Protooncogene *Bcl-2* Induces Apoptosis in Several Cell Lines

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Abstract. Using a recombinant vaccinia virus expressing protooncogene *Bcl-2*, we demonstrate opposite effects of the expressed *Bcl-2* in two cell lines: apoptosis induction in BSC-40 cells and apoptosis prevention in HeLa G cells. The apparent molecular weight of the expressed Bcl-2, its amounts and its effects on the mitochondrial membrane potential are comparable in both cell lines, suggesting that the consequences of *Bcl-2* expression depend on the cellular environment. To further support these findings we demonstrate the pro-apoptotic effect of the expressed *Bcl-2* in several other cell lines.

In all organisms, there is a well-controlled balance between cell proliferation and cell death. Cell death is a process underlying animal development, elimination of abnormal or dangerous cells, and preservation of the integrity of the organism. Two types of cell death are generally distinguished – apoptosis and necrosis. During apoptosis or programmed cell death (PCD), an intracellular death programme is activated and cells commit a pre-programmed suicide in a controlled way. Apoptotic cells shrink and are rapidly phagocytosed by neighbouring cells, before any leakage of their contents can occur. In contrast, cells that die accidentally in response to an acute injury often do so by an uncontrolled process called necrosis. They swell and burst, spilling their intracellular contents over the neighbouring cells and eliciting a damaging inflammatory response. Between the extremes of these two processes, many morphological and functional transitions exist (Cohen, 1993; Majno and Joris, 1995).

The *Bcl-2* gene product plays a crucial role in PCD (Kroemer, 1997). It is a membrane protein localized in outer mitochondrial, endoplasmic reticulum and perinuclear membranes (Hockenbery et al., 1990; Reed, 1999). There is a large family of Bcl-2-like proteins, some of which inhibit apoptosis (Bcl-2, Bcl-x_L, Bcl-w), while others are involved in its induction (Bax, Bak, Bad). Interestingly, the anti-apoptotic Bcl-2 protein can be converted into a pro-apoptotic factor upon proteolytic cleavage by caspases or other proteases (Strack et al., 1996; Grandgirard et al., 1998).

We have observed that vaccinia virus-driven expression of an apparently unmodified Bcl-2 oncoprotein in the BSC-40 epithelial cell line induced apoptosis. In contrast, infection of the HeLa G epithelial cell line with the same virus vector expressing Bcl-2 of the same apparent molecular weight revealed anti-apoptotic effects. Finally, we demonstrate that the pro-apoptotic effects of Bcl-2 are not unique for the BSC-40 cell line, as we could induce apoptosis by expression of Bcl-2 in several other cell lines.

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Abbreviations: $\Delta\Psi_m$ – mitochondrial membrane potential, AIF – apoptosis-inducing factor, BH – Bcl-2 homology, CAT – chloramphenicol acetyltransferase, $DiOC_6(3)$ – 3,3'-dihexyloxacarbocyanine iodide, DMEM – Dulbecco's modified Eagle medium, FBS – foetal bovine serum, FCCP – carbonyl cyanide p-(trifluoromethoxy)phenyl hydrazone, h.p.i. – hours after infection, iNOS – inducible nitric oxide synthase, IPTG – isopropyl-(-D-thiogalactopyranoside, LUC – luciferase, m.o.i. – multiplicity of infection, MTO – MitoTracker Orange, NAO – nonyl acridine orange, NCS – neonatal calf serum, PCD – programmed cell death, PFU – plaque-forming unit, PI – propidium iodide, PKR – interferon-inducible protein kinase, Rh 123 – Rhodamine 123, TMRE – tetramethylrhodamine ethyl ester, VV – vaccinia virus, WRBcl2 – VV recombinant with *Bcl-2* under VV late promoter p4b, WRCAT – VV recombinant with *CAT* under VV late promoter p4b, WRNOS – VV recombinant with *iNOS* under VV late promoter p4b, VVBcl2 – VV recombinant with *Bcl-2* under VV early/late promoter p7.5, VVLUC – VV recombinant with *LUC* under VV early/late promoter p7.5.

Material and Methods

Material

All the media and growth supplements were purchased from Gibco BRL Life Technologies (Paisley, Scotland), PAA Laboratories GmbH (Linz, Austria) or Sigma Chemical Co. (St. Louis, MO), unless otherwise specified. Isopropyl-β-D-thiogalactopyranoside (IPTG), Rhodamine 123 (Rh 123), 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$), carbonyl cyanide p-(trifluoromethoxy)phenyl hydrazone (FCCP), and Hoechst 33342 were purchased from Sigma; propidium iodide (PI) and tetramethylrhodamine ethyl ester (TMRE) from Fluka (Buchs, Switzerland); MitoTracker Orange (MTO), and nonyl acridine orange (NAO) from Molecular Probes (Eugene, OR). The kit M30 CytoDeath, Fluorescein was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Other chemicals were purchased from Sigma or Fluka, unless otherwise specified.

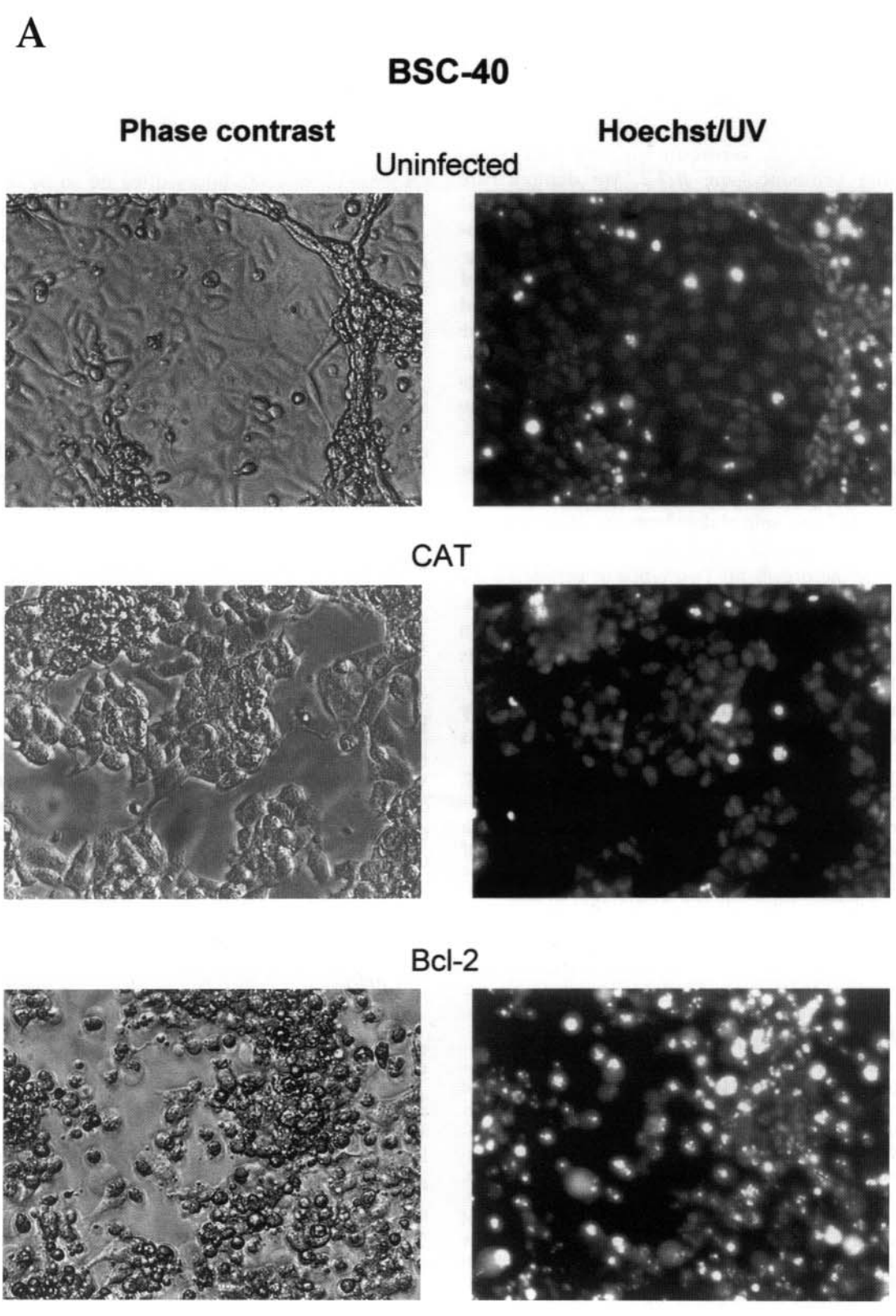
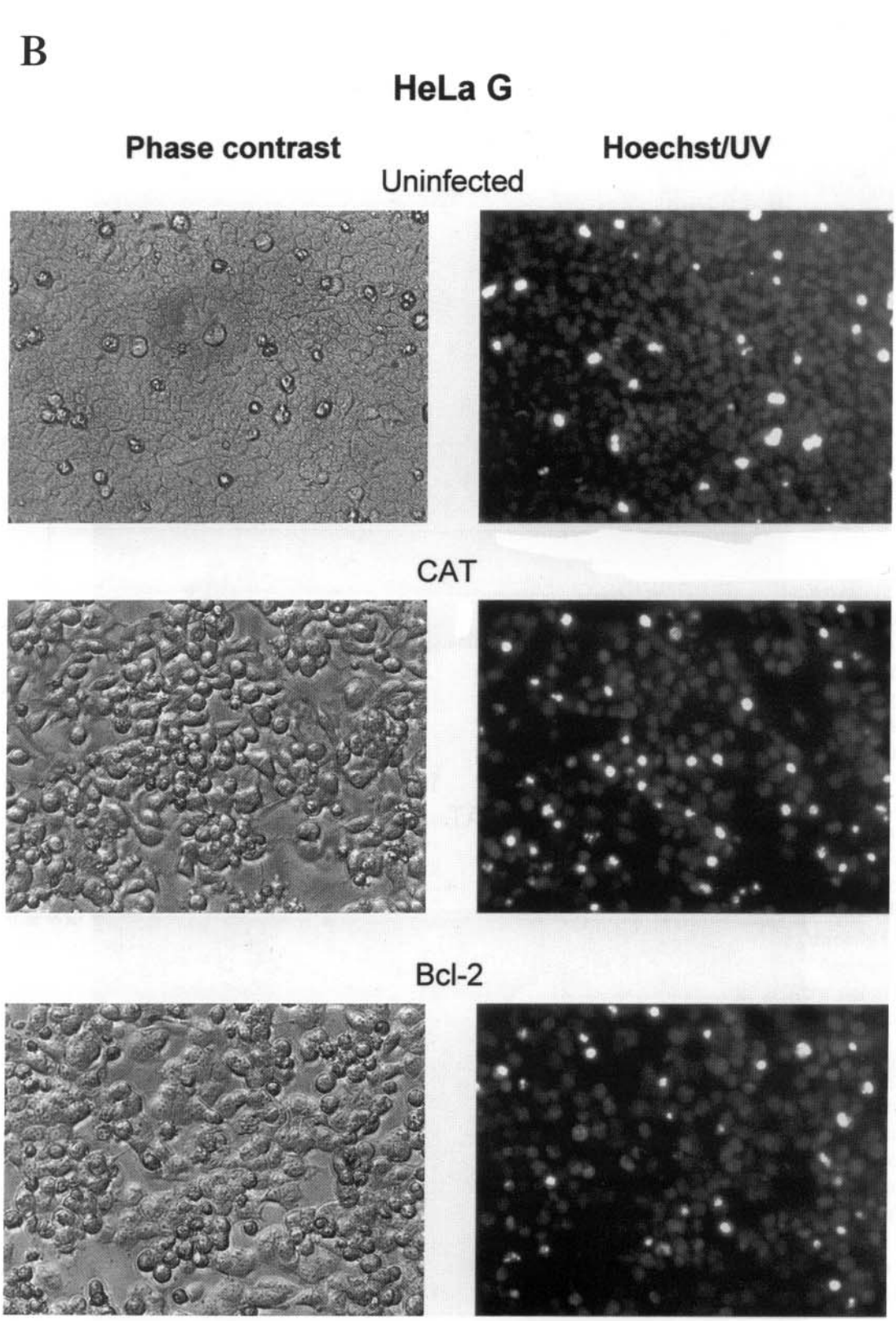
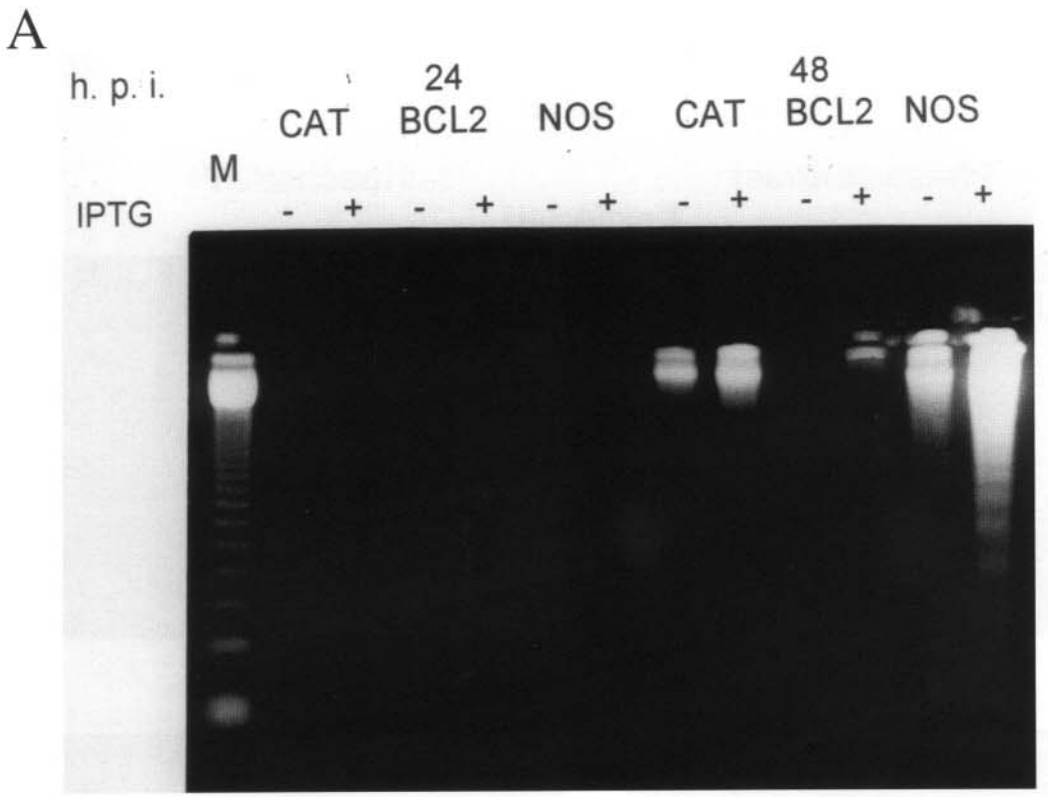


Fig. 1. Bcl-2 induces apoptosis of BSC-40 cells. A (this page): BSC-40 cells. B (opposite page): HeLa G cells. The cells were mock-infected or infected with 2 PFU/cell of WRCAT (CAT) or WRBcl2 (Bcl-2) in the presence of IPTG. At 24 h.p.i. (BSC-40) or at 48 h.p.i. (HeLa G), cell-permeable, DNA-binding dye Hoechst 33342 was added to the culture wells and the cells were observed using an inverted fluorescence microscope in phase contrast or in UV light. Original magnification 150×.


B
HeLa G
Phase contrast
Hoechst/UV
Uninfected
CAT
Bcl-2



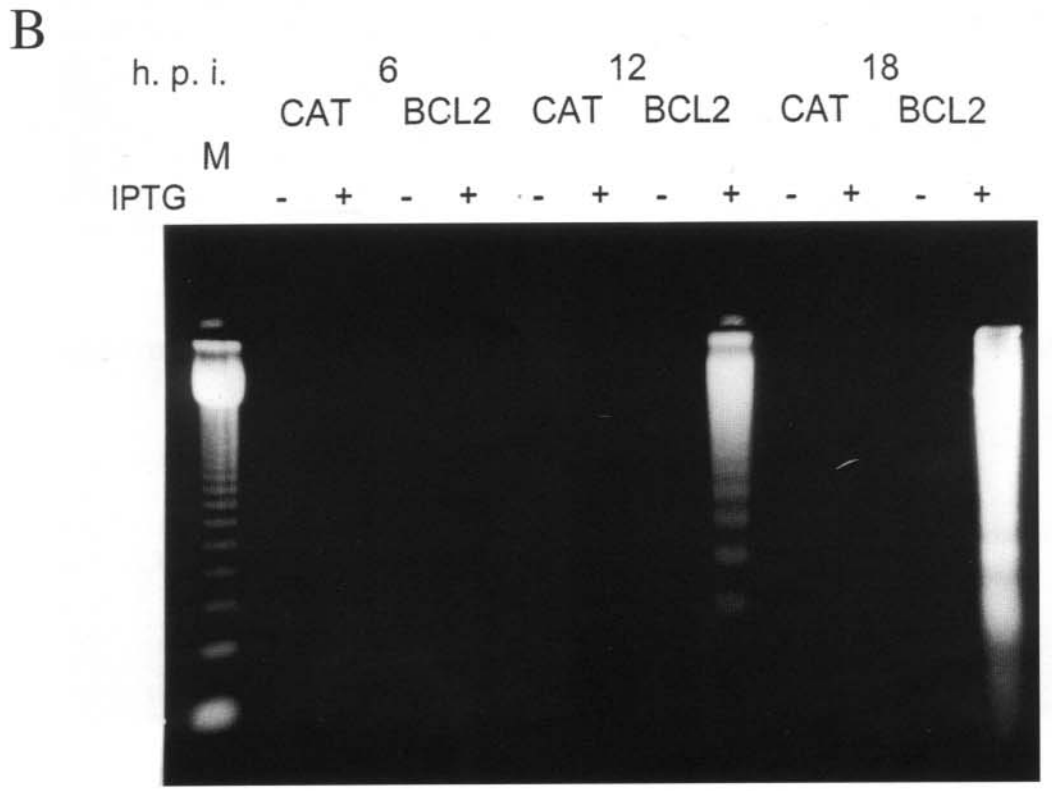


Fig. 2. DNA ladder. A: BSC-40 cells. B: HeLa G cells. The cells were infected with 2 PFU/cell of WRCAT (CAT), WRBcl2 (BCL2) or WRNOS (NOS) in the presence or absence of IPTG. At indicated times after infection (h.p.i.), the cells were collected, low molecular weight DNA was isolated and resolved by 2% agarose gel electrophoresis. M, 123 bp molecular weight marker.

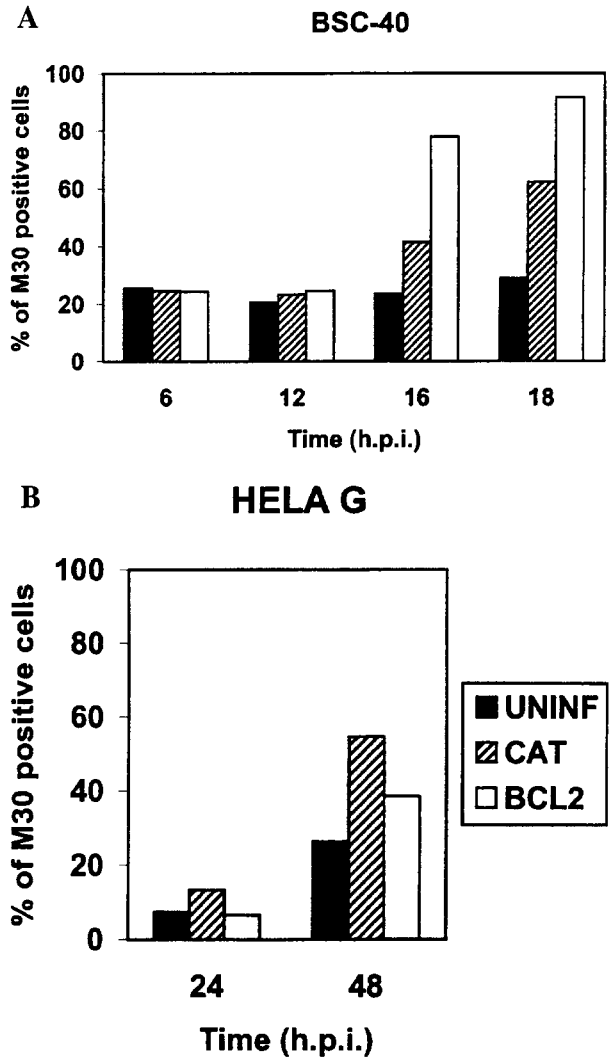


Fig. 3. Cleavage of cytokeratin 18. A: BSC-40 cells. B: HeLa G cells. The cells were mock-infected or infected with 2 PFU/cell of WRCAT (CAT) or WRBcl2 (BCL2) in the presence of IPTG. At indicated h.p.i., the cells were collected, fixed, incubated with M30 fluorescein antibody recognizing the cleaved cytokeratin 18, product of activated caspases, and analysed by flow cytometry. Mean of duplicate samples. Representative results of two experiments.

Cells

African green monkey kidney-derived BSC-40, and human cervical carcinoma HeLa G cell lines were grown in Dulbecco's modified Eagle medium (DMEM; glucose 4.5 g/l) supplemented with 10% heat-inactivated neonatal calf serum (NCS; 10% NCS-DMEM). Mouse monocyte/macrophage cell line J774.G8, human osteosarcoma cells 143 TK(-), SV40-transformed CV-1 simian kidney cells Cos-7, and adenovirus-transformed primary human embryonic kidney cells 293 were grown in DMEM supplemented with 10% heat-inactivated foetal bovine serum (FBS; 10% FBS-DMEM). Human T-cell derived Jurkat (E6-I) cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS (10% FBS-RPMI). All media included penicillin ($1x10^5$ U/l) and streptomycin (100 mg/l). The cells were maintained at 37°C, in a 5% CO_2 atmosphere with 95% humidity.

Recombinant viruses, virus growth, and titration

Recombinant vaccinia viruses (VV) expressing Bcl-2 (WRBcl2) or control chloramphenicol acetyltransferase (*CAT*; WRCAT) under the control of VV late promoter p4b were generated and kindly provided by Dr. S. B. Lee (Lee et al., 1993). Recombinant VV expressing mouse inducible nitric oxide synthase (*iNOS*; WRNOS) was described earlier (Melkova and Esteban, 1995). In these viruses, expression of the recombinant gene is repressed by *Lac*I and induced by IPTG. In the experiments with cell lines of haematopoietic origin, purified stocks of recombinant VV expressing *Bcl-2* (VVBcl2) (manuscript in preparation) or luciferase (*LUC*; VVLUC) (Rodriguez et al., 1988) under the control of VV early/late promoter p7.5 were used. The viruses were propagated in BSC-40 cells supplemented with 2% NCS-DMEM and crude or purified stocks of viruses were prepared (Joklik, 1962). Virus titres were determined by serial dilutions and plaque assays in BSC-40 cells. For experiments, virus inocula were added in serum-free media at multiplicity of infection (m.o.i.) specified in each experiment, and allowed to adsorb for 1 h in the presence of 1.5 mM IPTG. After removal of virus inocula, cells were once washed with the appropriate medium, and supplemented with the same medium containing 2% NCS or FBS and 1.5 mM IPTG. IPTG was omitted in experiments with VVLUC and VVBcl2 (its presence was found to have no effect on induction of apoptosis by VVBcl2 in BSC-40 cells). At indicated times after infection (h.p.i.), the cells were collected and processed as described below. For virus titrations, the cells were resuspended directly in the culture medium, lysed by two cycles of freezing-thawing and sonication, and virus yields were determined by serial dilutions and plaque assays in BSC-40 cells.

Optical microscopy

Cultured cells were visualized directly in tissue culture plates using an inverted epifluorescence microscope Olympus IX-70 and a computer-assisted image analysis system equipped with a CCD camera Cohu (image analysis software LUCIA 4.6; Laboratory Imaging Ltd., Prague, Czech Republic). The cells were observed in phase contrast and under UV after addition of a cell-permeable, DNA-binding dye Hoechst 33342, final concentration 0.2 μg/ml.

DNA ladder

Low molecular weight DNA was isolated as previously described (Lee et al., 1997). Briefly, at indicated times after infection the cells were collected by centrifugation, lysed in a buffer containing 20 mM Tris, 10 mM

EDTA, 1% Triton X-100, and high molecular weight DNA was removed by centrifugation at 10 000 *g* for 10 min. Supernatant containing low molecular weight DNA was extracted with phenol/chloroform/ isoamylalcohol (25 : 24 : 1); low molecular weight DNA was precipitated with ethanol, resuspended in 10 mM Tris-HCl, pH 8, 1 mM EDTA, and treated with 5 μg/ml of RNase A at 37°C for 1 h. DNA was resolved by 2% agarose gel electrophoresis in 1x TBE and visualized by UV after ethidium bromide staining.

Flow cytometry

The cells were collected in culture medium by pipetting and stained with appropriate fluorescent dyes. For $DiOC_6(3)$, TMRE, and NAO staining, the dyes were added directly to the cells collected in culture medium at final concentrations of 20 nM, 1 μM, and 100 nM, respectively, incubated for 15 min at 37°C in the presence of 5% CO_2, and analysed using a flow cytometer in FL1 (Becton-Dickinson FACScan, Becton-Dickinson, Mountain View, CA). For Rh 123 and MTO staining, the cells were pelleted and resuspended in prewarmed DMEM containing the appropriate concentration of NCS and Rh 123 or MTO at final concentrations of 800 nM or 100 nM, respectively. The samples were incubated for 15 min at 37°C in the presence of 5% CO_2, washed once with the medium, and resuspended again in DMEM containing the appropriate concentration of NCS; 10^4 or $5x10^3$ cells were then analysed using Becton-Dickinson FACScan in FL1 or FL2, respectively. The dependence of fluorescence of the dyes on the mitochondrial membrane potential ($\Delta\Psi_m$) was confirmed by inhibition of their accumulation by an uncoupler of oxidative phosphorylation (5 μM FCCP). Since infected cells are PI-positive due to VV-induced changes of cytoplasmic membrane permeability (Carrasco and Esteban, 1982), all measured cells were included in the analysis.

For quantitative analysis of apoptosis, the kit M30 CytoDeath, Fluorescein was used, and the samples were prepared according to the manufacturer's protocol. Briefly, the cells were collected, washed with PBS and fixed with ice-cold pure methanol for 30 min at –20°C. After fixation, the samples were washed with washing buffer (0.1% Tween-20 in PBS), and incubated for 1 h at room temperature in 100 μl of M30 CytoDeath working solution (M30 fluorescein antibody diluted 1 : 250 in PBS containing 1% BSA and 0.1% Tween-20). Consequently, the samples were washed twice with washing buffer, diluted in PBS and analysed using Becton-Dickinson FACScan in FL1. All the analysis of the flow cytometric data was performed using shareware utility WinMDI v2.8 (Joseph Trotter, The Scripps Research Institute, http://facs.scripps.edu/software.html).

Western blot analysis

Samples were collected, lysed, and resolved by 14 or 10% SDS-PAGE (for detection of Bcl-2 or VV polypeptides, respectively) (Laemmli, 1970). Western blot analysis was then performed as previously described (Harlow and Lane, 1988), using chemiluminescent or chromogenic substrates (ECL, Amersham, Buckinghamshire, UK, or α-chloronaphthol, respectively). Bcl-2 was detected with a Bcl-2-specific rabbit polyclonal antibody (dilution 1 : 500, Santa Cruz Biotechnology, Santa Cruz, CA). VV polypeptides were detected using rabbit polyclonal antiserum against VV (dilution 1 : 1000, kindly provided by Drs. D. and J. R. Rodriguez). Peroxidase-conjugated goat anti-rabbit IgG was used as a secondary antibody (dilution 1 : 5000 for enhanced chemiluminescence and 1 : 1000 for a chromogenic substrate, Cappel Research Products, Durham, NC).

Results

Bcl-2 induces apoptosis of BSC-40 cells

The protooncogene *Bcl-2* is well known for its anti-apoptotic effects (Gross et al., 1999). Using a VV expression system, we and others have previously shown that Bcl-2 could prevent apoptosis caused by over-expression of interferon-inducible protein kinase (*PKR*), *RNase L* or *iNOS* in HeLa G cells (Diaz-Guerra et al., 1997; Lee et al., 1997; Melkova et al., 1997). However, in contrast to HeLa G cells, we did not observe the anti-apoptotic effect of Bcl-2 in BSC-40 cells since its expression by a recombinant VV under the control of an IPTG-inducible VV late promoter p4b (WRBcl2) rapidly induced apoptosis of the infected cells. Apoptosis was first characterized by morphological changes of the cells (Fig. 1). In phase contrast, apoptotic cells can be recognized as small and shrunk cells, while the condensed nuclei of the same cells strongly accumulate the cell-permeable, DNA-binding dye Hoechst 33342 and appear very bright under UV. As shown in Fig. 1A, a relatively low number of apoptotic cells could be observed in uninfected BSC-40 cells as well as in cells infected with a control VV expressing *CAT* (WRCAT). In contrast, in BSC-40 cells infected with WRBcl2, many apoptotic cells with condensed and sometimes fragmented nuclei were found. On the other hand, comparable numbers of apoptotic cells were found in uninfected, WRCAT as well as WRBcl2-infected HeLa G cells, as demonstrated in Fig. 1B.

Apoptosis of WRBcl2-infected BSC-40 cells was further confirmed by a typical DNA ladder formation (Fig. 2A). At 12 h.p.i., when some cells started to shrink, a DNA ladder could be detected, while at 18 h.p.i., when most cells were shrunk, signs of DNA degradation were already observed. No DNA ladder

could be observed in cells infected at the same m.o.i. with control WRCAT. Similarly, apoptosis of BSC-40 cells was induced when the cells were infected with another recombinant VV expressing *Bcl-2* under the control of VV early/late promoter p7.5 (VVBcl2), while no apoptosis was caused by a control VV expressing *LUC* under the same promoter (VVLUC; data not shown). Again, HeLa G cells infected with WRBcl2 or with WRCAT did not reveal any signs of apoptosis characterized by a DNA ladder (Fig. 2B). These cells were, however, able to undergo apoptosis (Fig. 2B) that could be prevented by the expression of *Bcl-2* as demonstrated previously (Lee et al., 1997; Melkova et al., 1997) as well as reproduced in this lab (not shown).

Finally, apoptosis was characterized by the levels of cleaved cytokeratin 18, product of activated caspases recognized by monoclonal antibody M30, and analysed using flow cytometry. As shown in Fig. 3, infection with control VV expressing *CAT* increased the percentage of cells recognized by the M30 antibody compared to

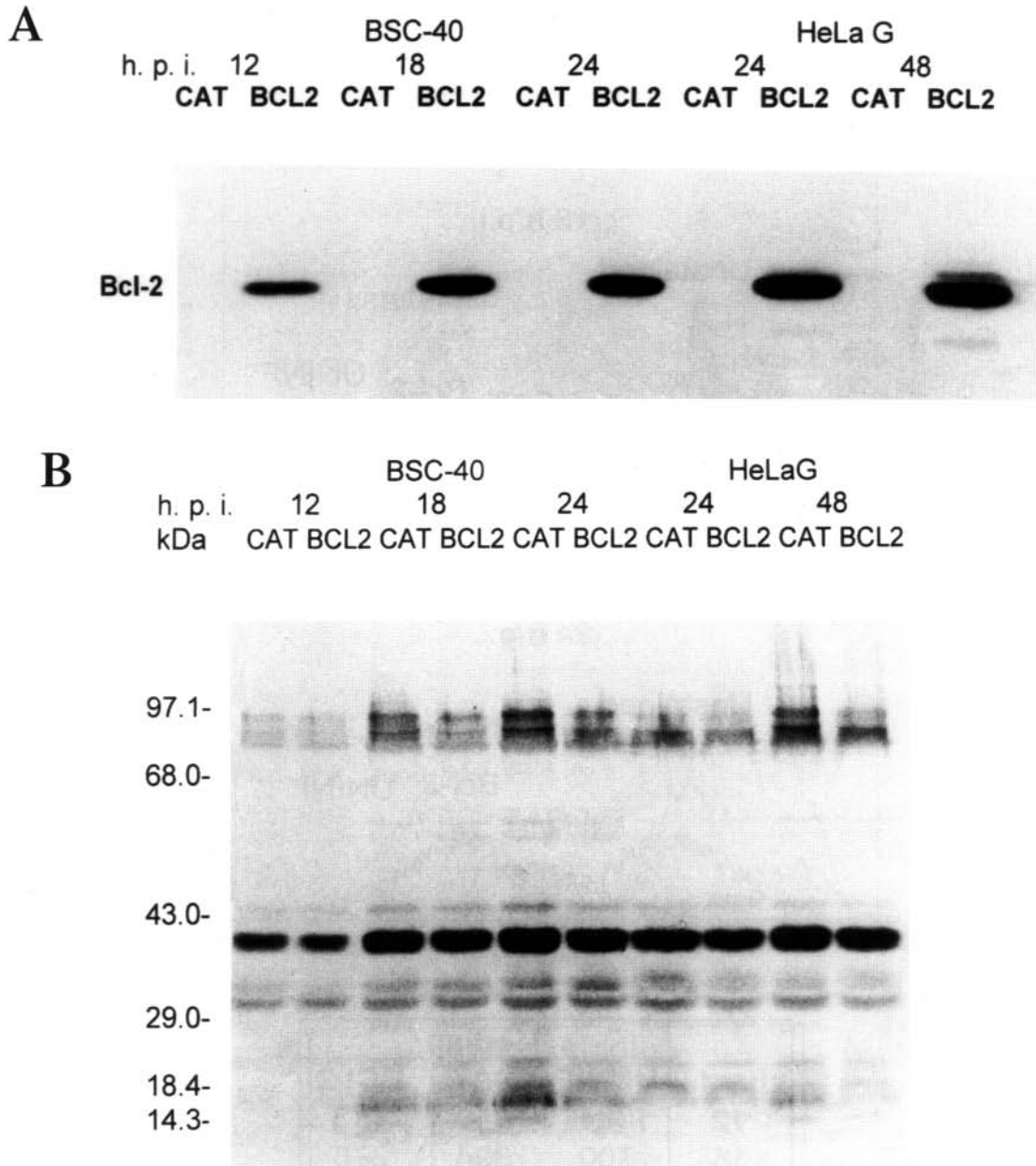
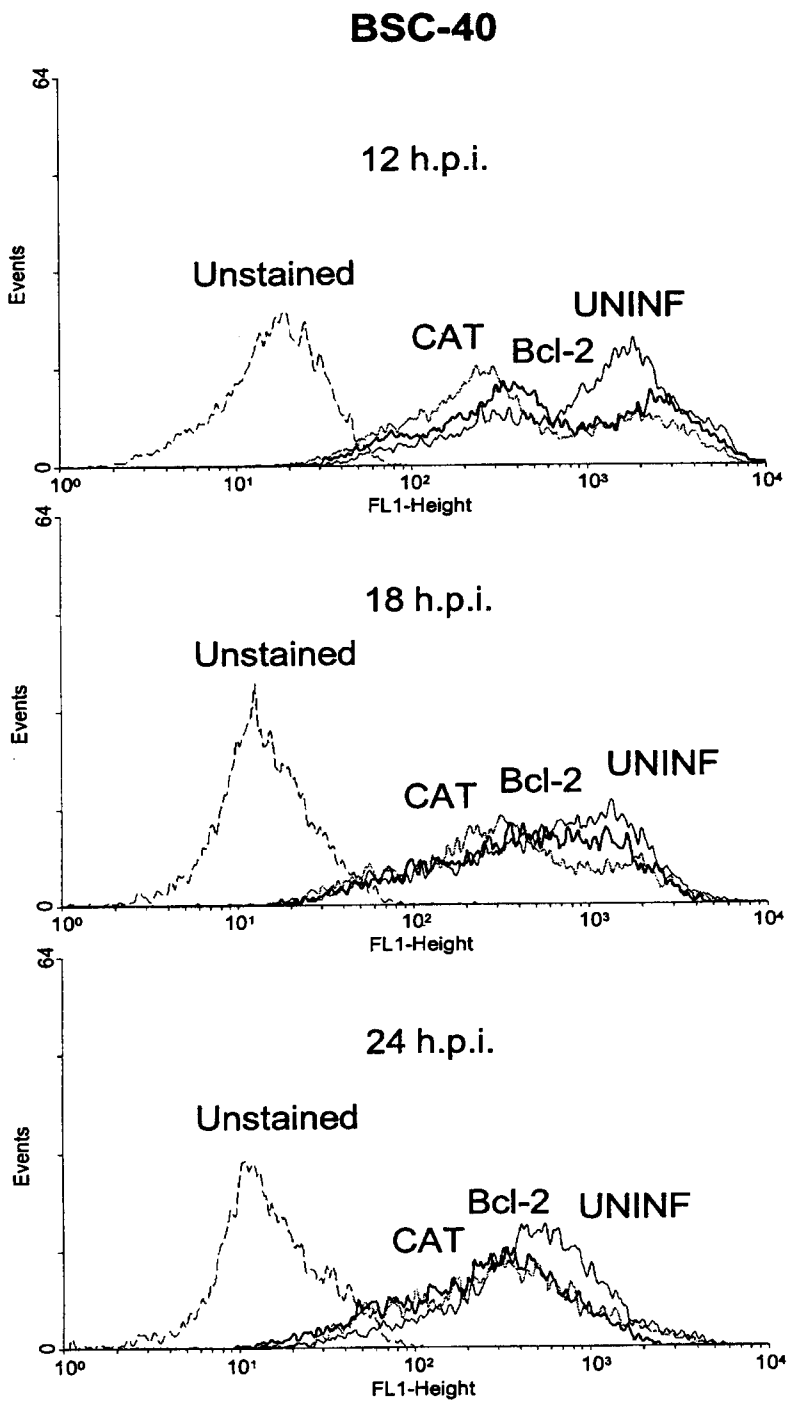


Fig. 4. Bcl-2 is not processed by caspases. A: time course of Bcl-2 expression in BSC-40 and HeLa G cells. BSC-40 and HeLa G cells were infected with 2 PFU/cell of WRCAT (CAT) or WRBcl2 (BCL2)) in the presence or absence of IPTG. At indicated h.p.i., the cells were collected and lysed in Laemmli sample buffer. Proteins were resolved by 14% SDS-PAGE. Bcl-2 was detected by Western blot analysis using Bcl-2-specific rabbit polyclonal antibody (dilution 1 : 500) and enhanced chemiluminescence. B: time course of VV protein expression in BSC-40 and HeLa G cells. BSC-40 and HeLa G cells were infected and processed as indicated above, except that proteins were resolved by 10% SDS-PAGE. VV polypeptides were detected using rabbit polyclonal antiserum against VV (dilution 1 : 1000) and immunoperoxidase staining.

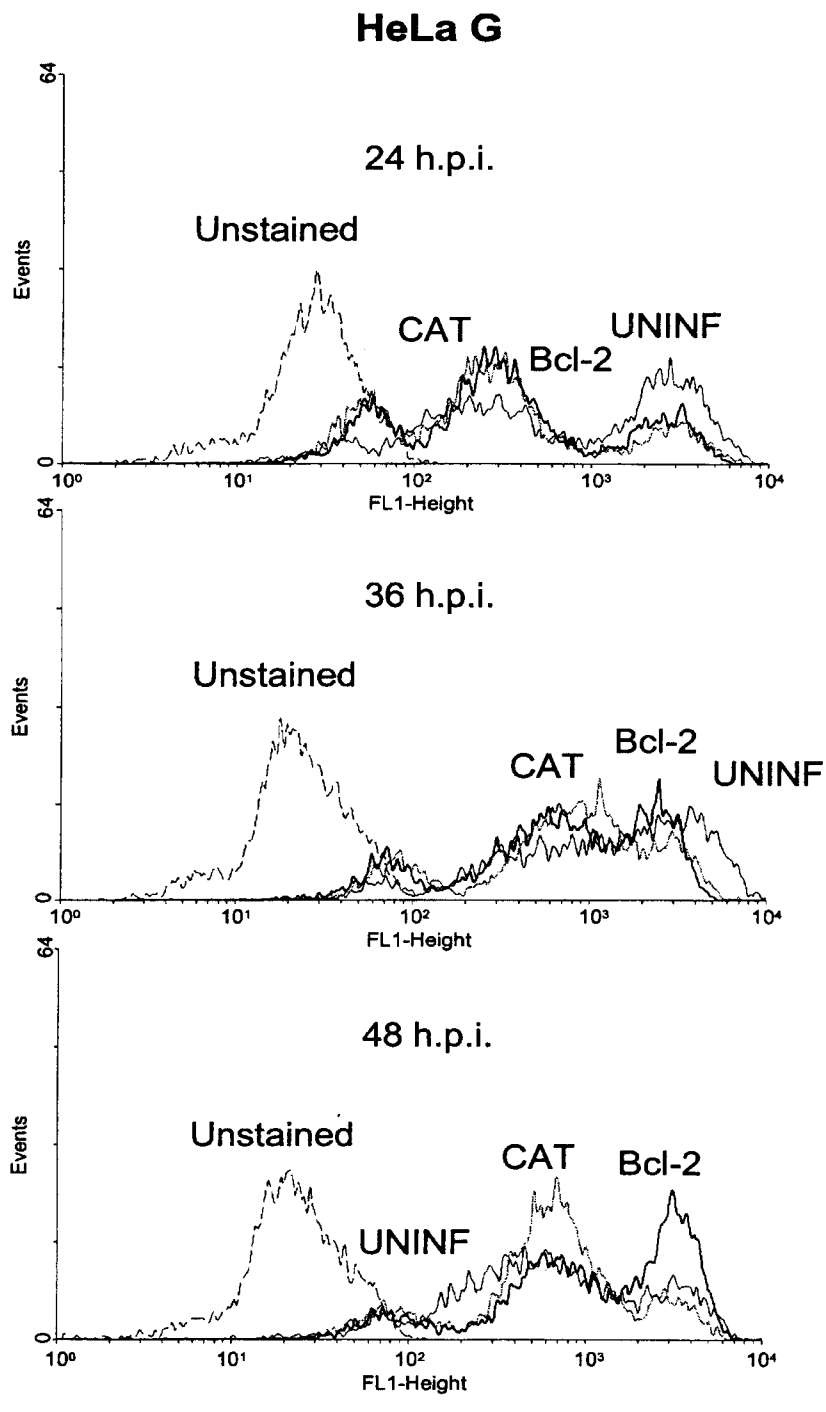
A



h.p.i.	Median		
	UNINF	CAT	BCL2
12	1034	340	413
18	700	304	376
24	481	266	268

Fig. 5. Bcl-2 causes an increase of mitochondrial membrane potential ($\Delta\Psi_m$). A (this page): BSC-40 cells – transient increase. B (opposite page): HeLa G cells – long-term effect. The cells were infected with 2 PFU/cell of WRCAT (CAT) or WRBcl2 (BCL2) in the presence of IPTG. At indicated times after infection (h.p.i.), the cells were collected, stained with $DiOC_6(3)$ and PI, and analysed by flow cytometry to determine $\Delta\Psi_m$. The table summarizes the mean of duplicate samples determined in this particular experiment. Representative result of one of three experiments performed in duplicate.

B



h.p.i.	Median		
	UNINF	CAT	BCL2
24	450	226	279
36	1366	775	628
48	731	606	1001

uninfected samples in both BSC-40 and HeLa G cells. However, infection with VV expressing *Bcl-2* further increased the percentage of positive cells in BSC-40, while it somewhat lowered this percentage in HeLa G cells.

Bcl-2 is not processed by caspases

It has been previously shown that in cells infected with alphaviruses or HIV-1, Bcl-2 can be processed by caspases or other proteases into a shorter homologue with a pro-apoptotic function (Strack et al., 1996; Grandgirard et al., 1998). However, as revealed by Western blot analysis (Fig. 4A), the apparent molecular weight of the expressed Bcl-2 was comparable in both BSC-40 and HeLa G cells, suggesting that apoptosis of BSC-40 cells was not caused by a proteolytic product of Bcl-2. Another Western blot analysis using polyclonal antiserum against VV (Fig. 4B) and determination of virus growth by titration demonstrated that infection by VV was comparable in both cell lines (titres obtained in the presence of IPTG in BSC-40 cells and HeLa G, respectively - WRCAT: 8.23 and 8.15 log PFU/ml, WRBcl2: 7.82 and 7.99 log PFU/ml). Similar results were observed when using increasing multiplicities of infection (2, 5, 10 PFU/cell).

Bcl-2 induces changes of mitochondrial membrane potential ($\Delta\Psi_m$)

Since Bcl-2 is believed to inhibit apoptosis at the level of mitochondria (Susin et al., 1996), we looked at Bcl-2-mediated changes of the mitochondrial membrane potential. $\Delta\Psi_m$ was characterized by flow cytometry after vital staining of the cells with potentiometric dyes, $DiOC_6(3)$, Rh 123, TMRE and MTO. These cationic lipophilic fluorochromes accumulate in mitochondria in dependence on $\Delta\Psi_m$ and their fluorescence intensity corresponds to this potential (Darzynkiewicz et al., 1982).

As shown in Fig. 5B, expression of *Bcl-2* in HeLa G cells caused an increase of $\Delta\Psi_m$ as compared to expression of *CAT* at 48 h.p.i. The Bcl-2-mediated shift of $\Delta\Psi_m$ to higher values resembled the distribution of fluorescence in uninfected cells, suggesting that Bcl-2 can preserve or stabilize $\Delta\Psi_m$ that is otherwise disturbed by VV infection. On the other hand, expression of *Bcl-2* in BSC-40 cells caused a transient increase of fluorescence of $DiOC_6(3)$ around 12–18 h.p.i. that was followed by a decrease due to cell death (Fig. 5A). Similar results were obtained with other potentiometric dyes Rh123, TMRE or MitoTracker Orange (data not shown). The fluorescence of the potentiometric dyes was determined to be FCCP-sensitive, demonstrating that the dye accumulation was $\Delta\Psi_m$-dependent.

The number of mitochondria is comparable in both BSC-40 and HeLa G cells

The level of fluorescence of the cells stained for $\Delta\Psi_m$ can be affected not only by the mitochondrial membrane potential, but also by the number of mitochondria. Therefore, the number of mitochondria in BSC-40 and HeLa G cells was characterized by NAO that interacted with cardiolipin in the inner mitochondrial membrane. As demonstrated in Fig. 6, the fluorescence of NAO was comparable in both cell lines, indicating that the number of mitochondria was comparable as well. In contrast, fluorescence of $DiOC_6(3)$ (Fig. 6A), as well as other potentiometric dyes (MTO; Fig. 6B) revealed higher $\Delta\Psi_m$ in HeLa G cells than in BSC-40 cells.

Other cell lines

To get further insight into the role of cellular background in the outcome of VV-driven expression of *Bcl-2*, we examined the effect of *Bcl-2* expression in several other cell lines of various origins. In addition to BSC-40 cells, infection by VV expressing *Bcl-2* caused apoptosis of human osteosarcoma cells 143 TK(-), SV40-transformed, CV-1-derived simian kidney cells Cos-7, adenovirus-transformed primary human embryonic kidney cells 293, human hepatoma-derived HepG2 cells and human T-cell-derived Jurkat cells, as characterized by DNA ladder formation. Additionally, depending on culture conditions, VV-driven expression of *Bcl-2* could either prevent or induce apoptosis of the mouse monocyte/macrophage J774.G8 cell line (not shown).

Discussion

Protooncogene *Bcl-2* has been considered as a prototype factor inhibiting apoptosis, mainly at the level of mitochondria. In contrast to this generally accepted function, we demonstrate *Bcl-2*-mediated induction of apoptosis in several cell lines. At the same time, we confirm the anti-apoptotic effects of *Bcl-2* in other cell lines.

We have characterized *Bcl-2* expression in detail mainly in two cell lines: in BSC-40 cells, where its expression induced apoptosis, and in HeLa G cells, where its expression did not cause any observable harmful effects and prevented apoptosis upon its induction by other stimuli (Diaz-Guerra et al., 1997; Lee et al., 1997; Melkova et al., 1997). According to Western blot analysis, the apparent molecular weight of the expressed Bcl-2 was comparable in both cell lines, suggesting that unlike in previously described cases (Strack et al., 1996; Grandgirard et al., 1998), Bcl-2 was not subject to proteolytic degradation or phosphorylation (Srivastava et al., 1999). Interactions between Bcl-2 family members occur via three domains, referred to as Bcl-2 homology (BH) regions. The crystal

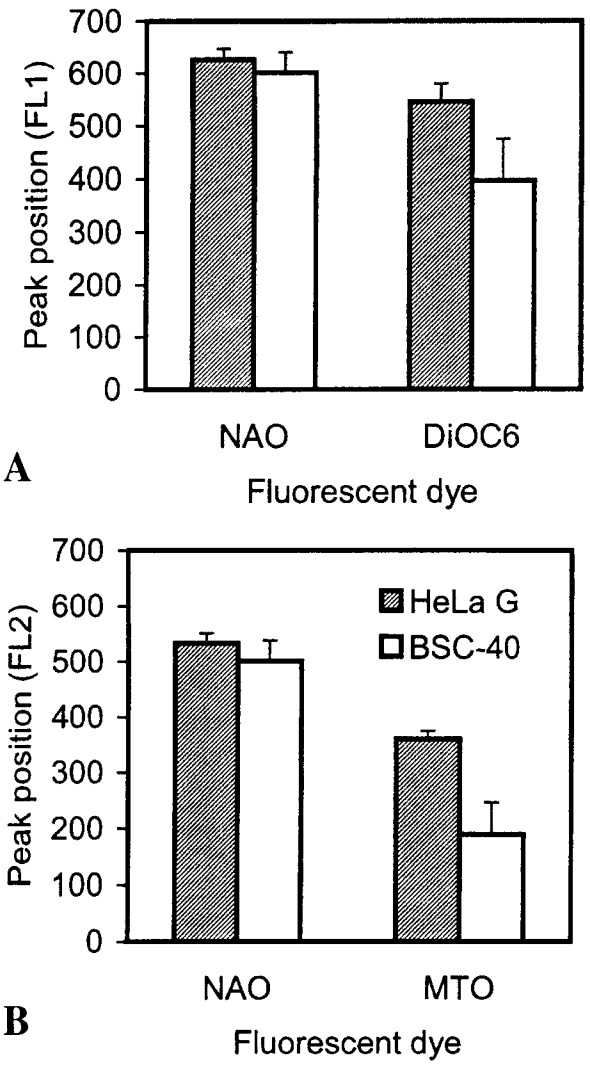


Fig. 6. The number of mitochondria is comparable in both BSC-40 and HeLa G cells, while $\Delta\Psi_m$ is higher in HeLa G cells. A: fluorescence of NAO and $DiOC_6(3)$ (FL1). B: fluorescence of NAO and MitoTracker Orange (FL2). Fluorescent indicators were added to BSC-40 and HeLa G cells collected after a 24 h period of growth at 37°C in the presence of 5% CO_2. The cells were analysed by flow cytometry to determine the peak position of fluorescence of each individual dye in FL1 or FL2. The peak position of control, unstained cells was subtracted as a background value. The results represent the mean of four independent experiments performed in duplicate; error bars indicate standard error of mean, S.E.M.

structure of the anti-apoptotic Bcl-x_L revealed that the three conserved domains form a hydrophobic pocket (Muchmore et al., 1996) that binds strongly to a BH3-containing peptide of a pro-apoptotic member of the Bcl-2 family Bak. In addition to BH1-3, anti-apoptotic members of the Bcl-2 family contain an N-terminal BH4 domain, which plays a critical role in suppressing apoptosis. Deletion or proteolytic cleavage of the N-terminus of Bcl-2 and Bcl-x_L by caspases or other proteases abolished their anti-apoptotic activities and converted them into potent pro-apoptotic proteins by exposure of the BH3 domain (Strack et al., 1996; Cheng et al., 1997; Grandgirard et al., 1998). Since we did not observe any changes in the apparent molecular weight of the expressed Bcl-2, it is conceivable to speculate that Bcl-2 might undergo conformational changes due to alteration of the intracellular microenvironment such as change of pH or ionic strength, or due to interactions with some other proteins. Alternatively, apoptosis might result from a normal function of Bcl-2. Indeed, we have shown that *Bcl-2* expression stabilized or perhaps increased the mitochondrial membrane potential in both BSC-40 and HeLa G cells (Fig. 5), suggesting that its effects on $\Delta\Psi_m$ were comparable. Nevertheless, the consequences were different, possibly due to differences in cellular environment. It should also be noted that unlike various kinds of transient transfection, the VV expression system allows high-level expression of recombinant genes in the majority of cells. The use of a complex virus like vaccinia however adds other, virus-specific changes to the host cell. Therefore, the results presented here should ideally be confirmed in permanently transfected cell lines using an inducible expression system that would allow the same level of expression as VV.

The exact mechanism of Bcl-2 action still remains unknown, but it is generally believed that Bcl-2 inhibits apoptosis by keeping the permeability transition pore of mitochondria closed (Susin et al., 1996). Using synthetic lipid bilayers, it has been suggested that Bcl-2 also had a channel-forming activity (Schendel et al., 1997), which might be responsible for Bcl-2-mediated regulation of ion transport that maintains $\Delta\Psi_m$ and prevents apoptosis (Shimizu et al., 1998). In contrast, the pro-apoptotic protein Bax can form ion channels with different specificity, leading to dissipation of $\Delta\Psi_m$ (Schlesinger et al., 1997). It is known that Bcl-2 and Bax form heterodimers and their relative ratio is thought to determine the release of apoptosis inducers such as cytochrome c (Yang et al., 1997), apoptosis-inducing factor (Susin et al., 1996) (AIF), Smac/DIABLO (Chai et al., 2000), several pro-caspases and other proteins from the mitochondrial intermembrane space (Hengartner, 2000). Possible other roles of Bcl-2 include antioxidative effects (reviewed in Korsmeyer et al., 1995), shift of cellular redox potential to a more reduced state (Ellerby et al., 1996), and regulation of Ca^{2+} levels in the cytoplasm, endoplasmic reticulum, and mitochondria (He et al., 1997; Foyouzi-Youssefi et al., 2000).

In both BSC-40 and HeLa G cells, infection by wild-type (wt) VV or VV recombinants expressing control genes, *CAT* or luciferase caused lysis (i.e. necrosis) of the cells, while VV recombinants expressing pro-apoptotic genes such as *PKR*, *RNase L* or *iNOS* induced apoptosis

of both BSC-40 and HeLa G cells (Diaz-Guerra et al., 1997; Lee et al., 1997; Melkova et al., 1997). In HeLa G cells, VV-driven expression of *Bcl-2* prevented all the above-mentioned types of apoptosis while in BSC-40 cells, expression of the same *Bcl-2* converted lytic infection of BSC-40 cells (i.e. necrosis) to apoptosis. The reason for this shift remains unclear, but it can be speculated that *Bcl-2* changes the rate and perhaps the course of VV-induced changes of the host cell metabolism that allow the cell to execute the death programme. In BSC-40 cells, VV-driven expression of *Bcl-2* induced apoptosis earlier than expression of the other pro-apoptotic genes (12–18 h.p.i. and 24–48 h.p.i., respectively). Recently, another example of apoptosis induced by relatively high levels of expression of *Bcl-2*, using the pcDNA3 expression vector or recombinant adenoviruses, has been published (Uhlmann et al., 1998). In contrast, our results indicate that comparably high levels of *Bcl-2* reveal opposite effects in different cell lines, suggesting that the amount of *Bcl-2* is not the main cause of apoptosis.

In summary, we used two different cell lines, BSC-40 cells derived from African green monkey kidney and HeLa G cells derived from human cervical carcinoma. Using VV expression vectors, we observed two opposite effects of *Bcl-2* expression: apoptosis prevention and apoptosis induction. In both cell lines, Bcl-2 increased fluorescence of potentiometric dyes that reflect $\Delta\Psi_m$, suggesting that the mode of its action is comparable in both cases. Importantly, no proteolytic degradation of Bcl-2 into a shorter, pro-apoptotic homologue was detected, and the levels of its expression were comparable in both BSC-40 and HeLa G cell lines. Fluorescence of NAO suggests that the number of mitochondria is equal in both cell lines as well. Finally, we described either pro-apoptotic or anti-apoptotic effects in several other cell lines.

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