Wild-type, mitochondrial and ER-restricted Bcl-2 inhibit DNA damage-induced apoptosis but do not affect death receptor-induced apoptosis

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SUMMARY

The proto-oncogene Bcl-2 is expressed in membranes of mitochondria and endoplasmic reticulum and mediates resistance against a broad range of apoptotic stimuli. Although several mechanisms of Bcl-2 action have been proposed, its role in different cellular organelles remains elusive. Here, we analyzed the function of Bcl-2 targeted specifically to certain subcellular compartments in Jurkat cells. Bcl-2 expression was restricted to the outer mitochondrial membrane by replacing its membrane anchor with the mitochondrial insertion sequence of ActA (Bcl-2/MT) or the ER-specific sequence of cytochrome b5 (Bcl-2/ER). Additionally, cells expressing wild-type Bcl-2 (Bcl-2/WT) or a transmembrane domain-lacking mutant (Bcl-2/ΔTM) were employed. Apoptosis induced by ionizing radiation or by the death receptors for CD95L or TRAIL was analyzed by determination of the mitochondrial membrane potential ($\Delta\Psi_m$) and activation of different caspases.

Bcl-2/WT and Bcl-2/MT strongly inhibited radiation-induced apoptosis and caspase activation, whereas Bcl-2/ΔTM had completely lost its anti-apoptotic effect. Interestingly, Bcl-2/ER conferred protection against radiation-induced mitochondrial damage and apoptosis similarly to Bcl-2/MT. The finding that ER-targeted Bcl-2 interfered with mitochondrial $\Delta\Psi_m$ breakdown and caspase-9 activation indicates the presence of a crosstalk between both organelles in radiation-induced apoptosis. By contrast, Bcl-2 in either subcellular position did not influence CD95- or TRAIL-mediated apoptosis.

Key words: Bcl-2, Mitochondria, Endoplasmic reticulum, Apoptosis, Radiation, CD95

INTRODUCTION

The *C. elegans* protein CED-9 and its related mammalian counterparts Bcl-2, Bcl-x_L, Mcl-1, Bcl-w and A1 comprise a highly conserved family of anti-apoptotic proteins. Studies on CED-9 function revealed the importance of the Bcl-2 family for ontogenesis of *C. elegans*. Gain-of-function mutants of CED-9 interfere with the apoptotic deletion of excess cells during embryonic development. CED-9 displays close physical and functional interactions with CED-4, CED-3 and EGL-1, which comprise the pro-apoptotic components of the worm death machinery. The functional cooperation between CED-3 and CED-4 results in the proteolytic activation of CED-3, which is abrogated by physical interaction of CED-9 with CED-4 (Chinnaiyan et al., 1997; Spector et al., 1997; Wu et al., 1997). On the contrary, EGL-1 can complex with CED-9 and thereby inhibits its anti-apoptotic function (Conradt and Horvitz, 1998). These mechanisms in *C. elegans* are relevant for apoptotic processes during somatic cell deletion as well as for cell death in response to DNA damage in germ cells (Gartner et al., 2000).

The search for human homologues revealed that CED-3 is a cysteine protease resembling the caspase protease family in higher eukaryotes (Miura et al., 1993), whereas CED-4 is both structurally and functionally related to APAF-1 (Zou et al., 1997). However, the linear model of apoptosis induction in *C. elegans* is opposed by more complex apoptosis systems in vertebrates. Up to now two major signaling cascades have been analyzed in greater detail.

A widely accepted mammalian pathway with significant similarities to the apoptotic cascade in *C. elegans* is involved in cell death in response to DNA damage and cellular stress. The transcriptional activation of Noxa or similar pro-apoptotic molecules are key events of apoptosis mediated by p53 (Lowe et al., 1993; Oda et al., 2000). Other pro-apoptotic members of the Bcl-2 protein family such as Bim may be involved in apoptosis since deletion of Bim was shown to confer partial resistance against apoptosis induced by gamma-irradiation (Bouillet et al., 1999).

Noxa and Bax induce mitochondrial damage with the breakdown of the mitochondrial membrane potential and release of cytochrome c (Jurgensmeier et al., 1998; Oda et al., 2000; Pastorino et al., 1998). The translocation of cytochrome c triggers the mitochondrial apoptosis pathway in a strictly Bcl-

2/Bclx$_L$-controlled manner (Hu et al., 1998; Yang et al., 1997). Cytoplasmic cytochrome c forms a complex with APAF-1 which oligomerizes and recruits the initiator caspase-9 for autoactivation (Li et al., 1997). Activated caspase-9 in turn activates caspase-3 and other effector caspases leading to the apoptotic phenotype through the cleavage of several vital proteins (Li et al., 1997).

The second well-characterized cascade is activated by binding of ligands of the tumor necrosis factor family (e.g. CD95/Fas/Apo-1 ligand (CD95L) or TRAIL/Apo-2L) to their cognate surface receptors. The CD95 signaling pathway is paradigmatic for the whole family of death receptors. Binding of CD95L to its receptor mediates the recruitment of the adapter molecule FADD, which in turn attracts procaspase-8, which undergoes autoproteolytic activation (Muzio et al., 1996). Subsequently, caspase-8 directly activates downstream effectors including caspase-3 (Stennicke et al., 1998). This simple model of CD95-mediated apoptosis is complicated by the existence of an amplification loop that is required for the rapid execution of cell death in cases of low initial caspase-8 activation (Scaffidi et al., 1998). In this scenario, caspase-8 activates the pro-apoptotic Bcl-2 member BID by removing its N-terminal domain. Truncated BID then translocates to mitochondria where it induces cytochrome c release (Li et al., 1998; Luo et al., 1998). Cytoplasmic cytochrome c in turn triggers the APAF1/caspase-9 cascade, thereby amplifying the initial death receptor signal. This amplification mechanism is particularly observed in so-called type-II cells, which are efficiently protected by Bcl-2, whereas in many other cell types (type-I cells) Bcl-2 does not strongly interfere with death receptor-mediated apoptosis (Strasser et al., 1995; Scaffidi et al., 1998; Huang et al., 1999; Belka et al., 2000; Engels et al., 2000).

Although it is generally accepted that Bcl-2 and Bcl-x$_L$ inhibit mitochondrial damage, a precise understanding of their function is hampered by the complexity of mammalian apoptotic cascades. The localization of Bcl-2/Bcl-x$_L$ in different subcellular compartments and a multitude of described interactions of Bcl-2/Bcl-x$_L$ with various components of the apoptotic machinery may contribute to the anti-apoptotic effects. Attempts have been made to predict functions from the molecular structure of Bcl-2/Bcl-x$_L$. Through interaction of the hydrophobic cleft that is formed by critical residues within the BH1, BH2 and BH3 domains, Bcl-x$_L$ interacts with the BH3 domain of pro-apoptotic molecules including Bax, Bak and Bid (Muchmore et al., 1996; Oltvai et al., 1993; Sattler et al., 1997; Wang et al., 1996). The ability to form heterodimers was shown to be crucial for the anti-apoptotic function of Bcl-x$_L$ in some models (Sedlak et al., 1995). By contrast, other studies (Cheng et al., 1996; Minn et al., 1999) provided evidence that those Bcl-x$_L$ mutants that do not interact with Bax, retain significant anti-apoptotic potency in yeast and higher eukaryotes. Thus, the anti-apoptotic capacity of Bcl-2 proteins may be only partially mediated by the functional sequestration of pro-apoptotic family members. Independently of the capability to heterodimerize with pro-apoptotic proteins, Bcl-2/Bcl-x$_L$ were shown to form ion channels in artificial lipid bilayers. Indeed, the 3D structures of Bcl-2 and Bcl-x$_L$ are related to pore-forming bacterial toxins. The ion channel activity of Bcl-2/Bcl-x$_L$ may maintain ion homeostasis across mitochondrial and other biological membranes. Thus, evidence exists that, in addition to the interaction with pro-apoptotic family members, the capability to form ion channels may be a key element of Bcl-2 function.

Although the analysis of the Bcl-2/Bcl-x$_L$ structure helps to clarify functional aspects of the anti-apoptotic action, several aspects remain unclear. For example, Bcl-2 and Bcl-x$_L$ interfere with many stages of the mitochondrial apoptosis pathway. Most importantly, it was shown that Bcl-2 blocks the release of cytochrome c from mitochondria (Kluck et al., 1997). However, other mitochondrial functions are influenced by Bcl-2/Bcl-x$_L$ as well. It has been shown that Bcl-x$_L$ abolished growth factor deprivation-induced cell death by facilitating mitochondrial ATP/ADP exchange (Vander Heiden et al., 1999). In addition, Bcl-x$_L$ prevents mitochondrial swelling and outer membrane rupture in response to CD95 stimulation or staurosporine treatment (Vander Heiden et al., 1997).

Apart from direct effects on mitochondria, Bcl-2 was shown to block apoptosis induction in response to microinjection of cytochrome c into cells, suggesting that Bcl-2 may also act downstream of cytochrome c (Brustugun et al., 1998). Since CED-9 directly interferes with CED-4 in *C. elegans* (Yang et al., 1998), Bcl-2/Bclx$_L$ were proposed to directly bind to and antagonize mammalian APAF-1. A specific mutation of CED-9, namely the G169E substitution in the CED-9(n1950) mutant, leads to a loss of CED-9 interaction with the BH3-only protein EGL-1. As a result of this impaired interaction, EGL-1-induced release of CED-4 from CED-4/CED-9 complexes is also impaired in the gain-of-function mutant (Parrish et al., 2000). In contrast to CED-4, it was shown recently that Bcl-2/Bcl-x$_L$ do not physically interact with APAF-1 and the proteins are not found in similar cellular locations (Hausmann et al., 2000; Moriishi et al., 1999). However, this does not exclude the possibility that additional CED-4-like adapter molecules might be inactivated by Bcl-2. Alternatively, the recently described protein Aven, which binds to both APAF-1 and Bcl-x$_L$, may act as bridging protein and thus potentially mediate Bcl-2/Bcl-x$_L$ effects downstream of cytochrome c (Chau et al., 2000).

In addition to mitochondria, Bcl-2 localizes to other biological membranes, in particular the endoplasmic reticulum and nuclear membrane (Lithgow et al., 1994), raising the possibility that Bcl-2 acts at extramitochondrial sites. However, the role of Bcl-2 in the ER is poorly understood. It was suggested that Bcl-2 and related proteins interfere with intracellular calcium stores and calcium release, which may be relevant for apoptosis induction and related to its ion channel activity. Bcl-2 decreases the ER calcium content by increasing the calcium permeability of the ER membrane (Foyouzi-Youssefi et al., 2000; Pinton et al., 2000). Other studies, by contrast, demonstrated that Bcl-2 causes an interference with ER calcium release and shift from the ER to mitochondria (Baffy et al., 1993; He et al., 1997).

Although a multitude of data on potential functions of Bcl-2 and other family members is available, the precise mechanisms still remain elusive. In particular, the contribution of the subcellular localization of Bcl-2 to its anti-apoptotic function is unclear. In order to gain further insight into the role of the Bcl-2 subcellular localization for its anti-apoptotic effects, we expressed organelle-specific mutants of Bcl-2 in Jurkat cells and investigated their effect on apoptosis induced

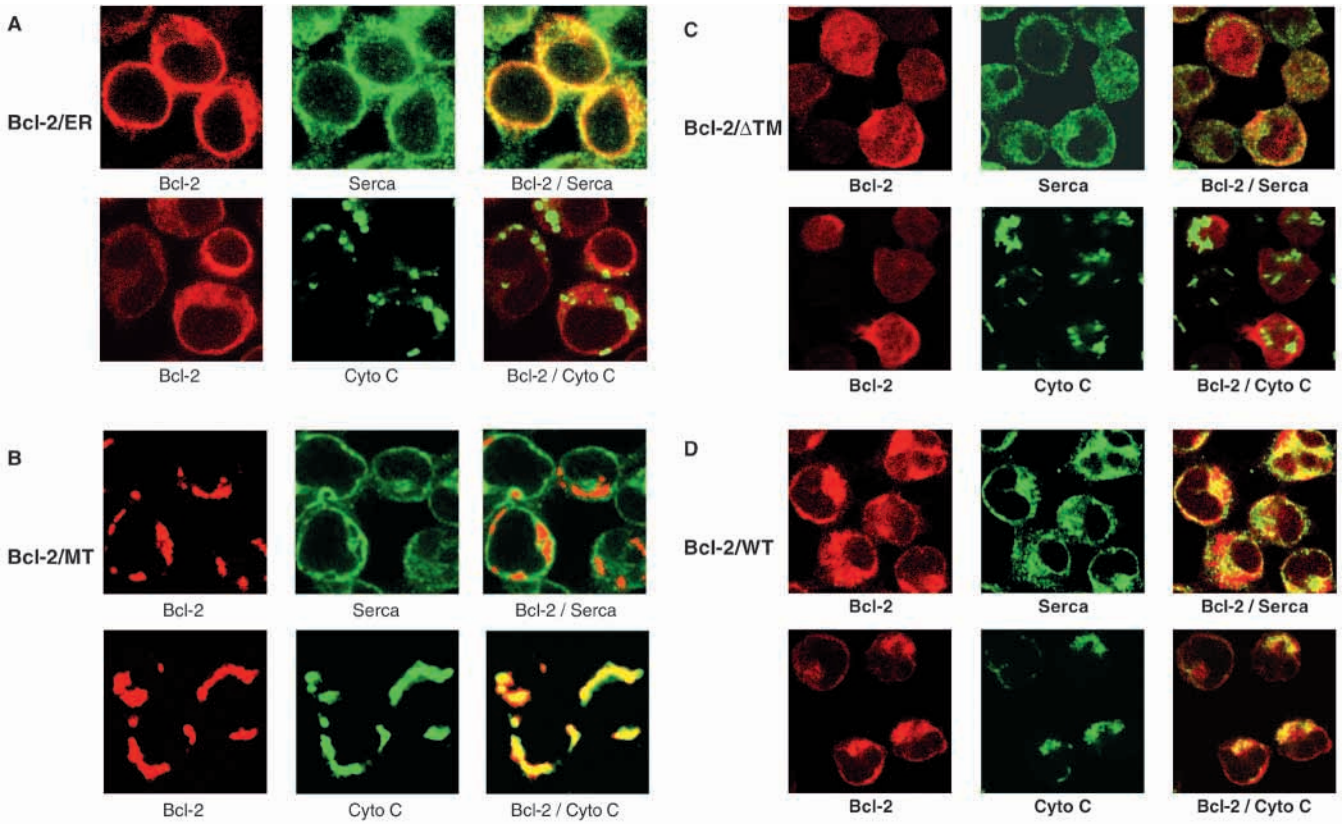

Fig. 1. Expression of wild-type Bcl-2 and Bcl-2 mutants with restricted subcellular location. Jurkat T-cells were stably transfected with expression constructs targeting Bcl-2 to the endoplasmic reticulum (Bcl-2/ER) or mitochondria (Bcl-2/MT), a construct lacking the transmembrane domain (Bcl-2/ΔTM) or with wild-type Bcl-2. Confocal microscopy was performed to confirm the subcellular localization of Bcl-2 in the appropriate compartment. The pool-transfected cells were co-stained with Bcl-2 (in red) and either with the calcium ATPase SERCA for endoplasmic staining or with cytochrome c for mitochondrial staining (in green). Colocalization of the molecules is indicated by the yellow color in the merged micrographs. In Bcl-2/ER cells (A) Bcl-2 colocalizes with SERCA but not with cytochrome c. In Bcl-2/MT cells (B) Bcl-2 clearly colocalizes with cytochrome c but not with SERCA. By contrast, Bcl-2/ΔTM (C) shows a diffuse expression pattern in the cytoplasm and nucleus. Wild-type Bcl-2 (D) is detectable in the perinuclear region, the ER and the mitochondria.

by either ionizing irradiation or death receptors. We assessed the effect of wild-type Bcl-2, a cytoplasmic version lacking the transmembrane domain and Bcl-2 mutants targeted to either mitochondria or the endoplasmic reticulum. Our data indicate the existence of a molecular crosstalk between the endoplasmic reticulum and mitochondria in radiation-induced apoptosis, which can be interrupted by Bcl-2 at the level of the endoplasmic reticulum.

MATERIALS AND METHODS

All biochemicals were from Sigma Chemicals (Deisenhofen, Germany) unless otherwise specified. Hoechst 33342, LEHD-fmk and zVAD-fmk were purchased from Calbiochem (Bad Soden, Germany).

Cell culture and transfections

Jurkat E6 T-lymphoma cells were from ATCC (Bethesda, MD). For all experiments cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco Life Technologies, Eggenstein, Germany) and maintained in a humidified incubator at 37°C and 5% CO_2. Expression vectors encoding wild-type Bcl-2 (Bcl-2/WT), cytoplasmic Bcl-2 lacking the transmembrane domain (Bcl-2/ΔTM) and Bcl-2 mutants restricted to the outer mitochondrial membrane (Bcl-2/MT) or endoplasmic reticulum (Bcl-2/ER) were kindly provided by B. Leber (Ontario, Canada). Mitochondrial and ER-specific targeting of Bcl-2 was achieved by exchanging the C-terminal insertion sequence of Bcl-2 with equivalent sequences of the listerial protein ActA and rat cytochrome b5, respectively (Zhu et al., 1996). Jurkat cells stably expressing the different versions of Bcl-2 and the respective vector control were prepared by electroporation using a Gene pulser II (BioRad, Munich, Germany) and were primarily used as pool transfectants. Parallel experiments were performed with individual clones derived from the bulk transfected culture by limiting dilution.

Radiation, CD95 and TRAIL stimulation

Cells were irradiated with 6 MV photons from a linear accelerator (LINAC SL25 Phillips) with a dose rate of 4 Gy/min at room temperature (RT). CD95 stimulation was performed using the agonistic IgM antibody CH11 (100 ng/ml; UBI Biomol, Hamburg, Germany). TRAIL receptors were stimulated using recombinant human flag-tagged TRAIL and a crosslinking antibody (Alexis Biochemicals, Grünberg, Germany)

Confocal microscopy

The subcellular localization of Bcl-2 was verified by confocal microcopy using a Leica TCS NT microscope. In brief, cells were immobilized on coverslips with 0.1% poly-L-lysine, fixed with 2.5% paraformaldehyde and permeabilized with 0.1% Triton X-100. After

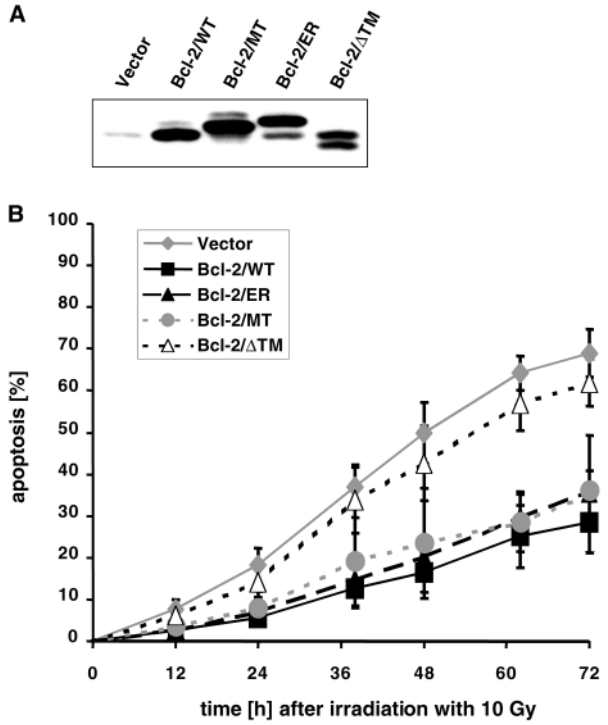

Fig. 2. Induction of apoptosis by ionizing irradiation. Immunoblot analysis with an anti-Bcl-2 antibody revealed that the transfectants expressed the different forms of Bcl-2 at roughly similar levels (A). Apoptosis induction in control or cells irradiated with 10 Gy was quantified by flow cytometry (B). Overexpression of Bcl-2/MT and Bcl-2/WT strongly protected cells against apoptosis. Similar to Bcl-2/MT, ER-targeted Bcl-2 conferred survival against irradiation-induced cell death. Cells expressing the cytosolic, non-membrane Bcl-2/ΔTM mutant revealed apoptosis to similar levels as vector control cells. The error bars indicate the standard deviations from independent measurements of the same cell batch (five independent experiments).

blocking with 10% fetal calf serum cells were stained with primary antibodies for 1 hour at RT. Cells were washed several times and incubated with secondary antibodies for 30 minutes. Finally, the coverslips were mounted with Mowiol (Sigma). Bcl-2 staining was performed with a specific rabbit antibody (Santa-Cruz-Biotech, Heidelberg, Germany). Localization of Bcl-2 in the ER or mitochondria was confirmed by costaining with a murine antibody against the calcium pump SERCA (UBI Biomol) and a mouse anti-cytochrome c antibody (PharMingen, Hamburg, Germany). As secondary antibodies Alexa Fluor™-conjugated anti-mouse (Molecular Probes, Mobitech, Göttingen, Germany) and Cy™5-conjugated anti-rabbit antibodies (Dianova, Hamburg, Germany) were employed.

Determination of apoptosis

Apoptosis was quantified using scatter characteristics in a FACS Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany). In addition, apoptosis induction was measured by DNA fluorescence microscopy. To this end, cells were incubated with Hoechst 33342 at a final concentration of 1.5 μM for 15 minutes. Cell morphology was then determined by fluorescence microscopy (Zeiss Axiovert 135, Carl Zeiss, Jena, Germany) using an excitation wavelength filter of 380 nm. At least five independent experiments were performed. The given error bars represent the standard deviation from independent measurements of the same cell batch.

Immunoblotting

Cells (1×10^5) were lysed in a buffer containing 25 mM Hepes, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 10 mM EDTA, 10 mM NaF and 125 mM NaCl. After removing insoluble material by centrifugation for 10 minutes at 12,000 ***g***, 20 μg of lysate was separated by SDS-PAGE. Blotting was performed in a tank blotting apparatus (BioRad) onto Hybond ECL membranes (Amersham, Braunschweig, Germany). Equal protein loading was confirmed by Ponceau S staining. Blots were blocked in TBS buffer containing 0.05% Tween-20 and 5% fetal calf serum at RT for 15 minutes. Then, the primary antibodies were applied for 1 hour. After repeated washings with TBS/Tween-20 (0.05%) the membrane was incubated with the secondary antibody (anti IgG-AP 1:20.000, Santa-Cruz-Biotech) in TBS/Tween for 30 minutes at RT and washed three times with TBS/Tween. Antibody binding was detected by enhanced chemoluminescence staining. The anti-caspase-3, anti-caspase-9 and anti-PARP antibodies were from cell signaling (NEB, Frankfurt, Germany). Caspase-8 activation was detected as described previously (Belka et al., 2000) employing a mouse monoclonal antibody directed against the p18 subunit at a 1:50 dilution of the hybridoma supernatant. Bcl-2 expression was tested using a Bcl-2 antibody (Santa-Cruz-Biotech). Caspase-12 was detected using a rat polyclonal serum against caspase-12 (Ab-2, Oncogene Research Products, San Diego, CA)

Determination of the mitochondrial potential

The mitochondrial transmembrane potential ($\Delta\Psi_m$) was analyzed using the $\Delta\Psi_m$-specific stain TMRE (Molecular Probes, Mobitech). In brief, 1×10^5 cells were stained at the indicated time points in a solution containing 25 nM TMRE for 30 minutes. Staining was quantified by flow cytometry using the FL2 channel and scatter characteristics.

RESULTS

Targeted subcellular expression of Bcl-2

To assess the role of the subcellular localization of Bcl-2, we expressed different Bcl-2 constructs in Jurkat cells. The Bcl-2 mutants were directed to certain subcellular compartments by specific targeting motifs. The specific distribution of either mutant was already shown by different authors (Hacki et al., 2000; Zhu et al., 1996). However, in the first set of experiments the assumed subcellular localization of either mutant was verified by confocal microscopy. Localization to the ER was tested by co-staining of Bcl-2 with the endoplasmic calcium ATPase SERCA. As shown in Fig. 1A, only the endoplasmic reticulum-specific mutant Bcl-2/ER colocalized with SERCA, displaying a punctuate staining pattern that excluded the nucleus. By contrast, expression of the mitochondrial construct Bcl-2/MT (Fig. 1B) as well as the transmembrane domain-lacking form Bcl-2/ΔTM (Fig. 1C) was not colocalized with SERCA.

In parallel, mitochondria were stained using an antibody directed against cytochrome c, and colocalization of Bcl-2 and the respective mutants was tested. In accordance with the Bcl-2/SERCA co-staining, only Bcl-2/MT colocalized with mitochondria, whereas no colocalization was observed with the Bcl-2/ER and Bcl-2/ΔTM constructs. Similar experiments were performed using the mitochondria-specific stain Mitotracker orange and revealed similar results (not shown). Interestingly, staining of Bcl-2/ΔTM did not exclude the nuclear region (Fig. 1C), suggesting that Bcl-2 without membrane anchor can freely enter the nucleus.

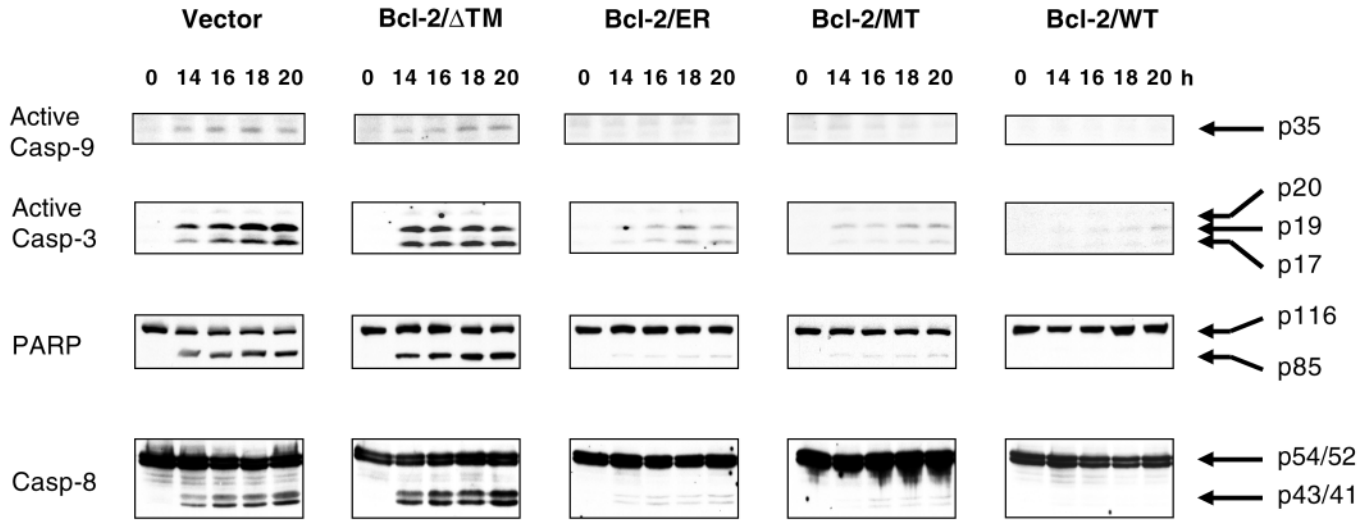

Fig. 3. Caspase activation after irradiation of Jurkat cells expressing different Bcl-2 mutants. Western blot analyses were performed with cell lysates prepared 14-20 hours after irradiation with 10 Gy. The blots show that activation of caspase-9, caspase-3 and caspase-8 as well as PARP cleavage was abrogated by overexpression of either mitochondrial (Bcl-2/MT), endoplasmic (Bcl-2/ER) or wild-type Bcl-2 (Bcl-2/WT). No differences in caspase activation were detectable when Bcl-2/ΔTM cells were compared with vector control cells.

Confocal analysis of cells overexpressing Bcl-2/WT revealed a localization at the ER, the perinuclear region and mitochondria (Fig. 1D). A discrete background staining detectable in all cell lines was due to a low-level expression of endogenous Bcl-2 (Fig. 2A).

Apoptosis induction in response to ionizing radiation

In contrast to initial ideas suggesting that ionizing radiation might employ CD95 signaling pathways (Belka et al., 1998; Reap et al., 1997), we and others have shown that the apoptosis pathways triggered by ionizing radiation and CD95 are different, although both pathways depend on the activation of caspases (Newton et al., 1998; Belka et al., 1999; Belka et al., 2000; Newton and Strasser, 2000). Thus, the use of cells deficient for CD95, FADD or caspase-8 allowed a clear distinction between the two cascades. The strict control of radiation-induced apoptosis by proteins of the Bcl-2 family in parallel to the requirement of APAF-1 and caspase-9 founded the hypothesis that radiation-induced apoptosis is controlled by mitochondrial mechanisms (Belka et al., 2000; Hakem et al., 1998; Kuida et al., 1998; Rudner et al., 2001; Yoshida et al., 1998).

We therefore asked whether an apoptotic pathway that is essentially controlled by mitochondria is affected by the subcellular distribution of Bcl-2. As shown in Fig. 2B, ionizing radiation induced time-dependent apoptosis in Jurkat cells, which was strongly inhibited by wild-type Bcl-2. The Bcl-2 mutant lacking the transmembrane domain was unable to interfere with radiation-induced apoptosis indicating a critical need for a membrane anchor. Surprisingly, however, mitochondrial and ER-targeted Bcl-2 inhibited radiation-induced cell death to a very similar extent, suggesting that both the ER and mitochondria are key elements in radiation-induced apoptosis.

We next analyzed whether the activation of caspases was

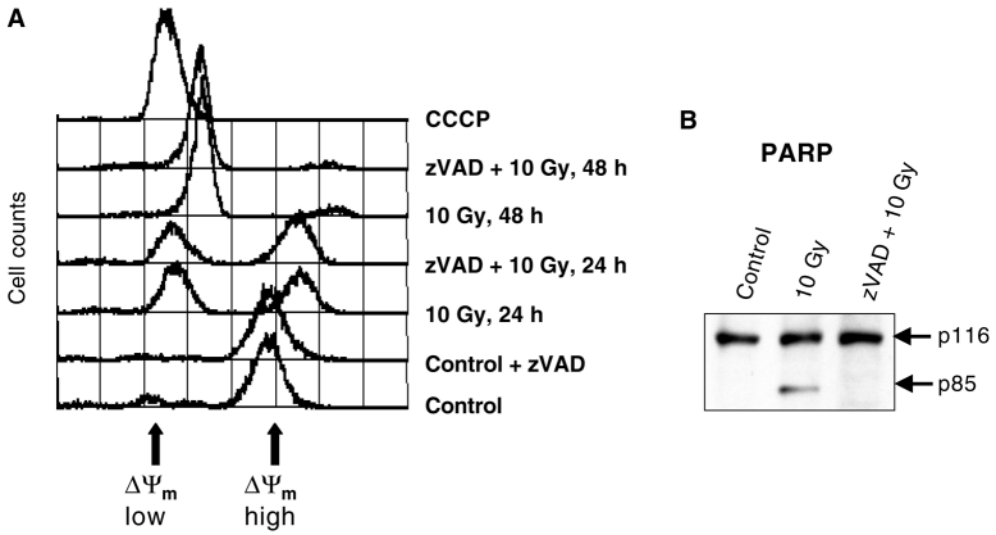

Fig. 4. Breakdown of the mitochondrial membrane potential after irradiation occurs prior to caspase activation. Prior to irradiation (10 Gy) cells were pretreated with the broad-spectrum caspase inhibitor zVAD (20 μM). No influence on the breakdown of $\Delta\Psi_m$ was detectable (A), although caspase activation indicated by the abrogation of PARP cleavage was blocked (B).

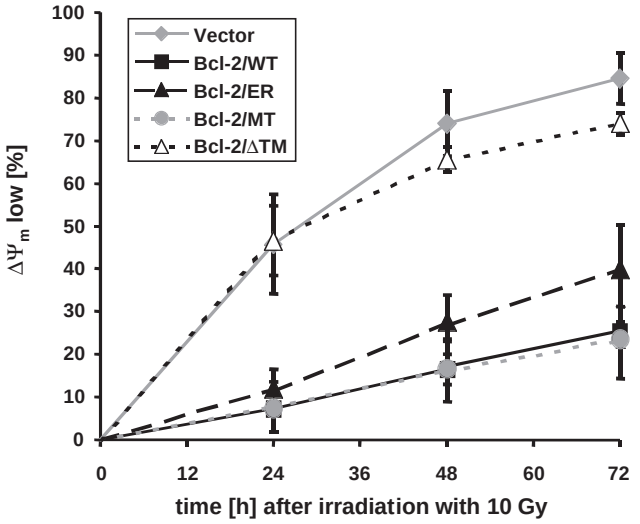

Fig. 5. Loss of mitochondrial membrane potential after irradiation. Cells were irradiated with 10 Gy and the mitochondrial membrane potential $\Delta\Psi_m$ was determined by flow cytometry. Bcl-2 overexpression at mitochondria (Bcl-2/MT), the ER (Bcl-2/ER) or in both compartments (Bcl-2/WT) protected cells from radiation-induced mitochondrial potential breakdown, whereas Bcl-2/ΔTM had no significant effects. The error bars indicate the standard deviations from independent measurements of the same cell batch.

influenced by overexpression of Bcl-2 in different subcellular compartments (Fig. 3). In contrast to the death receptor pathway, caspase-9 is the most apical caspase activated by ionizing radiation downstream of mitochondrial release of cytochrome c. Thus, we expected that overexpression of Bcl-2/WT and Bcl-2/MT would interfere with radiation-induced activation of caspase-9. Irradiation of Jurkat cells induced a time-dependent processing of caspase-9 as demonstrated by the appearance of the p35 cleavage fragment that is generated through autocatalytic activation of caspase-9 at D315. (Fig. 3). As expected, capase-9 activation was inhibited by Bcl-2/WT but not by Bcl-2/ΔTM. Interestingly, Bcl-2/ER and Bcl-2/MT efficiently prevented caspase-9 processing also.

The same results became even more evident when processing of caspase-3 was analyzed using an antibody directed against its active p20/p19/p17 cleavage products. In accordance, PARP cleavage was also abrogated (Fig. 3). Thus, caspase-9 and caspase-3 activation was completely inhibited by all Bcl-2 mutants except Bcl-2/ΔTM.

In addition, we analyzed the effect of Bcl-2 on radiation-induced caspase-8 activation which, in contrast to CD95, is not mediated by FADD-induced proximity but by a postmitochondrial event (Strasser et al., 1995; Huang et al., 1999; Belka et al., 2000; Huang et al., 1999; Newton and Strasser, 2000). Caspase-8 is synthesized as two isoforms of 54 and 52 kDa (caspase-8a and -8b) which, following formation of intermediate cleavage products of 43 and 41 kDa, are processed to a p18 and p10 heterodimer. Radiation induced a pronounced processing of caspase-8 that was strongly prevented by both mitochondrial and ER-targeted Bcl-2. The slight activation of caspases corresponded to the background level of apoptosis and was presumably due to a lower expression level of Bcl-2 and the mutants in a few cells of the pooled transfectants.

Since mitochondrial damage is considered as a key step for DNA damage-induced apoptosis, it was speculated that Bcl-2-like proteins generally act by protecting mitochondrial integrity. Our data suggest that at least some aspects of Bcl-2 action may be also regulated at the level of the ER. To determine how far mitochondrial damage is influenced by Bcl-2 in different subcellular positions, the integrity of mitochondrial function was analyzed by determining the mitochondrial potential ($\Delta\Psi_m$). $\Delta\Psi_m$ was measured using the potential-sensitive stain TMRE and subsequent flow cytometry. In a first set of experiments we analyzed how far radiation-induced breakdown of $\Delta\Psi_m$ in Jurkat cells is a primary event and does not occur secondarily to caspase activation.

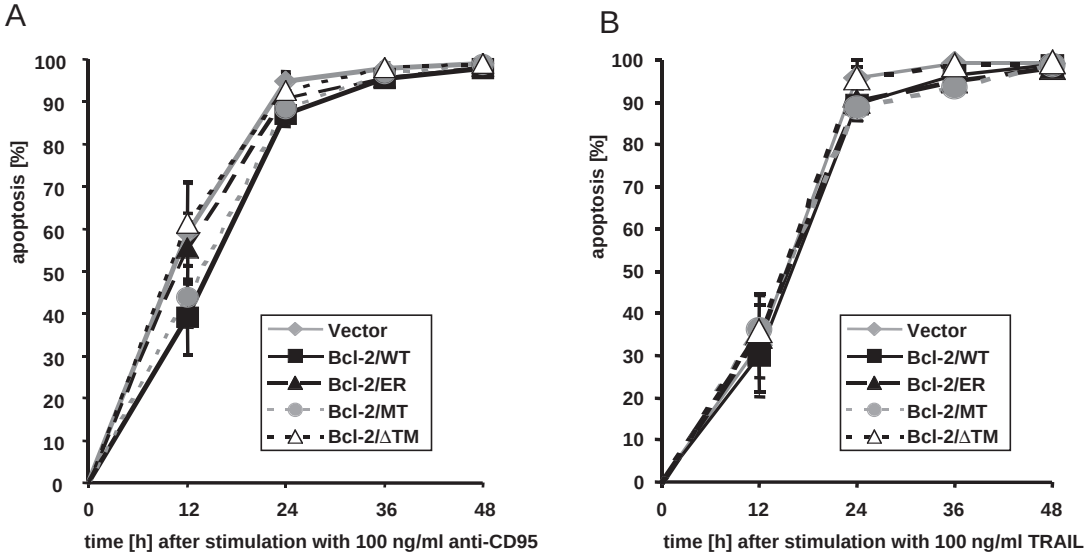

Fig. 6. CD95- and TRAIL-mediated apoptosis in Jurkat cells overexpressing Bcl-2 at different intracellular locations. Cells were stimulated with 100 ng/ml of either anti-CD95 (A) or TRAIL (B). After the indicated times apoptosis was quantified by flow cytometry. The experiments reveal that overexpression of the Bcl-2 mutants had no significant effect on death receptor-mediated apoptosis. The error bars indicate the standard deviations from independent measurements of the same cell batch (five independent measurements).

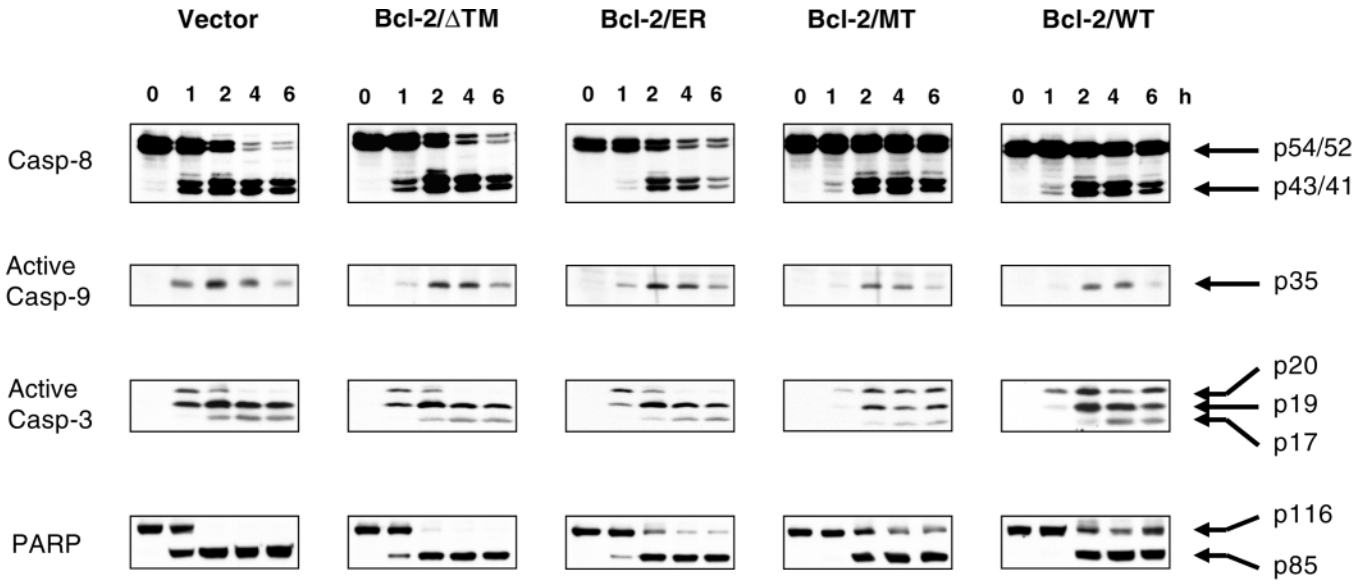

Fig. 7. Effect of the Bcl-2 mutants on CD95-mediated caspase activation. Cell lysates were prepared 1, 2, 4 and 6 hours after CD95 stimulation (100 ng/ml CH11) and analyzed for caspase processing and PARP cleavage by immunoblotting using antibodies against caspase-8, active caspase-9, active caspase-3 and PARP. The immunoblots show the proforms and p43/41 intermediate cleavage products of caspase-8, the p35 intermediate caspase-9 fragment, the different cleavage products of caspase-3, and the full-length and p85 fragment of PARP. In vector control cells as well as in Bcl-2/ΔTM- and Bcl-2/ER-expressing cells procaspase-8 was almost completely processed, whereas in cells expressing the wild-type and mitochondrial form of Bcl-2 the processing was attenuated. Caspase-9 and caspase-3 activation as well as PARP cleavage was also delayed in Bcl-2/WT and Bcl-2/MT cells.

Therefore, Jurkat cells were pretreated with the broad-spectrum caspase inhibitor zVAD (20 μM), leading to a complete inhibition of effector caspases during radiation-induced apoptosis (not shown). In parallel, PARP cleavage was fully abrogated (Fig. 4). However, no influence on the breakdown of $\Delta\Psi_m$ could be detected (Fig. 4A). Thus, the breakdown of $\Delta\Psi_m$ is an initial event during radiation-induced apoptosis and therefore suitable to determine the maintenance of mitochondrial integrity by Bcl-2.

In subsequent experiments we tested how far wild-type, mitochondrial Bcl-2 as well as the ER-targeted form interfered with the breakdown of the mitochondrial membrane potential in response to irradiation. Regardless of its localization, Bcl-2 inhibited the breakdown of the mitochondrial membrane

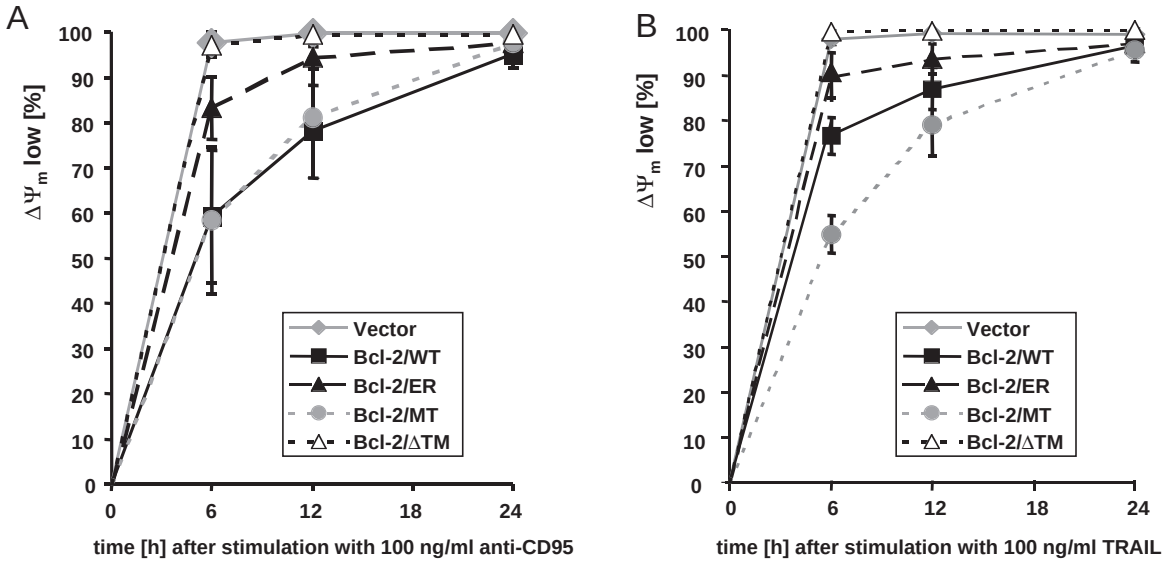

Fig. 8. Effect of the Bcl-2 mutants on the breakdown of the mitochondrial membrane potential after CD95 (A) or TRAIL (B) stimulation. Cells were stimulated for the indicated time points, and the mitochondrial potential ($\Delta\Psi_m$) was determined using the mitochondrial potential-specific dye TMRE. The amount of cells with a low $\Delta\Psi_m$ was quantified by FACS analysis. When overexpressed at mitochondria or as wild-type protein, Bcl-2 delayed $\Delta\Psi_m$ breakdown. However, after 24 hours almost all cells displayed a low $\Delta\Psi_m$ irrespective of the Bcl-2 status. Bcl-2/ER influenced the breakdown of $\Delta\Psi_m$ only modestly, whereas Bcl-2/ΔTM had no effect compared with vector control cells. The error bars indicate the standard deviations from independent measurements of the same cell batch.

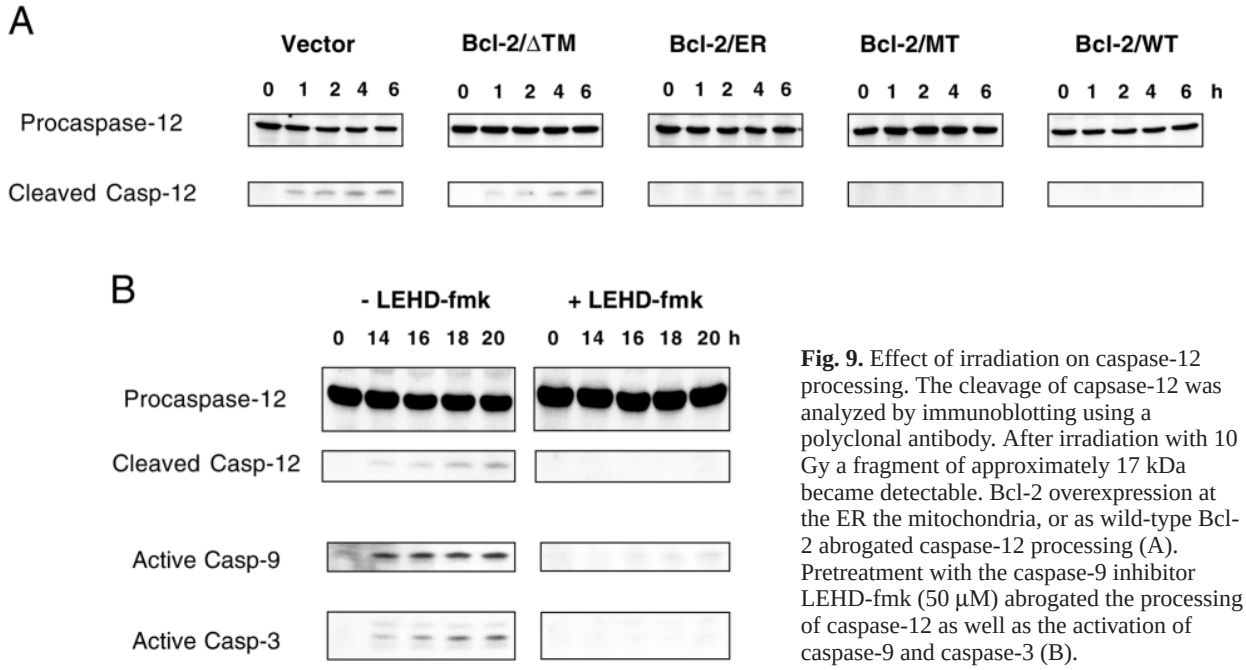

Fig. 9. Effect of irradiation on caspase-12 processing. The cleavage of capsase-12 was analyzed by immunoblotting using a polyclonal antibody. After irradiation with 10 Gy a fragment of approximately 17 kDa became detectable. Bcl-2 overexpression at the ER the mitochondria, or as wild-type Bcl-2 abrogated caspase-12 processing (A). Pretreatment with the caspase-9 inhibitor LEHD-fmk (50 μM) abrogated the processing of caspase-12 as well as the activation of caspase-9 and caspase-3 (B).

potential (Fig. 5). These results therefore suggest a molecular crosstalk between the ER and the mitochondria during radiation-induced apoptosis that is suppressed by Bcl-2 at the level of the ER and mitochondria upstream of $\Delta\Psi_m$ breakdown. Similar results were obtained using individual clones derived from the bulk-transfected cultures (not shown).

Apoptosis induction in response to death receptor stimulation

Conflicting data have been reported on the effect of Bcl-2 on CD95 and TRAIL-mediated cell death in different cell types. Jurkat T-cells were categorized as type-II cells that require an intact mitochondrial pathway for CD95-induced apoptosis that is inhibited by Bcl-2 (Scaffidi et al., 1998). However, we and others found that overexpression of wild-type Bcl-2 (Bcl-2/WT) only moderately changed the kinetics of CD95-induced cell death, but no requirement for the mitochondrial pathway was detectable (Belka et al., 2000; Strasser et al., 1995; Huang et al., 1999). In order to further define the role of Bcl-2 during death receptor-induced apoptosis, the influence of Bcl-2 localization was tested. As measured by flow cytometry, treatment of Jurkat control cells with 100 ng/ml of either the agonistic CD95 antibody CH11 or TRAIL rapidly induced apoptosis in approximately 90% of the cells after 24 hours (Fig. 6). No significant differences of apoptosis induction between Jurkat controls cells and Bcl-2 pool-transfected cells were detectable after 24 hours regardless of the subcellular localization of Bcl-2. Similar results were obtained with individual clones. Thus, mitochondrial pathways are not crucial for CD95 or TRAIL-induced apoptosis in Jurkat cells.

In a next set of experiments the activation of caspases in response to CD95 stimulation was investigated. During CD95-mediated apoptosis, the caspase cascade is initiated by the recruitment and cleavage of caspase-8 at the death-inducing signaling complex (DISC). As assessed with an antibody directed against the p18 subunit, CD95 ligation induced the rapid formation of the intermediate cleavage products, followed by a more or less complete consumption of procaspase-8 (Fig. 7). Consistent with the time course of apoptosis, processing of caspase-8 was slightly delayed in cells overexpressing Bcl-2/WT and Bcl-2/MT compared with control cells or cells expressing the endoplasmic or cytosolic form of Bcl-2. The proform of caspase-8 was almost completely converted after 4-6 hours in vector control and Bcl-2/ER or Bcl-2/ΔTM-expressing cells, whereas significant amounts of caspase-8 remained unprocessed in cells expressing wild-type or mitochondrially targeted Bcl-2. Similar results were obtained after stimulation with 100 ng/ml TRAIL (not shown).

Since caspase-8 directly activates caspase-3, the processing of caspase-3 was analyzed using an antibody directed against its active p20/p19/p17 cleavage products. Initial caspase-3 cleavage occurs at D175 giving rise to an intermediate p20 fragment and the p12 subunit. Secondary processing of p20 occurs at residues D9 and D28 generating the fragments p19 and p17. The latter processing is mediated through autoprocessing by caspase-3 or alternatively by caspase-9. As shown in Fig. 7, CD95 stimulation resulted in the rapid processing of procaspase-3 to the p20 and p19 fragments and the subsequent generation of the mature p17 subunit. Overexpression of Bcl-2/WT and Bcl-2/MT delayed the maturation of caspase-3. Whereas in vector control cells as well as in Bcl-2/ER and Bcl-2/ΔTM cells the p20 form was literally absent after 4 hours, it remained detectable in cells expressing wild-type or mitochondrial Bcl-2. Corresponding results were obtained after stimulation with 100 ng/ml TRAIL (not shown).

In addition, caspase-9 activation was analyzed using an antibody directed against its p35 subunit. Again, Bcl-2/WT and Bcl-2/MT delayed and weakened the activation of caspase-9 (Fig. 7). In parallel, cleavage of the caspase-3 substrate PARP to its characteristic 85 kDa fragment was determined (Fig. 7). In accordance with the previous data, Bcl-2 targeted to mitochondria and the wild-type form weakly interfered with the time course of caspase activation, whereas the soluble, cytosolic and ER-specific Bcl-2 mutants had almost no effect.

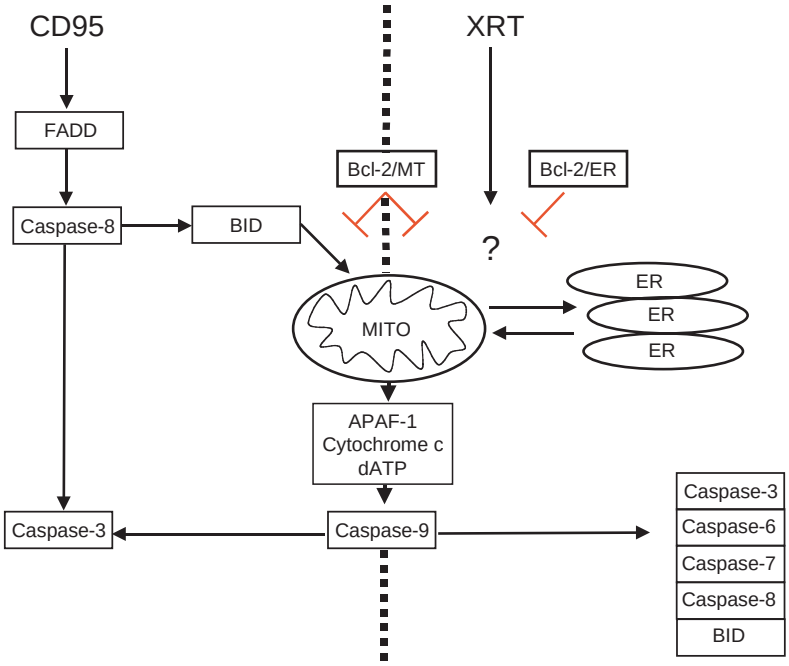

Fig. 10. Schematic model of the effect of Bcl-2 subcellular location in two forms of apoptosis. During death receptor-mediated apoptosis caspase-8 is the most apical caspase. Once activated, caspase-8 in turn triggers the downstream effector cascade either directly through caspase-3 or through engagement of the mitochondrial pathway. In this process, Bid triggers cytochrome c release, which is needed for caspase-9 activation. Bcl-2 located at mitochondria interferes with the activation of this amplification loop, whereas Bcl-2 at the ER has no influence on death receptor-mediated apoptosis. By contrast, the most apical caspase in radiation-induced apoptosis is caspase-9, which is activated in response to mitochondrial damage. A hypothetical crosstalk between mitochondria and the ER, which is upstream of caspase activation and may involve perturbations in calcium homeostasis or other ER-based pro-apoptotic molecules, may essentially control the initial steps of radiation-induced cell death.

An additional feature of CD95-induced apoptosis is the breakdown of the mitochondrial membrane potential ($\Delta\Psi_m$). Therefore, we tested how far Bcl-2 and the differentially expressed mutants interfered with $\Delta\Psi_m$ breakdown. As shown in Fig. 8, 24 hours after treatment of cells with anti-CD95 or TRAIL a complete loss of the mitochondrial potential was observed in almost all cells regardless of Bcl-2 expression. However, slight differences in the proportion of cells with low and high $\Delta\Psi_m$ were detectable 6 hours and still 12 hours after stimulation in those cells, in which either Bcl-2/WT or Bcl-2/MT were expressed.

Together, these data suggest that the slope of CD95 or TRAIL-mediated caspase activation is weakly attenuated in cells overexpressing wild-type or mitochondrial Bcl-2, whereas the non-membrane form or ER-targeted Bcl-2 do not provide protection. However, no durable inhibition of CD95 or TRAIL-induced apoptosis was observed at later time points of apoptosis, suggesting that the CD95 as well as the TRAIL pathway in Jurkat cells is not dependent on mitochondria.

Involvement of caspase-12

Up to this point our results suggested that ER-controlled steps are involved in radiation-induced apoptosis. Recent data suggested that the activation of caspase-12 is involved in a specific form of apoptosis in the ER unfolded protein response that can be induced by brefeldin A or tunicamycin (Nakagawa et al., 2000). In knockout mice it was shown that apoptosis induction by other stimuli including CD95 and staurosporine did not require the presence of caspase-12. Using a polyclonal serum directed against caspase-12 a single band corresponding to the p53 proform of caspase-12 was detected (Fig. 9). After irradiation with 10 Gy a smaller fragment of approximately 17 kDa became visible. Overexpression of Bcl-2 in either position abrogated the processing of caspase-12 (Fig. 9A). Since caspase-9 is considered as the key caspase for radiation-induced apoptosis, we tested whether the observed weak cleavage of caspase-12 might be secondary to the activation of caspase-9. As shown in Fig. 9B, pretreatment with 50 μM of the caspase-9 LEHD-fmk abrogated caspase-9, caspase-3 as well as caspase-12 processing, suggesting that the observed cleavage of caspase-12 was downstream of caspase-9.

DISCUSSION

In the present study, we investigated the importance of the subcellular localization of Bcl-2 for its anti-apoptotic activity. Our data provide evidence for distinct roles of different organelles in two models of apoptosis. Whereas Bcl-2 located at both the ER and mitochondria substantially inhibited radiation-induced apoptosis, none of the Bcl-2 forms significantly interfered with death receptor-induced death.

There are only a few data available regarding the importance of Bcl-2 localization for its anti-apoptotic effects. In our experiments, we overexpressed Bcl-2 mutants that had the hydrophobic C-terminus exchanged for that of cytochrome b5 or the mitochondrial protein ActA. Such Bcl-2 constructs have been previously shown to block apoptosis in certain but not all cell types. Whereas MDCK epithelial cells were protected from serum deprivation-induced apoptosis by wild-type and mitochondrial Bcl-2, no survival was conferred by Bcl-2 targeted to the ER (Zhu et al., 1996). By contrast, in Rat-1/Myc cells Bcl-2 located at the ER was more effective than Bcl-2 targeted to mitochondria (Zhu et al., 1996). A recent study provided evidence that ER stress triggered by brefeldin A, a compound that blocks the retrograde transport of Golgi vesicles, or tunicamycin, which induces aberrant protein folding, was inhibited by Bcl-2 in the ER (Hacki et al., 2000). It was also shown that ER-targeted Bcl-2 could inhibit c-Myc- but not etoposide-induced apoptosis in Rat-1 fibroblasts (Lee et al., 1999).

The role of membrane anchoring for the function of Bcl-2 has not been determined in detail. Our data provide evidence that the transmembrane domain is required for the anti-

apoptotic function of Bcl-2, since neither CD95- nor radiation-induced apoptosis was affected by the Bcl-2/ΔTM construct in Jurkat cells. This finding may be important as the apoptosis pathway in *C. elegans* suggested that Bcl-2 might eventually act via direct interference with cytosolic APAF-1. Indeed, coprecipitation experiments indicated that Bcl-2 interacts with APAF-1 (Pan et al., 1998), whereas later, more detailed studies excluded a physical interaction between molecules (Hausmann et al., 2000; Moriishi et al., 1999). Our data show that the presence of free cytoplasmic Bcl-2 is not sufficient for the anti-apoptotic function, suggesting that functional interactions with cytoplasmic APAF-1 are very unlikely.

The influence of Bcl-2 on death receptor-mediated apoptosis is still a matter of debate (Huang et al., 2000; Schmitz et al., 1999). Based on the lack of efficient DISC formation after CD95 stimulation Jurkat cells have been categorized as type-II cells (Scaffidi et al., 1998). According to this model, apoptosis in Jurkat cells at least partially relies on a Bcl-2-inhibitable mitochondrial pathway. We and others, however, have shown that stimulation with either the anti-CD95 antibody CH11 (Belka et al., 2000) or CD95L (Huang et al., 1999) clearly induced apoptosis in Bcl-2-overexpressing cells. Furthermore, Huang et al. suggested that differences between type-I and type-II cells might be solely attributed to the anti-CD95 antibody used (Huang et al., 1999; Huang et al., 2000). The data presented here suggest that Bcl-2 only weakly interferes with the time course of CD95-induced caspase activation. The effect of Bcl-2 was observed only in very early phases resulting in a reduced activation of caspase-8, whereas the final outcome of cell death was not affected by Bcl-2 expression. This finding is compatible with the notion that caspase-8 initially triggers both a direct pathway leading to activation of caspase-3 and an indirect pathway leading to activation of caspase-3 via BID and caspase-9. Bcl-2 overexpression only interferes with the activation of amplifying mitochondrial pathways, thereby reducing the initial activation of caspase-8. Therefore, our data do not favor a general role of Bcl-2 for the regulation of CD95-mediated apoptosis. Since Bcl-2 also did not interfere with TRAIL-induced apoptosis in Jurkat cells, a similar argumentation applies to the TRAIL receptor system.

Up to now most data suggest that the anti-apoptotic action of Bcl-2 relies on its interference with mitochondrial pathways, in particular cytochrome c release. Our data show that mitochondrial and ER-targeted Bcl-2 confer survival against radiation to a similar extent. Furthermore, we found that apoptosis induced by other stimuli, such as ER stress-inducing agents such as brefeldin A, was influenced by the two versions of Bcl-2 (data not shown). A protective effect by ER-targeted Bcl-2 and inhibition of mitochondrial cytochrome c release by staurosporine, brefeldin A and tunicamycin has been recently reported (Hacki et al., 2000), although in this study the impact of mitochondrial Bcl-2 was not assessed. Thus, our data reveal that, unlike the death receptor pathway, during irradiation-induced apoptosis Bcl-2 can also function in the ER to inhibit mitochondrial events. These findings therefore imply a molecular crosstalk between both organelles in radiation-induced apoptosis. It will be interesting to investigate whether Bcl-2 targeted to other membranes, such as lysosomes or the plasma membrane, retains its anti-apoptotic activity.

The role of the ER in apoptosis has not yet been analyzed extensively. It is not known how irradiation perturbs the ER and how these perturbations impinge on mitochondria and the caspase cascade. Several possible explanations can be considered. Caspase-8 could be a candidate since it can reside at the ER and mediate mitochondrial damage via BID activation. Bcl-2 has been shown to bind to and neutralize the endoplasmic pro-apoptotic caspase-8 substrate Bap31 (Ng et al., 1997; Ng and Shore, 1998). However, several data argue against a contribution of caspase-8. It has been demonstrated that neither a deficiency of caspase-8 nor of Bid abolishes radiation-induced apoptosis (Belka et al., 2000; Yin et al., 1999). Thus, an initial radiation-induced activation of caspase-8 at the ER with the propagation of this signal to mitochondria is unlikely. Furthermore, Nakagawa et al.'s results and our results do not favor an important role of caspase-12 for radiation-induced apoptosis. Only a weak processing of caspase-12 could be observed that was apparently downstream of caspase-9 activation.

An alternative way of crosstalk may involve calcium. Both the ER and mitochondria act as calcium stores controlling the capacitive calcium influx and cytoplasmic calcium homeostasis (Pozzan and Rizzuto, 2000). Indeed, Bcl-2 was shown to control calcium storage at mitochondria and the ER (Baffy et al., 1993; Foyouzi-Youssefi et al., 2000; He et al., 1997; Zhu et al., 1996). Bcl-2 may modulate ER permeability by forming an ion channel or regulating ER-based channels, such as the IP3 receptor or the calcium ATPase SERCA. In addition, other members of the Bcl-2 family were shown to interfere with calcium flux-mediated apoptosis. For instance, lymphocytes from mice lacking Bim did not undergo apoptosis when the calcium homeostasis was disrupted by calcium ionophores (Bouillet et al., 1999).

Only a few data are currently available on a potential calcium-mediated crosstalk between the ER and mitochondria during apoptosis. Apoptosis in response to thapsigargin, a specific inhibitor of the ER calcium pump, was shown to involve Bcl-2 inhibitable cytochrome c release (Srivastava et al., 1999). However, the contribution of Bcl-2 in either localization was not tested. In contrast to thapsigargin, the influence of ionizing radiation on cellular calcium homeostasis is unknown. Nevertheless, some molecules critically involved in calcium homeostasis appear to be mediators in radiation-induced apoptosis. IP3 receptor-1, which is involved in TCR receptor-mediated calcium response in T cells, also critically controls radiation-induced cell death (Jayaraman and Marks, 1997). In addition, the tyrosine kinase Lck, which is also required for the regulation of calcium release from the ER (Keizer et al., 1995; Straus and Weiss, 1992) is involved in the control of radiation and ceramide-induced apoptosis (Belka et al., 1999; Manna et al., 2000). By contrast, studies using the intracellular calcium chelator AM-BAPTA revealed contradictory results. Whereas in Ly cells calcium chelation interfered with apoptotic DNA degradation in response to ionizing radiation (Voehringer et al., 1997), only minor effects were observed in MOLT-4 cells (Takahashi et al., 1999). The use of calcium chelators is problematic, since calcium levels in all cellular compartments are affected. Thus, more precise techniques to measure and manipulate calcium within cellular compartments will have to be employed to assess the role of calcium in the crosstalk between ER and mitochondria during radiation-induced apoptosis.

Taken together, the following model system of radiation- and

receptor-induced apoptosis can be derived from the effects of Bcl-2 in cellular compartments (Fig. 10). In the death receptor pathway, triggering of CD95 leads to initial activation of caspase-8, which subsequently activates downstream molecules including BID and caspase-3. BID triggers an amplifying mitochondrial pathway leading to activation of caspase-9 and downstream caspases. The entire network is complicated by the existence of several feedback loops. For example, caspase-3 may also activate caspase-9, caspase-8 and BID, and caspase-8 may directly activate other caspases. Hence, inhibition of cytochrome c release will only affect the time course of caspase activation but not the final outcome of apoptosis in cases of optimal CD95/TRAIL stimulation. By contrast, radiation presumably initially triggers perturbations in the ER and mitochondria, which may involve changes of calcium homeostasis or activation of pro-apoptotic mediators that may crosstalk between both organelles. Such an ER/mitochondrial loop may lead to profound mitochondrial damage, cytochrome c release and activation of caspase-9 and downstream caspases. It is interesting to note that mitochondria have been observed to exist as a continuous interconnected reticulum closely associated with the ER. Changes in calcium homeostasis in the ER might thus be preferentially transferred into the mitochondrial matrix. Future experiments will therefore have to investigate whether the close proximity of mitochondria and the ER and the tight coupling of calcium signaling plays an essential role in the cell sensitivity to radiation-induced apoptosis.

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