c-Myc primed mitochondria determine cellular sensitivity to TRAIL-induced apoptosis

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Oncogenic c-Myc renders cells sensitive to TRAIL-induced apoptosis, and existing data suggest that c-Myc sensitizes cells to apoptosis by promoting activation of the mitochondrial apoptosis pathway. However, the molecular mechanisms linking the mitochondrial effects of c-Myc to the c-Myc-dependent sensitization to TRAIL have remained unresolved. Here, we show that TRAIL induces a weak activation of procaspase-8 but fails to activate mitochondrial proapoptotic effectors Bax and Bak, cytochrome c release or downstream effector caspase-3 in non-transformed human fibroblasts or mammary epithelial cells. Our data is consistent with the model that activation of oncogenic c-Myc primes mitochondria through a mechanism involving activation of Bak and this priming enables weak TRAIL-induced caspase-8 signals to activate Bax. This results in cytochrome c release, activation of downstream caspases and postmitochondrial death-inducing signaling complex-independent augmentation of caspase-8-Bid activity. In conclusion, c-Myc-dependent priming of the mitochondrial pathway is critical for the capacity of TRAIL-induced caspase-8 signals to activate effector caspases and for the establishment of lethal caspase feedback amplification loop in human cells.

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Introduction

The TNF-family death ligands TNF- α , CD95L/FasL and TRAIL promote apoptosis in many types of tumor and virus-infected cells. Remarkably, primary or non-transformed cells are often resistant to the death receptor-induced apoptosis, yet they express functional receptors for these ligands (LeBlanc and Ashkenazi, 2003; Fesik, 2005). The death receptor ligands can promote regression of tumors *in vivo*, which in part is attributable to the selective tumor cell killing by these agents. However, only TRAIL induces tumor regression without

exhibiting significant systemic toxicity *in vivo*, and currently both TRAIL and agonistic antibodies to TRAIL receptors are in clinical trials for the treatment of cancer (Fesik, 2005). Despite years of intense study, only little is known about mechanisms that render tumor cells sensitive to TRAIL.

TRAIL kills cancer cells by binding specific cell surface death receptors, which are TNFRSF10A (DR4) and TNFRSF10B (DR5). After binding TRAIL, these transmembrane receptors form trimeric complexes and assemble a death-inducing signaling complex (DISC) in their cytosolic parts (LeBlanc and Ashkenazi, 2003). DISC is a primary signaling complex in which an adaptor protein FADD mediates recruitment of cysteine protease procaspase-8 to the receptors. FADD interacts with procaspase-8 through homotypic death effector domain interactions and promotes oligomerization-dependent auto-activation of this caspase. The active caspase-8 can initiate an apoptotic caspase cascade. In addition, TRAIL may also induce activation of other signaling pathways. For example, JNK, p38 MAPK and IKK/NF- κ B kinase pathways are activated downstream of DISC assembly and caspase-8 activation (Varfolomeev *et al*, 2005). The kinase pathways may control apoptosis but also mediate non-apoptotic, for example proliferative or inflammatory, effects of death ligands (Algeciras-Schimmich *et al*, 2002; LeBlanc and Ashkenazi, 2003; Wajant *et al*, 2003; Secchiero *et al*, 2005). The DISC-activated caspase-8 connects to the downstream apoptotic death machinery in two ways. In certain cells (Type I), DISC assembly generates large amounts of active caspase-8, which is sufficient to directly cleave and activate downstream effector caspases, such as caspases-3, -6 and -7, that execute the apoptotic death program. In other cell types (Type II), death receptor-induced apoptosis requires engagement of the mitochondria-mediated pathway into the process of cell death (Scaffidi *et al*, 1998, 1999; Fulda *et al*, 2002; Rudner *et al*, 2005). The active caspase-8 can cleave proapoptotic Bcl-2 family protein Bid into an active form called truncated Bid (tBid). The tBid, in turn, recruits the mitochondrial pathway by activating the distal proapoptotic Bcl-2 family proteins Bax and Bak at the mitochondrial membranes, which leads to the release of apoptosis promoting factors such as holocytochrome c (cyt c), Smac/DIABLO and Omi/HtrA2 from mitochondria to the cytosol (Lowe *et al*, 2004). Once released into the cytosol, cyt c activates via APAF-1/caspase-9 complex effector caspases that execute apoptosis. TRAIL-induced apoptosis is often crucially dependent on the intact mitochondrial pathway (Deng *et al*, 2002; LeBlanc *et al*, 2002).

Activation of c-Myc renders primary and non-transformed cells sensitive to TNF- α , CD95L and TRAIL-induced apoptosis (Hueber *et al*, 1997; Klefstrom *et al*, 1997; Ricci *et al*, 2004; Wang *et al*, 2004). The molecular mechanisms underlying this apoptotic sensitization are not well understood, but they may involve an inhibitory action of c-Myc towards TNF-induced NF- κ B activation, which normally counteracts the apoptotic action of TNF (Klefstrom *et al*, 1997; You *et al*,

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2002). c-Myc may also augment TRAIL-dependent activation of caspase-8 through transcriptional inhibition of FLIP (FLICE inhibitory protein), which antagonizes generation of active caspase-8 in DISC (Ricci *et al*, 2004). However, as c-Myc sensitizes cells to a wide variety of apoptotic insults including death receptors, serum-deprivation and genotoxic agents, it is conceivable that the mechanisms of c-Myc-dependent sensitization derive from a common root. The anti-apoptotic Bcl-2 family proteins, Bcl-2 and Bcl-x_L, accelerate c-Myc-induced tumorigenesis by inhibiting c-Myc-dependent apoptosis (Pelengaris *et al*, 2002). These data implicate a close link between c-Myc and the mitochondrial apoptosis pathway. Consistent with this, activation of a conditional form of Myc (MycERTM) induces a rapid cyt *c* release in growth factor-deprived cells (Juin *et al*, 1999; Iaccharino *et al*, 2003). The upstream mechanisms triggering cyt *c* release involve conformational change and oligomerization of Bax and Bak proteins at the mitochondrial surface. More upstream, the activation of Bax/Bak complexes is promoted by upstream proapoptotic messengers tBid and Bim and antagonized by Bcl-2 and Bcl-x_L (Lowe *et al*, 2004). Bax appears to orchestrate the apoptotic c-Myc pathway, as ablation of bax genes renders cells resistant to c-Myc-dependent apoptosis and vice versa the Bax protein promotes the apoptotic activity of c-Myc (Mitchell *et al*, 2000; Juin *et al*, 2002). c-Myc may transcriptionally regulate Bax levels or more indirectly, regulate Bax pathway by controlling Bax oligomerization (Mitchell *et al*, 2000; Annis *et al*, 2005). In some cells c-Myc may also sensitize cells to apoptosis by downregulating Bcl-2 or Bcl-x_L expression levels (Eischen *et al*, 2001; Maclean *et al*, 2003). Taken together, there is substantial evidence that c-Myc primarily sensitizes cells to apoptosis by activating the mitochondrial apoptosis pathway.

In this study, we show that treatment of fibroblasts and mammary epithelial cells with TRAIL weakly activates caspase-8 without killing the cells. However, c-Myc promotes activation of the mitochondrial proapoptotic Bcl-2 protein Bak, and we show evidence that in such conditions even weak TRAIL-induced caspase-8-Bid signals are sufficient to activate Bak/Bax complexes. The ensuing cyt *c* release and activation of effector caspases then postmitochondrially augment caspase-8 activation independently of DISC. Thus, c-Myc-mediated priming of the mitochondria pathway enables weak caspase-8 signals to activate effector caspases and establish a death executing caspase feedback amplification loop.

Results

c-Myc sensitizes cells to TRAIL by activating the mitochondrial apoptosis pathway

To explore mechanisms whereby c-Myc sensitizes cells to TRAIL, we retrovirally introduced a hydroxytamoxifen (4-OHT)-inducible MycdeltaERTM construct into human immortal, non-transformed MCF10A mammary epithelial cells. Addition of 4-OHT to growth factor-deprived MCF10A-MycERTM cells induced cell cycle re-entry, whereas the controls expressing N-terminally deleted and functionally inactive Myc mutant (MycERTM) remained quiescent (Partanen *et al*, manuscript in preparation). Thus, we conclude that MycERTM is functional in the studied cells. In addition to epithelial cells, we also used telomerase-immor-

talized human MRC5hT fibroblasts expressing MycERTM. Neither of these cell types showed apoptotic response to the activation of MycERTM in cultures maintained in normal growth medium (Figure 1A). However, if c-Myc was preactivated and subsequently TRAIL was added to the cells, a widespread apoptosis occurred in both cell cultures. TRAIL alone, without c-Myc, did not induce any significant apoptosis. In further control experiments, the 4-OHT-induced activation of MycdeltaERTM failed to sensitize cells to TRAIL (data not shown). Thus, c-Myc and TRAIL synergistically induced apoptosis. Apoptosis was blocked by the pan-caspase inhibitor zVAD-fmk, which indicates a caspase-dependent form of apoptosis (Figure 1A).

To determine whether the mitochondrial apoptosis pathway was activated, we analyzed the conformational activation of Bax (6A7 antibody, Trevigen) at the mitochondria and the sub-cellular localization of cyt *c*. Figure 1B shows that both Bax activation and release of cyt *c* from mitochondria to the cytosol required active c-Myc and TRAIL. To determine whether activation of the mitochondrial apoptosis pathway was required for the apoptotic cooperation of c-Myc and TRAIL, we generated MRC5-hT-MycERTM and MCF10A-MycERTM cells overexpressing Bcl-x_L (Figure 1B and C). Experiments with these cells showed that Bcl-x_L blocks or strongly inhibits the ability of c-Myc and TRAIL to induce Bax activation, cyt *c* release (Figure 1B, D and E) and apoptosis (data not shown). Thus, we conclude that c-Myc and TRAIL induce apoptosis by synergistically activating Bax and cyt *c* release.

c-Myc augments TRAIL-induced activation of procaspase-8 by a mechanism requiring activation of the mitochondrial apoptosis pathway

To gain insight into the activation pattern of caspase machinery, we analyzed whether c-Myc influences the ability of TRAIL to induce proteolytic activation of proximal caspase-8, its target Bid and downstream effector caspase-3.

In fibroblasts, TRAIL induced weak but reproducible cleavage of procaspase-8 into the active p43/p41 and p18 subunits (Figure 2A; note lane MYC OFF, 50 ng/ml TRAIL). Although processing of caspase-8 occurred without active c-Myc, it was notable that preactivation of c-Myc markedly enhanced the TRAIL-induced processing of procaspase-8 in these cells (Figure 2A). As cells did not undergo apoptosis without active c-Myc, we determined whether TRAIL alone induced not only processing, but also the activity of caspase-8. Cells were treated as indicated in Figure 2A and the IETD-pNa (preferred caspase-8 substrate) cleavage activity present in cell lysates was measured. In fibroblasts, TRAIL induced about 2.4-fold increase in the proteolytic activity towards IETD peptide (Figure 2A). In comparison, TRAIL and active c-Myc induced over six-fold increase in the IETDase activity. In mammary epithelial cells, 50 ng/ml of TRAIL induced caspase-8 cleavage and 1.6-fold increase in the IETD cleavage activity (Figure 2A). However, if c-Myc was active, over 2.5-fold increase was observed. Furthermore, 10 ng/ml concentration of TRAIL induced detectable procaspase-8 cleavage in mammary cells only if c-Myc was active (Figure 2A). Thus, c-Myc is not required for TRAIL-induced activation of procaspase-8, but the presence of active c-Myc strongly augments the TRAIL-induced activation of this caspase. Downstream of caspase-8, we observed that the activity of

Figure 1 TRAIL-induced activation of Bax and release of cytochrome *c* (cyt *c*) requires active c-Myc and is blocked by Bcl-x_L. (A) c-Myc sensitizes MCF10A mammary epithelial cells and MRC5 fibroblasts to TRAIL-induced apoptosis. To activate Myc, MCF10A-MycERtm and MRC5-hT-MycERtm cells were treated for 24 h with 10 nM 4-OHT. Control cells were treated with ethanol carrier. Subsequently, the cells were treated with 0, 10 or 50 ng/ml of TRAIL for 2 h. Representative photomicrographs of the cell cultures are shown in the picture. zVAD-fmk (50 μM) was used to block caspases. (B) c-Myc was activated in the control and Bcl-x_L-overexpressing MCF10A-MycERtm cells and the cells were treated with 50 ng/ml of TRAIL as described in (A). Fixed cells were immunostained either with anti-active Bax or anti-cyt *c* antibody and counterstained with Hoechst 33258. Shown are immunofluorescence images of the cells treated with ethanol carrier (MYC OFF) or 4-OHT (MYC ON) and TRAIL. Note the punctate, mitochondrial staining pattern of active Bax in the cells with active c-Myc and TRAIL pathways (white arrow). Cyt *c* normally exhibits punctate mitochondrial staining pattern, which becomes diffuse upon release of cyt *c* to the cytosol (white arrow). (C) Western immunoblot analysis showing ectopic expression level of Bcl-x_L. (D, E) Quantitation of cells with active Bax or cytosolic cyt *c*. Cells were treated (MYC +/–, 50 ng/ml TRAIL +/–) and stained as in B. Images were collected and analyzed as described in Materials and methods. Between 300–700 cells were analyzed at each treatment. The graph values represent mean ± s.d. of minimum of three independent experiments.

Figure 2 TRAIL induces activation of caspase-8, which is augmented by active c-Myc via Bcl-x_L regulated pathway. **(A)** The cells were treated as in Figure 1A and the cell lysates were analyzed for procaspase-8 cleavage and for proteolytic activity towards Ac-IETD-pNa. The immunoblot analysis shows that active c-Myc enhances TRAIL-induced cleavage of procaspase-8 into the active subunits (p43/p41 and p18). The caspase-8 antibody used recognized full-length procaspase-8 in MRC5 cells, but for some reason only weakly in MCF10A cells. However, the cleavage products that were diagnostic for caspase-8 activation were reliably detected in both cell types. The boxed values represent relative caspase-8 activities extracted from the datasets in Figure 4B. **(B)** Bid and caspase-3 activation. The immunoblot analyses of Bid cleavage using an antibody recognizing both full length and truncated forms of Bid and caspase-3 activation with an antibody recognizing active processed form. **(C)** The cells overexpressing Bcl-x_L were treated as in A and analyzed for procaspase-8 processing. The immunoblots demonstrate that c-Myc fails to augment TRAIL-induced processing of procaspase-8 in the cells overexpressing Bcl-x_L.

both c-Myc and TRAIL was required for the induction of detectable proteolytic processing of Bid and activation of caspase-3 (Figure 2B).

Next, we wished to determine whether the mechanism by which c-Myc augments TRAIL-induced caspase-8 activation operate upstream or downstream of the activation of the mitochondrial pathway. We reasoned that if c-Myc augments the processing of caspase-8 upstream of the mitochondria, for example in DISC, the blockade of the mitochondrial pathway would not inhibit this augmentation. Strikingly, Bcl-x_L completely blocked the ability of c-Myc to augment TRAIL-induced caspase-8 processing and activation in fibroblasts (Figure 2C; see Figure 4B for IETDase activity). In addition, Bcl-x_L also blocked the c-Myc-dependent processing of Bid and activation of caspase-3 by TRAIL (Figure 2C). In the epithelial cells, Bcl-x_L inhibited the c-Myc-dependent augmentation of caspase-8 processing and reduced the ability of c-Myc and TRAIL to generate active caspase-3. These results

agree with the previously published data showing that c-Myc can augment the processing of procaspase-8 into its active forms in TRAIL-treated cells (Ricci *et al*, 2004). However, in contrast to the previously published data suggesting that the augmented processing of caspase-8 results from genetic interaction between FLIP and c-Myc affecting the DISC, our present data imply that postmitochondrial mechanisms, which act downstream of the activation of the mitochondrial pathway, primarily result in the enhancement of caspase-8 activity.

Caspase-8-Bid axis activates Bax and cyt c release during c-Myc and TRAIL-induced apoptosis

TRAIL-induced activation of caspase-8 occurred without c-Myc, whereas surprisingly, the cleavage of Bid required both TRAIL and active c-Myc (Figure 2A and B). However, it is possible that the immunoblot analyses failed to detect a weak processing of Bid by the non-augmented caspase-8

activity (Myc OFF, TRAIL ON). Therefore, we undertook a genetic approach to determine whether the caspase-8-Bid axis is essential for Bax activation.

For this purpose, we created lentiviral constructs expressing short-hairpin (sh) RNAs targeting either caspase-8 or Bid (Figure 3). The analysis of transduced cells showed that

Figure 3 Caspase-8 and Bid are required for induction of cytochrome c release and Bax activation by c-Myc and TRAIL. (A) Western immunoblot demonstrates lentiviral shRNA silencing of endogenous procaspase-8. The efficiency of lentiviral infection was about 80%. (B) Immunofluorescence detection of cytochrome c release specifically in EGFP-positive transduced cells. Control and caspase-8 shRNA-transduced MCF10A-MycERtm cells were immunostained with anti-cytochrome c antibody (red) and the nuclei were counterstained with Hoechst (blue). Transduced EGFP expressing cells are green in the images. In merged images, diffuse yellow staining pattern (white arrow) indicates cytosolic cytochrome c in EGFP-positive cells. (C) Quantitation of transduced cells with cytosolic cytochrome c. The cells were treated as in Figure 1 and scored from merged images. The graph values show percentage of EGFP-positive cells with cytosolic cytochrome c. (D) Western immunoblot demonstrating lentiviral shRNA silencing of endogenous Bid. (E) Control and Bid shRNA-transduced cells were treated and analyzed as described in Figure 1. Representative immunofluorescence images are shown. (F, G) Quantitation of Bid-deficient cells with active Bax or cytosolic cytochrome c. Cells were scored as in Figure 1. All graph values show percentages and represent mean $\pm$ s.d. of at least three independent experiments.

Figure 4 c-Myc fails to augment TRAIL-induced activation of procaspase-8 in the cells with impaired mitochondrial Bid pathway. **(A)** c-Myc and TRAIL pathways were activated in the cell strains lacking Bid or overexpressing Bcl-x_L as described in Figure 1 and the cell lysates were analyzed for procaspase-8 cleavage. **(B)** The cell lysates were analyzed for proteolytic activity towards Ac-IETD-pNa substrate. Lysates (40 g) from the above experiments were analyzed. The graph values represent mean $\pm$ s.d. of three independent experiments (**P*-value < 0.05).

silencing of procaspase-8 (Figure 3A) abrogates c-Myc and TRAIL-induced cyt *c* release (Figure 3B and C) and apoptosis (data not shown). To suppress Bid expression, we used pDSL lentivector, allowing hygromycin selection of the transduced cells. Following selection, no detectable expression of Bid could be observed (Figure 3D). In the absence of Bid, the combined action of TRAIL and c-Myc failed to activate Bax or induce cyt *c* release (Figure 3E–G) or induce apoptosis (data not shown). In summary, these results show that the c-Myc-dependent induction of apoptosis by TRAIL requires caspase-8-Bid axis to mediate the signals that activate Bax at the mitochondria and release cyt *c*.

c-Myc fails to augment TRAIL-induced procaspase-8 activation in Bid-deficient cells

The results in Figure 2 suggest that mechanisms operating downstream of the mitochondrial pathway lead to augmentation of procaspase-8 processing. Thus, blockade of Bid that mediates apoptotic signaling from caspase-8 to mitochondrial Bax and Bak complexes should also prevent the c-Myc-dependent augmentation of caspase-8 processing and activity. Indeed, in the Bid-deficient cells, TRAIL and c-Myc induced only basal level of caspase-8 processing (Figure 4A) and IETD cleavage activity (Figure 4B). Furthermore, both Bid knockdown and overexpression of Bcl-x_L produce very similar augmentation deficiency (Figure 4A and B). The results strongly suggest that elements downstream of the mitochondrial pathway activation result in the augmented caspase-8 activation in the cells with both c-Myc and TRAIL pathways active.

c-Myc augments caspase-8 activity independently of the DISC

In DISC, FADD coordinates oligomerization of inactive procaspase-8 zymogens, and it is considered that the zymogen dimerization is the crucial event needed for formation of active caspase-8 (Boatright *et al*, 2003). However, alternative mechanisms for caspase-8 activation have also been proposed—for example, the mitochondria-mediated pathway activates caspase-8 through a mechanism involving an inter-chain cleavage of procaspase-8 by effector caspases-3 and -6 (Sohn *et al*, 2005). Our results linking the c-Myc-dependent augmentation of caspase-8 activation to the mitochondrial pathway led us to explore whether caspase-8 was processed outside of the DISC. For these experiments, we created a conditionally active form of procaspase-8, called Fv-caspase-8. The construct consists of heterologous Fv (modified FKBP) dimerization domains fused to protease domains of procaspase-8. The Fv domains bring the whole construct under dimerization control of small molecule FKBP dimerizers (Muzio *et al*, 1998; Yang *et al*, 1998). However, as the construct is lacking death effector domains (DED) needed for interaction with FADD, it cannot be recruited to DISC. The Fv-caspase-8 construct was cotransfected with control or wild-type c-Myc expression plasmid into 293FT cells and a small molecule AP20187 (AP) dimerizer was administered to the cells to activate Fv-caspase-8. The microscopic analysis of transfected cells showed that active Fv-caspase-8 construct synergized with c-Myc overexpression in the induction of apoptosis (Figure 5A). In control experiments, the catalytically inactive Fv-mut-caspase-8 failed to synergize with

Figure 5 c-Myc augments cleavage of procaspase-8 outside the DISC. The chemically dimerizable Fv-caspase-8 expression constructs were cotransfected with mock or c-Myc expression plasmid into 293FT cells. After 48 h of transfection, the Fv constructs were dimerized by incubation of the cells with 100 nM AP20187 for four additional hours. (A) Phase contrast photomicrographs of the cells. (B) The immunoblots demonstrate that a chemically induced, non-DISC mediated processing of Fv-caspase-8 into the active subunits is enhanced in the cells overexpressing c-Myc (compare + AP, -Myc and + AP, + Myc). The blots also show that active Fv-caspase-8 cooperates with c-Myc to induce activation of caspase-3. (C) caspase-8-like activity (Ac-IETD-pNa) and (D) caspase-3-like activity (Ac-DEVD-pNa). The caspase activity assays were performed as described in Figure 4.

c-Myc (Figure 5A). Immunoblot analysis of Fv-caspase-8 processing showed that AP treatment generates p44 and p18 active subunits, indicating formation of active caspase-8 (Figure 5B). Strikingly, overexpression of c-Myc with AP-induced Fv-caspase-8 strongly enhanced the generation of p44 and p18 subunits and IETD cleavage activity (Figure 5B and C), which coincided with prominent caspase-3 activity (Figure 5B and D). The caspase-3 activation in these conditions is consistent with the widespread cell death observed in the cultures (Figure 5A). These results indicate that c-Myc augments caspase-8 processing independently of the DISC platform.

Loss-of antiapoptotic Bcl-2/Bcl-x_L activity sensitizes cells to TRAIL and augments the caspase-8 activity

A plausible explanation for the above results is that c-Myc enables TRAIL to activate Bax/Bak complexes and the subsequent activation of the mitochondria pathway and downstream effector caspases catalyzes caspase-8 activation in a feedback loop fashion. To test the hypothesis, we explored whether the sensitizing action of c-Myc can be phenocopied by chemical sensitization of Bax/Bak complexes. In these experiments, we used a cell permeant small molecule ABT-737, which lowers the activation threshold of Bax/Bak by antagonizing major antiapoptotic Bcl-2 family members

(Bcl-2, Bcl-x_L and Bcl-w) (Oltersdorf *et al*, 2005; Certo *et al*, 2006). We pretreated MCF10A cells with ABT-737 and subsequently, added TRAIL to the cultures. Figure 6 shows a prominent apoptosis in the cells with activated TRAIL pathway and sensitized Bax/Bak complexes (Figure 6A). Neither TRAIL nor ABT-737 induced any significant apoptosis alone. We also observed that ABT-737 strongly promoted the ability of TRAIL to induce caspase-8 processing (Figure 6B) and IETDase activity (Figure 6C). Furthermore, the level of augmentation obtained with ABT-737 was comparable to that observed with c-Myc (Figure 6C). Thus, ABT-737 can both sensitize cells to TRAIL and enhance the activation of caspase-8. Therefore, c-Myc and ABT-737 may sensitize cells to TRAIL via similar Bax and Bak involving mechanisms.

c-Myc induces and activates Bak protein, which mediates sensitization to TRAIL

Finally, we sought to find mechanisms through which c-Myc enables activation of the mitochondrial pathway by TRAIL. We first explored whether c-Myc activation affects the protein expression levels of six different Bcl-2 family proteins Bim_{EL}, Bid, Bax, Bak, Bcl-x_L and Puma (Figure 7A). c-Myc strongly upregulated the levels of Bak protein in both fibroblasts and epithelial cells. The upregulated levels peaked after 10 h of c-Myc activation and within 24 h the Bak expression

Figure 6 Chemical inhibition of antiapoptotic Bcl-2 proteins (Bcl-x_L, Bcl-2 and Bcl-w) sensitizes cells to TRAIL. (A) Photomicrographs of controls and MCF10A-MycERtm cells pretreated with 1 μM ABT-737 compound for 24 h followed by further 2 h treatment with 50 ng/ml TRAIL. (B) The immunoblots demonstrate that ABT-737 augments TRAIL-induced processing of procaspase-8. (C) The cell lysates were analyzed for proteolytic activity towards Ac-IETD-pNa substrate as described in Figure 4. The graph values represent mean ± s.d. of three independent experiments (**P*-value < 0.05).

was returned back to the basal level. The results expose Bak as a novel BH protein target for transient c-Myc regulation, and that Bak upregulation occurs without concomitant induction of apoptosis. To clarify whether a transient upregulation of Bak by c-Myc could be linked to TRAIL sensitization, we analyzed in parallel experiments the pattern of Bak and Bax activation using N-terminal antibodies (anti-Bak Ab-1, Calbiochem). We first observed that both Bak and Bax proteins were strongly activated by combined action of

c-Myc and TRAIL (Figure 7B). When the effects of TRAIL and c-Myc were examined separately, we also found that c-Myc activation alone activated Bak (Figure 7B). The immunostaining signal indicating Bak N-terminal exposure was not as strong in the cells with active c-Myc as compared with the cells with active c-Myc and TRAIL, but the signal was clearly detected in over 20% of the cells after 24 h c-Myc activation (Figure 7C). Next, we silenced Bak by shRNA lentiviruses to determine whether Bak is required for c-Myc-induced sensitization to TRAIL (Figure 8A). Loss-of Bak almost completely inhibited TRAIL and c-Myc-induced Bax activation, cytochrome *c* release and caspase-3 activation (Figure 8B–D). Furthermore, c-Myc failed to augment TRAIL-induced caspase-8 processing in the Bak-deficient cells (Figure 8E). Our results suggest that the c-Myc-dependent activation of Bak is part of the machinery through which c-Myc primes mitochondrial pathway for activation (Figure 9).

Discussion

There is emerging evidence that crosstalk between the mitochondrial and death receptor apoptosis pathways determines whether cells survive or die in response to TRAIL. For example, impairment of the mitochondrial pathway through spontaneous loss of Bax allows tumor cells to escape the therapeutic cytotoxicity of TRAIL (LeBlanc *et al*, 2002). However, it has remained unclear why TRAIL-induced signaling pathways in some cells activate the mitochondrial pathway and kill, whereas other cell types survive from TRAIL treatment. Do ligand-bound TRAIL receptors activate completely different set of pathways in cells that survive as compared with cells that die and if not, at which point the signaling pathways bifurcate? Interestingly, in cell-free assays, re-addition of a mitochondrial fraction into cell lysates has been shown to significantly lower the concentration of active recombinant caspase-8 needed to activate downstream caspases (Kuwana *et al*, 1998). Therefore, the mitochondrial pathway does not just passively route death receptor signals to the effector caspases, but instead the mitochondria-specific mechanisms may actually set a threshold for caspase-8 activity that is needed to activate effector caspases.

TRAIL-caspase-8-Bid and c-Myc-Bak signaling modules cooperate to release cytochrome *c*

c-Myc sensitizes cells to all death receptors that use caspase-8 for signaling (Hueber *et al*, 1997; Klefstrom *et al*, 1997; Aza-Blanc *et al*, 2003; Ricci *et al*, 2004), and there is previous evidence that c-Myc activates the mitochondrial pathway for cyt *c* release (Juin *et al*, 2002; Klefstrom *et al*, 2002). Therefore, we postulated that the apoptotic cooperation of c-Myc and TRAIL involves differential sensitivity of 'mitochondria primed' and 'mitochondria non-primed' cells to caspase-8. Consistent with this model, our data show that TRAIL induces a weak activation of caspase-8, but these signals fail to activate the mitochondrial pathway as indicated by lack of Bax or Bak activation or release of cyt *c*. However, activation of c-Myc enables TRAIL-caspase-8-Bid axis to induce Bax activation, which subsequently promotes cyt *c* release, leading to caspase-3 activation and finally apoptosis. How would c-Myc enable weak TRAIL-caspase-8 signaling to activate Bax? Our results show that activation of c-Myc transiently upregulates Bak protein and exposes the

Figure 7 c-Myc transiently upregulates Bak protein expression and induces Bak activation (**A**) Western immunoblot showing effects of c-Myc activation on the protein expression levels of six Bcl-2 proteins. Cells were treated with 4-OHT for 0–24 h before lysis. (**B**) MCF10A-MycERtm cells were treated with ethanol carrier or 4-OHT for 24 h and subsequently, 50 ng/ml of TRAIL was added for 2 h as indicated. The cells were fixed and costained with anti-active Bax and anti-active Bak antibodies. Note the weak Bak antibody signal in the cells with active c-Myc and an increased strength of the signal in cells exposed to Myc and TRAIL. (**C**) Quantitation of cells exhibiting immunoreactivity towards active Bax or active Bak. Cells treated as in (**B**) were fixed and immunostained and images were collected from randomly chosen fields using fixed exposure time on each channel. Positive cells were scored from non-merged images (examples of images shown in **B**). The graph values represent mean $\pm$ s.d. of three independent experiments.

N terminus of Bak, which reflects activation or transition of Bak towards active state. Furthermore, c-Myc-induced Bak remained in this active state long-term (>24 h) and after the Bak protein levels were already returned to the basal level. As Bak is required for c-Myc and TRAIL-induced Bax activation (Figure 8), we believe that Bak is part of the machinery

through which c-Myc 'primes' the mitochondrial pathway. tBid can promote oligomerization of both Bax and Bak (Wei *et al*, 2000), and the detailed nature of complexes forming at the mitochondria during c-Myc and TRAIL induced apoptosis remains to be clarified. However, we speculate that TRAIL-induced increase in the immunoreactivity of Bak N terminus,

Figure 8 Bak is required for apoptotic events induced by c-Myc and TRAIL. (A) Western immunoblot demonstrating lentiviral shRNA silencing of endogenous Bak in MCF10A-MycERtm cells. Lysates were made after hygromycin selection. (B–D) Bax activation, cyt *c* release and caspase-3 activation were quantitated as described in Figure 1. The graph values in (B, C) represent mean $\pm$ s.d. of three independent experiments and of two experiments in (D). (E) The immunoblot shows that c-Myc fails to augment TRAIL-induced processing of procaspase-8 in Bak-deficient cells. The analyses were performed as in Figure 4.

Figure 9 Mitochondria-priming model. Binding of TRAIL to its cognate receptors induces sublethal level of caspase-8 activity in human epithelial and fibroblast cells. In healthy cells, this weak caspase-8 activity is insufficient to trigger Bid-mediated Bax or Bak activation. However, oncogenic c-Myc or specific drugs can preactivate Bak and in these conditions even a weak activation of caspase-8 and Bid fully activates formation of Bak/Bax complexes, which triggers release of cyt *c* and subsequently recruits downstream effector caspases. Downstream effector caspases cleave vital cellular substrates and generate substantially more caspase-8 activity through interchain cleavage, which results in strong Bid activation. We propose that the onset of such caspase feedback loop represents a phase transition, where apoptosis becomes a TRAIL-independent cell autonomous process. These mechanisms may have evolved to ensure that the progression of late stage apoptosis is not dependent on the extracellular availability of death ligands. The model is illustrated in the figure as a process diagram with graphical notation system (Kitano *et al*, 2005). The symbols are: closed arrow, state transition; open arrow, translocation; closed arrow and dotted line, unknown transition; circle-headed line, promotion of transition; solid line surrounding protein complexes, known protein complexes; dotted line surrounding protein complexes, hypothetical protein complexes; dotted line surrounding single protein, active protein.

which specifically occurred in the cells with c-Myc is due to formation of high-order Bak complexes (Mikhailov *et al*, 2003; Ruffolo and Shore, 2003). Therefore, the weak c-Myc-

induced immunostaining may indicate formation of preactive Bak mono- or oligomers (Zhang *et al*, 2004). It is notable, that previous studies have already implicated a role for c-Myc in

the regulation of Bax oligomerization (Soucie *et al*, 2001; Annis *et al*, 2005). Previous reports have documented that c-Myc elevates protein expression levels of Bim and Bax (Mitchell *et al*, 2000; Hemann *et al*, 2005) or downregulates Bcl-x_L (Maclean *et al*, 2003), which changes were not observed in this study. It is very likely that c-Myc alters the balance of pro- and antiapoptotic Bcl-2 proteins in a different manner depending on the cell type or cellular response to c-Myc in given conditions (apoptotic vs non-apoptotic).

TRAIL-induced caspase-8 activity is augmented at the postmitochondrial stage of apoptosis

Recent data have suggested that a direct transcriptional downregulation of FLIP by c-Myc results in enhanced caspase-8 processing in DISC (Ricci *et al*, 2004). However, our transcriptional profiling of MCF10A-MycERtm cells failed to detect any c-Myc-dependent alterations in FLIP levels (data not shown). Furthermore, the absence of FLIP in a public database of compiled c-Myc target genes may indicate that FLIP is not a common target for transcriptional regulation by c-Myc (<http://www.mycancergene.org/>). The present data show that c-Myc fails to augment caspase-8 activity in the cells containing mitochondrial pathway defects related to Bid- or Bak-deficiency or Bcl-x_L overexpression. Therefore, in fibroblasts and epithelial cells, mechanisms acting downstream or at the level of activation of the mitochondrial pathway augment caspase-8 cleavage.

Sohn *et al* (2005) have shown evidence that during drug-induced apoptosis, caspase-8 is activated in the absence of DISC formation by a mechanism involving interchain cleavage performed by effector caspases-3 and -6. This and previous studies by Slee *et al* (2000) postulate that the effector caspases, which are activated at the postmitochondrial stage of apoptosis, activate the death receptor proximal effectors caspase -8 and Bid (Sohn *et al*, 2005). The significance of such caspase feedback amplification loop for apoptosis has been recently underscored by findings that mouse embryonic fibroblasts deficient in caspase-3 and -7 show pronounced delay in the kinetics of cyt *c* release and Bax translocation in response to UV radiation (Lakhani *et al*, 2006). In the present study, we show that c-Myc-induced augmentation of caspase-8 cleavage and activity requires an intact mitochondrial pathway but not DISC. Therefore, most likely the action of effector caspases acting at the postmitochondrial stage of apoptosis caused the augmented cleavage and activation of caspase-8 in the present model system.

The reason why caspase feedback loop is coupled to apoptotic TRAIL machinery could be related to the use of TRAIL-caspase-8 signaling not only for death induction, but also for non-apoptotic TRAIL responses (Varfolomeev *et al*, 2005; Trauzold *et al*, 2006). We suggest that in normal cells it is important to keep the caspase-8 activity carefully in check to control non-apoptotic TRAIL responses and to avoid death. However, when TRAIL-induced activation of caspase-8-Bid axis leads to cyt *c* release, for example in transformed cells, consequent activation of effector caspases establishes a caspase feedback amplification loop, which now recruits caspase-8 into death execution process. Thereafter, the process of dying is not anymore controlled by the extracellular availability of TRAIL ligand, but instead, the apoptotic feedback loop drives apoptosis in an autonomous manner ensuring completion of death process. The cell-autonomous

execution phase in apoptosis may ensure that once cells have entered the point-of-no-return stage, changing extracellular conditions do not interrupt the process of dying.

Mitochondria-priming model

In summary, we propose a mitochondria-priming model in Figure 9 to explain mechanisms whereby c-Myc enables TRAIL to fully activate caspase machinery in human cells. The presented model can generally explain the synergistic induction of apoptosis by active c-Myc and any death receptor recruiting caspase-8 for signaling.

Materials and methods

Generation of cDNA and shRNA expressing stable cell lines

Human non-transformed MCF10A mammary epithelial cell line and MRC5 human fibroblast cells were obtained from ATCC. Immortal MRC5 cells (MRC5hT) were generated by retroviral introduction of human telomerase reverse transcriptase subunit/hTERT into these cells (the pWZL-hTERT construct was a kind gift from Drs Martin McMahon, UCSF and Robert A Weinberg, Whitehead/MIT). The 4-OHT (but not estrogen)-inducible MycERtm construct in pBabepuro retrovirusvector was a kind gift from Gerard Evan, UCSF. The pBabeMycERtm construct was retrovirally introduced in the studied cells and the cells were selected in 2.5 µg/ml puromycin. The clones surviving in selection were pooled for assays. The pBabehygro Bcl-x_L (gift from Gerard Evan, UCSF) overexpressing cells were generated with similar procedures except for the selection carried out in 200 µg/ml hygromycin. For caspase-8 silencing, we used pLentiLox3.7 (pLL3.7) vector (<http://web.mit.edu/ccr/labs/jacks/protocols/rnaresources.htm>) with shRNA targeting sequence 5'-CAGGGTCATGCTCTATCAGATTT-3'. For Bid and Bak silencing, we used pDSL_hpUGIH (AFCS, <http://www.signaling-gateway.org>) vector expressing shRNA targeting Bid sequence 5'-ACA GAATCCGCACTGTGCTcc-3' and Bak sequence 5'-CATAGCGTCGTT GATGTCgt-3'.

Generation of Fv-caspase-8 constructs

The protease domains of caspase-8 and caspase-8 C-A mutant (catalytic site cysteine mutated into an alanine) were PCR amplified from pcDNA3.1-Caspase 8 and pcDNA3.1-Caspase 8 C-A vectors. Resulting fragments were digested and subcloned into pC4M-Fv2E (Ariad Pharmaceuticals). The two Fv (FKBP variant) domains in the chimeric Fv2E-caspase-8 construct substitute for the natural DED dimerization domains of caspase-8 and allow ectopic dimerization of the construct with chemical dimerizer AP20187. The sequences of pC4M-Fv2E-caspase-8 and pC4M-Fv2E-caspase-8 C-A constructs were verified by sequencing.

Reagents

To activate MycERtm, cells were treated with 10 nM 4-hydroxytamoxifen (Sigma). The recombinant human soluble TRAIL was from R&D Systems. To activate Fv constructs, cells were treated with 100 nM AP20187 (Ariad). Cells were incubated with 50 µM of the pan-caspase inhibitor z-VAD-fmk (Enzyme Systems Products) to inhibit caspases. To inhibit Bcl-2, Bcl-x_L and Bcl-w, we used a small molecule inhibitor ABT-737 at 1 µM concentration (a kind gift from Dr Steven W Elmore, Abbott Laboratories).

Immunofluorescence microscopy, image acquisition and data analysis

Cells were stained with anti-cytochrome *c* (Pharmingen), anti-active Bax (6A7, Trevigen), anti-active Bak (Ab-1, Calbiochem), anti-active caspase-3 (Cell Signaling) and Hoechst 33258 (Sigma). Secondary antibodies used were Alexa-488 or -546 (Molecular Probes). Staining protocols, image acquisition and data analysis are explained in Supplementary data.

Western immunoblot analyses

Cell lysates were prepared using non-ionic Triton-X cell lysis buffer (10 mM Tris, pH 7.5, 130 mM NaCl, 1 % Triton X-100, 10 mM NaP_i and 10 mM Nappi). The monoclonal antibodies used for immunodetection were anti-caspase-8 (Cell Signaling Technology, abbr.

CST), anti-active caspase-3 (CST), anti-Bax (6A7, Trevigen), anti-Bak (Ab-1, Calbiochem), anti-Myc (9E10 a kind gift from G Evan) and anti- α -actin (Sigma). The polyclonal antibodies used were anti-Bid (CST), anti-Bcl-x_L (BD Pharmingen), anti-Bim (CST) and anti-Puma (CST).

Caspase-8 and caspase-3 activity assay

Cells were lysed in Triton-X cell lysis buffer (10 mM Tris, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM NaP_i and 10 mM NaPP_i) and the amount of caspase-8 activity was estimated by measuring proteolytic activity towards synthetic caspase-8 substrate Ac-IETD-pNA or caspase-3, Ac-DEVD-pNA peptide (Calbiochem). To measure caspase-8 or caspase-3 activity, 40 μ g aliquot of each lysate was incubated in a 100 μ l total volume of 1 $\times$ Salvesen optimal caspase buffer (Stennicke and Salvesen, 1997) supplemented with 200 μ M colorimetric pNA-peptide. Samples were incubated at 37°C and read

at 405 nm with an automated plate-reading spectrophotometer at 1-h intervals.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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