Failure of Bcl-2 to block cytochrome *c* redistribution during TRAIL-induced apoptosis

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Abstract Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family of cytokines that promotes apoptosis and NF-κB activation. Here we show that recombinant hu-TRAIL initiates the activation of multiple caspases, the loss of mitochondrial transmembrane potential, the cleavage of BID and the redistribution of mitochondrial cytochrome *c*. However, whereas Bcl-2 efficiently blocked UV radiation-induced cytochrome *c* release and consequent apoptosis of CEM cells, it failed to do either in the context of TRAIL treatment. Thus, TRAIL engages a death pathway that is at least partially routed via the mitochondria, but in contrast with other stimuli that engage this pathway, TRAIL-induced cytochrome *c* release is not regulated by Bcl-2.

Key words: Apoptosis; Bcl-2; BID; Caspase; Cytochrome *c*; Tumor necrosis factor-related apoptosis-inducing ligand

1. Introduction

Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family of cytokines that can promote apoptosis and NF-κB activation by binding to the membrane receptors TRAIL-R1/death receptor (DR)4 and TRAIL-R2/DR5 [1–8]. Related TRAIL receptors (TRAIL-R3/DcR1/TRID and TRAIL-R4/DcR2) have also been described that do not promote apoptosis but may function as decoy receptors that reduce the amount of ligand available for binding to TRAIL receptors 1 and 2 [5–12]. TRAIL induces apoptosis in a variety of transformed cell lines but is far less effective in promoting the death of untransformed cells [13–15]. In addition, in contrast with the potent cytotoxicity seen upon administration of agonistic anti-CD95 (APO-1/Fas) antibodies in mice [16], the administration of leucine zipper forms of TRAIL is relatively non-toxic in vivo [15]. Taken together, these observations suggest that TRAIL may have considerable potential as a cancer therapeutic agent.

The intracellular mechanism of TRAIL-induced apoptosis is unclear at present. Predictably, TRAIL-induced apoptosis is caspase-dependent but the repertoire and order of caspases activated in response to TRAIL receptor engagement remain ill-defined [14,16–18]. Similarly, the adapter molecule(s) that couple the cytoplasmic tail of the TRAIL receptors to the death machinery has been the subject of debate [3,5,10,17,19,20]. In particular, studies differ with respect to the role of Fas-associated protein with death domain (FADD) in transducing TRAIL receptor-initiated death signals [3,5,10,17,19–21]. However, in this context it is relevant to note that apoptosis in response to overexpression of TRAIL-R1/DR4 remained intact in embryonic fibroblasts from FADD knockout mice, whereas CD95, TNFR1 and DR3-initiated apoptosis were absent [22]. This suggests that FADD is not essential for coupling TRAIL-R1 to the cytoplasmic effectors of apoptosis but does not rule out the possibility that this adapter could participate in signalling events initiated by TRAIL-R2/DR5 and even TRAIL-R1 in other cell types.

In the context of other death receptors such as CD95 and TNFR1, it has been established that caspase-8 is the most proximally activated caspase within the death signalling pathway [23–25]. Caspase-8 is thought to propagate the death signal further in two alternative ways that are not necessarily mutually exclusive: (1) by direct proteolytic processing of downstream caspases such as caspase-3 [26–28] and (2) by cleaving BID (a Bcl-2 family protein) which then targets the mitochondria and promotes release of cytochrome *c* into the cytosol [29–32]. Upon entry into the cytosol, cytochrome *c* then propagates the caspase cascade by binding to Apaf-1 and activating its latent caspase-9 activating potential [33–36]. Bcl-2 and Bcl-x have been shown to regulate the BID-dependent pathway, but it is unclear whether they can also intervene in the more direct mitochondria-independent pathway [30–32].

To explore the mechanism of TRAIL-induced apoptosis, we investigated the range of intracellular events that took place in response to TRAIL receptor engagement in the Jurkat and CEM T leukaemia cell lines. Exposure of Jurkat cells to recombinant TRAIL triggered apoptosis that was accompanied by activation of multiple caspases, cleavage of several caspase substrates, loss of mitochondrial transmembrane potential, cleavage of BID, release of cytochrome *c* from the mitochondrial intermembrane space and exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane. Interestingly, although Bcl-2 was effective in attenuating BID cleavage, cytochrome *c* release and consequent apoptosis in response to UV irradiation, it failed to do so in the context of TRAIL-induced apoptosis. These data suggest that TRAIL

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Abbreviations: DR, death receptor; FADD, Fas-associated protein with death domain; PARP, poly(ADP-ribose) polymerase; PS, phosphatidylserine; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; z-VAD-fmk, *Z*-Val-Ala-DL-Asp-fluoromethylketone

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engages a Bcl-2-insensitive cell death pathway involving mitochondrial cytochrome *c* release.

2. Materials and methods

2.1. Materials

Annexin V-FITC was purchased from Clontech (Palo Alto, CA, USA), 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) from Molecular Probes (Eugene, OR, USA) and *Z*-Val-Ala-DL-Asp-fluoromethylketone (z-VAD-fmk) from Bachem (UK). Anti-caspase-3 (CPP32), anti-caspase-7 (Mch-3) and anti-gelsolin monoclonal antibodies (mAbs) were purchased from Transduction Laboratories (Lexington, KY, USA), anti-actin mAb from ICN (UK), anti-caspase-8 (FLICE) and anti-caspase-6 mAbs from Pharmingen (San Diego, CA, USA), anti-Fodrin mAb from Chemicon International (Temecula, CA, USA), anti-caspase-2 (ICH-$1_{S/L}$) polyclonal Ab from Santa Cruz (Santa Cruz, CA, USA), anti-poly(ADP-ribose) polymerase (PARP) polyclonal Ab from Boehringer Mannheim (Germany) and anti-FLAG M2 mAb from Sigma (UK). Anti-caspase-9 rabbit polyclonal, anti-FLICE/caspase-8 mAb, anti-BID rat polyclonal and anti-cytochrome *c* rabbit polyclonal were generously provided by Dr Doug Green (La Jolla Institute for Allergy and Immunology, San Diego, CA, USA), Dr Marcus Peter (German Cancer Research Center, Tumor Immunology Division, Heidelberg, Germany), Dr Junying Yuan (Harvard Medical School, Boston, MA, USA) and Dr Don Newmeyer (La Jolla Institute for Allergy and Immunology), respectively. Recombinant hu-FLAG-TRAIL was produced by co-transfecting COS7 cells with pCDNA3.hu-FLAG-TRAIL and pCDNA3.CrmA expression constructs. Serum-free supernatants of transfected cells were collected after 1 week, concentrated 20-fold and used as stock solution (approximately 50 μg/ml hu-FLAG-TRAIL). The specificity of the TRAIL-induced apoptosis was determined by inhibition of TRAIL activity in the COS7 cell supernatants with TRAIL-R2-Fc.

2.2. Cell culture and induction of apoptosis

Cells were cultured in RPMI 1640 containing 5% foetal calf serum (FCS). To initiate apoptosis, cells were either exposed to a UVB light source for 20 min or incubated with indicated dilutions of culture supernatant containing recombinant FLAG-TRAIL, in the presence of 50–1000 ng/ml anti-FLAG antibody. Where z-VAD-fmk was used to block caspase activity, cells were pre-incubated with the inhibitor for 1 h prior to exposure to the apoptosis-promoting stimulus.

2.3. Flow cytometry

Apoptosis was quantitated by staining with annexin V-FITC, which detects PS externalisation on the outer leaflet of the plasma membrane, as previously described [37]. Briefly, cells were resuspended at 10^6/ml in HEPES buffer (10 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) and incubated for 10 min followed by data collection on a FACScan flow cytometer. Changes in mitochondrial membrane potential ($\Delta\Psi_m$) were measured using $DiOC_6$, as follows. Cells were incubated for 30 min at 37°C in culture medium containing 50 nM $DiOC_6$, followed by data collection on a FACScan flow cytometer.

2.4. SDS–PAGE and Western blotting

Cells were lysed by incubation for 10 min at 10^8 cells/ml in ice-cold lysis buffer (50 mM HEPES-NaOH, pH 7.2, 150 mM NaCl, 5 mM EDTA, 0.1% NP40). Lysates were then spun at $20\,000\times g$ for 10 min to remove insoluble material. Protein assays (Bio-Rad, UK) were performed to ensure that equal amounts of protein (120 μg per lane) were loaded on each lane of the gel. Proteins were then separated under reducing conditions in SDS–PAGE gels, as described previously [36,38]. Separated proteins were blotted onto nitrocellulose membrane at 40 mA for 14 h. Blots were blocked for 1 h in TBST (10 mM Tris–HCl, pH 8, 150 mM NaCl, 0.05% Tween 20) containing 5% non-fat dried milk and then probed for 2 h with an appropriate amount of the primary antibody diluted in the same buffer. Blots were then washed for 30 min in several changes of TBST, followed by probing for a further 1 h with the appropriate peroxidase-coupled secondary antibodies (Amersham, UK). Bound antibody was detected by enhanced chemiluminescence using the Supersignal reagent (Pierce, UK). Blots were erased for re-probing by incubation in 0.2 M NaOH for 5 min at room temperature to strip antibodies, followed by brief washing in TBST. Blots were then re-blocked and probed with fresh antibody as described above.

2.5. Cytochrome c release assays

Cells (1×10^7) were induced to undergo apoptosis by exposure to UV irradiation or FLAG-TRAIL and harvested at times indicated in the appropriate figure legend by centrifugation at $800\times g$ for 10 min. Cell pellets were resuspended at $1–2\times10^8$ cells/ml in mannitol-sucrose extraction buffer containing protease inhibitors (220 mM mannitol, 68 mM sucrose, 50 mM PIPES/KOH, pH 7.5, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin) and after 30 min incubation on ice, were disrupted in a Dounce homogeniser with 40 strokes of a B-type pestle. Cell homogenates were spun at $20\,000\times g$ for 20 min, resuspended in the same supernatant and spun again as before. Protein assays were performed on the cytosolic fractions using the Bio-Rad reagent (Bio-Rad, UK). Equal amounts of each lysate (100–200 μg per lane) were then separated by SDS–PAGE and immunoblotted for cytochrome *c*, followed by the re-probing of blots for other proteins where appropriate.

3. Results and discussion

3.1. TRAIL induces multiple caspase activation events in Jurkat cells

The exposure of Jurkat T lymphoblastoid cells to recombinant hu-FLAG-TRAIL, in the presence of anti-FLAG crosslinking antibody, resulted in a dose-dependent increase in cells undergoing morphological features of apoptosis (membrane

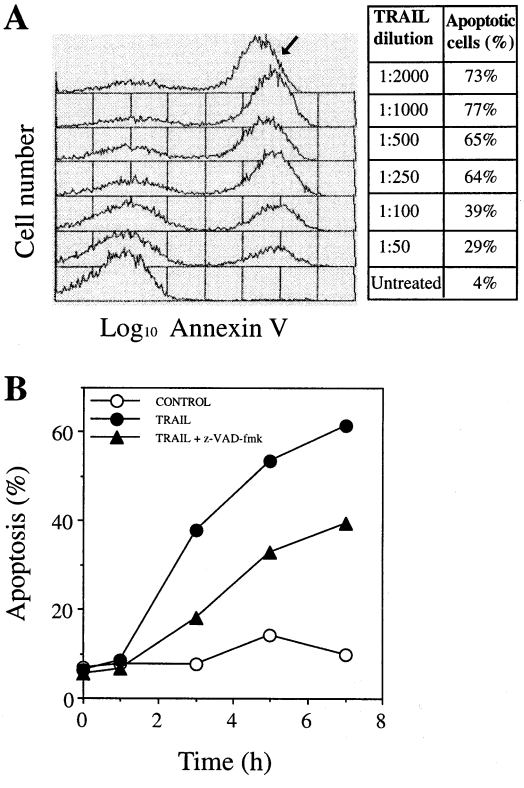

Fig. 1. TRAIL-induced apoptosis is caspase-dependent. A: Jurkat cells were cultured for 6.5 h in the presence of the indicated dilutions of hu-FLAG-TRAIL supernatant and 50 ng/ml crosslinking anti-FLAG antibody, followed by assessment of annexin V-FITC binding by flow cytometry. B: Time course of PS externalisation in untreated Jurkat cells or cells cultured in the presence of TRAIL (1:1000 dilution, plus 50 ng/ml of anti-FLAG mAb) in the presence or absence of 50 μM z-VAD-fmk. Data shown are derived from analyses of 5000 cells at each data point and are representative of three separate experiments.

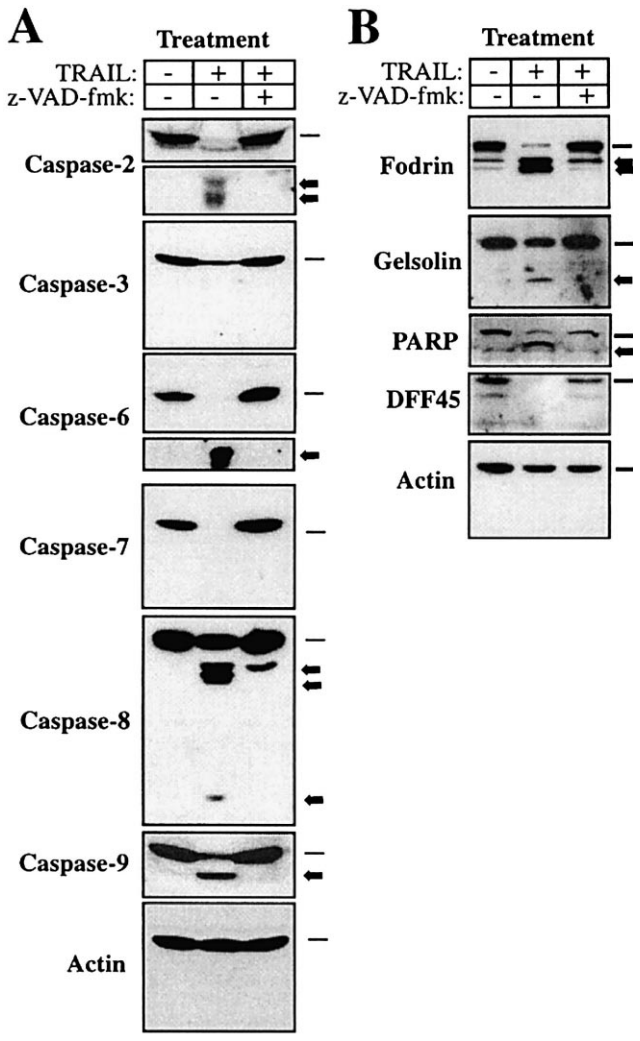

Fig. 2. TRAIL-induced activation of multiple caspases and cleavage of caspase substrate proteins. A: Jurkat cells were cultured for 4.5 h in medium alone, or in the presence of 50 ng/ml of hu-FLAG-TRAIL plus 50 ng/ml of crosslinking anti-FLAG mAb. Where indicated, z-VAD-fmk was also added to the medium at a final concentration of 50 μM. Cell lysates were then prepared for SDS–PAGE followed by immunoblotting for the detection of the indicated caspases. Bars represent caspase pro-forms and arrows indicate processed forms. In some cases, in order to detect the processed form the lower portions of the blots were overexposed. In these cases, the longer exposure is shown as a separate panel. B: Jurkat cells were treated as in A, followed by immunoblotting for the indicated caspase substrates.

blebbing and DNA condensation; data not shown), as well as exposure of PS on the outer leaflet of the plasma membrane, as detected by annexin V binding (Fig. 1A). In the presence of limiting amounts of anti-FLAG crosslinking antibody, the lower concentrations of FLAG-TRAIL resulted in increased levels of apoptosis (Fig. 1A). This is most likely because the crosslinking of FLAG-TRAIL with limiting amounts of anti-FLAG antibody becomes less efficient when saturating amounts of ligand are present. As expected, TRAIL-induced apoptosis was significantly inhibited in the presence of the broad spectrum caspase inhibitor, z-VAD-fmk (Fig. 1B), confirming that TRAIL-induced apoptosis is caspase-dependent.

To explore the range of caspase activation events initiated in response to TRAIL receptor engagement, we probed cell lysates from TRAIL-treated Jurkat cells with a panel of anti-caspase antibodies. As illustrated in Fig. 2A, TRAIL treatment resulted in the maturation of the zymogen forms of caspase-2, -3, -6, -7, -8 and -9 to their corresponding processed forms. A number of well characterised caspase substrates, fodrin, gelsolin, PARP and DFF45, were also cleaved in response to TRAIL treatment (Fig. 2B). The caspase inhibitor, z-VAD-fmk, blocked essentially all of these TRAIL-induced caspase activation events, although a small amount of caspase-8 processing was still observed in the presence of this inhibitor suggesting that caspase-8 may be the most proximally activated caspase in this context (Fig. 2A).

3.2. TRAIL-initiated mitochondrial events

Numerous recent studies have implicated the mitochondrion as a key target in the program leading to collapse and death of the cell [39–42]. Mitochondria respond to numerous apoptosis-promoting signals by releasing pro-apoptotic molecules such as cytochrome *c* and apoptosis-inducing factor into the cytosol [41,43,44]. Mitochondria also exhibit a dramatic reduction in transmembrane potential ($\Delta\Psi_m$), as a result of either partial disruption of the outer mitochondrial membrane or the opening of discrete membrane channels [39,40,45]. To ask whether TRAIL-initiated apoptosis was associated with perturbations in $\Delta\Psi_m$ and the release of mitochondrial cytochrome *c*, we exposed Jurkat cells to TRAIL and assessed changes in $\Delta\Psi_m$ and cytosolic cytochrome *c* in the presence or absence of z-VAD-fmk. As shown in Fig. 3, TRAIL-treated Jurkat cells exhibited a dramatic reduction in $\Delta\Psi_m$ that could be inhibited by z-VAD-fmk. TRAIL also triggered the caspase-dependent release of mitochondrial cytochrome *c* (Fig. 3B).

In the context of the CD95 and TNF receptor pathways, it was recently demonstrated that cytochrome *c* release is instigated by the Bcl-2 family member, BID [30–32]. In response to engagement of the latter death receptors, caspase-8 is rapidly activated at the membrane and targets cytosolic BID for proteolytic cleavage. Cleaved BID then translocates to the mitochondrial membrane where it triggers cytochrome *c* release via a mechanism that has yet to be elucidated [30–32]. Thus, we asked whether BID also played a role in the cytochrome *c* release seen during TRAIL-induced death. As illustrated in Fig. 3B,C, TRAIL-induced cytochrome *c* redistribution in Jurkat cells was associated with a dramatic reduction in the levels of intact BID (the antibody used did not efficiently recognise the cleaved form of BID). The inhibition of caspase activity partially restored the levels of full-length BID, in parallel with a concomitant reduction in cytochrome *c* release (Fig. 3B). To confirm that decreases in the levels of intact BID did not reflect a general decrease in cellular protein levels as cells underwent apoptosis, a time course experiment was performed and levels of BID were compared with actin. As illustrated in Fig. 3C, whereas BID levels decreased by almost 50% within 3 h of exposure to TRAIL, actin expression levels did not change significantly over the same time course.

3.3. Bcl-2 fails to regulate TRAIL-induced apoptosis in CEM cells

Bcl-2 is capable of repressing apoptosis initiated by numerous death-promoting stimuli and is thought to act primarily within the mitochondrial outer membrane where it has been

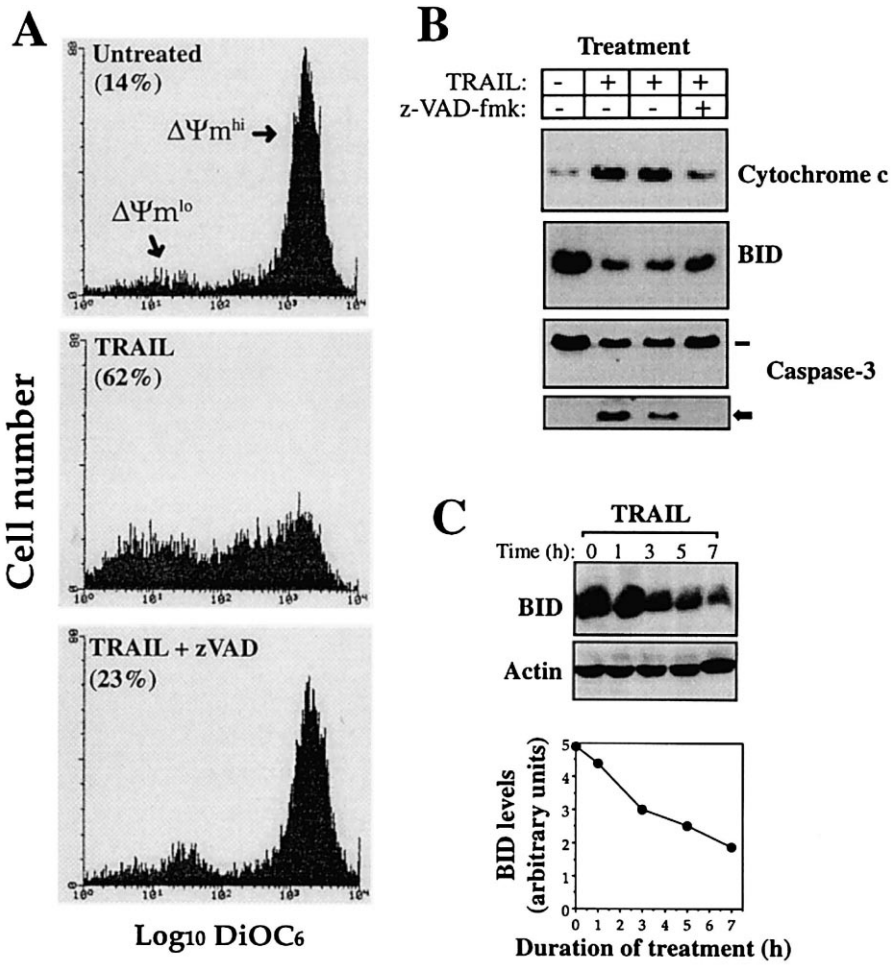

Fig. 3. TRAIL-induced mitochondrial changes, cleavage of BID and redistribution of cytochrome *c* are caspase-dependent. A: Jurkat cells were incubated for 4 h either in medium alone or in the presence of hu-FLAG-TRAIL (50 ng/ml, plus 50 ng/ml anti-FLAG mAb) or hu-FLAG-TRAIL plus 50 μM z-VAD-fmk, as indicated. Changes in mitochondrial transmembrane potential ($\Delta\Psi_m$) were measured using $DiOC_6$ uptake in conjunction with flow cytometry. B: Jurkat cells were treated as in A, except that cells were incubated for 6 h under the indicated conditions. Cytosolic extracts were then made, mitochondria were pelleted and extracts were probed for cytochrome *c*, BID and caspase-3, as indicated. The arrow indicates the processed form of caspase-3. C: Time course of BID cleavage in Jurkat cells cultured in the presence of hu-FLAG-TRAIL (25 ng/ml plus 50 ng/ml anti-Flag mAb). After probing for BID, the blot was re-probed for actin to assess protein loadings. For quantitation of BID protein levels, densitometry was performed on the blot shown using NIH-Image software. Data shown are representative of at least three experiments.

shown to regulate cytochrome *c* release by an unknown mechanism [43,44,46]. Limited evidence also suggests that Bcl-2 may be capable of acting downstream of this point under certain circumstances [47,48]. It is not known whether Bcl-2 is capable of regulating apoptosis initiated by TRAIL receptor ligation.

To explore this question, CEM.neo and CEM.bcl-2 cells were compared with respect to their response to TRAIL or exposure to UV radiation. As illustrated in Fig. 4A, Bcl-2-expressing CEM cells were very resistant to apoptosis initiated by UV irradiation, as well as several other stimuli, as previously reported [37,49]. However, Bcl-2 transfectants were as sensitive to TRAIL as their vector-transfected counterparts, at all concentrations tested (Fig. 4B,C).

3.4. *TRAIL-induced cytochrome c release in CEM cells is not regulated by Bcl-2*

Because TRAIL-induced apoptosis was associated with the redistribution of cytochrome *c* to the cytosol, an event that is inhibited by Bcl-2 in the context of several other pro-apoptotic stimuli, we asked whether the failure of Bcl-2 to regulate TRAIL-induced apoptosis could be attributed to a failure to block cytochrome *c* release. Fig. 5 illustrates that Bcl-2 failed to block caspase-8 activation and BID cleavage in response to TRAIL receptor engagement on CEM cells, suggesting that these events take place upstream of the point of Bcl-2 action, as in the CD95R and TNFR pathways. However, whereas Bcl-2 efficiently blocked UV-radiation induced cytochrome *c* release, it failed to do so in cells exposed to TRAIL. These data suggest that TRAIL engages a novel pathway leading to cytochrome *c* release and apoptosis that can bypass the protective effects of Bcl-2. We also assessed the Bcl-2 status in CEM.bcl-2 cells exposed to various concentrations of TRAIL to assess whether TRAIL receptor engagement resulted in either phosphorylation, proteolysis or downregulation of Bcl-2, however, no overt changes were found by Western blotting (data not shown).

Here we have shown that TRAIL engages a cell death pathway that results in multiple caspase activation events, the cleavage of the Bcl-2 family member BID, the release of cy-

tochrome *c* and the loss of mitochondrial transmembrane potential. All of these events were blocked by caspase inhibitors, suggesting that early caspase activation drives the mitochondrial changes that take place in the TRAIL-initiated cell death pathway, much in the same way that these events are coordinated in the Fas/CD95 and TNFR pathways. However, TRAIL-initiated cytochrome *c* release and consequent apoptosis failed to be regulated by Bcl-2. This contrasts sharply with what has been observed with other apoptosis-promoting signals, such as UV radiation and staurosporine, that also trigger cytochrome *c* release [43,44]. In the latter cases, Bcl-2 can block the release of cytochrome *c* from the mitochondrial intermembrane space and consequently confers protection from these agents [43,44]. This suggests that TRAIL engages a unique pathway leading to cytochrome *c* release that can bypass the inhibitory effects of Bcl-2, possibly by inactivating the latter. These data may partially explain how many tumour cell types are uniquely susceptible to the cytotoxic effects of this cytokine.

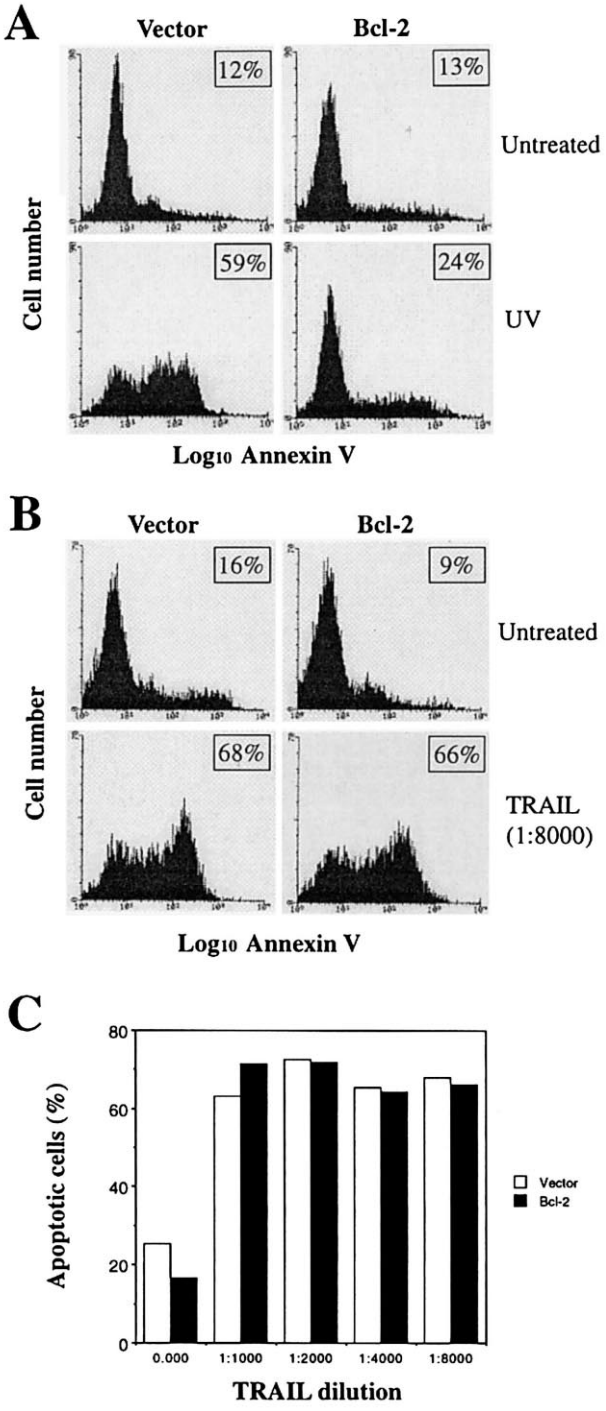

Fig. 4. Bcl-2 protects against UV- but not TRAIL-induced apoptosis. CEM cells stably transfected with either vector or Bcl-2 expression plasmids were exposed to UV radiation (10 min) (A) or (B) to hu-FLAG-TRAIL (6.25 ng/ml, plus 50 ng/ml anti-FLAG mAb), followed by incubation for 3.5 h. C: CEM.neo and CEM.bcl-2 cells were cultured for 3.5 h in the presence of the indicated concentrations of hu-FLAG-TRAIL (50 ng/ml anti-FLAG mAb was included in all treatments) followed by assessment of apoptosis. Apoptosis-associated annexin V-FITC binding was quantitated by flow cytometry. Data shown are representative of six independent experiments.

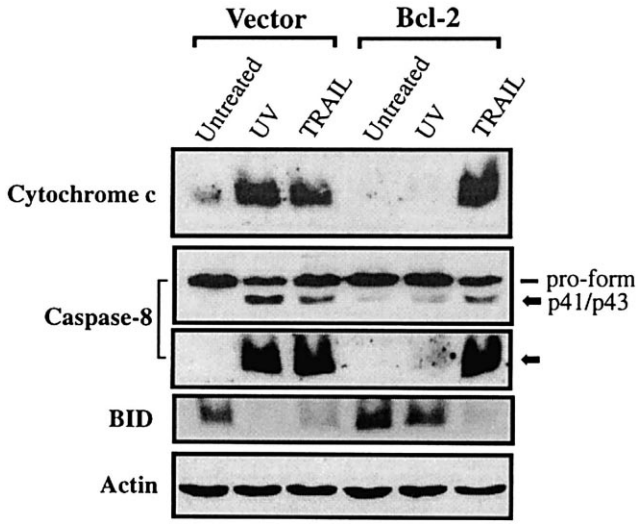

Fig. 5. Failure of Bcl-2 to regulate TRAIL-induced cytochrome *c* release. CEM.neo and CEM.bcl-2 cells were either left untreated, were UV-irradiated for 10 min or exposed to 50 ng/ml hu-FLAG-TRAIL (plus 50 ng/ml anti-FLAG mAb), followed by incubation for 7 h. Cytosolic extracts were then made followed by probing for the indicated proteins. The lower panel of the caspase-8 blot was overexposed to facilitate the detection of the processed form of caspase-8.

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