncogenic levels of c-Myc can stabilize p53 protein levels, and c-Myc expression resulted in the stabilization of p53 in the *bax*$^{-/-}$ cells (Figure 2A). To test the importance of p53 in mediating c-Myc sensitization to TRAIL, we performed p53 siRNA knockdown experiments. We found that significant reduction in p53 levels did not change the ability of c-Myc to sensitize *bax*$^{-/-}$ cells to TRAIL-induced death (see Figure S1 in the Supplemental Data available with this article online).

c-Myc Represses TRAIL-Induced Mcl-1 and cIAP2 Expression

Myc expression in *bax*$^{-/-}$ cells showed reduced Mcl-1 and cIAP2 proteins and mRNAs following TRAIL treatment (Figures 2A and 2B). Time-course analysis revealed that TRAIL rapidly induced Mcl-1 and cIAP2 mRNA and protein

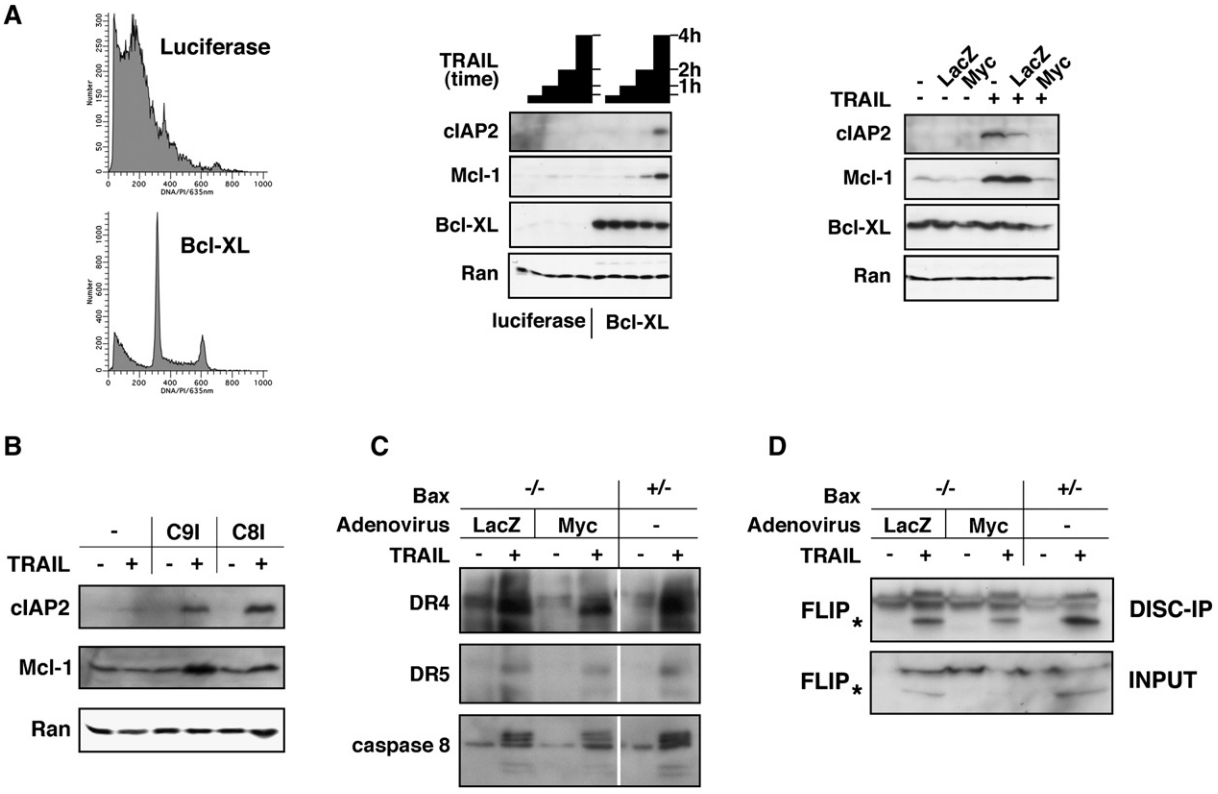


Figure 3. TRAIL Signaling Increases cIAP2 and Mcl-1 Expression when Caspase Signaling Is Blocked

(A) HCT116 *bax*$^{+/-}$ cells stably expressing Bcl-xL or firefly luciferase were generated by retroviral insertions. (Left) Cells were treated with TRAIL for 4 hr. (Center) Cells treated with TRAIL for the times indicated were collected and immunoblot analysis performed. (Right) HCT116-Bcl-xL cells were infected with the indicated adenoviruses for 24 hr, and then treated with TRAIL for 4 hr. Immunoblots are shown.

(B) HCT116 *bax*$^{+/-}$ cells were treated with caspase 8 (C8I) or caspase 9 (C9I) inhibitors for 30 min prior to TRAIL exposure. Cells were collected 6 hr later.

(C and D) Cells were treated with TRAIL for 15 min, collected and DISC immunoprecipitation was performed. In (D), the asterisk indicates the 43 kDa cleaved form of c-Flip.

expression, and this induction was substantially reduced by c-Myc (Figures 2B and 2C). In contrast to *bax*$^{-/-}$ cells, the *bax*$^{+/-}$ cells showed only a slight induction of Mcl-1 protein that quickly disappeared following TRAIL treatment and no induction of cIAP2 (Figure 2C). Examination of c-Myc expressing cells following TRAIL treatment showed a clear dose-dependent relationship between c-Myc and the reduction in Mcl-1 and cIAP2 (Figure 2D).

TRAIL Induces Mcl-1 and cIAP2 in Bax-Expressing Cells When Apoptosis Is Blocked

To help determine the role mitochondrial release of factors plays in TRAIL-induced gene expression, we established HCT116 *bax*$^{+/-}$ cells stably expressing Bcl-xL. Cells expressing Bcl-xL were resistant to TRAIL-induced death (Figure 3A). TRAIL induced Mcl-1 and cIAP2 in Bcl-xL-expressing cells, and c-Myc repressed their induction (Figure 3A). Therefore, when the intrinsic pathway is blocked by Bcl-xL overexpression or Bax-deficiency, TRAIL mediates induction of the antiapoptotic factors Mcl-1 and cIAP2.

To test whether caspase 9 activation contributes to TRAIL induction of Mcl-1 and cIAP2, we examined lysates of *bax*$^{+/-}$ cells treated with TRAIL plus caspase inhibitors. Caspase 8 and 9 inhibitors block TRAIL-mediated death of the *bax*$^{+/-}$ cells (Figure 1E) and permit the expression of Mcl-1 and cIAP2 (Figure 3B). HCT116 *bax*$^{+/-}$ cells are classified as type II cells, and as such, caspase 8 activity is not principally responsible for activating executioner caspases in these cells (Kim et al., 2004; Ozoren and El-Deiry, 2002). Indeed, inhibition of caspase 9 resulted in almost complete ablation of TRAIL-induced caspase 8 activity (data not shown), which might result from the inhibition of feedback activation of caspase 8 by executioner caspases such as caspase 6 (Cowling and Downward, 2002). Therefore, we postulated that if c-Myc alters Death Inducing Signaling Complex (DISC) formation in *bax*$^{+/-}$ or *bax*$^{-/-}$ cells, this is not the principal mechanism for c-Myc-mediated repression of TRAIL-induced gene expression. We tested whether c-Myc expression altered DISC formation by performing DISC immunoprecipitations and found that there were only modestly smaller amounts of FLIP, DR4, DR5, and caspase 8 at the TRAIL

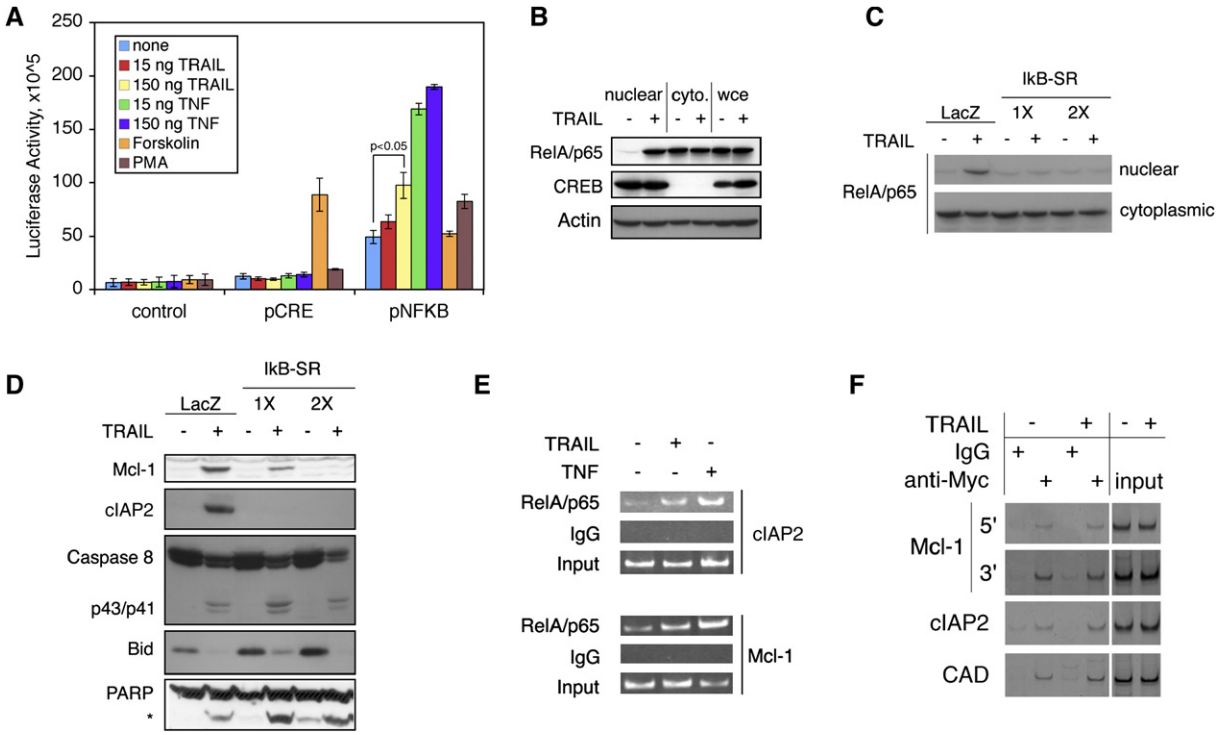


Figure 4. NF-κB Mediates TRAIL Activation of Mcl-1 and cIAP2 Transcription

(A) *bax*$^{-/-}$ cells transfected with luciferase reporter plasmids responsive to CREB, NF-κB, or a control were treated with TRAIL for 24 hr. Forskolin was used as a positive control for CREB activation. Significance of difference for the NF-κB reporter was calculated by Student's t test analysis. Results are shown as the mean (bar) ± SD.

(B) *bax*$^{-/-}$ cells treated with 150 ng TRAIL for 2 hr were collected and cytosolic and nuclear extracts prepared. Equal protein amounts of each were loaded along with whole cell extracts (wce).

(C) *bax*$^{-/-}$ cells were infected with adenovirus expressing IκB-SR or LacZ for 24 hr. Cells were treated with TRAIL (150 ng/ml) for 2 hr, collected, and cytosolic and nuclear extracts were prepared.

(D) *bax*$^{-/-}$ cells were infected with adenovirus expressing IκB-SR or LacZ for 24 hr. Cells were treated with TRAIL for 6 hr and total lysates prepared.

(E) *bax*$^{-/-}$ cells treated with TNF (10 ng/ml) or TRAIL (50 ng/ml) for 4 hr were subject to chromatin immunoprecipitation analysis using a RelA/p65 antibody or IgG isotype control.

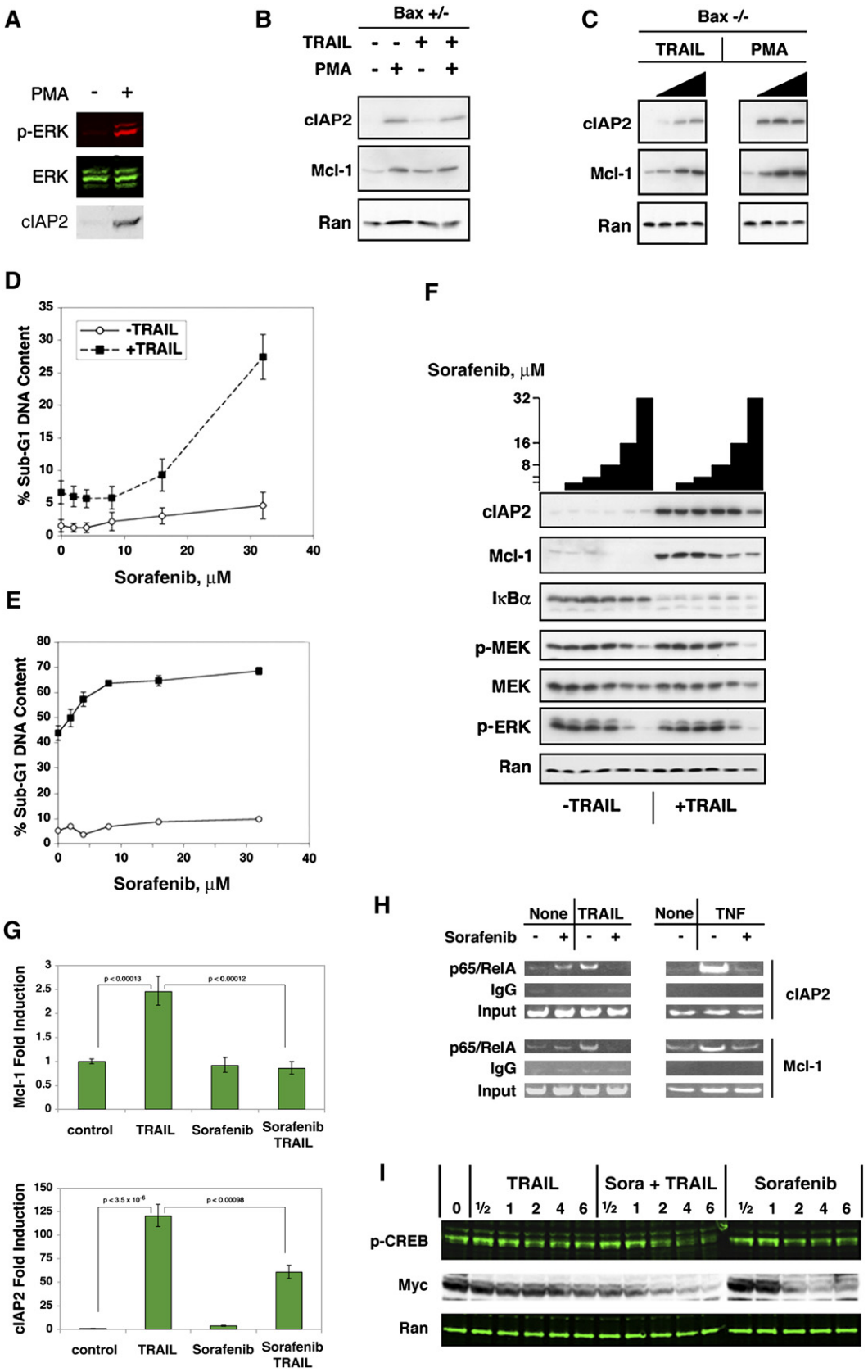
(F) *bax*$^{-/-}$ cells were infected with Ad-Myc for 24 hr then treated with TRAIL for 1.5 hr. ChIP analysis was performed. The two sequences for Mcl-1 refer to upstream (5′) and downstream (3′) of the Mcl-1 transcriptional start site. CAD is a known Myc target gene.

DISC in c-Myc-expressing cells (Figures 3C and D). Diminished FLIP levels also had no effect on TRAIL induced Mcl-1 and cIAP2 expression (Figure S4).

Transcriptional Control of Mcl-1 and cIAP2 by TRAIL

ciap2 is a well-characterized transcriptional target of NF-κB, potently induced by TNF-α (Wang et al., 1998). Transcriptional control of *mcl-1* is less understood, but putative binding sites for NF-κB were identified in its promoter region (Moshynska et al., 2004) and both *mcl-1* and *ciap2* were found to be c-AMP response element-binding protein (CREB) targets (Nishihara et al., 2004; Wang et al., 1999). Multiple kinase signaling pathways, including Raf/MEK/ERK, phosphorylate and activate CREB. TRAIL has been shown to activate both NF-κB and the Raf/MEK/ERK kinase pathway (Harper et al., 2001; Hu et al., 1999; Morel et al., 2005; Tran et al., 2001; Zhang et al., 2003). We observed TRAIL rapidly induces ERK phosphorylation and IκBα degradation, an activating step for NF-κB (Figure 2C). To directly examine whether TRAIL activated NF-κB or CREB signaling in *bax*$^{-/-}$ cells, we used luciferase reporter plasmids containing binding sites for CREB or NF-κB (Figure 4A). These data showed that TRAIL induced NF-κB transactivation but had no effect on CREB.

We further examined the role of NF-κB in mediating TRAIL action. We observed TRAIL induced a rapid decrease in IκBα protein expression after 30 min, and IκBα levels returned to normal after 2 hr (Figure 2C). Given that *IκBα* and *ciap2* are known NF-κB target genes, these data suggest that NF-κB is activated by TRAIL in *bax*$^{-/-}$ cells, and NF-κB may also regulate *mcl-1* (Ito et al., 1994; Wang et al., 1998). To test this, we examined nuclear extracts from TRAIL-treated cells and observed the translocation of the RelA/p65 subunit of NF-κB into the nucleus (Figure 4B). We expressed a nondegradable mutant of IκBα (IκB-Super Repressor) to test whether NF-κB is required for TRAIL induction of Mcl-1 and cIAP2. IκB-SR blocked TRAIL-activation of NF-κB in *bax*$^{-/-}$ cells


A
PMA - +
p-ERK
ERK
cIAP2
B
Bax +/-
TRAIL - - + +
PMA - + - +
cIAP2
Mcl-1
Ran
C
Bax -/-
TRAIL
PMA
cIAP2
Mcl-1
Ran
D
-TRAIL
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% Sub-G1 DNA Content
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E
% Sub-G1 DNA Content
Sorafenib, μM
F
Sorafenib, μM
32
16
8
cIAP2
Mcl-1
IκBα
p-MEK
MEK
p-ERK
Ran
-TRAIL
+TRAIL
G
Mcl-1 Fold Induction
p < 0.00013
p < 0.00012
control
TRAIL
Sorafenib
Sorafenib TRAIL
cIAP2 Fold Induction
p < 3.5 x 10-6
p < 0.00098
control
TRAIL
Sorafenib
Sorafenib TRAIL
H
None
TRAIL
None
TNF
Sorafenib - + - + - - +
p65/RelA
IgG
Input
cIAP2
p65/RelA
IgG
Input
Mcl-1
I
TRAIL
Sora + TRAIL
Sorafenib
0 ½ 1 2 4 6 ½ 1 2 4 6 ½ 1 2 4 6
p-CREB
Myc
Ran

(Figure 4C) and TRAIL-mediated induction of Mcl-1 and cIAP2 (Figure 4D). NF-κB inhibition did not alter the cleavage of caspase 8 or Bid, but it did result in increased TRAIL-induced death, visualized by an increase in PARP cleavage. The cleavage patterns of caspase 8 and Bid in the presence of IκB-SR are consistent with the interpretation that the effects of TRAIL occur downstream of and in spite of Bid processing. We further examined whether NF-κB can directly activate *mcl-1* transcription. *ciap2* is a well-characterized NF-κB target gene that can be activated by TNF (Wang et al., 1998). We performed chromatin immunoprecipitation using antibody to the RelA/p65 subunit of NF-κB following TRAIL and TNF treatment and found that it binds to both the *mcl-1* and *ciap2* promoters (Figure 4E).

The northern blot data suggest that Myc regulates *mcl-1* and *ciap2* at the level of transcription (Figure 2B). To confirm this, we performed ChIP using Ad-Myc infected *bax*$^{-/-}$ cells and found Myc bound to both the *mcl-1* and *ciap2* promoters (Figure 4F). Addition of TRAIL did not significantly alter Myc binding. This suggests that TRAIL does not alter Myc regulation of *mcl-1* and *ciap2*, but that Myc may alter TRAIL action on NF-κB. There are several reports showing Myc suppression of NF-κB function, but a clear consensus on the mechanism is lacking (Keller et al., 2005; Klefstrom et al., 1997; Sitcheran et al., 2005; Tanaka et al., 2002; You et al., 2002). For example, one report shows that B cells derived from Eμ-Myc mice have reduced NF-κB expression and DNA-binding activity (Keller et al., 2005). We examined whether Myc regulates NF-κB expression in *bax*$^{-/-}$ cells, and found that Myc does not significantly diminish NF-κB subunit protein levels or their translocation to the nucleus following TRAIL treatment (Figure S2A).

The Kinase Inhibitor Sorafenib Sensitizes *bax*$^{-/-}$ Cells to TRAIL

In parallel with our experiments examining transcriptional control of *mcl-1* and *ciap2* following TRAIL treatment, we explored the importance of Raf/MEK/ERK signaling downstream of TRAIL. We tested whether Raf/MEK/ERK signaling altered *mcl-1* and *ciap2* transcription in *bax*$^{-/-}$ cells by treating them with phorbol 12-myristate 13-acetate (PMA), a known inducer of Raf/MEK/ERK signaling (Figure 5A) (Ueda et al., 1996). PMA rapidly and potently induced Mcl-1 and cIAP2 in *bax*$^{+/-}$ and *bax*$^{-/-}$ cells (Figures 5B and 5C). Interestingly, PMA activated NF-κB signaling but not CREB using the luciferase reporter (Figure 4A), suggesting that p-ERK signaling does not activate cREB in *bax*$^{-/-}$ cells. We tested whether Raf signaling was directly involved in mediating TRAIL activation of Mcl-1 and cIAP2 by blocking the kinase pathway using sorafenib, a potent inhibitor of Raf1 (Wilhelm et al., 2004). *bax*$^{-/-}$ cells treated with the combination of TRAIL plus increasing concentrations of sorafenib showed a dose-dependent sensitization to TRAIL by sorafenib (Figure 5D). *bax*$^{+/-}$ cells were also sensitized to TRAIL by sorafenib (Figure 5E). Doses of sorafenib that sensitized *bax*$^{-/-}$ cells to TRAIL also resulted in reduced levels of TRAIL-induced Mcl-1 and cIAP2 (Figure 5F). We made similar observations of the sensitizing effects of sorafenib on TRAIL-induced apoptosis using TRAIL-resistant Calu6, HT29, and RKO human tumor cells (Figure 8A). Confirming the functionality of the Raf inhibitor, we observed that sorafenib reduced constitutively high levels of phosphorylated MEK and ERK1/2 in the *bax*$^{-/-}$ cells (Figure 5F). TRAIL alone does change the status of phosphorylated CREB (p-CREB), which is in agreement with results using the luciferase reporters (Figure 4A), but its combination with sorafenib results in its decrease (Figure 5I). Therefore, the reduction of p-CREB may contribute to the regulation of Mcl-1 expression by the combination of TRAIL and sorafenib. Because GSK3β can regulate both Myc and Mcl-1 protein levels (Gregory et al., 2003; Maurer et al., 2006), we examined whether sorafenib might act on both Myc and Mcl-1 through this pathway. We did not see any observable effects on phosphorylation of GSK3β by TRAIL, sorafenib, or their combination (data not shown). Examination of whether Myc has a role in mediating sensitization by sorafenib showed a dramatic loss of endogenous Myc following sorafenib treatment (Figure 5I). This suggests that the signaling pathways leading to loss of Mcl-1 and cIAP2 expression activated by sorafenib do not involve upregulation of endogenous c-Myc.

Because NF-κB mediates *mcl-1* and *ciap2* transcriptional activation and inhibition of Raf/MEK/ERK can block TRAIL action, we examined whether sorafenib can disrupt TRAIL signaling through NF-κB. We found that sorafenib reduces the amount of TRAIL activated NF-κB bound to the *mcl-1* and *ciap2* promoters using ChIP (Figure 5H). Sorafenib also significantly decreased TRAIL-induced mRNA levels, as would be expected with a loss of

Figure 5. TRAIL signals through the Raf/MEK/ERK pathway to induce cIAP2 and Mcl-1 expression

(A) *bax*$^{-/-}$ cells were treated with 50 ng/ml PMA for 6 hr and immunoblot analysis was performed.

(B) HCT116 *bax*$^{+/-}$ cells were treated with TRAIL (10 ng/ml) and PMA (50 ng/ml) for 6 hr.

(C) *bax*$^{-/-}$ cells were treated with 35, 63, or 125 ng/ml TRAIL, or 12.5, 25, or 50 ng/ml PMA for 6 hr.

(D) Sorafenib was added to *bax*$^{-/-}$ cells at the concentration shown. TRAIL was added 1 hr later. Fifteen hours later, cells were collected and stained with propidium iodide. Results are shown as the mean ± SD.

(E) *bax*$^{+/-}$ cells were treated with sorafenib for 1 hr prior to addition of TRAIL (10 ng/ml) for 6 hr. Results are shown as the mean (bar) ± SD.

(F) Cells treated as in D. were collected, lysed and immunoblotting was performed.

(G) *bax*$^{-/-}$ cells were treated with TRAIL (50 ng/ml) or sorafenib (16 μM) for 4 hr, RNA collected and quantitative RT-PCR analysis performed. mRNA levels normalized to GAPDH are shown. Results are shown as the mean (bar) ± SD. Significance of difference was calculated using Student's t test analysis.

(H) Chromatin immunoprecipitation analysis using *bax*$^{-/-}$ cells treated for 4 hr was performed for the treatments indicated.

(I) *bax*$^{-/-}$ cells were treated with TRAIL or sorafenib for the times indicated.

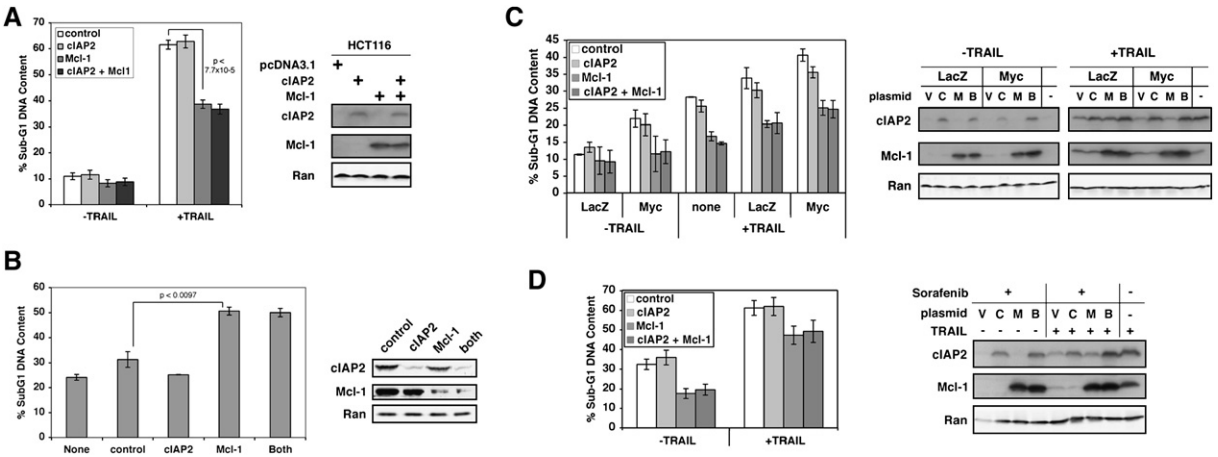

Figure 6. Mcl-1 Is a Critical Mediator of TRAIL Resistance

(A) HCT116 *bax*$^{+/-}$ cells were transiently transfected with expression plasmids for cIAP2, Mcl-1, both or the empty vector (C, M, B, or V) for 24 hr. Cells then were treated with TRAIL for 6 hr. An expression plasmid for GFP-Spectrin was also added at 10% of the total DNA. The percentages of apoptotic cells expressing GFP are shown ($p < 7.7 \times 10^{-5}$). Immunoblots are shown in on the right.

(B) *bax*$^{-/-}$ cells transfected with siRNA were treated as shown for 5 hr. Cells were collected and analyzed for DNA content or protein expression ($p < 0.0097$).

(C) *bax*$^{-/-}$ cells were transfected as in (A) for 6 hr then infected with the indicated adenoviruses (LacZ or Myc) for 15 hr. After this, cells were treated with TRAIL for 6 hr, collected and stained with propidium iodide (left panel), or extracts were analyzed by immunoblotting (right).

(D) *bax*$^{-/-}$ cells were transfected, and 24 hr later treated with sorafenib (16 μM) for 1 hr followed by TRAIL treatment for 6 hr. Cells were collected and stained (left panel) or extracts were analyzed by immunoblotting (right). The data represents the average of 6 measurements collected from three separate experiments and Mcl-1 overexpression is significantly different from control in TRAIL + sorafenib treated cells ($p < 0.0044$). Sub G1 results are shown as the mean (bar) ± SD in (A)–(D). Significance of difference was calculated by Student's t test analysis.

promoter-bound NF-κB (Figure 5G). Use of reporter plasmids containing the *mcl-1* or *ciap2* promoters showed similar decrease of TRAIL-induced activity by sorafenib (Figure S6). TRAIL-induced luciferase activity was lost when NF-κB binding sites were mutated in either *mcl-1* or *ciap2* promoters. The importance of the NF-κB binding site in the *mcl-1* promoter was confirmed by ChIP analysis of the five potential relevant NF-κB sites in the Mcl-1gene (Figure S7). This region of the *mcl-1* promoter showed increased acetylation of histone H3 following TRAIL treatment, and both anti-p65/RelA and anti-Acetyl-H3 antibodies showed reduced binding with sorafenib treatment. Examination of the effect of sorafenib on both TRAIL-induced degradation of IκBα and NF-κB translocation into the nucleus showed no significant changes (Figure S3). These data show sorafenib sensitizes *bax*$^{-/-}$ cells to TRAIL, in part, by disrupting NF-κB transcriptional activation of *mcl-1* and *ciap2*.

Mcl-1 Is a Critical Mediator of TRAIL Resistance in *bax*$^{-/-}$ Cells

To assess the importance of Mcl-1 and cIAP2 in mediating resistance to TRAIL, we expressed these proteins in *bax*$^{+/-}$ cells and tested for changes in TRAIL sensitivity. Mcl-1 provided significant protection from TRAIL, but cIAP2 expression did not (Figure 6A). We used siRNA to assess the importance of Mcl-1 and cIAP2 in mediating TRAIL resistance and observed Mcl-1 knockdown increased TRAIL-induced apoptosis by close to 2-fold (Figure 6B), whereas knockdown of cIAP2 in *bax*$^{-/-}$ cells did not enhance TRAIL-mediated death (Figure 6B). Because control siRNA showed some sensitization to TRAIL, we included siRNA to Bak as an additional negative control. These results suggest that Mcl-1 may be more important than cIAP2 in mediating TRAIL-induced resistance to the TRAIL death signal.

Mcl-1 Expression Protects Cells from c-Myc and Sorafenib Sensitization to TRAIL

We tested whether Mcl-1 and cIAP2 were critical mediators of TRAIL sensitization by Myc or sorafenib. Transient transfection of *bax*$^{-/-}$ cells with Mcl-1 and cIAP2, followed by infection using LacZ- or c-Myc-expressing adenovirus produced a large amount of background apoptosis that was reduced by Mcl-1 (Figure 6C). Addition of TRAIL to these cells recapitulated the Myc sensitization, but the dramatic differences seen compared to the controls was masked by the high level of background death. Nevertheless, Mcl-1 clearly exerted protection against the various apoptotic signals: TRAIL, c-Myc, and introduction of exogenous DNA. We used the same approach to examine the significance of Mcl-1 and cIAP2 in protecting cells from sensitization to TRAIL by sorafenib. Again, Mcl-1 protected *bax*$^{-/-}$ cells from sorafenib, and DNA transfection mediated death while also protecting the cells from this combination plus TRAIL (Figure 6D). These experiments indicate that cIAP2 may not be critical for TRAIL resistance in *bax*$^{-/-}$ cells and that Mcl-1 likely plays a

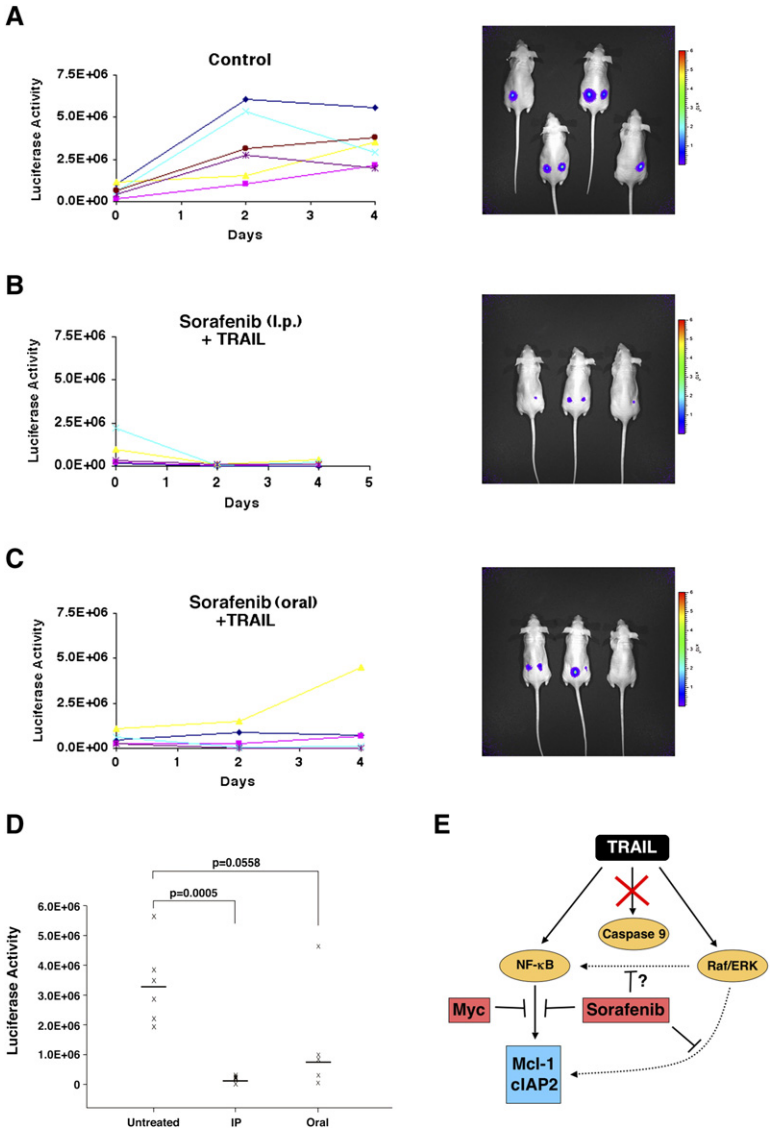

Figure 7. TRAIL Combined with Sorafenib Prevents Growth of *bax*$^{-/-}$ Tumor Xenografts

Mice bearing luciferase-expressing HCT116 *bax*$^{-/-}$ tumor xenografts received TRAIL plus sorafenib. TRAIL was administered once on days 1 and 3. Sorafenib was administered once on each day for 5 days (days 0–4). Luciferase activity was measured on days 0, 2, and 4.

(A) Control (n = 7).

(B) TRAIL plus sorafenib, i.p. administration (n = 5).

(C) TRAIL plus sorafenib, oral administration (n = 5).

(D) Summary of data from experiments shown in (A)–(C). Mean value of each group was marked as a short horizontal solid line. Significance of difference was calculated by Student's t test analysis.

(E) Model of TRAIL action. TRAIL can activate caspase 9, NF-κB, and Raf/ERK signaling. If caspase 9 activation is blocked (i.e., by Bax deficiency), NF-κB acts directly to increase Mcl-1 and cIAP2 transcription, which can be blocked by Myc or sorafenib. Sorafenib inhibits Raf/ERK, affecting multiple targets, including NF-κB. Decreased levels of TRAIL-induced antiapoptotic proteins, including Mcl-1 and cIAP2, override the defect in intrinsic apoptotic signaling resulting in cell death.

major role in offering protection from TRAIL-induced death.

Sorafenib Combined with TRAIL Caused Regression of TRAIL-Resistant Tumors In Vivo

To assess the in vivo efficacy of combining TRAIL and sorafenib, we tested these compounds using mouse tumor xenograft models monitored with in vivo bioluminescence technology. Mice bearing luciferase-expressing *bax*$^{-/-}$ tumors were treated with daily doses of sorafenib for 5 days, and two doses of TRAIL (Figure 7). We administered sorafenib either by i.p. injection or oral gavage. In both treatment methods, sorafenib plus TRAIL significantly inhibited the continued growth of *bax*$^{-/-}$ tumor xenografts (i.p. injection: untreated versus treated *bax*$^{-/-}$ tumors, p = 0.0005; oral administration: untreated versus treated *bax*$^{-/-}$ tumors, p = 0.0558). We also examined the combination of TRAIL plus sorafenib against *bax*$^{+/-}$ cells, and observed enhancement of TRAIL-mediated apoptosis with sorafenib (Figure S5).

To confirm these results in another system, we used TRAIL-resistant HT29 colon tumor cells. Mice bearing luciferase-expressing HT29 tumors were treated with sorafenib, TRAIL, or both. Comparison of the bioluminescence signal before and after treatments showed a nearly 10-fold decrease (p = 0.0002) in tumors from sorafenib + TRAIL treated mice (Figure 8D). Evidence for apoptosis was demonstrated by treatment-induced PARP cleavage (Figure 8E) and nearly completes disappearance of tumor cell mass accompanied by substantial fibrosis (Figure 8F). Sorafenib alone showed no change in mean tumor size (Figure 8C). PARP cleavage and fibrosis substitution of tumor mass was observed, but to a lesser degree than in tumors from the sorafenib + TRAIL treated group. Although there was some PARP cleavage in the sorafenib alone treatment group and this may have contributed to

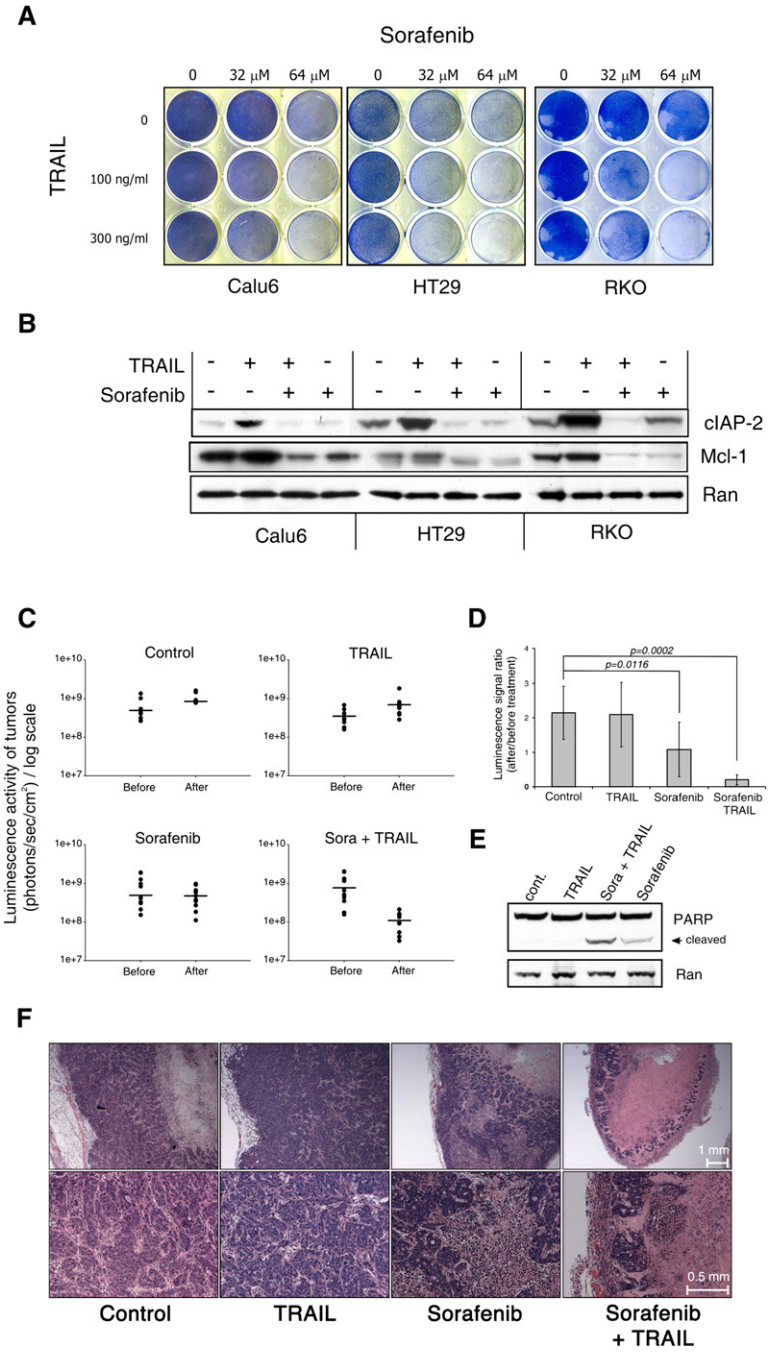

Figure 8. TRAIL Combined with Sorafenib Killed Tumor Cells in HT29 Xenografted Tumors

Cell lines indicated were treated with TRAIL or sorafenib for 24 hr. (A) Plates of cells were stained and fixed with Coomassie Blue, and (B) lysates were collected and immunoblotting performed. (C) Bioluminescence activity from each tumor nodule was plotted in log scale. Mean value of each group was marked as a short horizontal solid line. Control group received no treatment. (D) Relative ratio of bioluminescence signal was obtained by dividing the bioluminescence signal after treatment by the signal before treatment. Results are shown as the mean (bar) ± SD. Significance of difference was calculated by Student's t test analysis. (E) After bioluminescence imaging, tumor tissues were harvested and subjected to preparation of protein lysates and paraffin embedding. Apoptosis in the tumor tissues was detected by measuring cleavage of human PARP. (F) H&E staining of tumor tissues.

a delay in growth compared to control and TRAIL-treated tumors, there was no obvious effect of sorafenib alone in terms of tumor shrinkage. TRAIL treatment alone did not affect tumor growth kinetics. Control- and TRAIL-treated tumors showed tumor masses separated intermittently by supporting connective tissues. Sorafenib-treated tumors showed decreased size and an increase in fibrotic area. There were very little tumor cell masses in sorafenib + TRAIL treated tumors, and massive fibrosis was observed. These in vivo results using *bax*$^{-/-}$ and p53 null HT29 tumor xenografts suggest significant promise for combining sorafenib with TRAIL to treat cancers harboring defects in apoptotic signaling.

DISCUSSION

Most cancer cells have defects in their ability to die by apoptosis. Activation of death receptor signaling, particularly through TRAIL receptors, provides an exciting approach for cancer therapy because of its potency, lack of significant toxicity, and ability to kill cells with defective intrinsic pathway apoptotic signaling. In some cells, referred to as

Type I, TRAIL signaling does not require the mitochondria to kill (Ozoren and El-Deiry, 2002). Type I cells initiate sufficient executioner caspase activity through the activity of caspase 8/10 (Scaffidi et al., 1998). Type II cells require activation of the intrinsic pathway following death receptor activation through a process mediated by caspase cleavage of Bid.

Identifying cancer cell requirements for TRAIL sensitivity assumes greater significance now that TRAIL therapy is in clinical trials. This may be crucial for the success of TRAIL as a cancer therapy because many cancer cell lines are not sensitive to TRAIL. A potential biomarker for identification of TRAIL sensitivity is oncogenic expression of Myc (Ricci et al., 2004; Wang et al., 2004). However, a high level of c-Myc expression is not the only requirement for TRAIL sensitivity. Cancer cells still need a functional apoptotic signaling pathway for TRAIL to activate a death response. While identifying patients with functional TRAIL signaling pathways prior to treatment remains as a goal in clinical trial design, one approach to optimize TRAIL therapy is the pharmacological enhancement of a cancer's sensitivity to TRAIL. One way to overcome TRAIL resistance is to convert Type II cells to Type I cells, thereby bypassing the need for intact intrinsic pathway. For example, DNA-damaging chemotherapy can bypass HCT116 *bax*$^{-/-}$ cell deficiency in mitochondrial signaling by increasing p53 levels, leading to the increased expression of DR5 and increased extrinsic pathway signaling (LeBlanc et al., 2002; Wang et al., 2004).

An exciting finding presented here is that the combination of sorafenib and TRAIL is effective not only in tumor cells with wild-type p53, but those that are p53-deficient (e.g., HT29). This finding has important clinical implications for tumors with mutant p53 that are TRAIL-resistant. Our initial examination of whether Myc can bypass Bax deficiency to convert Type II tumor cells to a Type I phenotype led us to identify sorafenib's ability to disable antiapoptotic mechanisms activated by TRAIL in cells harboring defects in intrinsic apoptotic signaling. c-Myc can sensitize *bax*$^{-/-}$ cells to TRAIL, but in order to die by apoptosis, these cells still require the intrinsic apoptotic pathway. Therefore, even though c-FLIP expression is repressed by c-Myc (Ricci et al., 2004), this suppression is not sufficient to sustain activation of effector caspases downstream of caspase 8 in the *bax*$^{-/-}$ cells.

Our examination of c-Myc action on proteins involved in maintaining mitochondrial membrane stability and preventing caspase-mediated proteolysis led us to identify that TRAIL induced Mcl-1 and cIAP2 expression in TRAIL resistant cells (Figure 2). The ability of TRAIL to induce Mcl-1 and cIAP2 appears dependent on suppression of the mitochondrial pathway of apoptosis and appears to be independent of whether His-tagged or native TRAIL is used (Figure S8). Blocking-activated caspase 9 in HCT116 *bax*$^{+/-}$ cells with a pharmacologic inhibitor, the stable expression of Bcl-xL, or through the complete loss of Bax, resulted in TRAIL induction of Mcl-1 and cIAP2 (Figure 3). c-Myc repressed TRAIL-mediated induction of Mcl-1 and cIAP2 mRNA and protein, indicating their potential importance in suppressing TRAIL sensitivity.

We also examined mediators of TRAIL signaling previously identified to be targets of c-Myc, either directly or through p53 stabilization. We did not observe significant alterations in the expression of Bcl-2, Bcl-xL, or Bid, but observed repression of TRAIL-induced Bim with c-Myc expression, an observation that should not contribute to Myc sensitization to TRAIL given the proapoptotic nature of Bim (Figure 2). Puma was not further examined because neither exogenously expressed Myc nor TRAIL treatment alone resulted in dramatic changes in its expression. The loss of Bak following TRAIL treatment is intriguing, and its relevance is under further investigation. c-Myc stabilized p53, but knockdown studies in *bax*$^{-/-}$ cells (Figure S1) and our previous observations indicate that c-Myc does not require p53 to sensitize cells to TRAIL (Ricci et al., 2004).

Examination of the relative importance of Mcl-1 and cIAP2 in mediating resistance to TRAIL signaling showed that Mcl-1 can exert significant protection against TRAIL, but cIAP2 appears less important (Figure 6). cIAP2 can inhibit caspases 3, 7, and 9 activity (Roy et al., 1997), but can be inhibited by Smac/DIABLO and Omi/Htr2A (Creagh et al., 2004; Yang et al., 2003; Yang and Du, 2004). Therefore, it is possible that cIAP2 alone is not sufficient to block TRAIL signaling when the mitochondrial membrane is destabilized.

Our investigations into how TRAIL increased Mcl-1 and cIAP2 expression led us to identify that both NF-κB and Raf signaling play major roles. TRAIL activates both NF-κB and Raf/MEK/ERK signaling in *bax*$^{-/-}$ cells. *ciap2* is a well-characterized NF-κB target gene (Wang et al., 1998), and both *ciap2* and *mcl-1* were shown to be CREB targets (Nishihara et al., 2004; Wang et al., 1999). We found that TRAIL activation of NF-κB results in its transcriptional activation of both *ciap2* and *mcl-1*.

We tested whether TRAIL could activate *ciap2* and *mcl-1* transcription through Raf/MEK/ERK signaling by using the multikinase inhibitor, sorafenib. Sorafenib as a single agent had little effect on the killing of *bax*$^{-/-}$, HT29, Calu6, or RKO cells, but combining it with TRAIL resulted in high levels of cell death (Figures 4 and 8). As with c-Myc, we found that sorafenib effectively prevents TRAIL-mediated induction of Mcl-1 and cIAP2. Reintroduction of Mcl-1 or cIAP2 by transient transfection confirmed that Mcl-1 plays an important role in sorafenib control of TRAIL-resistance (Figure 6).

How do both sorafenib and Myc diminish TRAIL-induced Mcl-1 and cIAP2? Decreased Mcl-1 and cIAP2 mRNA steady-state levels suggest that both sorafenib and Myc affect transcriptional activation of these genes. One potential mechanism is both diminish NF-κB signaling. There are multiple reports showing Myc suppression of NF-κB function (Keller et al., 2005; Klefstrom et al., 1997; Sitcheran et al., 2005; Tanaka et al., 2002; You et al., 2002). We tested directly whether sorafenib can alter NF-κB transcription and found it reduced the amount of TRAIL-induced RelA/p65 bound to these genes promoters (Figure 5H). While we did not observe sorafenib

to change nuclear levels of NF-κB following TRAIL treatment, there is substantial evidence that the DNA binding and transactivation abilities of NF-κB are regulated by multiple phosphorylation modifying events of the NF-κB subunits (Viatour et al., 2005). Numerous kinases have been identified that can phosphorylate NF-κB subunits. These modifications also affect the ability of NF-κB to recruit chromatin remodeling enzymes. Therefore, it is quite probable that sorafenib acts to diminish NF-κB DNA binding to the *mcl-1* and *ciap2* promoters by disrupting kinase signaling important to the modification and enhanced transactivation function of NF-κB subunits.

Two recent reports showed sorafenib treatment can result in downregulation of Mcl-1 levels in a variety of human tumor cell lines (Rahmani et al., 2005; Yu et al., 2005). Neither study found that sorafenib affects steady-state Mcl-1 mRNA levels, but rather altered Mcl-1 expression at the translational or posttranslational levels. No previous study has examined the effects of sorafenib on cIAP2 or Mcl-1 levels in TRAIL-treated cells. Taken together, sorafenib kinase inhibition may affect not only TRAIL-mediated NF-κB activation of Mcl-1 transcription (Figure 5G), but it may also diminish Mcl-1 through posttranscriptional mechanisms. Furthermore, the combination of sorafenib plus TRAIL also reduces p-CREB levels and this also may contribute to the regulation of Mcl-1 expression.

Increased expression of Mcl-1 can mediate significant protection against activation of the mitochondrial death pathway. In the absence of Bax, the antiapoptotic properties of Mcl-1 take on greater significance because of the high affinity binding between Mcl-1 and Bak and the importance of this interaction in apoptosis (Cuconati et al., 2003). Our observations that c-Myc or sorafenib can prevent TRAIL-mediated induction of Mcl-1 and cIAP2 levels, combined with our overexpression and knockdown studies, show that TRAIL protects cells from its own death signal when caspase signaling is blocked. These results also open up new areas of investigation including kinase targets of sorafenib that ultimately impact on p65 binding to DNA as well as which activities of this multikinase inhibitor are ultimately responsible for its potent antitumor effect when combined with TRAIL. These results also establish the clinical potential for combining TRAIL with an orally bioavailable, low-toxicity kinase inhibitor, sorafenib/Nexavar, thus providing an exciting approach for attacking cancer cells that harbor defective intrinsic apoptotic machinery.

EXPERIMENTAL PROCEDURES

Cell Death Assays

Sub-G1 DNA Content

Cells collected, fixed, and stained with propidium iodide were analyzed by flow cytometry using a Coulter-Beckman Elite Epics sorter.

Mitochondrial Membrane Potential

Cells were collected and incubated with 20 nM rhodamine 123, 3,3′-dihexiloxadicarbocyanine [DiOC6] (Molecular Probes, Eugene OR) for 30 min then analyzed by flow cytometry. Percentage of cells with subnormal concentrations of DIOC6 have decreased mitochondrial membrane potential are shown.

Annexin V Staining

Cells were collected and incubated with red-shifted phycoerythrin-conjugated recombinant human annexin V (CalTag, Burlingame, CA) and analyzed by flow cytometry.

RNA Analysis

Five micrograms of total RNA were used in northern blot analysis performed as previously described (Ricci et al., 2004). Radiolabeled DNA probes using PCR-amplified products Mcl-1 and cIAP2 cDNAs were used for hybridizations. Reverse transcription was done using Superscript III reverse transcriptase (Invitrogen) and oligo-dT primers with 1 μg of total RNA at 50°C for 1 hr in a 20 μl reaction. Real-time PCR was performed using using ABI TaqMan Universal PCR Master Mix PCR and the Applied Biosystems 7700 sequence detector. Primer sequences are provided in the Supplemental Data.

siRNA-Mediated Knockdown

Double-stranded RNA oligonucleotides directed toward cIAP2, Mcl-1, and a negative control (Allstars Negative siRNA) were purchase from Qiagen. Transfections were carried out using Lipofectamine 2000 according to manufacturer's instructions (Invitrogen).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation experiments were performed essentially as described (Ricci et al., 2004)but with the following changes: NF-κB ChIP was done with one round of immunoprecipiation using a RelA/p65 antibody (H-286, Santa Cruz). Primers used are provided in Supplementary Data.

Tumor Xenograft Experiments

Female athymic NCr-*nu/nu* mice (Taconic Farms, Germantown, NY) were used for all studies. The mice were housed and maintained in accordance with the University of Pennsylvania Institutional Animal Care and Use Committee and state and federal guidelines for the humane treatment and care of laboratory animals.

bax$^{-/-}$ *Xenografts*

Two million *bax*$^{-/-}$ cells were injected s.c. into the right and left flanks of each mouse and allowed to grow for three days. Noninvasive bioluminescence (photons/sec/cm^2/steradian) signals were visualized by intraperitoneal injection of 5 mg D-luciferin into anesthetized mice (ketamine/xylazine), followed by detection of live images using a Xenogen IVIS System as described (Wang and El-Deiry, 2003). A Cremophor EL/ethanol/water solution (12.5% Cremophor EL/ 12.5% ethanol/ 75% water) containing sorafenib was prepared as previously described (Wilhelm et al., 2004) and 60 mg/kg was administered by intraperitoneal injection or by oral gavage. TRAIL was prepared in PBS and 100 μg/mouse was administered by intravenous tail vein injection.

HT29 Xenografts

HT29 Cells were suspended in 50% Matrigel and injected subcutaneously into two sites per mouse. Three million cells were used per injection. Mice were subjected to imaging within 15–30 min after intraperitoneal injection of D-luciferein under anesthesia. Treatment was given daily with 30 mg/kg of sorafenib and/or 5 mg/kg of his-TRAIL for 4 consecutive days. Treatment was started 11 days after injection. Sorafenib was given IP and TRAIL by IV. Control and TRAIL treated group consist of 4 mice/8 tumors; and sorafenib and sorafenib plus TRAIL groups had 5 mice/10 tumors. Bioluminescence imaging was taken before the first treatment and 24 hr after final treatment.

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures and eight supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/12/1/66/DC1/.

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