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Bcl-x_L can inhibit apoptosis in cells that have undergone Fas-induced protease activation

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ABSTRACT **Programmed cell death or apoptosis provides an irreversible mechanism for the elimination of excess or damaged cells. Several recent studies have implicated the activation of the interleukin 1β-converting enzyme/Ced-3 (ICE/Ced-3) family of proteases as the "point of no return" in apoptotic cell death, while others have suggested that loss of mitochondrial membrane potential ($\Delta\Psi_m$) is the ultimate determinant of cell death. The temporal relationship of these two events during apoptosis and the role of Bcl-2 proteins in inhibiting these steps has not been defined. To examine these issues, control and Bcl-x_L-transfected Jurkat T cells were treated with Fas antibodies in the presence and absence of the ICE protease inhibitor zVAD-FMK. ICE/Ced-3 protease activity was monitored by following the cleavage of poly(ADP-ribose) polymerase (PARP) and $\Delta\Psi_m$ was followed by rhodamine 123 fluorescence. Although Bcl-x_L expression did not block Fas-induced protease activation, it substantially inhibited the subsequent loss of $\Delta\Psi_m$ and cell death in Fas-treated cells. In contrast, zVAD-FMK blocked PARP cleavage as well as loss of $\Delta\Psi_m$ and cell death. Together these data demonstrate that Bcl-x_L can maintain cell viability by preventing the loss of mitochondrial membrane potential that occurs as a consequence of ICE/Ced-3 protease activation.**

The Bcl-2 family is comprised of a number of genes that play critical roles in the regulation of programmed cell death (1). Some members of the Bcl-2 family such as *bax*, *bak*, and *bad* appear to promote apoptosis. In contrast, expression of other family members including *bcl-2*, *bcl-x_L*, and *ced-9* can prevent apoptosis. The anti-apoptotic members of the Bcl-2 family have been shown to prevent apoptosis in response to a wide variety of stimuli. One of the best characterized forms of receptor-mediated death is initiated by the cell surface receptor Fas, a member of the tumor necrosis factor (TNF) receptor family (2). Recent evidence suggests that the crosslinking of Fas results in the recruitment and direct activation of a novel member of the interleukin 1β-converting enzyme/Ced-3 (ICE/Ced-3) protease family, FLICE/Mach (3–5). Furthermore, overexpression of FLICE/Mach can lead directly to the induction of apoptosis in transiently transfected cells (4, 5). Fas crosslinking *in vivo* results in lethality due to fulminant hepatic failure (6–8). Expression of Bcl-2 in the liver can prevent Fas-induced hepatocyte apoptosis (7, 8). These data are compatible with previous genetic evidence that *ced-9* can prevent programmed cell death in *Caenorhabditis elegans* by inhibiting the action of the Ced-3 protease (1, 9). Although these data do not determine whether Ced-9 functions biochemically to prevent Ced-3 protease activation or to prevent active Ced-3 from effecting the downstream events of apoptosis, ICE/Ced-3 protease activation has been suggested to represent the first irreversible step in the induction of programmed cell death (9–12). An opposing view has recently been proposed by Kroemer and colleagues (13–17), who have suggested that the first irreversible step in programmed cell death may involve the loss of mitochondrial integrity through the activation of the permeability transition pore complex. The opening of this pore appears to inactivate mitochondrial function (18). Kroemer and colleagues (13) have demonstrated that Bcl-2 overexpression can prevent mitochondrial permeability transition. In support of this hypothesis there is biochemical evidence that Bcl-2 is localized to the outer mitochondrial membrane (19, 20) as well as recent molecular evidence that the structure of Bcl-x_L resembles that of a pore-forming domain (21). Such domains have been implicated in the regulation of membrane permeability. Therefore, we sought to examine the relationship of Bcl-x_L expression to ICE/Ced-3 protease activation and mitochondrial permeability transition in response to Fas crosslinking.

MATERIALS AND METHODS

Cell Culture and Viability Assays. Jurkat transfectants were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 20 mM Hepes, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 mg/ml G418. Cells were seeded at 5×10^5/ml in fresh medium containing the Fas mAb CH-11 (Panvera, Madison, WI) at 100 ng/ml. Viability was determined by fluorescence-activated cell sorter (FACS) analysis (FACSort and Lysis II; Becton Dickinson) of propidium iodide exclusion as described (22). For determination of $\Delta\Psi_m$, cells were treated as indicated. At each time point three 0.5-ml aliquots of cells were removed for viability analysis. Cells were isolated by centrifugation and resuspended in 0.5 ml of buffer containing 5 μg/ml rhodamine 123 (rh123; Molecular Probes). Cells were incubated for 30 min at 37°C, washed with FACS buffer, and resuspended in 0.5 ml of FACS buffer containing propidium iodide at 2 μg/ml. rh123 and propidium iodide staining was determined by analysis on a FACSort flow cytometer utilizing Lysis II software (Becton Dickinson).

Poly(ADP-ribose) Polymerase (PARP) Analysis. For PARP analysis, Jurkat transfectants were resuspended at 5×10^5 cells/ml and treated with medium or anti-Fas at 100 ng/ml in the presence or absence of 50 μM zVAD-FMK (zVAD; Enzyme Systems Products, Dublin, CA) for the indicated times. Cells (10 ml) were washed with PBS and lysed in 150 μl of RIPA buffer (150 mM NaCl/1.0% Nonidet P-40/0.5%

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Abbreviations: ICE/Ced-3, interleukin 1β-converting enzyme/Ced-3; FACS, fluorescence-activated cell sorter; PARP, poly(ADP-ribose) polymerase; rh123, rhodamine 123; zVAD, zVAD-FMK.

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deoxycholic acid/0.1% SDS/50 mM Tris, pH 8.0/protease inhibitors). Lysates were clarified by centrifugation and the supernatants were stored at −80°C until analysis. Protein was quantitated using a commercially available kit (BCA, Pierce) and 100 μg of protein per sample were subjected to SDS/PAGE (10% acrylamide) and transferred to nitrocellulose. Blots were blocked and incubated with 2C-10 at a dilution of 1:10,000 (provided by Guy Poirier, Laval, Quebec) as described (22). Quantitation of PARP cleavage was determined by scanning densitometry.

RESULTS AND DISCUSSION

Previous studies have demonstrated that human Jurkat T cells undergo apoptosis in response to Fas receptor crosslinking (23). To examine the role of Bcl-2 proteins in regulating Fas-mediated cell death, Jurkat T cells were transfected with a Bcl-x_L expression vector or vector alone, and stable clones that expressed equivalent levels of Fas on the cell surface were isolated (data not shown). The expression level of Bcl-x_L was confirmed by Western blot analysis. The susceptibility of these cells to death in response to Fas crosslinking was analyzed by treating the cells with a Fas mAb and determining viability by propidium iodide exclusion (Fig. 1*A*). Essentially all control transfected cells had undergone apoptotic death by 48 hr. In contrast, Bcl-x_L-overexpressing cells remained 73.8 ± 0.7% viable at 24 hr and 50.1 ± 1.0% remained viable even after 96 hr of continuous anti-Fas treatment.

One substrate that has been identified for the FLICE/Mach protease is the enzyme PARP (5). Therefore, to monitor the activation of ICE/Ced-3 proteases in response to Fas crosslinking, the extent of PARP cleavage was examined at various times after the addition of Fas antibody. As expected, PARP cleavage could be detected as an early event following Fas crosslinking. Six hours after anti-Fas addition, all of the PARP present in the control transfectants had been cleaved into an 85-kDa fragment characteristic of ICE/Ced-3 protease degradation (Fig. 1*B*). Unexpectedly 85.5 ± 2.7% (mean ± SD of three independent experiments) of the PARP protein in the Bcl-x_L transfectants was also found to be cleaved 6 hr after anti-Fas addition. This high degree of PARP cleavage in Bcl-x_L transfectants in response to Fas stimulation was observed in three independent clones (data not shown). No further PARP cleavage was observed between 6 and 24 hr in Bcl-x_L transfectants. Equivalent amounts of uncleaved PARP were found in cell extracts taken either 6 or 24 hr after anti-Fas addition (uncleaved PARP, 14.5 ± 2.7% of untreated control at 6 hr versus 12.6 ± 6.2% of untreated controls at 24 hr; mean ± SD of three independent experiments). However, a significant amount of cleaved PARP product was eliminated from the Bcl-x_L-transfected cells over this period. This suggests that the cells were actively clearing the PARP cleavage product during this time period. The amount of cleaved product observed at 24 hr represents only 12.0 ± 0.5% (mean ± SD of three independent experiments) of the uncleaved PARP in untreated cells. No decrease in the amount of cleaved PARP is evident in the Neo transfectants. In these cells there is quantitative recovery of the initial PARP as an 85-kDa cleavage product at both 6 and 24 hr after anti-Fas addition.

FLICE/Mach-induced PARP cleavage and cell death have been shown to be inhibited by the ICE/Ced-3 protease inhibitor zVAD (5). To confirm that PARP cleavage was the result of Fas-induced protease activation, control and Bcl-x_L transfectants were treated with anti-Fas in the presence of 50 μM

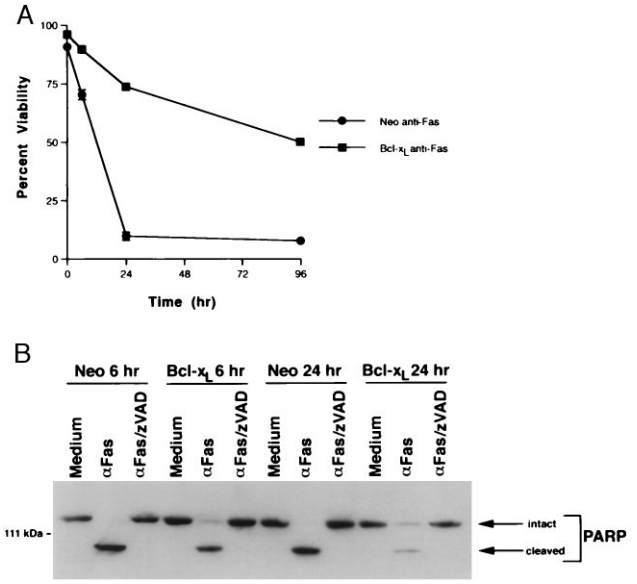

FIG. 1. Bcl-x_L protects against anti-Fas-induced cell death, but not PARP cleavage. (*A*) Jurkat cells transfected with either a *bcl-x_L* (Bcl-x_L, ■) expression vector or the vector control (Neo, ●) were treated with anti-Fas for a 96-hr time course. Viability was determined at indicated time points by propidium iodide exclusion. The data presented are the mean and standard deviation of three independent determinations and are representative of four independent experiments. (*B*) Jurkat cells transfected with a *bcl-x_L* expression vector (Bcl-x_L) or vector control (Neo) were treated for 24 hr with nothing (Medium), anti-Fas (αFas), or anti-Fas and zVAD (αFas/zVAD). At 6 and 24 hr, cells were removed and lysates were produced for Western blot analysis. Western blot analysis of PARP was performed with the PARP antibody 2C-10, which recognizes the 116-kDa intact PARP as well as an 85-kDa cleaved product observed during apoptotic death (14). The data are representative of four independent experiments and have been reproduced using two additional clones of each transfectant.

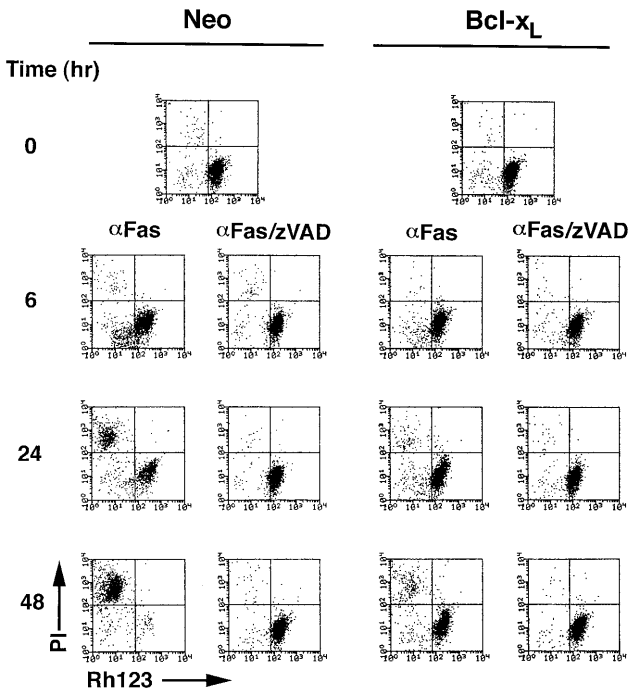

FIG. 2. Bcl-x_L and protease inhibition can block loss of mitochondrial membrane potential. Jurkat T cells were transfected with a *bcl-x_L* expression vector (Bcl-x_L) or control vector (Neo) and treated with anti-Fas in the presence and absence of zVAD as described. At the indicated time points, cells were removed for FACS analysis of viability by propidium iodide staining (*y* axis; PI) and changes in mitochondrial membrane potential by rh123 staining (*x* axis; Rh123). The data are presented as one of three determinations from a representative experiment. Similar data were obtained in at least five independent experiments performed with three independent clones of each transfectant.

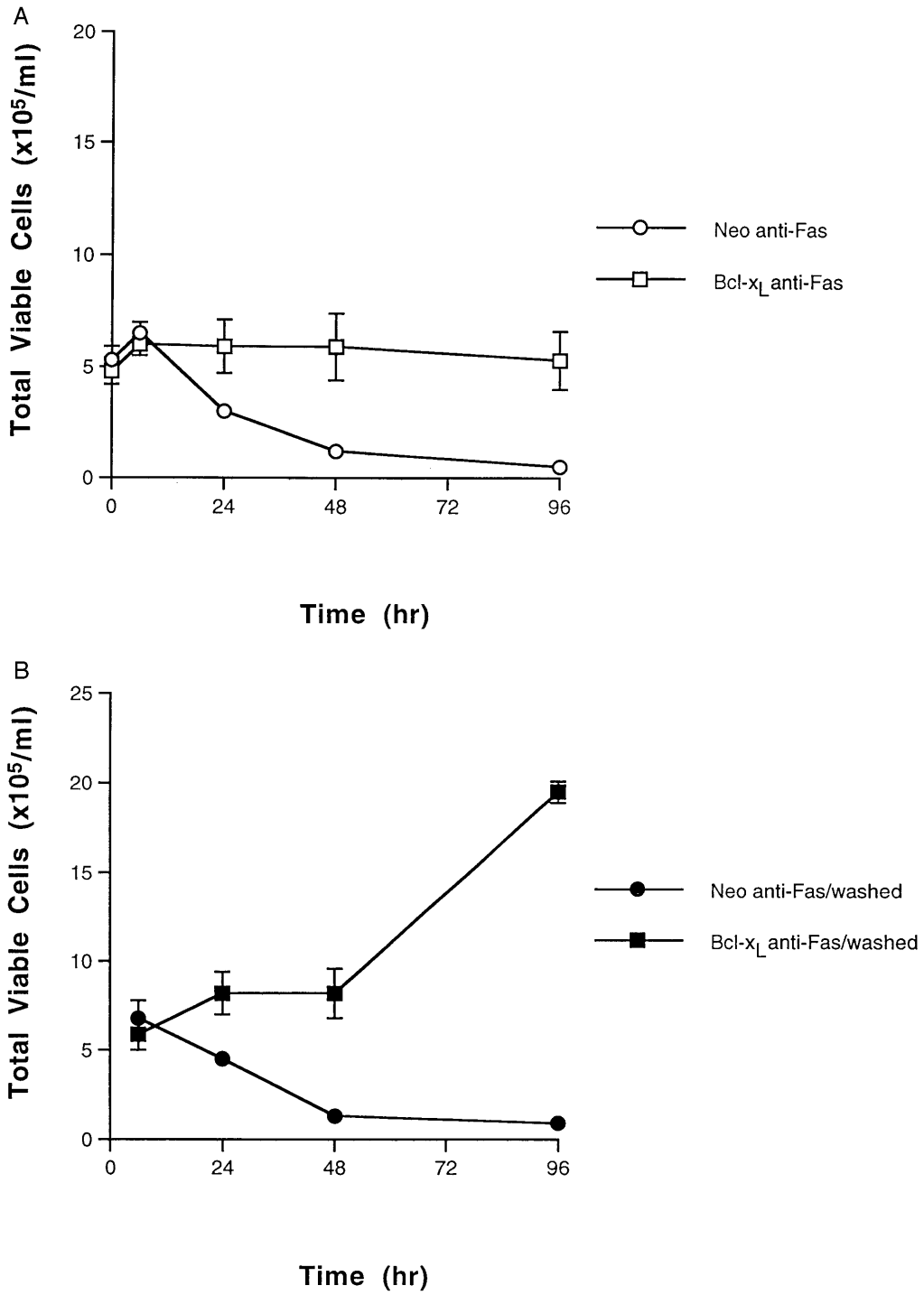

FIG. 3. Bcl-x_L-transfected cells can proliferate following removal of anti-Fas. (*A*) Jurkat T cells transfected with a *bcl-x$_L$* expression vector (Bcl-x_L, □) or a control vector (Neo, ○) were incubated with 100 ng/ml of anti-Fas for a 96-hr time course. At indicated time points, cells were removed for determination of viability by propidium iodide exclusion and for cell counts. The number of viable cells was determined by multiplying the cell count by the fraction of cells that exclude propidium iodide. (*B*) Jurkat T cells transfected with a *bcl-x$_L$* expression vector (Bcl-x_L, ■) or a control vector (Neo, ●) were treated with 100 ng/ml of anti-Fas for 6 hr. Cells were isolated by centrifugation, washed three times, and resuspended in fresh medium. Cell counts were performed after washing to determine cell loss due to washing and counts adjusted accordingly. Viable cell number was determined as described in *A*. The data are presented as the mean ± standard deviation of three separate determinations from a representative of two individual experiments.

zVAD. PARP cleavage was completely inhibited in both control and Bcl-x_L transfectants in the presence of zVAD (Fig. 1*B*).

Together the above data suggest that Bcl-x_L does not act primarily by inhibiting the induction of protease activity in response to Fas ligation. At 6 hr, over 85% of PARP is cleaved in a cell population that remains 90% viable as measured by dye exclusion. Loss of PARP by itself is not likely to irreversibly lead to cell death since genetic disruption of the PARP gene is not lethal (24). These data suggest that Bcl-x_L may prevent or at least delay apoptosis in the presence of Fas-activated proteases. The ability of Bcl-x_L to protect cells from death following a substantial amount of PARP cleavage was not unique to Jurkat T cells. Similar results were obtained studying

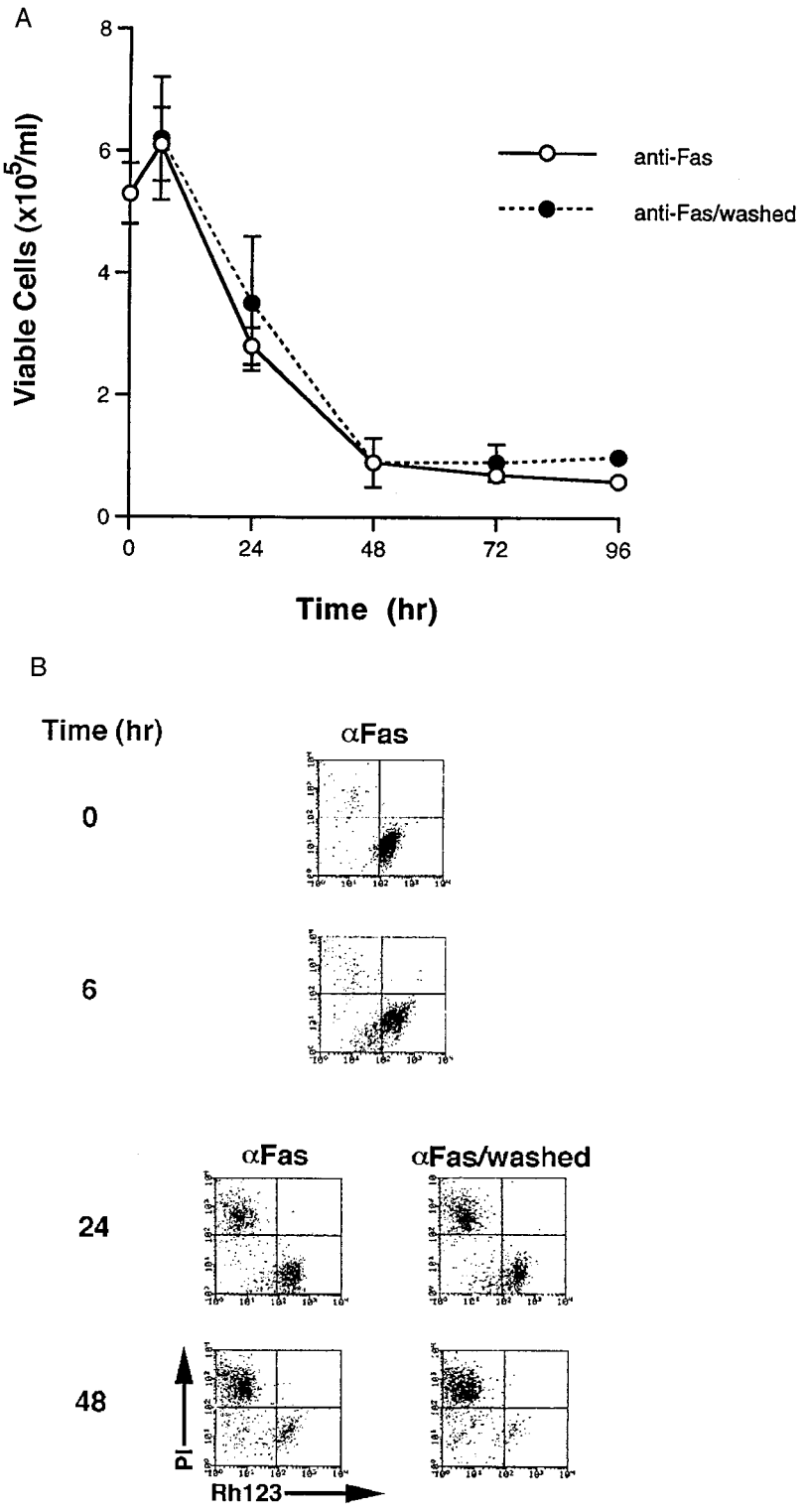

FIG. 4. Commitment to Fas-induced cell death occurs at an early time point relative to loss of viability as measured by $\Delta\Psi_m$ or dye exclusion. (*A*) Jurkat T cells were treated with anti-Fas for 6 hr at which point one-half of the cells were removed, washed three times in fresh medium, and resuspended in fresh medium. The number of total viable cells was determined, as described in Fig. 3, at indicated time points. The cells which remained continually in anti-Fas are represented by open symbols and solid lines while the washed cells are represented by filled circles and dashed lines. The data are presented as the mean ± standard deviation of six independent determinations and are representative of two independent experiments. (*B*) Jurkat T cells were treated as described in *A*, and viability and $\Delta\Psi_m$ were monitored by staining cells with propidium iodide (*y* axis; PI) and rh123 (*x* axis; Rh123) at the indicated time points. The data represent one of three replicate samples from a representative experiment. The data are representative of four independent experiments.

apoptotic cell death in the pro B cell line FL5.12 (data not shown).

One recently proposed mechanism of Bcl-x_L action is the prevention of mitochondrial depolarization (13–17). Mitochondria lose their membrane potential as a result of the opening of permeability transition pores (18). Isolated mitochondria that undergo permeability transition in response to any of a variety of treatments have been shown to release

substance(s) that are capable of inducing apoptotic changes in isolated nuclei (13, 25). Mitochondria isolated from cells overexpressing Bcl-2 are resistant to membrane depolarization in response to oxidizing agents or drugs that uncouple oxidative phosphorylation, but undergo membrane depolarization when treated with calcium ions or the thiol-crosslinking agent diamide (13). To examine mitochondrial depolarization following Fas crosslinking, $\Delta\Psi_m$ was monitored by rh123 fluorescence (15) in Neo and Bcl-x_L cells treated with anti-Fas in the presence and absence of zVAD (Fig. 2). Loss of $\Delta\Psi_m$ was detected in 23.4 ± 0.5% of control transfected cells 6 hr after anti-Fas addition. At 6 hr, the majority of the cells that have undergone a loss of $\Delta\Psi_m$ as measured by decreased rh123 fluorescence still exclude propidium iodide. Because changes in cell membrane permeability have been found to occur prior to the nuclear changes of apoptosis (A. Minn, L.H.B., and C.B.T., unpublished data), these data are consistent with the hypothesis that the loss of $\Delta\Psi_m$ marks an early step in the irreversible phase of apoptosis in response to Fas activation. Over the next 42 hr essentially all of the rest of the control cells underwent a loss of $\Delta\Psi_m$ followed by loss of the ability to exclude propidium iodide. In contrast, the majority of Bcl-x_L transfectants do not undergo a loss of $\Delta\Psi_m$ despite having undergone extensive PARP cleavage as documented in Fig. 1. Even after 48 hr of anti-Fas treatment, 60.6 ± 0.9% of cells have an intact $\Delta\Psi_m$. In both control and Bcl-x_L transfectants the loss of $\Delta\Psi_m$ was prevented by culturing the cells in 50 μM zVAD suggesting that the loss of $\Delta\Psi_m$ is dependent on ICE/Ced-3 protease activation. Based on these data, Bcl-x_L appears to function downstream of ICE/Ced-3 protease activation by preventing loss of $\Delta\Psi_m$.

Although the above data suggest that Bcl-x_L can prevent apoptosis in cells that have undergone Fas-mediated protease activation, it remained possible that Bcl-x_L did not actually prevent death but merely delayed the time it took for cells to undergo the morphologic changes associated with apoptosis. Consistent with this possibility we found that under the conditions in which the previous assays were performed, the absolute number of viable Bcl-x_L-transfected cells failed to increase during extended culture in the presence of Fas antibodies (Fig. 3*A*). One explanation for the failure of Bcl-x_L transfectants to expand under these conditions is the constant presence of the anti-Fas in the culture medium resulting in the loss of viability shown in Fig. 1*A*. To address this possibility, control and Bcl-x_L-transfected cells were cultured with anti-Fas for 6 hr to allow for maximal PARP cleavage and then washed three times to remove the anti-Fas antibodies. After washing, the cells were resuspended in complete medium without anti-Fas and cell viability and cells counts were analyzed daily. The resulting data were compared with that obtained from cells cultured in the continuous presence of anti-Fas. Both the cell counts and cell viability rose daily in the cultures of Bcl-x_L transfectants in which the anti-Fas antibodies were removed after 6 hr (Fig. 3*B*). The number of total viable cells in the washed cultures at 96 hr were only 22.6% less than the number of anti-Fas-treated Bcl-x_L-transfected cells cultured in the continuous presence of zVAD ($19.5 \pm 0.6 \times 10^5$ cells/ml versus $25.2 \pm 1.2 \times 10^5$ cells/ml, respectively, at 96 hr of culture).

One surprising feature of the previous experiment is that while the removal of anti-Fas at 6 hr demonstrated that Bcl-x_L-protected cells remained capable of recovery and proliferative expansion following Fas-induced protease activation, none of the control transfected cells were rescued by removal of anti-Fas at 6 hr of culture. To examine this issue further, additional experiments were performed to directly compare the survival and loss of mitochondrial membrane potential between control cells continuously treated with anti-Fas and control cells treated for 6 hr with anti-Fas (Fig. 4). No difference in the rate or extent of cell death was noted. Surprisingly, although PARP cleavage was complete by 6 hr and the cells were washed free of unbound anti-Fas, 31.2 ± 0.8% of the washed cells had intact $\Delta\Psi_m$ at 24 hr. Nevertheless these cells underwent mitochondrial depolarization and became propidium iodide-permeable over the next 24 hr. These data suggest that the time between initial ICE/Ced-3 protease activation and the final irreversible events of apoptosis may be much longer than previously supposed.

The possibility that Bcl-x_L can function in the cell death pathway downstream of ICE/Ced-3 protease activation is consistent with several previous observations. Although the ICE/Ced-3 protease FLICE/Mach is recruited to the Fas receptor complex and activated by Fas engagement, additional ICE/Ced-3 proteases have been implicated in a proteolytic cascade during Fas-induced cell death (23, 26, 27). Because the various proteases have different kinetics of activation in response to Fas (27), one could envision that the net effects of this cascade may be cumulative rather than immediate. Recent studies have suggested that several of the early targets of ICE/Ced-3 proteases are cellular repair proteins (28). Some investigators have suggested that it is the loss of the homeostatic function of repair enzymes such as PARP or the catalytic subunit of DNA-dependent protein kinase that ultimately leads to the demise of the cell (28, 29). Given the extended period of time between PARP cleavage and the loss of $\Delta\Psi_m$, the exact point at which Bcl-x_L function intervenes is not clear. Bcl-x_L may function to prevent the activation or activity of ICE/Ced-3 proteases downstream of the initial proteases induced by Fas, thus regulating the amplification phase of ICE/Ced-3 protease activation. Alternatively, the structure of Bcl-x_L suggests that it could be a pore-forming protein. Such a protein could regulate organelle homeostasis and prevent the initiation of irreversible changes such as loss of mitochondrial $\Delta\Psi_m$ in response to protease activation. These possibilities may not be mutually exclusive. Loss of mitochondrial $\Delta\Psi_m$ may correlate with the release of cytochrome *c* from the mitochondrial intermembrane space (30). Cytochrome *c* has been reported to be an important cofactor for both the activation of CPP32, a protease thought to act late in the ICE/Ced-3 protease cascade (12, 27), and in the induction of apoptotic changes in isolated nuclei (25). Thus, Bcl-x_L may prevent the release of cytochrome *c* by maintaining mitochondrial homeostasis.

In conclusion, the data presented here demonstrate that activation of ICE/Ced-3 protease(s) do not irreversibly lead to programmed cell death. In addition, Bcl-x_L can function downstream of Fas-induced protease activation to prevent the loss of mitochondrial membrane potential, an early event in the irreversible phase of programmed cell death.

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