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Bcl-2 mediated inhibition of erucylphosphocholine-induced apoptosis depends on its subcellular localisation

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Abstract

The synthetic phospholipid derivative erucylphosphocholine (ErPC) is a potent inducer of apoptosis in human tumor cell lines. This membrane-targeted drug induces apoptosis independently from death receptor signaling through a mitochondrial pathway that is inhibited by over-expression of Bcl-2.

Within the cell, Bcl-2 resides in membranes of mitochondria, endoplasmic reticulum (ER) and the nucleus. However, the importance of its subcellular localisation in distinct organelles for protection against apoptosis is not completely understood.

To investigate the impact of Bcl-2 localised at defined subcellular compartments on its protective effects against ErPC-induced apoptosis, Bcl-2 expression was directed to the outer membrane of the mitochondria or the ER of Jurkat T Lymphoma cells, using Bcl-2 mutants with modified membrane anchors. The mitochondrial insertion sequence of ActA directed Bcl-2 to the mitochondria (Bcl-2/MT), the ER-specific sequence of cytochrome b5 to the ER (Bcl-2/ER). Additionally, Jurkat cells expressing wild-type Bcl-2 (Bcl-2/WT) or a transmembrane domain-lacking mutant (Bcl-2/ΔTM) were employed.

While restricted expression of Bcl-2 either at membranes of the mitochondria or the ER strongly interfered with ErPC-induced mitochondrial damage and apoptosis, cytosolic Bcl-2/ΔTM exhibited only reduced protection. Thus, membrane localisation of Bcl-2 is a prerequisite for substantial protection against ErPC-induced apoptosis. For efficient long-term inhibition of ErPC-induced apoptosis Bcl-2 had to be present in the membranes of both compartments, the ER and the mitochondria.

The finding that ER-targeted Bcl-2 interferes with ErPC-induced mitochondrial damage points to an involvement of the ER in apoptosis signaling upstream of the mitochondria and to a crosstalk between both compartments.

Keywords: Erucylphosphocholine; Mitochondria; ER; Bcl-2; Apoptosis

1. Introduction

Induction of cell death in tumor cells is a major objective of anticancer treatment with DNA-damaging anticancer drugs and/or ionizing radiation. Unfortunately, many tumors are characterised by acquired or intrinsic treatment resistance. It has become evident that apoptosis contributes to the cytotoxic effects of genotoxic therapies implicating that apoptosis resistance may limit treatment efficacy [1,2]. Thus, aberrant apoptosis pathways of tumor cells constitute an attractive target for the modulation of therapy response [3,4]. In this regard, the exact knowledge on molecular mechanisms of apoptosis signaling and resistance constitutes a prerequisite for the exploitation of apoptosis as a target for the modulation of therapy response.

In general, apoptosis is characterised by defined morphological and biochemical alterations that are mainly triggered by specialized intracellular proteases, the caspases. Caspases are synthesized as inactive precursors and become activated through proteolytic cleavage. Dependent on the stimulus these central executioners of apoptosis can be activated via two main pathways that converge at the level of the effector caspase-cascade [5].

Abbreviations: APC, alkylphosphocholine; Bcl-2/ER, Bcl-2 with targeted expression at the endoplasmic reticulum; Bcl-2/ΔTM, Bcl-2 mutant lacking the transmembrane domain; Bcl-2/MT, Bcl-2 with targeted expression at the mitochondria; Bcl-2/WT, Bcl-2 with wild type subcellular distribution; DISC, death inducing signaling complex; ErPC, erucylphosphocholine; ER, endoplasmic reticulum; FADD, Fas associated protein with death domain; HePC, hexadecylphosphocholine

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The death receptor pathway is initiated at the cellular surface by ligation of death receptors (e.g. CD95, TRAIL-receptors) by their respective ligands (CD95-ligand, TRAIL) [6]. Ligand binding triggers rapid multimerization of the receptor and recruitment of the adapter protein Fas associated protein with death domain (FADD) and an inactive initiator caspase (pro-caspase-8 or pro-caspase-10) generating the so-called death inducing signaling complex (DISC). Upon auto-proteolytic cleavage the activated initiator caspase cleaves downstream effector caspases (e.g. caspase-3, -6 and -7) that finally execute cell death [7].

In contrast, the mitochondrial death pathway becomes activated upon application of cellular stress such as hypoxia, irradiation or chemotherapeutic treatment. This signaling pathway critically involves alterations of mitochondrial function with breakdown of the mitochondrial membrane potential and release of pro-apoptotic proteins including cytochrome *c* from the mitochondrial intermembrane space into the cytosol. Cytosolic cytochrome *c* triggers activation of the pro-caspase-9 within the apoptosome, a cytosolic high molecular death inducing complex composed of the adapter protein Apaf-1, cytochrome *c*, dATP and pro-caspase-9. The active initiator caspase-9 then cleaves downstream effector caspases with subsequent proteolytic degradation of cellular death substrates [8].

These two main apoptotic pathways are interconnected via caspase-8 mediated cleavage of the pro-apoptotic Bcl-2 protein Bid which translocates to the mitochondria triggering alteration of mitochondrial functions [9].

Erucylphosphocholine (ErPC) constitutes the prototypical intravenously applicable derivative of the alkylphosphocholines (APC), a structural class of antineoplastic synthetic phospholipid derivatives. In contrast to standard chemotherapeutic drugs these lipophilic agents target cellular membranes without direct interaction with the DNA [10]. They primarily interfere with membrane permeability and fluidity, membrane lipid composition, and metabolism of phospholipids thereby affecting cell proliferation, cell cycle progression, differentiation, invasion and angiogenesis (for a review, see [11,12]). Importantly, these membrane-targeted drugs are potent inducers of apoptosis in tumor cells. In this regard, ErPC was recently shown to induce apoptosis in highly chemo- and radiation resistant glioblastoma cell lines in vitro [13]. We have shown that ErPC induces apoptosis via a mitochondrial death pathway that is inhibited by over-expression of anti-apoptotic members of the Bcl-2 family, Bcl-2 and Bcl-x_L [14].

It is generally accepted that Bcl-2 family proteins function as crucial apoptosis regulators at the level of the mitochondria. In this regard, pro-apoptotic Bcl-2 family members (e.g. Bax, Bak, Puma, Noxa) trigger mitochondrial damage, which is counteracted by anti-apoptotic Bcl-2 proteins (e.g. Bcl-2, Bcl-x_L, Bcl-w). Thus, in a simple model the ratio of pro- and anti-apoptotic family members may define the cellular fate upon apoptosis initiation [15]. However, the multitude of described interactions of pro- and anti-apoptotic Bcl-2 proteins and other apoptosis regulatory molecules within the complex cellular signaling network complicates the precise definition of their function.

There is increasing evidence that apart from the molecular structure the subcellular localisation of Bcl-2 proteins in intracellular membranes may define their apoptosis modulating activity. Bcl-2 resides in the outer membrane of the mitochondria, the endoplasmic reticulum (ER) and the nucleus [16,17]. Various reports point to a role of mitochondrial Bcl-2 in the protection of the cells from stress-induced mitochondrial damage. Furthermore, recent data reveal that organelle-specific expression of Bcl-2 at the ER may similarly confer protection against stress-induced apoptosis [18–20].

To gain further insight into upstream signaling events of apoptosis-induction by the membrane active drug ErPC and the role of Bcl-2 localisation in this process the impact of Bcl-2 specifically targeted to the mitochondria and the ER, respectively, on its protective effect against ErPC-induced apoptosis was studied in a Jurkat T Lymphoma cell model.

2. Material and methods

2.1. Chemicals and drugs

Erucylphosphocholine (ErPC) was synthesized by H. Eibl, Max-Planck-Institute of Biophysical Chemistry, Göttingen, Germany. ErPC was dissolved in 200 μl ethanol, and diluted with RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum to a concentration of 10 mM (stock solution). The final ethanol concentrations in the tissue culture experiments were below 0.05% (v/v).

TMRE was from Molecular Probes (Mobitec, Goettingen, Germany). The proton shuttle carbonylcyanide-*m*-chloro-phenylhydrazone (CCCP) was from Sigma. Hoechst 33342 (Calbiochem, Bad Soden, Germany) was dissolved in distilled water as a 1.5 mM stock solution. Monoclonal mouse antibody against Bcl-2 was from Santa Cruz Biotechnology (Heidelberg, Germany). Rabbit anti-full length and rabbit anti-cleaved caspase-3 as well as rabbit anti-PARP and rabbit anti-cleaved PARP were from Cell Signaling (New England Biolabs, Schwalbach/T., Germany). Anti-cytochrome *c* mouse monoclonal antibody was obtained from Pharmingen, Becton Dickinson (Heidelberg, Germany).

Caspase-8 was detected using a mouse monoclonal antibody (BioCheck, Münster, Germany). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology.

All other chemicals were purchased from Sigma–Aldrich (Deisenhofen, Germany) if not otherwise specified.

2.2. Cell culture, transfections and cellular treatment

Jurkat E6 T lymphoma cells were from ATCC (Bethesda, Maryland, USA). For all experiments cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (Gibco Life Technologies, Eggenstein, Germany) and maintained in a humidified incubator at 37 °C and 5% CO_2. Cells were treated with 12.5, 25 and 50 μM ErPC for 1–48 h.

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 [20]. 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.

Only bulk transfectants and Jurkat cell clones expressing comparable levels of the different versions of Bcl-2 in addition to the low levels of endogenous Bcl-2 were used. The subcellular localisation of Bcl-2 was verified by confocal microcopy as described and shown elsewhere [18]. Bcl-2 staining was performed with a specific rabbit antibody (Santa Cruz Biotech). Localisation of Bcl-2 in the ER or mitochondria was confirmed by costaining with a murine antibody against the SERCA calcium ATPase (UBI, Biomol, Germany) and a mouse anti-cytochrome *c* antibody (Pharmingen, Becton Dickinson). As secondary antibodies Alexa Fluor™-conjugated anti-mouse (Molecular Probes) and Cy™5-conjugated anti-rabbit antibodies (Dianova, Hamburg, Germany) were employed.

2.3. Determination of apoptosis

Cell death was determined by FACS using light scatter characteristics employing a FACS Calibur flow cytometer (Becton Dickinson).

For quantification of nuclear fragmentation, cells were submitted to FACS-analysis upon staining with propidium iodide (PI) in a hypotonic buffer [21]. In brief, cells were washed, incubated for 60 min at room temperature in the dark in 0.1% (w/v) sodium citrate plus 50 μg/ml PI and 0.1% (v/v) Triton X-100 and subsequently subjected to FACS analysis.

When indicated, apoptosis was analysed after staining of the cells with Hoechst 33342 and subsequent fluorescence microscopy. In brief, cells were incubated with Hoechst 33342 at a final concentration of 1.5 μM for 15 min. Cell morphology was then determined by fluorescence microscopy (Zeiss Axiovert 200, Carl Zeiss, Jena, Germany) using a G365/FT395/LP420 filterset. Cells were analysed with 40× magnification and documented using a CCD camera device (Zeiss Axiocam MR).

2.4. Determination of mitochondrial transmembrane potential and cytochrome c-release

The mitochondrial transmembrane potential ($\Delta\psi_m$) was analysed by FACS using the $\Delta\psi_m$-specific stain tetramethylrhodamine-ethylester-perchlorate (TMRE) (Molecular Probes). To this end, cells were loaded for 30 min at 37 °C with 25 nM TMRE and subsequently analysed by flow cytometry. Preincubation with 1 μM of the proton ionophore carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) was used as a positive control for complete depolarization of the mitochondrial membrane potential.

The release of cytochrome *c* from the mitochondria was determined by fluorescence microcopy using a Zeiss Axiovert 200 microscope. In brief, cells were immobilized on cover slips with 0.1% (w/v) poly-L-lysine, fixed with 2.5% (v/v) formaldehyde in PBS and permeabilized with 0.1% (v/v) Triton X-100 in PBS. After blocking with 10% (v/v) fetal calf serum cells were stained with the anti-cytochrome *c* primary antibody (Pharmingen, Becton Dickinson) for 1 h at room temperature. Cells were washed several times and incubated with the secondary antibody (Alexa Fluor™ 488-conjugated anti-mouse antibody, Molecular Probes) for 45 min. Finally, the cover slips were mounted with Mowiol (Sigma, Deisenhofen, Germany). Cells were analysed with 100× magnification. Green fluorescence was documented using filter set 09 (BP450–490/FT510/LP515 Zeiss, Germany) and an CCD camera device (Photometrix SensysCam). Unspecific fluorescence and blurring was removed by nearest neighbour deconvolution (MetaMorph, Visitron Systems GmbH, Germany) using the following settings: Filtersize: 20, *xy* spacing: 2.0, *z* spacing: 0.5, deconvolution wavelength: 540 nm, noise suppression on. Raw-tiffs were background reduced before processing.

Alternatively, translocation of cytochrome *c* was quantified by FACS-analysis (Becton Dickinson) following a modified protocol of Waterhouse and Trapani [22]. In brief, 10^6 cells were fixed for 15 min in 4% (w/v) paraformaldehyde, washed in PBS and permeabilized for 15 min in ice cold PBS supplemented with 0.1% (v/v) Triton X-100. Cells were washed again and blocked for 10 min in PBS with 5% (v/v) FCS (blocking buffer). Upon staining with a mouse monoclonal anti-cytochrome *c* antibody (1:200 in 5%, v/v, FCS) cells were washed two times (blocking buffer) and incubated with a conjugated anti-mouse Alexa Fluor™488 antibody (1:400 in blocking buffer). Upon microscopic evaluation of fluorescence localisation cells were subsequently analysed by flow cytometry.

2.5. Determination of caspase-activation

Caspase-activation was determined by Western blot analysis of cytosolic extracts upon ErPC treatment. To this end, cells (1×10^7 ml^{-1}) were lysed for 10 min at 99 °C in CST lysis buffer (62.5 mM Tris–HCl (pH 6.8); 2%, w/v, SDS; 10%, v/v glycerol; 50 mM DTT; 0.01%, w/v, bromphenolblue). 20 μg lysate were separated by SDS-PAGE and blotted onto PVDF-membranes (Amersham-Pharmacia Biotech, Freiburg, Germany). Blots were blocked for 1 h in PBS buffer containing 0.05% (v/v) Tween 20 and 5% (w/v) non-fat dried milk. The membrane was incubated over night at 4 °C with the respective primary antibody. After repeated washings with PBS/Tween-20 (0.05%, v/v) the membrane was incubated for 1 h at room temperature with the secondary antibody (anti-IgG-HRP 1:4.000, Amersham Biosciences, Buckinghamshire, UK) in PBS/Tween and again washed several times with PBS/Tween. The detection of antibody binding was performed by enhanced chemiluminescence staining (ECL Western blotting analysis system, Amersham-Pharmacia Biotech, Freiburg, Germany). Equal protein loading was confirmed by Coomassie stain.

In addition, activation of caspases was quantified by flow cytometry using the CaspAce® detection system (Promega, Mannhein, Germany), which allows in vivo staining of active caspases. The CaspAce® staining was performed according to the manufacturer's guidelines.

3. Results

3.1. ErPC induces apoptosis in Jurkat E6 T lymphoma cells

We have recently shown that ErPC potently induces apoptosis in T- and B-lymphoma cell lines independently from death receptor signaling via a mitochondrial death pathway that was inhibited by over-expression of Bcl-2. Apoptosis induction involved early depolarisation of the mitochondrial membrane potential, release of cytochrome *c*, activation of caspases-9, -3 and -8 as well as nuclear fragmentation [14].

To approve our earlier findings in the T lymphoma cell model used in the present study, kinetics and extent of ErPC-induced apoptosis in Jurkat E6 cells were verified in a first set of experiments. As depicted in Fig. 1A–D ErPC-induced substantial depolarisation of the mitochondrial membrane potential, caspase-activation and nuclear fragmentation in a time- and concentration-dependent manner.

Furthermore, ErPC-induced apoptotic nuclear morphology in Jukat vector control cells as well as in cells lacking essential components of the death receptor pathway such as FADD or caspase-8. In contrast, defects in the mitochondrial pathway due to expression of a dominant negative caspase-9 mutant or expression of Bcl-2 efficiently protected against ErPC-induced apoptosis (Fig. 1E). Similarly, over-expression of Bcl-x_L also protected against ErPC-induced mitochondrial damage, caspase-activation and apoptosis (data not shown). Altogether these data highlight the importance of the mitochondrial death pathway for ErPC-induced apoptosis.

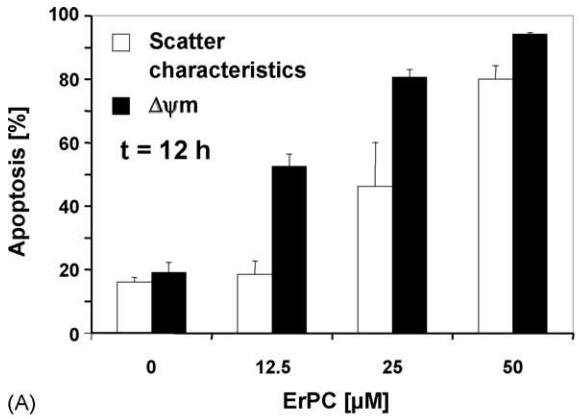
3.2. Targeted expression of Bcl-2 at the mitochondria and the ER interferes with ErPC-induced apoptosis

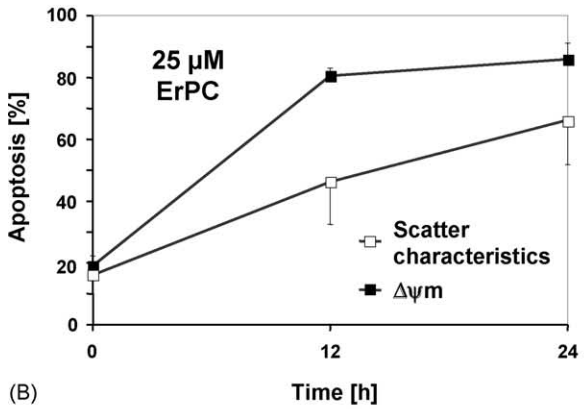
Thus, ErPC induces apoptosis via a mitochondrial death pathway that is strongly inhibited by over-expression of anti-apoptotic members of the Bcl-2 family, namely Bcl-2 and Bcl-x_L. Since recent investigations revealed that subcellular localisation of Bcl-2 may interfere with its apoptosis modulating activity [18–20] the aim of the present study was to analyse the impact of targeted Bcl-2 expression at specific subcellular membranes on ErPC-induced cell death in the Jurkat E6 cell lymphoma model.

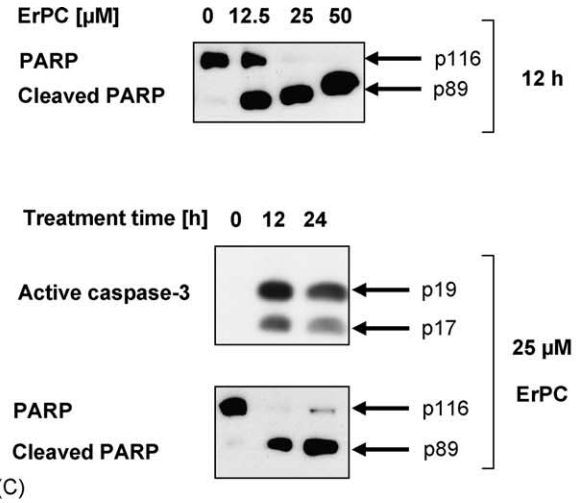
To this end, Bcl-2 constructs with defined membrane targeting anchors were used to direct Bcl-2 expression specifically to the mitochondria (Bcl-2/MT) or the ER (Bcl-2/ER), respectively. In addition, Jurkat cells over-expressing either wild-type Bcl-2 that localises at the mitochondria, the ER and the perinuclear region (Bcl-2/WT) or a transmembrane domain-lacking mutant (Bcl-2/ΔTM) that resides in the cytosol and the nucleus were used (Fig. 2A). Over-expression of the respective Bcl-2 constructs was verified by Western blotting (Fig. 2B). Multiple Bcl-2 bands that appeared in addition to the dominant Bcl-2 mutants result from detection of basal endogenous Bcl-2 (see Jurkat vector cells for basal expression) and phosporylated Bcl-2/Bcl-2 mutant forms (respective bands with increased molecular weight). The specific subcellular distribution of the four Bcl-2 mutants was verified by confocal microscopy using co-staining with specific marker proteins of the mitochondria and the ER, respectively, as already demonstrated in a recent publication [18].

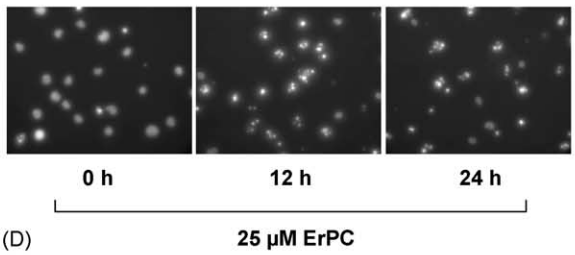
In a first set of experiments with bulk transfected cells ErPC-induced time-dependent apoptotic cell death in vector control cells as determined by FACS-analysis using light scatter characteristics (Fig. 2C). All Bcl-2 mutants with defined localisation in subcellular membranes (Bcl-2/WT, Bcl-2/MT, Bcl-2/ER) conferred efficient protection against ErPC-induced apoptosis. Surprisingly, at early time points (12 h) restricted expression of Bcl-2 at the ER inhibited ErPC-induced apoptosis with similar efficiency as Bcl-2/WT and Bcl-2/MT. In contrast, the transmembrane domain lacking Bcl-2 mutant Bcl-2/ΔTM failed to inhibit ErPC-induced cell death.

To further corroborate our data on the influence of Bcl-2 targeted to different cellular organelles and to exclude artefacts of data acquired with bulk transfected cells single cell clones were selected from bulk transfected cells by limited dilution. Expression levels of Bcl-2 mutants were analysed by Western blotting and clones with comparable









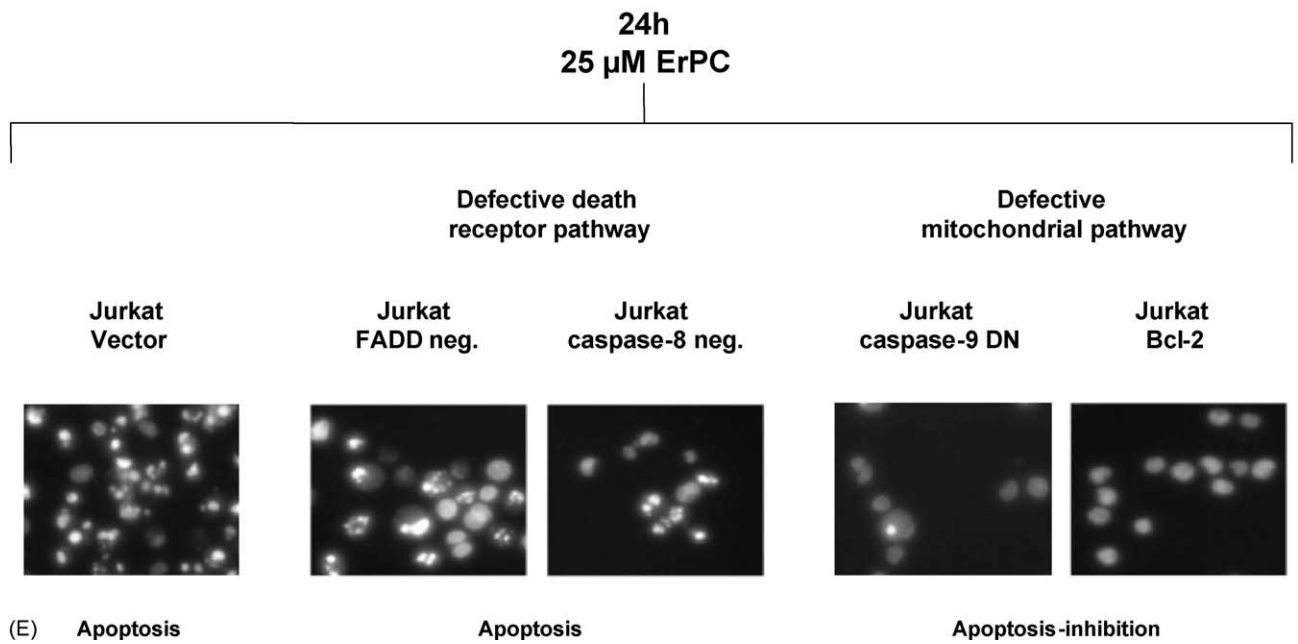


Fig. 1. ErPC induces apoptosis in Jurkat E6 cells. Jurkat E6 cells were treated for 12 h with 0, 12.5, 25 or 50 μM ErPC or alternatively for 0, 12 and 24 h with 25 μM ErPC. ErPC-induced apoptosis in Jurkat E6 cells was then quantified by flow cytometry (A and B) using light scatter characteristics (white bars, open squares). In addition, breakdown of the mitochondrial outer membrane potential ($\Delta\psi_m$) was determined by flow cytometry upon staining with the potential sensitive dye TMRE (black bars, filled squares). Dose-dependent (A) and time-dependent (B) ErPC-effects are shown. The error bars indicate the standard deviations from independent experiments ($n = 3$). Caspase-activation upon ErPC-treatment was verified by Western blotting (C). Dose-dependent (C, upper panel) and time-dependent (C, lower panel) effects of ErPC are depicted from a representative experiment. Nuclear fragmentation upon treatment with 25 μM ErPC for 0, 12 or 24 h was visualized by fluorescence microscopy upon staining with Hoechst 33342 (D). Data from a representative experiment are shown. ErPC induces a mitochondrial death pathway in Jurkat T lymphoma cells (E). Chromatin condensation and nuclear fragmentation typical for apoptosis induction were visualized by fluorescence microscopy of Hoechst 33342-stained cells 24 h after treatment with 25 μM ErPC in Jukat vector control cells, in FADD- or caspase-8-negative Jurkat A3 cells as well as in Jurkat cells expressing a dominant negative caspase-9 mutant or Bcl-2, respectively.

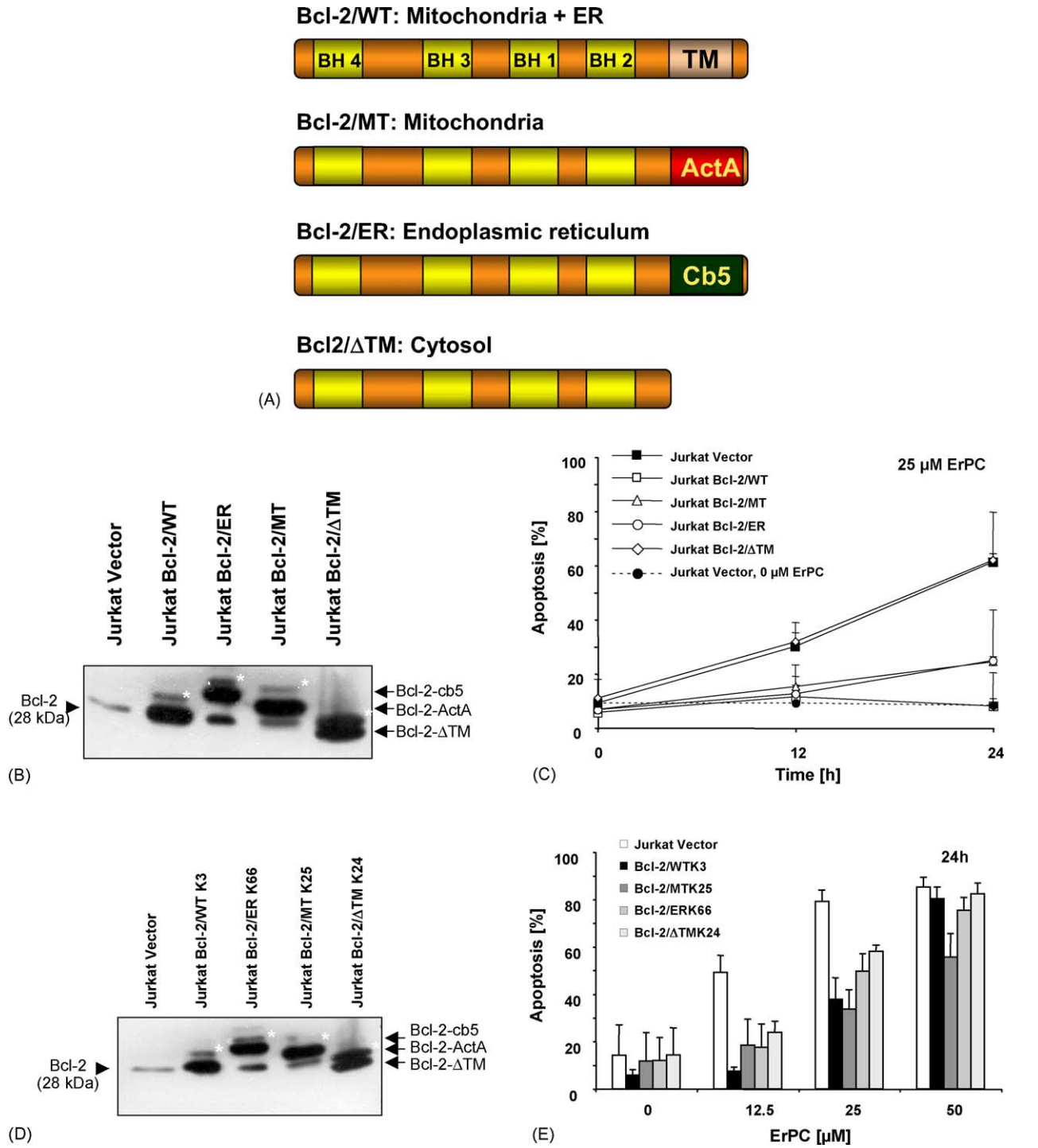


Fig. 2. Expression of Bcl-2 with defined localisation in subcellular compartments inhibits ErPC-induced apoptosis. Jurkat E6 were stably transfected with wild-type Bcl-2 (Bcl-2/WT), Bcl-2 targeted to the mitochondrial membrane (Bcl-2/MT), Bcl-2 targeted to the ER (Bcl-2/ER) or with a transmembrane domain lacking Bcl-2 mutant (Bcl-2/ΔTM). Model structures of Bcl-2 constructs for targeted expression of Bcl-2 used in the present study are shown (A). Expression levels of Bcl-2 and targeted mutants in bulk transfectants were analysed by Western blotting using a monoclonal anti-Bcl-2 antibody (B; asterisk indicates phosphorylated protein). Data reveal an almost similar expression level of Bcl-2 mutants in bulk transfected cells. Apoptosis induction in bulk transfected cells upon treatment for 0, 12 or 24 h with 25 μM ErPC was quantified by flow cytometry using light scatter characteristics (C). Data show means ± standard deviations (S.D.) from independent experiments ($n = 3$). Expression levels of Bcl-2 and targeted mutants in selected cell clones were analysed by Western blotting revealing almost similar expression levels of Bcl-2 in selected clones (D). Apoptosis induction upon treatment of selected clones with 0, 12.5, 25 or 50 μM ErPC for 24 h was quantified by flow cytometry using light scatter characteristics (E). Data show means from at least five independent experiments. Error bars indicate the standard deviations.

expression levels were used for further analysis (Fig. 2D). Similar to the data obtained with bulk transfected cells, ErPC-induced apoptosis in Jurkat vector cells in a dose-dependent manner as determined by FACS-analysis using light scatter characteristics (Fig. 2E). Bcl-2/WT, Bcl-2/MT and Bcl-2/ER efficiently inhibited ErPC-induced apoptosis at low (12.5 μM) and intermediate concentrations (25 μM) while Bcl-2/ΔTM was less effective. However, at 50 μM ErPC, even full length Bcl-2/WT failed to prevent cell death to a substantial amount suggesting that the protective effects of the Bcl-2 constructs may depend on the respective ErPC concentration.

3.3. Targeted expression of Bcl-2 influences Bcl-2-mediated protection against ErPC-induced mitochondrial damage

Up to now our data indicated that targeted expression of Bcl-2 at defined subcellular membranes strongly interferes with ErPC-induced apoptosis. Since signaling pathways of apoptosis execution upon ErPC-treatment have already been defined previously, we next aimed to clarify the impact of restricted expression of Bcl-2 on different mediators and phases of apoptosis execution.

In this regard, ErPC-induced apoptosis critically involves mitochondrial alterations. To elucidate the role of subcellular Bcl-2 localisation on its protective effects against ErPC-induced mitochondrial damage, breakdown of mitochondrial membrane potential ($\Delta\psi_m$) and cytochrome *c*-release in response to ErPC-treatment were tested in Jurkat vector cells as well as in Bcl-2/WT, Bcl-2/MT, Bcl-2/ER and Bcl-2/ΔTM over-expressing Jurkat cell clones (Fig. 3). In vector control cells, treatment with ErPC even at low concentrations (12.5 μM) induced breakdown of $\Delta\psi_m$ in a substantial part of the population within 12 h as determined by flow cytometric analysis of cells loaded with the fluorescent potential sensitive dye tetramethylrhodamine-ethylester-perchlorate (TMRE) (Fig. 3A and B). Upon treatment with 12.5 μM ErPC all membrane-targeted Bcl-2 mutants completely inhibited ErPC-induced mitochondrial damage regardless of the respective subcellular localisation of Bcl-2. Interestingly, at increased ErPC concentrations (25 μM) the protective effect of Bcl-2/ER became less prominent compared to Bcl2/WT or Bcl-2/MT (Fig. 3A and B). Similar to the results above, at 50 μM ErPC even Bcl-2/WT and Bcl-2/MT were almost not protective. In contrast to the membrane-targeted Bcl-2 mutants, the truncated Bcl2/ΔTM mutant showed always decreased inhibitory effects when compared to the membrane-targeted Bcl-2 mutants including Bcl-2/ER.

A second early event of ErPC-induced mitochondrial apoptosis signaling that is counteracted by Bcl-2 is the release of pro-apoptotic factors such as cytochrome *c* from the mitochondrial intermembrane space. We therefore analysed whether targeted Bcl-2 mutants also interfere with ErPC-triggered release of cytochrome *c* using fluorescence-mediated detection of the loss of cytochrome *c* staining from the mitochondria (Fig. 3C). In untreated cells high intensity focal cytochrome *c* staining pattern was representative for the predominant localisation of cytochrome *c* in intact mitochondria as detected by fluorescence microscopy (Fig. 3C, left panel). As expected, treatment of Jurkat vector cells for 12 h with 12.5 μM or even more pronounced with 25 μM ErPC triggered release of cytochrome *c* from the mitochondria into the cytosol resulting in the loss of mitochondria-associated intense focal cytochrome *c* staining and appearance of cells with homogeneous low intensity cytosolic fluorescence staining (Fig. 3C, left panel). Similar to our results obtained for the $\Delta\psi_m$ breakdown, restricted expression of Bcl-2 at the ER inhibited ErPC-induced cytochrome *c*-release into the cytosol with almost similar potency as Bcl-2/WT and Bcl-2/MT while Bcl-2/ΔTM was almost not protective (Fig. 3C, left panel). These observations were corroborated by flow cytometric data revealing a strongly increased amount of Jurkat vector cells with low fluorescence intensity upon ErPC-treatment that may be explained by fluorescence dilution and leakage from the cell upon its release into the cytosol (Fig. 3C, right panel; M1 depicts cells with low fluorescence intensity; values for M1 of the experiment shown are given in the table). Again, Bcl-2/WT, Bcl-2/MT and Bcl-2/ER efficiently inhibited cytochrome *c*-release upon ErPC-treatment while Bcl-2/ΔTM was less protective (Fig. 3C, right panel).

3.4. Targeted expression of Bcl-2 influences the inhibitory effects of Bcl-2 on ErPC-induced caspase-activation

Activation of caspases-9, -3 and -8 downstream of the mitochondria constitutes an integral part of ErPC-induced apoptosis signaling [14,23]. Therefore, in a next set of experiments we examined the impact of restricted Bcl-2 over-expression in different subcellular compartments on ErPC-induced caspase-activation (Fig. 4). In vector control cells ErPC induced a prominent and dose-dependent processing of caspase-3 as demonstrated by appearance of the p19 kDa cleavage fragment and decrease of pro-caspase-3 in Western blot analysis (Fig. 4A, upper panel). Similarly, a concentration-dependent cleavage of the caspase-3 substrate PARP into p89 kDa cleaved PARP and of the two pro-caspase-8 isoforms of 54 and 52 kDa into the corresponding 41/43 kDa cleavage fragments was observed in vector control cells upon ErPC-treatment (Fig. 4A, middle and lower panel, respectively). However, caspase-8 processing occured only after substantial caspase-3 activation supporting earlier results on caspase-8 processing downstream of the mitochondria. Consistent with these data FACS-analysis using an activation-specific caspase detection system (CaspACE®) as fluorochrome revealed a concentration-dependent increase in the amount of vector

control cells with increased fluorescence intensity indicative for the activation of caspases upon treatment with ErPC (Fig. 4B). In addition, Western blotting experiments also demonstrated time-dependent caspase-activation in ErPC-treated vector control cells (Fig. 4C). As expected, caspase-processing and PARP-cleavage were strongly inhibited by over-expression of Bcl-2/WT and Bcl-2/MT. Interestingly, restricted expression of Bcl-2 in the outer membrane of ER reduced ErPC-induced activation of caspase-3 and -8 and cleavage of PARP with almost similar potency as Bcl-2/WT and Bcl-2/MT, while Bcl-2/ΔTM was less protective especially in terms of PARP-cleavage (Fig. 4A and C).

The data on inhibitory effects of membrane-targeted Bcl-2 mutants on ErPC-mediated caspase-activation were further corroborated by FACS-analysis using the activation-specific CaspACE® detection system. While ErPC triggered a concentration-dependent increase in the amount of cells with active caspases in Jurkat vector and Jurkat Bcl-2/ΔTM cells, at least at low ErPC-concentrations (12.5 μM) ErPC-induced caspase-activation was reduced by targeted expression of Bcl-2 in subcellular membranes (Bcl-2/WT, Bcl-2/MT and Bcl-2/ER) (Fig. 4B). However, at increased ErPC-concentrations (25 and 50 μM) the protective effect of Bcl-2/ER was less pronounced compared to Bcl-2/WT and Bcl-2/MT (Fig. 4B).

3.5. Targeted expression of Bcl-2 influences the inhibitory effects of Bcl-2 on ErPC-induced nuclear fragmentation

Our earlier data revealed that ErPC-induced apoptosis culminates in chromatin condensation and nuclear

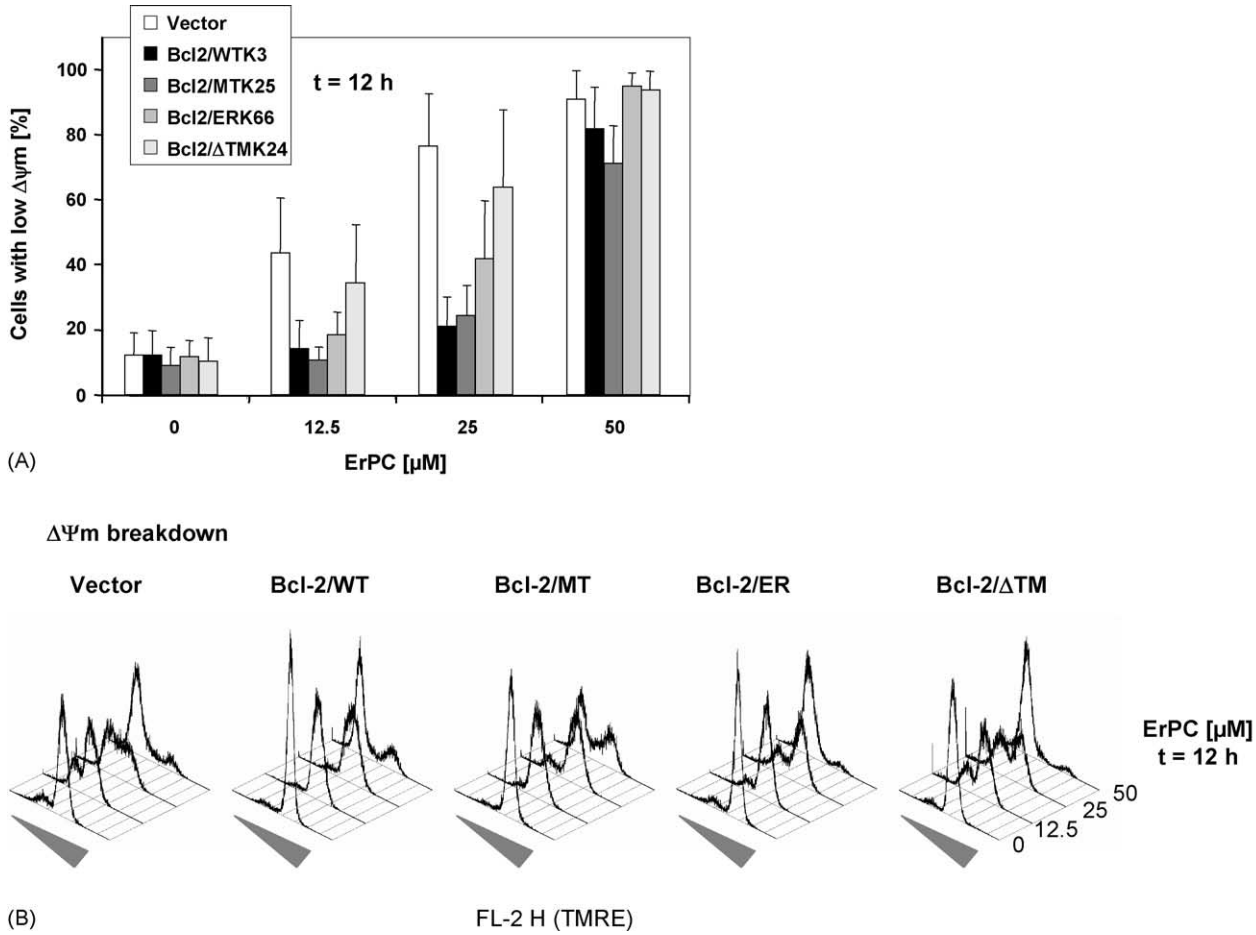


Fig. 3. Targeted expression of Bcl-2 at membranes of the mitochondria or the ER inhibits ErPC-induced mitochondrial damage. Selected cell clones expressing targeted Bcl-2 mutants were treated for 12 h with 12.5, 25 or 50 μM ErPC. Breakdown of $\Delta\psi_m$ was quantified by flow cytometry of TMRE-stained cells (A and B). Results are presented as means ± S.D. (n = 14) (A) or original histograms from one representative set of similar experiments (B). Cytochrome *c*-release from mitochondrial intermembrane space into the cytosol was determined by immunofluorescence microscopy (C, left panel) and flow cytometry (C, right panel) using cytochrome *c* specific primary antibodies and Alexa Fluor™488 labeled secondary antibodies, respectively. Blurring and unspecific fluorescence were removed by nearest neighbour deconvolution. Focal high intensity cytochrome *c* staining pattern (fluorescence microscopy) and high fluorescence intensity (FACS), respectively, were representative for the predominant localisation of cytochrome *c* in intact mitochondria as seen in untreated cells. In contrast, cytochrome *c*-release from the mitochondria into the cytosol, e.g. upon ErPC-treatment of Jurkat vector and Bcl2/ΔTM cells resulted in homogeneous low intensity staining of the cellular cytosol. Leakage of cytosolic cytochrome *c* from the cells may be causative for decreased cytochrome *c* fluorescence levels obtained by FACS-analysis (C). A phase contrast image (insert) of the same section is given for vector control cells treated with 25 μM ErPC showing the presence of intact cells in the area with low homogenous cytosolic cytochrome *c* staining. Data from one representative experiment are presented.

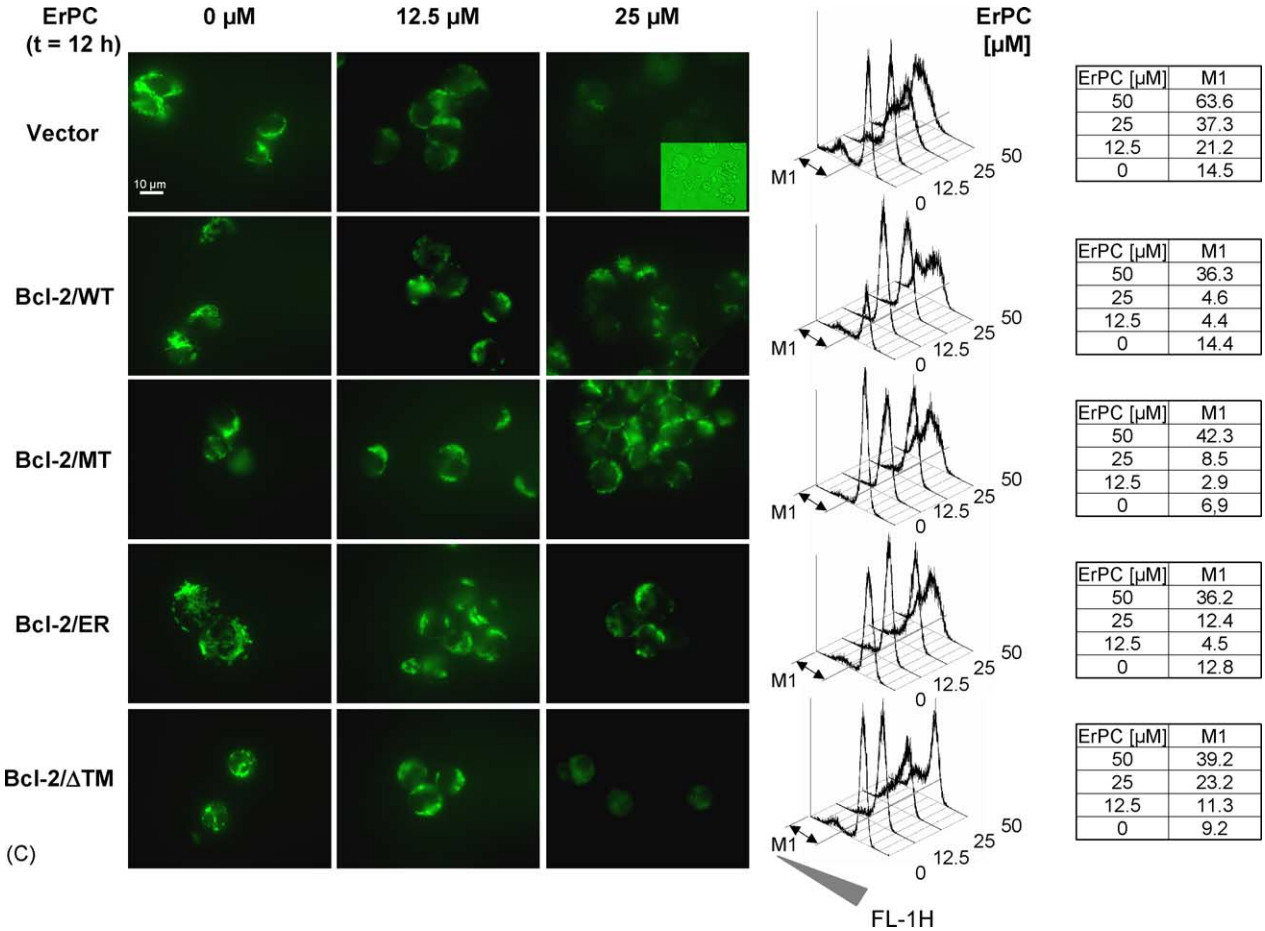


Fig. 3. (*Continued*).

fragmentation. In the last set of experiments, we therefore analysed the impact of subcellular localisation of Bcl-2 on this final step of apoptosis execution.

Analysis of nuclear morphology by fluorescence microscopy revealed substantial apoptosis induction in vector control cells showing chromatin condensation and nuclear fragmentation as typical features of apoptosis even at low ErPC-concentrations (12.5 μM). A comparable pattern of ErPC-induced nuclear morphology was observed in cells over-expressing Bcl-2/ΔTM. In contrast, Jurkat E6 cell clones over-expressing membrane-targeted Bcl-2 irrespective of its subcellular localisation (Bcl-2/WT, Bcl-2/MT and Bcl-2/ER) revealed strongly decreased ErPC-induced apoptotic morphology (Fig. 5A).

Quantification of nuclear fragmentation by FACS-analysis of PI-stained nuclei gave similar results (Fig. 5B). Interestingly, at low ErPC-concentrations Bcl-2/WT, Bcl-2/MT and Bcl-2/ER conferred almost similar protection while at increased ErPC-concentrations (25 μM) Bcl-2/ER had decreased inhibitory effects compared to Bcl-2/WT and Bcl-2/MT (Fig. 5B). These data support the above mentioned finding that the protective effect of distinct Bcl-2 mutants may depend on the respective ErPC-concentration.

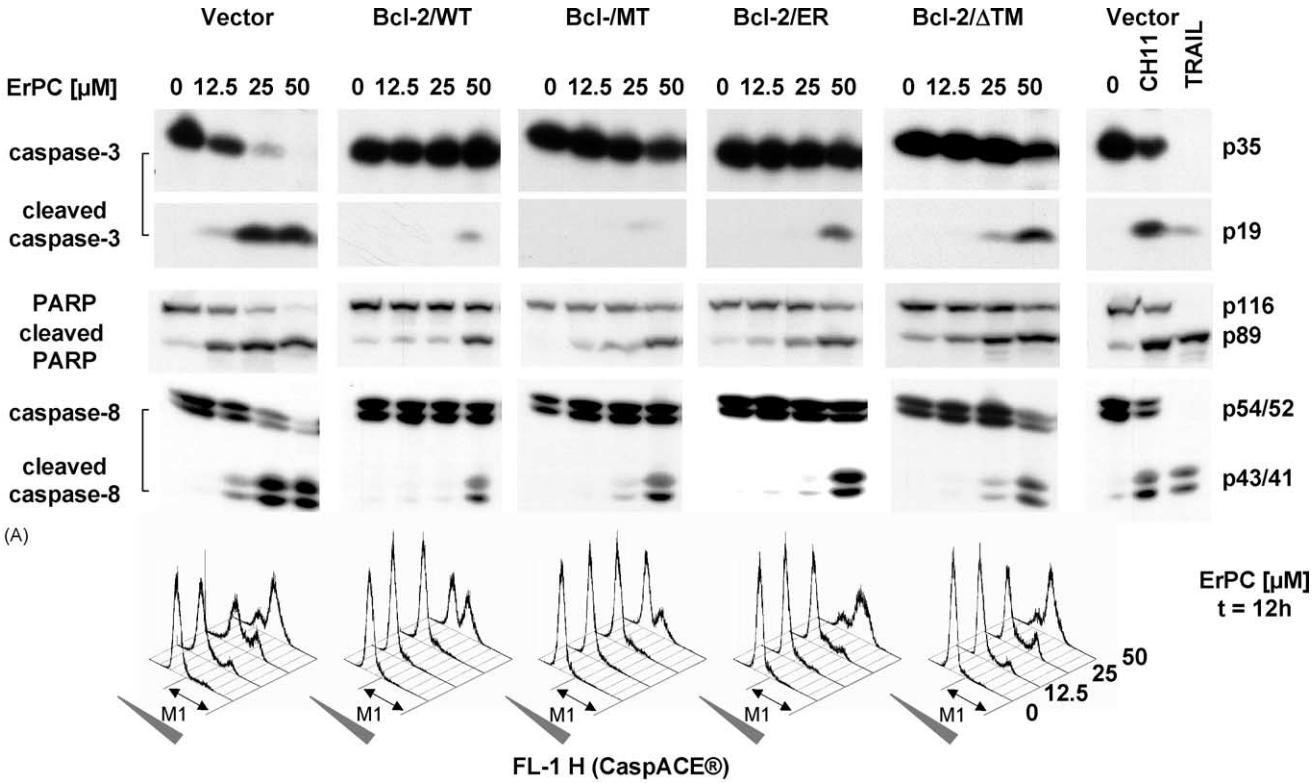
4. Discussion

We have shown earlier that the membrane-targeted drug ErPC induces a mitochondrial death pathway that is inhibited by over-expression of Bcl-2 [14]. In the present study, we analysed the importance of subcellular localisation of Bcl-2 for its protective effects against ErPC-induced apoptosis. Our data reveal that expression of Bcl-2-mutants with a defined membrane anchor inhibits all steps of apoptosis execution in response to ErPC, including breakdown of mitochondrial membrane potential, cytochrome *c*-release, caspase-activation, chromatin condensation and nuclear fragmentation regardless of the subcellular localisation of the Bcl-2 mutants in the mitochondria, the ER or in both compartments. In contrast, the transmembrane domain lacking Bcl-2/ΔTM conferred only limited protection against ErPC-induced mitochondrial damage, caspase-activation and apoptosis. As recently shown this transmembrane domain lacking Bcl-2 mutant resides mainly in the cytosol and the nucleus but fails to integrate into subcellular membranes [18].

Our data implicate that localisation of Bcl-2 to cytoplasmic membranes facing the cytosol such as the membranes of the mitochondria or the ER constitutes a

prerequisite for efficient inhibitory action on ErPC-induced cell death. The observation that Bcl-2 targeted to the mitochondria confers potent protection against ErPC-induced apoptosis together with the finding that protection is not restricted to Bcl-2 but that Bcl-x_L exerts similar effects is consistent with earlier findings on inhibitory effects of Bcl-2 and Bcl-x_L and further underlines the importance of mitochondria in APC-induced apoptosis signaling [14,24]. In contrast to Bcl-2 Bcl-x_L contains a specific mitochondrial targeting sequence suggesting a specific function at the mitochondria [25]. Up to now mitochondria are believed to be a crucial target for mediators of stress-induced apoptosis pathways and the role of mitochondrial Bcl-2 in the protection against cellular stress is widely accepted. Mitochondrial Bcl-2 is suggested to sequester pro-apoptotic BH3-only family members thereby preventing Bax or Bak mediated mitochondrial damage [26]. In addition, the anti-apoptotic effect of Bcl-2 has been attributed to its ability to maintain threshold values in the mitochondrial Ca(2+) concentration [27].

Here we show that restricted expression of Bcl-2 to the membrane of the ER inhibited ErPC-induced caspase-activation and nuclear fragmentation with almost similar potency as Bcl-2/WT and Bcl-2/MT pointing to a relevant effect of the ER in apoptosis signaling upon ErPC-treatment. Importantly, Bcl-2/ER could substitute for Bcl-2/MT even regarding inhibition of ErPC-induced mitochondrial alterations. The finding that localisation of Bcl-2 in the mitochondria is not required for efficient protection from ErPC-induced mitochondrial damage at least at low ErPC-concentrations suggests a molecular interaction between the ER and the mitochondria in ErPC-induced apoptosis signaling upstream of $\Delta\psi_m$. The observation of less efficient protective effects of Bcl-2/ER compared to Bcl-2/WT and Bcl-2/MT at increased drug-concentrations and later time-points further supports the assumption that the ER constitutes an upstream regulator of mitochondrial function during ErPC-induced apoptosis and emphasizes at the same time the fundamental role of the mitochondria in this process downstream of the ER.



(B) Specific caspase activation (M = M_1-M_0)

Vector [%]	Bcl-2/WT [%]	Bcl-2/MT [%]	Bcl-2/ER [%]	Bcl-2/ΔTM [%]	ErPC [μM]
7	0	0	3.8	10.8	12.5
22	0	1.1	12.4	25.3	25
71.2	37.5	21	72.3	72.3	50

Fig. 4. Membrane-targeted Bcl-2 mutants inhibit ErPC-induced caspase-activation. ErPC-triggered activation of caspases in Jurkat vector control cells as well as selected cell clones with defined subcellular Bcl-2 localisation was determined by Western blotting (A and C) as well as by FACS-analysis employing the fluorescent CaspaseACE® (FITC-VAD-fmk) substrate (B). Dose- and time-dependent appearance of the respective caspase-cleavage products (Western blotting) or increased fluorescence due to CaspACE®-binding (FACS) upon treatment of the indicated Jurkat cell clones for 12 h with 0, 12.5, 25 and 50 μM ErPC (A and B) or for 0, 1, 2, 4, 6 and 12 h with 25 and 50 μM ErPC, respectively, were analysed (C). Data from representative experiments are shown. As a control, caspase-activation via the death-receptor pathway was triggered in Jurkat E6 vector control cells by application of TRAIL (1 ng/ml) or the activating CD95-antibody CH11 (100 ng/ml) and revealed significant caspase-cleavage (A). Specific caspase-activation [$M = M_1 - M_0$ (0 μM)] as quantified from flow cytometric data (B lower panel).

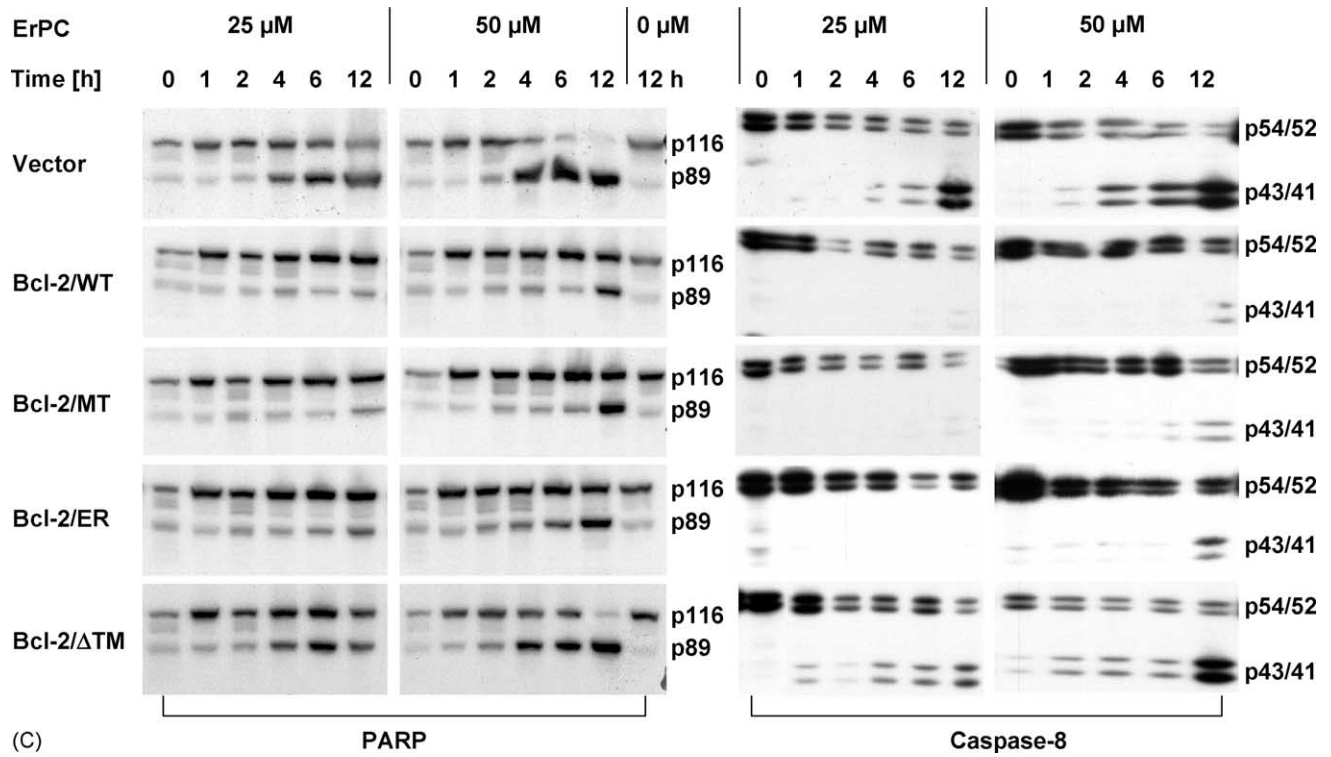


Fig. 4. (*Continued*).

Our results are consistent with previous data revealing equivalent protective effects of Bcl-2/ER on mitochondrial damage and apoptosis induction by ionizing radiation [18]. Furthermore, similar Bcl-2/ER constructs inhibited myc-induced apoptosis in Rat-1 fibroblasts and protected Rat-1/myc cells against apoptosis induction by growth factor withdrawal [27,28]. In addition, Bcl-2/ER protected cells from mitochondrial damage and apoptosis induced by ceramide, the protein kinase inhibitor staurosporine as well as by two drugs that induce ER-stress via the production of aberrant proteins in the secretory system, brefeldin A and tunicamycin [19,29]. Bcl-2/ER also interfered with cytochrome *c*-release from the mitochondria and apoptosis induction triggered by thapsigargine, a specific inhibitor of the ER calcium ATPase SERCA [30,31]. Together with these earlier findings our data point to a more general role of Bcl-2 expressed at the ER in the regulation of mitochondrial death pathways. However, the function of the ER in apoptosis signaling and the role of Bcl-2 at the ER is not completely understood.

It has been shown that the ER constitutes an important checkpoint for Ca(2+) dependent death stimuli. In this regard, persistant ER stress, e.g. through disturbance of Ca(2+) homeostasis or aberrant protein folding can result in apoptosis initiation at the ER [32,33]. In addition to this direct pro-apoptotic function, several findings argue for a role of the ER in the regulation of mitochondrial death pathways involving a Ca(2+)-mediated crosstalk between ER and mitochondria. These two organelles both function as Ca(2+) stores and contribute to the regulation of cellular Ca(2+) homeostasis [34]. It has been suggested that a Bcl-2 controlled efflux of Ca(2+) from the ER which is mostly associated with subsequent Ca(2+) uptake into the mitochondria may regulate cytochrome *c*-release from mitochondria at least in some apoptosis models. In this scenario, Bcl-2 at the ER may either increase leakyness of the ER membrane [35–38] or decrease stimulus-induced Ca(2+) release [39], thereby lowering the amplitude of pro-apoptotic signals generated at the ER. Moreover, Bcl-2 localised at the ER was able to inhibit the oligomerization of a Bax mutant that was constitutively present in the mitochondrial outer membrane [40]. Interestingly, the pro-apoptotic Bcl-2 proteins Bax, Bak, Bim and Bik were also shown to localise at the ER and to participate in the control of mitochondrial apoptosis via the suggested Ca(2+)-dependent ER checkpoint [41–47].

The mechanism of ErPC-induced effects at the ER are not yet defined. On the one hand, synthetic phospholipid derivatives have been shown to interfere with lipid biosynthesis. Since the ER was shown to be involved in this process it may be speculated that ErPC-induced alterations in lipid generation may trigger ER stress and subsequent ER-mediated apoptosis [48]. Consequently, Bcl-2/ER may inhibit ErPC-induced direct apoptosis initiation at the ER.

On the other hand, earlier findings revealed that formation of ceramide that triggers a Bax-dependent mitochondrial death pathway can contribute to apoptosis signaling by synthetic phospholipid derivatives [11,49,50]. Upstream of the mitochondria ceramide triggered a Bcl-2- and $[Ca(2+)_{ER}]$-controlled release of Ca(2+) from the ER

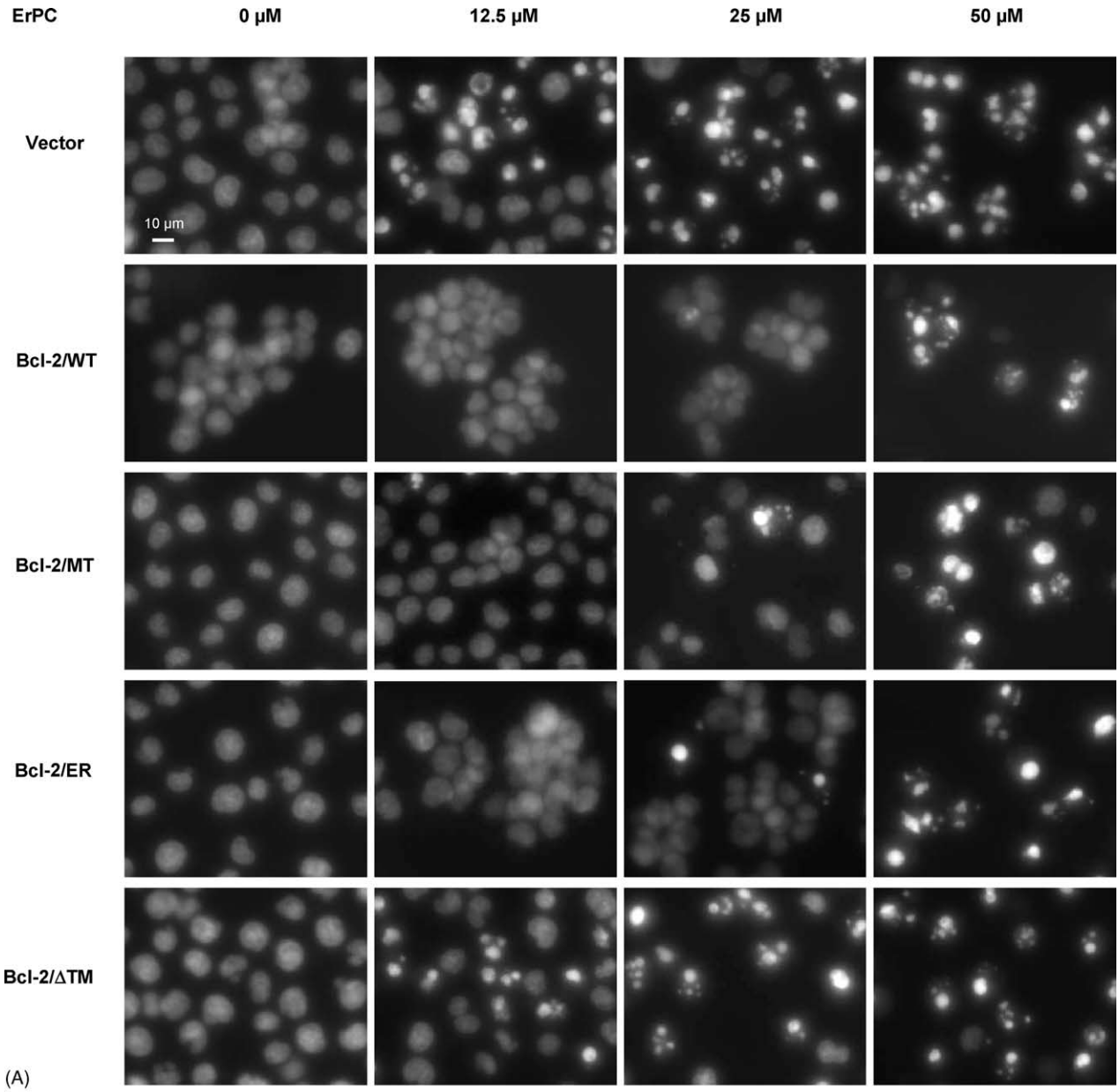


Fig. 5. Targeted expression of Bcl-2 at membranes of the ER and the mitochondria inhibit ErPC-induced nuclear fragmentation. Jurkat vector cells and selected cell clones with targeted expression of Bcl-2 were treated for 24 h with 0, 12.5, 25 and 50 μM ErPC. Effects of targeted expression of Bcl-2 on nuclear morphology were determined by fluorescence microscopy of Hoechst 33342 stained nuclei (A). Data from a representative experiment are depicted. Putative inhibitory action of Bcl-2-mutants on integrity of cellular DNA was analysed by flow cytometry upon staining of the nuclei with propidium iodide (B). Results are presented as means ± S.D. ($n = 6$).

with subsequent uptake into the mitochondrial matrix [43,51]. Thus, ErPC-induced apoptosis may involve a ceramide-dependent Ca(2+)-signal that is generated at ER and sensitive to inhibition by Bcl-2/ER.

Generally, apoptosis induction by synthetic phospholipid derivatives has been suggested to involve alterations of intracellular Ca(2+) concentrations, but the data are still conflicting. Several reports revealed an early increase in intracellular free Ca(2+) and subsequent apoptosis upon treatment that were shown to result from drug-induced release of Ca(2+) from intracellular stores [24,52,53]. In this regard, drug-induced alterations of the lipid environment may affect activity of membrane located enzymes such as Ca(2+)-channels [52] or Ca(2+)-ATPase [54] triggering Ca(2+) influx across the cytoplasmic membrane. However, synthetic phospholipid derivatives had also been shown to function as inhibitors of inositol phosphate mediated Ca(2+) signaling [55].

As mentioned above, the protective effect of Bcl-2 was dependent on its localisation at subcellular membranes. Interestingly, the protection by membrane-localised Bcl-2 decreased with increasing concentrations of ErPC. APC have been shown to affect membrane lipid composition, membrane fluidity and membrane permeability. In this regard, composition, physicochemical characteristics and integrity of natural or synthetic membranes can affect recruitment, multimerisation and function of pro-apoptotic Bcl-2 family members [56–58]. It may be suggested that altered membrane lipid composition could similarly affect insertion of anti-apoptotic Bcl-2 proteins into subcellular membranes as well as their anti-apoptotic function. Thus, drug-induced reorganisation of Bcl-2 and Bcl-x_L within subcellular membranes may contribute to the reduced protective effects at increased drug concentrations.

In conclusion, we demonstrate that similar to Bcl-2 located at the mitochondria Bcl-2 located at the ER potently inhibits ErPC-induced apoptosis. Apart from supporting our earlier findings on the importance of the mitochondria for ErPC-induced apoptosis our data implicate for the first time an involvement of the ER in apoptosis signaling triggered by this membrane-targeted drug. These findings support the recently established concept on the importance of the ER as cellular compartment participating in the regulation of stress-induced apoptosis. The influence of ErPC on the putative Ca(2+)-mediated cross-talk between the mitochondria and the ER will be analysed in future functional studies.

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