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Alphaviruses induce apoptosis in Bcl-2-overexpressing cells: evidence for a caspase-mediated, proteolytic inactivation of Bcl-2

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Bcl-2 oncogene expression plays a role in the establishment of persistent viral infection by blocking virus-induced apoptosis. This might be achieved by preventing virus-induced activation of caspase-3, an IL-1β-converting enzyme (ICE)-like cysteine protease that has been implicated in the death effector phase of apoptosis. Contrary to this model, we show that three cell types highly overexpressing functional Bcl-2 displayed caspase-3 activation and underwent apoptosis in response to infection with alphaviruses Semliki Forest and Sindbis as efficiently as vector control counterparts. In all three cell types, overexpressed 26 kDa Bcl-2 was cleaved into a 23 kDa protein. Antibody epitope mapping revealed that cleavage occurred at one or two target sites for caspases within the amino acid region YEWD^{31}↓AGD^{34}↓A, removing the N-terminal BH4 region known to be essential for the death-protective activity of Bcl-2. Preincubation of cells with the caspase inhibitor Z-VAD prevented Bcl-2 cleavage and partially restored the protective activity of Bcl-2 against virus-induced apoptosis. Moreover, a murine Bcl-2 mutant having Asp31, Asp34 and Asp36 substituted by Glu was resistant to proteolytic cleavage and abrogated apoptosis following virus infection. These findings indicate that alphaviruses can trigger a caspase-mediated inactivation of Bcl-2 in order to evade the death protection imposed by this survival factor.
Keywords: alphaviruses/apoptosis/Bax/Bcl-2/caspase, ICE-like protease/Semliki/Sindbis

Introduction

Programmed cell death (apoptosis) plays a crucial role in the proper embryonic development and later life of multicellular organisms because it removes damaged, expended and misplaced cells (Jacobson *et al.*, 1997). When dysregulated, apoptosis contributes to many diverse human diseases that are either caused by too many unwanted cells, for example cancer and autoimmunity, or by insufficient cell numbers, as seen in neurodegeneration and AIDS (Bellamy *et al.*, 1995). Thus, it has become a major challenge to understand the biochemical and molecular events that control apoptosis in the hope that such knowledge may ultimately be used in the treatment of diseases.

The apoptotic process can be divided into three phases: initiation, effector and degradation (Kroemer, 1997). The initiation phase comprises death stimulus-specific signalling pathways that converge on a common effector phase whose role it is to execute death by degrading various cellular components (Fraser and Evan, 1996). Crucial molecular players in these phases are interleukin 1β-converting enzyme (ICE)-like cysteine proteases—recently renamed cysteine-aspartate proteases (caspases) (Yuan, 1995; Alnemri *et al.*, 1996). These enzymes are activated from zymogens and cleave substrates by proteolysis at aspartate residues (Martin and Green, 1995), thus forming protease cascades similar to those seen in clotting and complement activation. Whereas most caspases participate in death stimulus-specific signalling, a subgroup founded by caspase-3 (formerly CPP32) controls the common death effector phase (Nicholson *et al.*, 1995; Kumar and Lavin, 1996). Importantly, death effector caspases are the closest homologues of ced-3, a caspase that has previously been shown to be essential for apoptosis in the development of the nematode worm *Caenorhabditis elegans* (Shaham and Horvitz, 1996). Numerous substrates of the ced-3/caspase-3 subfamily have so far been identified (reviewed in Martin and Green, 1995), among them poly (ADP-ribose) polymerase (PARP), an enzyme implicated in DNA repair (Lazebnik *et al.*, 1994; Orth *et al.*, 1996).

The caspase death effector machinery is under negative control by members of the Bcl-2 family (Yang and Korsmeyer, 1996). These are homologues of the *C.elegans* ced-9 gene product known to suppress apoptosis during nematodal apoptosis (Shaham and Horvitz, 1996). The prototype of this family is Bcl-2, a proto-oncogene product that was originally identified as overexpressed membrane protein in follicular lymphomas carrying a t(14;18) chromosomal translocation (Cleary *et al.*, 1986). Meanwhile, overexpression of Bcl-2 has been shown to delay or block apoptosis induced by numerous, often unrelated physiological and pathological stimuli (Reed, 1994). How Bcl-2 performs this action is about to be revealed. Through its hydrophobic C-terminus, it is anchored to the outer membranes of mitochondria, the endoplasmic reticulum (ER) and the nucleus (Krajewski *et al.*, 1993), where it may form a cation-selective ion channel (Minn *et al.*, 1997; Schendel *et al.*, 1997), block the release of cytochrome c from mitochondria into the cytosol (reviewed in Kroemer, 1997), and attract cytosolic adapter molecules (reviewed in Reed, 1997). This multifunctional action of Bcl-2 probably serves to prevent the activation of the ced-3/caspase-3 subfamily in response to apoptotic stimuli and thus to suppress the death effector machinery (Boulakia *et al.*, 1996; Chinnaiyan *et al.*, 1996;

Monney *et al.*, 1996). For its activity Bcl-2 requires four protein domains (BH1–4) that are highly homologous between members of the Bcl-2 family. Among them is a 30 amino acid stretch at the N-terminus of Bcl-2, called BH4 (Borner *et al.*, 1994).

There are many examples of viruses that kill cells apoptotically. In most cases, apoptosis is a defence mechanism beneficial for the host because it curtails the infectious cycle and prevents neighbouring cells from being infected with progeny virions (Vaux *et al.*, 1994). However, pathological situations of viral infections exist. At one extreme, the host defence system is circumvented by the presence of anti-apoptotic proteins that are either endogenous to host cells (for example Bcl-2; Levine *et al.*, 1993) or brought into the cells by viruses [viral latent membrane protein-1 (LMP-1) of Epstein–Barr virus, E1B of adenovirus; Henderson *et al.*, 1991; Rao *et al.*, 1992]. In these cases, host cells survive viral infections and persistently produce progeny virions (viral persistence). At the other extreme, viruses provoke pathological lesions because they benefit from the anti-apoptotic properties of the host cells for reproduction but then kill these cells by overcoming their survival potential at a later stage (Ubol *et al.*, 1994).

Our particular interest is in unveiling the molecular mechanisms that govern the survival and death of cells infected by the alphaviruses Sindbis (SIN) and Semliki Forest virus (SFV). Alphaviruses are RNA viruses that can be severe pathogens for a broad range of mammals, including humans (reviewed in Strauss and Strauss, 1994). Following endocytotic uptake by the host cell the viral RNA is delivered to the cytoplasm where first non-structural and then structural proteins are synthesized. The capsid protein is a serine protease that cleaves itself from the nascent structural polyprotein and contributes to the dramatic shut-off of host cell protein synthesis early after infection (reviewed in Strauss and Strauss, 1994; Favre *et al.*, 1996). This allows the production of high titres of progeny virions. Depending on cell type and viral strain, the host cell undergoes apoptosis or develops persistent infection (Levine *et al.*, 1991). While apoptosis involves the activation of caspase-3 and PARP cleavage by so far unknown viral factors (Ubol *et al.*, 1996), viral persistence appears to be due to the overexpression of Bcl-2 or other survival factors that block these activations (Levine *et al.*, 1993).

During the study of the molecular mechanisms underlying viral persistence in Bcl-2-overexpressing cells, we discovered that alphaviruses can actively break this persistence by inducing the cleavage and inactivation of Bcl-2 in a caspase-dependent manner. To our knowledge this is the first time that an apoptotic stimulus such as alphaviruses is shown to exploit caspase proteases to destroy the death-protective action of a survival factor.

Results

Cells overexpressing Bcl-2 are rapidly killed by infection with SIN or SFV

The infection of subconfluent rat 6 (R6) embryo fibroblasts with SFV [multiplicity of infection (m.o.i.) 30] leads to a time-dependent loss of cell adherence (Figure 1A and B) and viability (Figure 2A). To examine whether viability loss could be blocked by the survival factor Bcl-2, we overexpressed this protein in R6 cells (R6-Bcl-2). Unexpectedly, R6-Bcl-2 cells were similarly sensitive to killing by SFV as control counterparts (Figure 1C and D and Figure 2A). This was also true for vector control and Bcl-2-overexpressing U937 monocytes and L929 fibroblasts as well as for the respective pairs of R6 cells infected with SIN (Figure 2). To ensure that Bcl-2 was functional as a survival factor in R6 cells, control and Bcl-2-overexpressing cells were treated with 50 mM NH_4Cl. This agent provoked a loss of control R6 cell viability that was markedly delayed by Bcl-2 overexpression (Figure 2C). Similar death-protection by Bcl-2 has been reported in R6, U937 and L929 cells exposed to numerous other agents that induce apoptosis (Borner, 1996; Monney *et al.*, 1996; Olivier *et al.*, 1997). Thus, in spite of being capable of conferring cell death resistance to other stimuli, Bcl-2 did not provide significant protection from death induced by alphavirus infection.

The death of control and Bcl-2-overexpressing cells induced by SFV exhibits the characteristic hallmarks of apoptosis

To determine the kind of cell death induced by alphaviruses, we analysed the genomic DNA of control R6 and R6-Bcl-2 cells infected with 30 m.o.i. of SFV. As shown in Figure 3, both cell types exhibited a marked, time-dependent cleavage of their DNA into nucleosome-sized fragments, a hallmark of apoptosis (Duvall and Wyllie, 1986). This fragmentation was initially delayed by 4 h in R6-Bcl-2 cells as compared with control R6 cells but occurred as efficiently in both cell types at later time points of infection (Figure 3A and B). Again, the inability of Bcl-2 to prevent DNA fragmentation was a peculiarity of viral infection, since Bcl-2 potently interfered with the degradation when induced by 50 mM NH_4Cl (Figure 3C) or other apoptotic agents (Borner, 1996; Monney *et al.*, 1996; Olivier *et al.*, 1997).

Infection of SFV results in caspase-3 activation and PARP cleavage that cannot be inhibited by Bcl-2 overexpression

It has been reported previously that apoptosis induced by viral infection is preceded by the activation of the death protease caspase-3 and cleavage of its major substrate PARP (Ubol *et al.*, 1996). Moreover, Bcl-2 is known to prevent caspase-3 activation in response to apoptotic stimuli (Boulakia *et al.*, 1996; Chinnaiyan *et al.*, 1996; Monney *et al.*, 1996). We therefore examined whether SFV infection triggered caspase-3 activation in our cells and whether Bcl-2 was somehow defective in interfering with the caspase activation process in this particular case. As shown in Figure 4A, infection of control R6 cells with SFV leads to a time-dependent cleavage of the 32 kDa pro-caspase-3 zymogen into the 17 kDa active protease. Similar kinetics of activation were noted upon SFV infection of control U937 cells (Figure 4B). In addition, simultaneously with caspase-3 maturation, the latter cells displayed a cleavage of the 116 kDa caspase-3 substrate PARP into the 85 kDa fragment (Figure 4B), typically seen in apoptotic cells (Lazebnik *et al.*, 1994; Orth *et al.*, 1996). As found previously for DNA fragmentation, R6-Bcl-2 cells exhibited a delay in caspase-3 activation by 4 h as compared with the respective vector control cells (compare Figure 3A and B and Figure 4). Bcl-2 also

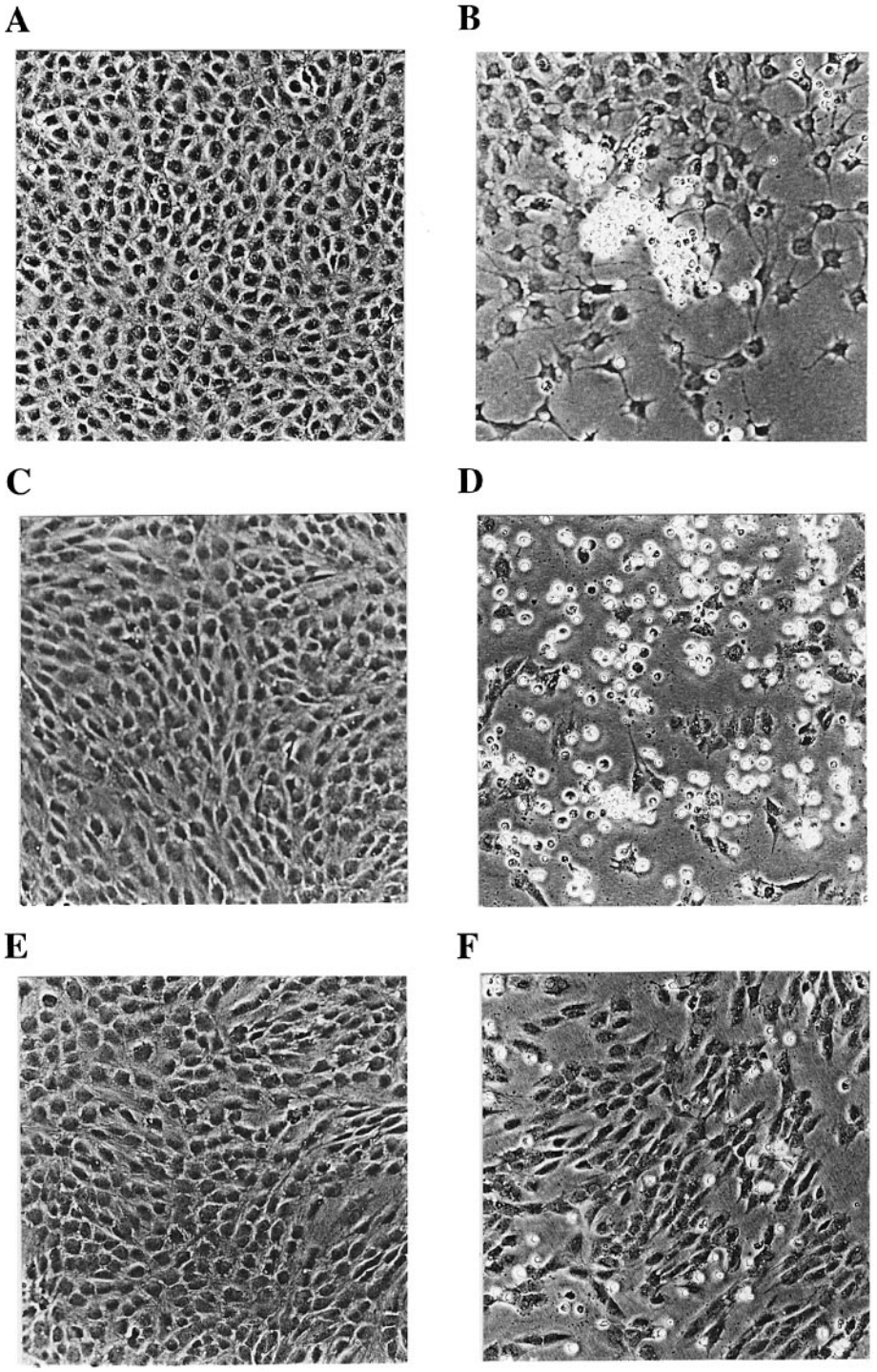

Fig. 1. Phase-contrast microscopy of R6 and R6-Bcl-2 cells following infection with SFV at a m.o.i. of 30. (**A**) Mock-infected R6 control cells; (**B**) SFV-infected R6 control cells; (**C**) mock-infected R6-Bcl-2 cells; (**D**) SFV-infected R6-Bcl-2 cells; (**E**) mock-infected R6-Bcl-2 cells treated with Z-VAD; (**F**) SFV-infected R6-Bcl-2 cells treated with Z-VAD. All pictures taken at 48 h post-infection.

delayed caspase-3 activation and PARP cleavage in U937 cells (Figure 4B). However, neither of these processes could be prevented by Bcl-2 overexpression (Figure 4). By contrast, in both R6 and U937 cells, Bcl-2 markedly intervened with caspase-3 activation and PARP cleavage induced by NH_4Cl (Figure 4A and unpublished data) and other apoptotic agents (Monney *et al.*, 1996 and unpublished data). These results indicate that although Bcl-2 is functional in preventing caspase-3 activation in response to various apoptotic stimuli, it is defective in fully exerting this activity in cells infected with alphaviruses.

Apoptosis induced by SFV and SIN in Bcl-2-overexpressing cells is preceded by proteolytic cleavage of Bcl-2

To identify the deficiency in the death-protective action of Bcl-2 in alphavirus-infected cells we monitored the

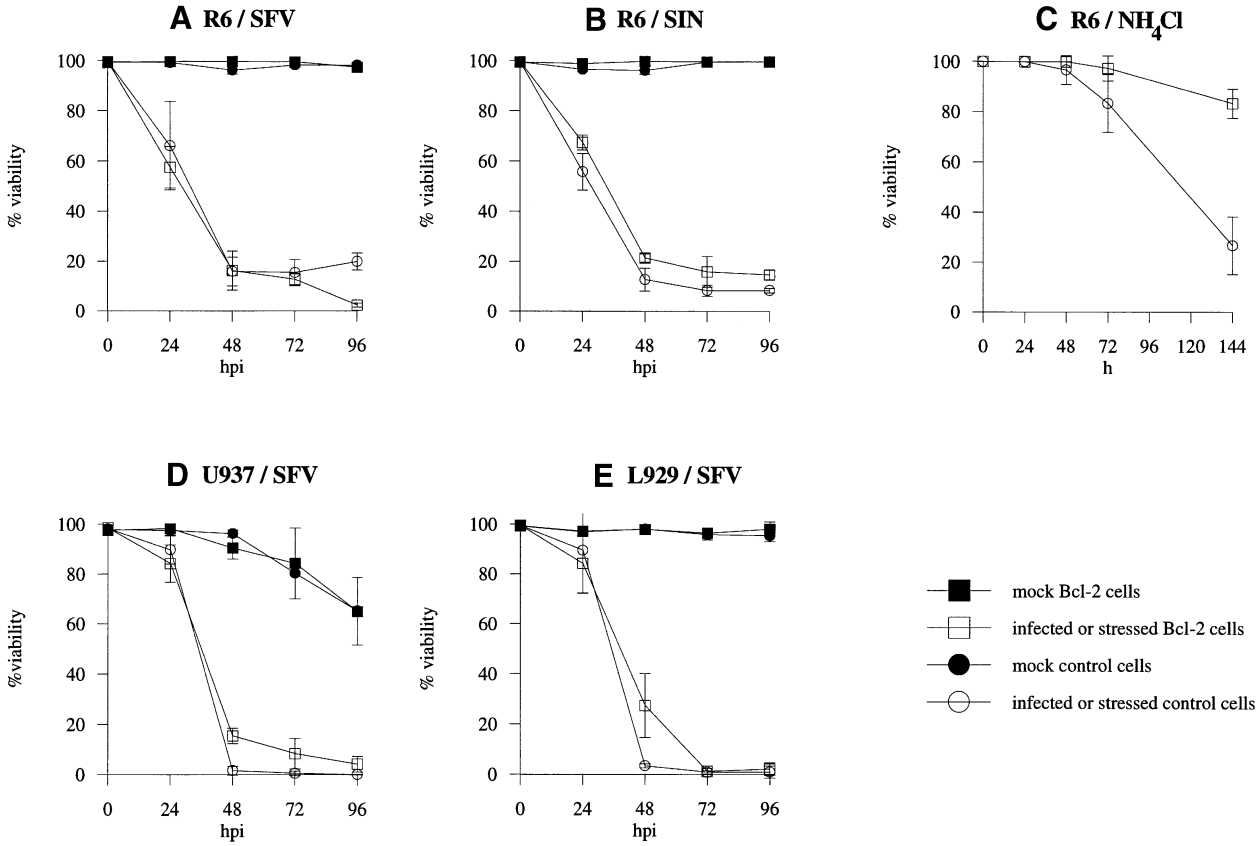

Fig. 2. Cell viability determined by trypan blue exclusion of (**A**) SFV-infected R6 control and Bcl-2 cells, (**B**) SIN-infected R6 control and Bcl-2 cells, (**C**) NH_4Cl-treated R6 control and Bcl-2 cells, and SFV-infected U937 (**D**) and L929 (**E**) control and Bcl-2 cells. Error bars indicate the SEM.

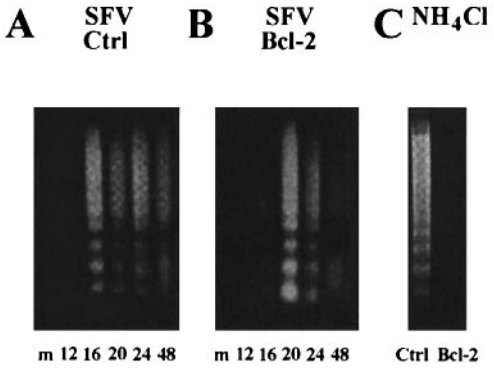

Fig. 3. DNA fragmentation in response to SFV infection. R6 control cells (Ctrl, **A**) and R6-Bcl-2 cells (Bcl-2, **B**) were infected with SFV and DNA fragmentation was determined by 1% agarose gel electrophoresis at serial time points after infection as described in Materials and methods. (**C**) DNA laddering of R6 and R6-Bcl-2 cells stressed with 50 mM NH_4Cl for 48 h. m, mock-infected.

presence and/or integrity of the Bcl-2 protein by Western blot analysis. Bcl-2 is highly expressed as a 26 kDa protein in both R6-Bcl-2 and U937-Bcl-2 cells (Figure 5). Infection of these cells with SFV led to a time-dependent cleavage of the 26 kDa Bcl-2 resulting in a 23 kDa protein (Figure 5A and D). The beginning of the cleavage coincided approximately with the detection of caspase-3 activation and PARP cleavage (16–24 h post-infection) and preceded morphological signs of apoptosis. Similar results were obtained with R6-Bcl-2 infected with SIN (Figure 5C) and L929-Bcl-2 infected with SFV (unpublished data), indicating that the cleavage of Bcl-2 was neither host cell- nor virus-type specific. Importantly, Bcl-2 was not cleaved at any time point following exposure of Bcl-2-overexpressing R6, U937 or L929 cells to NH_4Cl (unpublished data) or other apoptotic agents (Borner, 1996; Monney *et al.*, 1996; Olivier *et al.*, 1997). These findings indicate that infection of eukaryotic cells with alphaviruses, but no other as yet known apoptotic stimuli, activates a protease that cleaves Bcl-2 into a 23 kDa product.

Cleavage of Bcl-2 occurs at membranes and also affects the heterodimeric Bcl-2 partner Bax

Previous subcellular and immunofluorescence analyses have shown that Bcl-2 mainly localizes to the ER/nuclear membranes in R6-Bcl-2 cells (Krajewski *et al.*, 1993 and unpublished data). Consistent with this notion, the 26 kDa Bcl-2 was detected in a nuclear cell extract from which it could be entirely extracted with Nonidet P-40 (NP-40) (Figure 6). This was also the case for the 23 kDa proteolytic fragment of Bcl-2 in SFV-infected cells (Figure 6), indicating that both wild-type and cleaved Bcl-2 were integral membrane proteins on nuclear membranes and that the protease in question probably acted on these membranes. Immunofluorescence analysis of infected R6-Bcl-2 cells confirmed that Bcl-2 did not alter its nuclear/ER membrane localization upon cleavage (unpublished data).

Bax is a pro-apoptotic Bcl-2 homologue that heterodimerizes with Bcl-2. It can be co-immunoprecipitated with Bcl-2 from cell extracts (Oltvai *et al.*, 1993) and co-localizes with Bcl-2 on mitochondria (Zha *et al.*, 1996;

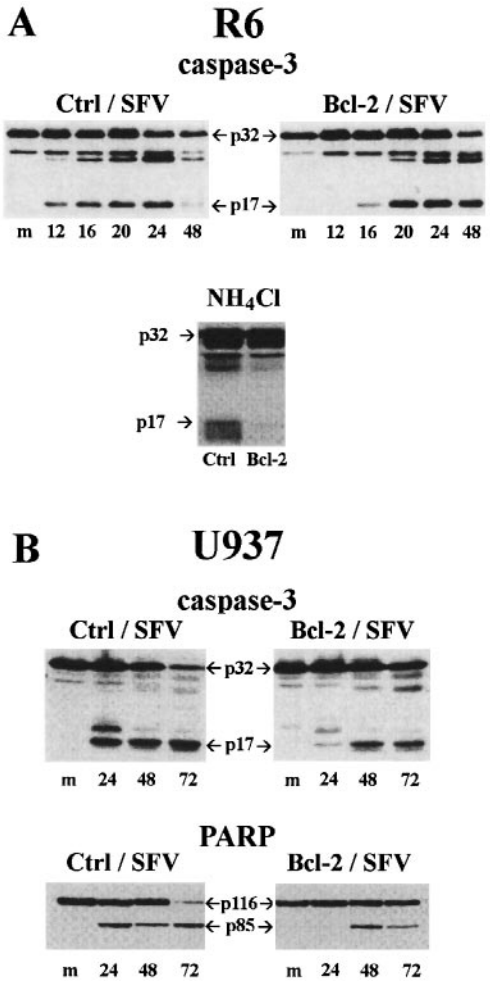

Fig. 4. Time course of the activation of caspase-3 and cleavage of PARP following infection with SFV. (**A**) Anti-caspase-3 immunoblots from total extracts of R6 control (Ctrl) and R6-Bcl-2 (Bcl-2) cells. Caspase-3 activation was also examined by stressing R6 control and Bcl-2 cells with 50 mM NH_4Cl for 48 h. (**B**) Anti-caspase-3 and anti-PARP immunoblots from total extracts of U937 control (Ctrl) and Bcl-2 (Bcl-2) cells. m, mock-infected.

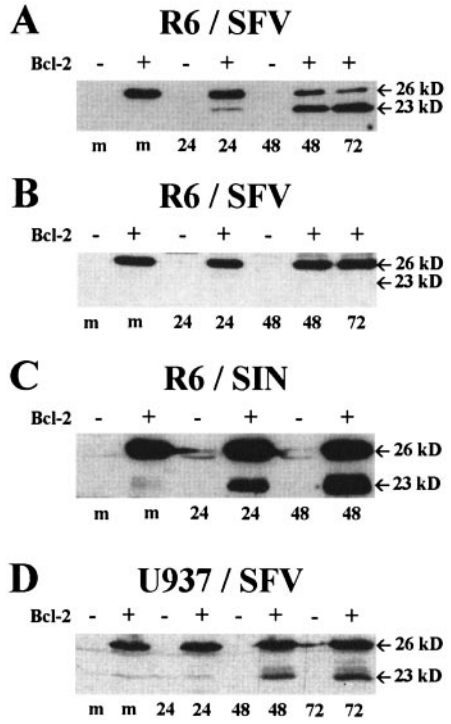

Fig. 5. Time course of the cleavage of Bcl-2 following infection with SFV and SIN, respectively, revealed by immunoblots of total cellular extracts. (**A**) SFV-infected R6 and R6-Bcl-2 cells using an antibody directed against the N-terminal amino acid residues 41–54 of Bcl-2 (27-6). (**B**) SFV-infected R6 and R6-Bcl-2 cells using an antibody directed against the N-terminal amino acid residues 20–34 of Bcl-2 (Ab-2). (**C**) SIN-infected R6 and R6-Bcl-2 cells using the 27-6 antibody. (**D**) SFV-infected U937 and U937-Bcl-2 cells using the 27-6 antibody.

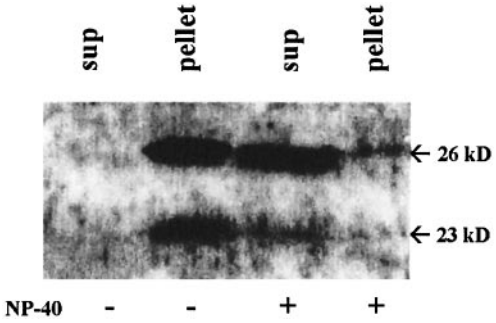

Fig. 6. The truncated 23 kDa Bcl-2 remains membrane bound. The nuclear pellets obtained 48 h post-infection of SFV-infected R6-Bcl-2 cells were treated (+) or not (–) with NP-40 (see Materials and methods) and the supernatants (sup) and pellets (pellet) obtained after centrifugation were subjected to anti-Bcl-2 (27-6) immunoblot analysis.

Rosse *et al.*, 1998), ER and nuclei in intact cells (our unpublished data). In agreement with the notion that a virus-induced protease acted on Bcl-2, endogenous 21 kDa Bax was found to be cleaved into a 18 kDa fragment with similar kinetics to that of Bcl-2 following infection of R6-Bcl-2 cells with SFV (Figure 7A and B). A similar cleavage of Bax has recently been reported in lymphatic cells following treatment with cytotoxic agents (Thomas *et al.*, 1996). Cleavage of Bax was also noted in SFV-infected L929-Bcl-2 (our unpublished data) and SIN-infected R6-Bcl-2 cells (Figure 7C), but not in R6-Bcl-2 cells treated with NH_4Cl (unpublished data) or other apoptotic agents (Borner, 1996; Monney *et al.*, 1996; Olivier *et al.*, 1997). Since Bax cleavage occurred as efficiently in infected vector control and Bcl-2 over-expressing cells (Figure 7), the responsible protease was not attracted to Bax because of Bcl-2 overexpression but by an as yet unknown alphavirus-induced mechanism. Importantly, alphavirus-induced proteolytic cleavage of both Bcl-2 and Bax appeared to be specific, because there was no bulk degradation of total protein in response to virus infection as judged from protein analysis on SDS–PAGE (unpublished data).

Both Bcl-2 and Bax are cleaved at their N-termini during virus-induced apoptosis

Next, we determined the cleavage sites of Bcl-2 and Bax by antibody epitope mapping. Both Bcl-2 and Bax have been shown to insert into membranes via their C-terminal, hydrophobic amino acid sequence. Because the cleaved 23 kDa Bcl-2 (Figure 6) and 18 kDa Bax (unpublished data) were still membrane-bound, we assumed that cleavage occurred at their N-termini. We therefore searched for

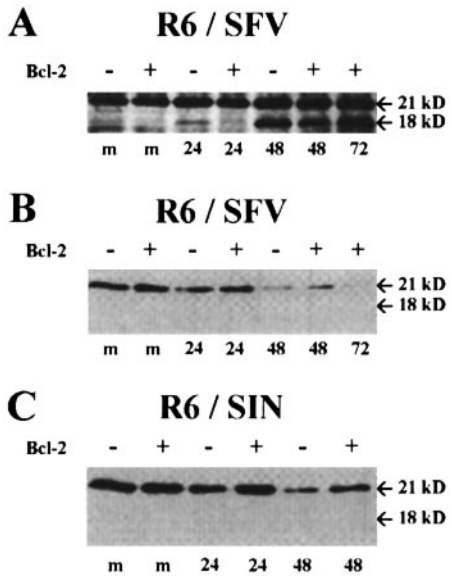

Fig. 7. Time course of the cleavage of Bax following infection with SFV and SIN, revealed by immunoblots of total cellular extracts. (**A**) SFV-infected R6 and R6-Bcl-2 cells using an antibody directed against amino acid residues 43–61 of Bax (Pharmingen). (**B**) SFV-infected R6 and R6-Bcl-2 cells using an antibody directed against the N-terminal amino acid residues 1–21 of Bax (UBI). (**C**) SIN-infected R6 and R6-Bcl-2 cells using the antibody UBI.

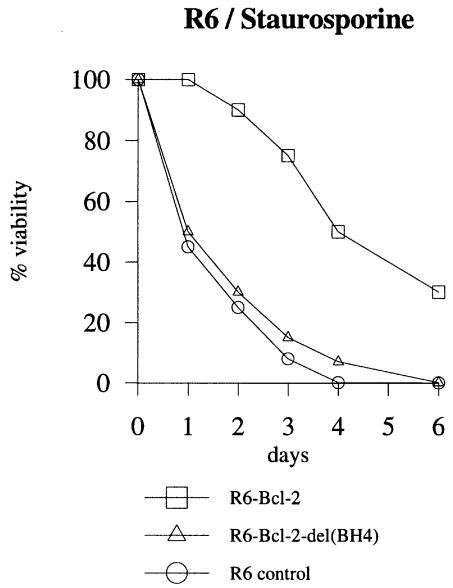

Fig. 8. Time course of the viability of R6, R6-Bcl-2(wt) and mutant R6-Bcl-2ΔBH4 cells, as determined by trypan blue exclusion. Cells were treated with 1 μM staurosporine.

antibodies that reacted with different epitopes in the N-terminal parts of Bcl-2 and Bax. As expected, whereas the polyclonal anti-Bcl-2 antibody 27-6 directed against residues 41–54 of Bcl-2 detected the proteolytic 23 kDa fragment (Figure 5A, C and D), no reactivity of this fragment was seen with the anti-Bcl-2 antibody Ab-2, recognizing amino acids 20–34 (Figure 5B). This indicates that the 23 kDa Bcl-2 was devoid of the N-terminus including the BH4 domain. As shown before in neurones (Borner *et al.*, 1994), a Bcl-2 mutant lacking the BH4 was incapable of protecting R6 cells from apoptosis induced by staurosporine (Figure 8), suggesting that the 23 kDa cleaved Bcl-2 is most likely inactive as a cell survival factor in SFV- and SIN-infected R6-Bcl-2 cells. As with Bcl-2, Bax was cleaved at its N-terminus in both control R6 and R6-Bcl-2 cells infected with SFV. While an antibody reactive against residues 43–61 of Bax detected the 18 kDa Bax fragment (Figure 7A), this was not the case with an antibody towards the first 21 amino acids of the N-terminus (Figure 7B and C). Since it is not yet known whether endogenous Bax plays an active role in toxin- or virus-induced apoptosis, we cannot determine whether the 18 kDa Bax fragment is an active or inactive species. Because this fragment has retained the BH3 region known to be crucial for the pro-apoptotic activity of Bax when overexpressed (Chittenden *et al.*, 1995), it is possible that cleavage of Bax in virus-infected cells does not abrogate its potential cytotoxic activity.

Alphavirus-induced cleavage of Bcl-2 and apoptosis are prevented by the caspase inhibitor Z-VAD

Calculation of the molecular mass of the proteolytic fragments and antibody epitope mapping revealed that a protein domain between approximately amino acids 30 and 40 of mouse Bcl-2 served as cleavage site for proteolysis in response to SFV and SIN infection. The sequence YEWDAGDADAAPL of this region contains three putative target sites for caspases, one for caspase-1 (YEWD$^{31}\downarrow$A), one for caspase-3 (DAGD$^{34}\downarrow$A) and one for another caspase (GDAD$^{36}\downarrow$A) (Figure 9). Since at least caspase-3 (but also maybe other caspases) was activated in response to SFV and SIN infection (Figure 4), we tested whether Bcl-2 cleavage was caspase-mediated and could be prevented by caspase inhibition. As anticipated, treatment of R6-Bcl-2 cells with 100 μM benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD) 12 h before and during infection with SFV (see Materials and methods) completely blocked Bcl-2 cleavage (Figure 10C). Bcl-2 appeared to be functional in these cells because activation of caspase-3 was barely detected following SFV infection (Figure 10B) and the cells were partially protected from virus-induced apoptosis (Figure 10, compare Figure 1D and F). However, since Z-VAD also delayed caspase-3 activation and apoptosis in control R6 cells (Figure 10B), it was impossible to distinguish whether the protective effect of Z-VAD was due to the lack of caspase-3 activation or to the maintenance of functional Bcl-2. This is in agreement with previous reports showing that the death-protective effects of Bcl-2 and Z-VAD are often non-additive because they probably target similar molecules (Boulakia *et al.*, 1996; Chinnaiyan *et al.*, 1996; Monney *et al.*, 1996). Importantly, the effect of Z-VAD on R6 cells was not due to an interference with viral infection, replication and/or progeny production as similar amounts of viral structural proteins and titres of infectious progeny virions were made in the presence or absence of Z-VAD after SFV infection (unpublished data). These findings indicate that R6-Bcl-2 cells may be susceptible to SFV- or SIN-induced apoptosis because the viruses have developed ways to activate a Z-VAD-sensitive caspase(s) that directly or indirectly cleave(s) and inactivate(s) Bcl-2.

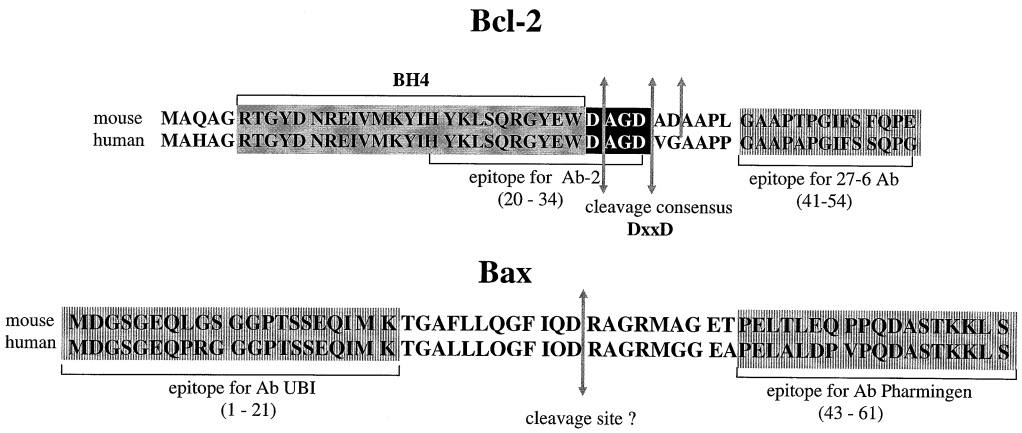

Fig. 9. Putative caspase cleavage sites in Bcl-2 and Bax showing the epitopes of all antibodies described in this study.

Mutation of Asp31, Asp34 and Asp36 to glutamic acid in the BH4 region prevents cleavage of Bcl-2 and abrogates apoptosis

To confirm that alphavirus infection inactivates Bcl-2 by caspase cleavage within the amino acid region YEWDA-GDADAAPL, we changed the caspase target residues Asp31, Asp34 and Asp36 to glutamic acid by site-directed mutagenesis. The mutant Bcl-2 (D31E, D34E, D36E) protein had the same molecular mass (26 kDa) and could be stably overexpressed in R6 cells at similar levels to wild-type Bcl-2 in the R6-Bcl-2 cell line (Figures 5 and 11). However, in contrast to R6-Bcl-2 cells, R6 clones overexpressing the mutant Bcl-2 were resistant to alphavirus-induced apoptosis and did not exhibit cleavage of the mutant Bcl-2 protein at any time following alphavirus infection (Figures 1, 5 and 11). These data clearly show that alphaviruses are capable of killing Bcl-2-overexpressing host cells by a caspase-mediated proteolytic inactivation of the survival factor Bcl-2.

Discussion

The present study shows that the alphaviruses SFV and SIN can kill three different host cell lines by apoptosis in spite of the fact that the cells overexpress Bcl-2. This is because the viruses activate a caspase-dependent pathway to cleave Bcl-2 into a protein which is devoid of its N-terminus and thus inactive in conferring cell death protection. Consistent with this notion, cells overexpressing a Bcl-2 variant mutated at three putative caspase cleavage sites do not exhibit Bcl-2 cleavage and are protected against alphavirus-induced apoptosis. These effects are reproduced by treating R6 cells overexpressing wild-type Bcl-2 with the caspase inhibitor Z-VAD. Our data unravel a so far unanticipated mode of action of viruses to bypass the survival potential of the host: the destruction of a host-encoded survival factor.

Bcl-2 has been shown previously to be susceptible to proteolysis *in vitro*. In one report purified Bcl-2 could be cleaved with trypsin-like, but not caspase-like proteases within the non-conserved, flexible loop region that links the BH4 and BH3 domains (Vance *et al.*, 1996). In another report, a site between the BH3 and BH1 regions of Bcl-2 (Phe112-Ala113) was found to be targeted by the HIV aspartic protease *in vitro* and when the protease and Bcl-2 were co-overexpressed in intact cells (Strack *et al.*, 1996). In both cases, cleavage gave rise to forms of Bcl-2 that were devoid of their essential BH4 and/or BH3 domains and thus most likely inactive in conferring death protection (Strack *et al.*, 1996; Vance *et al.*, 1996). However, no evidence has yet been presented that Bcl-2 is cleaved by trypsin-like proteases in intact cells exposed to apoptotic agents. In addition, although it was reported that the cellular expression level of Bcl-2 dropped in response to HIV infection (Strack *et al.*, 1996), it was unclear whether this was due to proteolysis by the HIV protease or by a down-regulation of Bcl-2 on the transcriptional or post-transcriptional level. In fact, overexpression of Bcl-2 protected against apoptosis induced by HIV infection and the overproduced Bcl-2 protein was not cleaved (Strack *et al.*, 1996). Thus, the data presented here are much different from those previously published and show for the first time that Bcl-2 can be proteolytically cleaved under a physiologically relevant condition: the infection of host cells by alphaviruses.

It is now widely accepted that Bcl-2 functions to prevent the activation of caspases implicated in the execution phase of apoptosis (caspase-3-like enzymes) (Boulakia *et al.*, 1996; Chinnaiyan *et al.*, 1996; Monney *et al.*, 1996) rather than being attacked and/or inactivated by these proteases. Indeed, our analysis on caspase-3 activation and PARP cleavage following alphavirus infection shows that overexpression of Bcl-2 initially delays these events by 4 h as compared with vector control cells consistent with the known function of Bcl-2. However, at later time points, no protection on caspase-3 activation, PARP cleavage or apoptosis was seen in Bcl-2-overexpressing cells and this time period coincided approximately with the proteolytic cleavage of Bcl-2. These results indicate that in addition to activating caspase-3 and the death machinery, alphaviruses can launch a somehow retarded, caspase-dependent pathway that targets and inactivates Bcl-2. Since no other apoptotic stimulus has yet been shown to provoke Bcl-2 cleavage, we propose that the pathway comprises at least one virally encoded factor that is brought into the host cell upon infection.

The infection of mammalian cells with alphaviruses results in a drastic inhibition of host protein synthesis, allowing the efficient intracellular replication of viral progeny (reviewed in Strauss and Strauss, 1994). In most cases viral production is associated with cytopathological changes that ultimately lead to cell lysis within 10–20 h

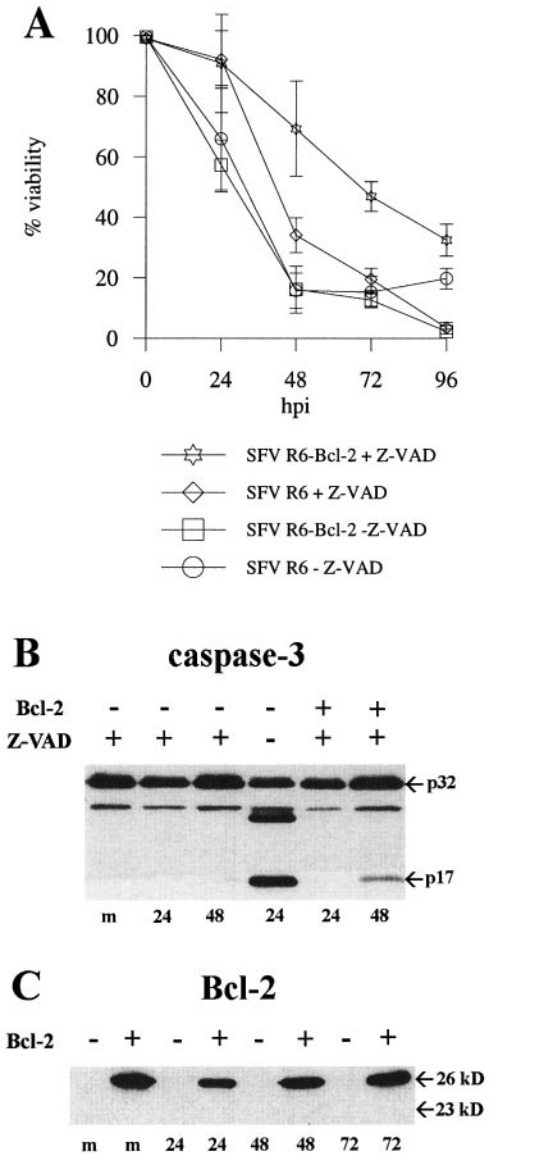

Fig. 10. Effect of Z-VAD on SFV-induced apoptosis and Bcl-2 cleavage in R6 and R6-Bcl-2 cells. (**A**) R6 and R6-Bcl-2 cells were either incubated or not with 100 μM Z-VAD for 12 h prior to and during infection with SFV and viability was determined by trypan blue exclusion. (**B**) Anti-caspase-3 immunoblots of total extracts of SFV-infected R6 control and Bcl-2 cells treated (+) or not (–) with Z-VAD. (**C**) Anti-Bcl-2 (27-6) immunoblots of total extracts of SFV-infected R6 control and Bcl-2 cells treated with Z-VAD. m, mock-infected.

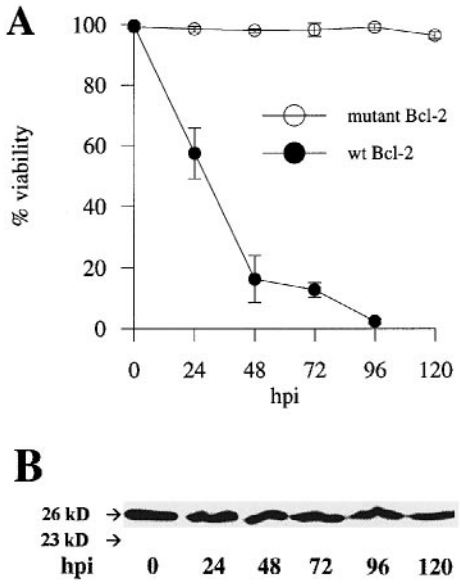

Fig. 11. Effect of caspase cleavage site-mutated Bcl-2 on alphavirus-induced apoptosis and Bcl-2 cleavage. Following infection with SFV (30 m.o.i.), R6 cells overexpressing the mutant Bcl-2 (D31E, D34E, D36E) were tested for cell viability by trypan blue exclusion (**A**) and Bcl-2 cleavage by anti-Bcl-2 immunoblot analysis of total cellular extracts (**B**). The epitope for the anti-Bcl-2 antibody used (27-6) was entirely maintained in the Bcl-2 mutant, i.e. cleaved Bcl-2 should be detected by the antibody.

post-infection (lytic infection) (reviewed in Kääriäinen and Söderlund, 1978). The cytopathological changes resemble apoptosis both biochemically and morphologically (Levine *et al.*, 1993, and this study). The viral factors that trigger the activation of the cell death machinery have, however, not yet been identified. A likely candidate for such an action is the virus-encoded capsid protein. We have previously shown that this protein elicits several biological responses after being synthesized from viral RNA: (i) it is able to shut down protein synthesis at high expression levels by provoking the phosphorylation and activation of the interferon-induced, double-stranded RNA-activated serine/threonine protein kinase (PKR) (Favre *et al.*, 1996), that in turn phosphorylates and inhibits the action of the α-subunit of eukaryotic initiation factor 2α (eIF2α) (reviewed in Merrick, 1992); (ii) it rapidly translocates into the nucleus and associates with nucleolar structures (Michel *et al.*, 1990; Favre *et al.*, 1994); and (iii) it has a chymotrypsin-like activity that acts in *cis* to release itself from the nascent structural polyprotein (reviewed in Strauss and Strauss, 1994). Although it is yet unclear which of these capsid protein activities would contribute to apoptosis, there is evidence for all of them. Firstly, overexpression of PKR has recently been shown to trigger caspase-3 activation and apoptosis in HeLa (Lee and Esteban, 1994) and U937 cells (Yeung *et al.*, 1996) and $PKR^{-/-}$ mice are resistant to apoptosis induced by double-stranded RNA, tumour necrosis factor-α (TNF-α) and lipopolysaccharide (Der *et al.*, 1997). In addition, activated PKR can phosphorylate the NFκB inhibitor IκB, leading to the release and activation of the nuclear transcription factor NFκB, a necessary component for SIN-induced apoptosis (Kumar *et al.*, 1994; Lin *et al.*, 1995). Secondly, the accumulation of the progeny capsid protein in the nucleus following infection with SFV coincides with apoptosis (Michel *et al.*, 1990), suggesting that the capsid protein may have nuclear targets crucial for apoptosis. Thirdly, and most importantly, the capsid protein may use its serine protease activity in *trans* to proteolytically cleave and activate caspases. Caspases are usually activated by autoproteolysis or proteolysis by heterologous caspases at internal aspartic acid residues (Martin and Green, 1995; Alnemri *et al.*, 1996). However, it has recently been reported that the caspase-7 zymogen can also be processed and activated by serine proteases that cleave at amino acids different from aspartic acids (Zhou and Salvesen, 1997). Experiments in our laboratories are in progress to examine whether the activation of caspases in response to alphavirus infection is indeed mediated by the chymotrypsin-like serine protease activity of the capsid protein.

Unfortunately, pretreatments of infected cells with chymotrypsin inhibitors such as tosylphenylalanine-chloromethyl ketone (TPCK) have so far failed to resolve this issue, mainly because such inhibitors are highly toxic to mammalian cells and potentially interfere with the replication of alphaviruses (Zhirnov *et al.*, 1986).

Regardless of how caspases are activated in response to alphavirus infection, at least one of these enzymes appears to be implicated in the cleavage of Bcl-2. The question remains whether this caspase is only one element of a protease signalling cascade or directly interacts with and cleaves Bcl-2. Although definitive proof is still missing, our data support the latter possibility. Based on molecular mass determination and antibody epitope mapping we have been able to define a putative target sequence for Bcl-2 proteolysis that contains three cleavage sites for caspases in mouse Bcl-2 (^{28}YEWD$^{31}\downarrow$AGD$^{34}\downarrow$AD$^{36}\downarrow$AAPL^{40}) and two in human Bcl-2 (^{28}YEWD$^{31}\downarrow$AGD$^{34}\downarrow$VGAAPP^{40}) (Figure 9). Since human Bcl-2 is similarly cleaved in response to alphaviral infection as mouse Bcl-2 (unpublished data), cleavage is unlikely to occur at the mouse-specific GDAD$^{36}\downarrow$A cleavage site. Out of the remaining two cleavage sites, the YEWD$^{31}\downarrow$A sequence may be recognized by caspase-1 (ICE)-like enzymes (YxxD$\downarrow$y; x = any amino acid, y = small hydrophobic amino acid) while the DAGD$^{34}\downarrow$A sequence is a perfect caspase-3/-7 target site (DxxD$\downarrow$y). Preliminary evidence from our laboratory excludes the involvement of caspases-3 and/or -7 in Bcl-2 cleavage. Firstly, Ac-DEVD-CHO, a caspase-3/-7-specific tetrapeptide inhibitor, does not block virus-induced Bcl-2 cleavage even when given to cells before infection, at high doses (up to 100 μM) and/or repetitively during infection (unpublished data). Secondly, Z-VAD has recently been reported to be an inefficient inhibitor of caspases such as caspase-3 and -7 that prefer DxxD$\downarrow$y target sites (Slee *et al.*, 1996). Thirdly, we also detected Z-VAD- but not DEVD-CHO-inhibitable, proteolytic cleavage of Bax, a heterodimeric partner of Bcl-2, following alphavirus infection. As determined by antibody epitope mapping, the only possible caspase cleavage site in Bax is FIQD$^{33}\downarrow$R (Figure 9), an amino acid sequence that does not conform with a classical DxxD$\downarrow$y caspase-3 consensus sequence. We therefore propose that, following alphaviral infection, Bcl-2 is cleaved at YEWD$^{31}\downarrow$A (and Bax at FIQD$^{33}\downarrow$R) by as yet unidentified Z-VAD-inhibitable caspases. Substitution of the Asp31 residue by Glu (in addition to mutating Asp34 and Asp36) indeed creates a Bcl-2 mutant that is resistant to cleavage in response to alphavirus infection. The question remains how viral infection, but no other apoptotic agent so far, manages to target a specific caspase(s) to Bcl-2. We have shown in this report that the cleaved 23 kDa Bcl-2 fragment remains attached to the nuclear/ER membrane, suggesting that Bcl-2 cleavage occurs at the membranes where it functions. Thus, some viral component either activates a caspase that consistently co-localizes with Bcl-2 or guides a caspase into the vicinity of Bcl-2 following infection. Interestingly, a neurovirulent strain of SIN containing a mutation in the E2 glycoprotein was recently shown to overcome the death-protective effect of Bcl-2 and induced apoptosis of Bcl-2-over-expressing host cells (Ubol *et al.*, 1994). It was suggested that E2 may usually interact with Bcl-2 on the ER membrane in order to allow viral persistence and that the mutant E2 may not do so. Alternatively, the mutant E2 may have facilitated the access of caspases to Bcl-2 and triggered Bcl-2 cleavage, a possibility that was not envisaged in the previous study, but is now worth examining.

Contrary to our data here, Bcl-2 was reported to protect effectively against apoptosis induced by the infection with SIN (Levine *et al.*, 1993), SFV (Scallan *et al.*, 1997), vaccinia (Hinshaw *et al.*, 1994), HIV (Antoni *et al.*, 1995; Strack *et al.*, 1996) and reoviruses (Rodgers *et al.*, 1997). We do not know the exact reason for this discrepancy but assume that there might be cell type- or virus-specific differences among the experiments. In their report on the conversion of lytic to persistent alphavirus infection by Bcl-2, Levine *et al.* (1993) used N-18 mouse neuroblastoma and AT-3 rat prostatic adenocarcinoma cells as hosts for SIN infection. These are transformed, tumorigenic cell lines that may differ from the normal fibroblasts used here in the expression, activation or subcellular localization of the caspase(s) involved in Bcl-2 cleavage. In support of the idea of virus-specific differences, Liao *et al.* (1997) have shown that Bcl-2-overexpressing N-18 cells were protected from SIN-induced, but not from Japanese encephalitis virus (JEV)-induced apoptosis. In addition, for SIN infection Levine *et al.* (1993) applied viral loads that were 10–30 times lower than ours (m.o.i. = 1). It may be that in their case, Bcl-2 cleavage did not take place because the caspase-activating viral components were insufficiently expressed or activated. However, we detected no marked difference in viability when R6-Bcl-2 cells were infected with SIN at a m.o.i. of 1 instead of 30 (unpublished data). Irrespective of these experimental differences, high viral loads may occur during infections in natural settings. Our data therefore offer the provocative possibility that, under the latter conditions, viral persistence may not develop because alphaviruses can actively destroy the survival potential of their host. This may be pivotal for the induction of pathogenic lesions by alphaviruses.

Materials and methods

Reagents

Phenylmethylsulfonyl fluoride (PMSF) was purchased from Fluka, and aprotinin and pepstatin from Sigma. The tetrapeptide inhibitor of ICE, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD) was from Bachem, Switzerland. Ac-Asp-Glu-Val-Asp-CHO (Ac-DEVD-CHO) and staurosporine were obtained from Juro Supply, Switzerland. Superfect™ was from Qiagen and the pcDNA3 vector from Invitrogen. Rabbit polyclonal Bcl-2 antibody (27-6) recognizing amino acid residues 41–54 of mouse Bcl-2 has previously been described (Borner *et al.*, 1994). Anti-Bcl-2 N-terminal antibody (Ab-2) recognizing amino acid residues 20–34 of human and mouse Bcl-2 (rabbit polyclonal IgG) was obtained from Oncogene Research Products. Anti-Bax antibody recognizing amino acid residues 1–21 of human, mouse and rat Bax (UBI) was from Upstate Biotechnology Inc., Lake Placid, USA. Polyclonal rabbit anti-Bax antibody, recognizing amino acid residues 43–61 of mouse and rat Bax was from Pharmingen. The rabbit polyclonal anti-caspase-3 antibody recognizing the human, mouse and rat 32 kDa and 17 kDa forms of caspase-3 (Darmon *et al.*, 1995) was kindly provided by Donald W.Nicholson, Merck Frosst, Quebec, Canada. The mouse monoclonal anti-PARP antibody (C-2-10) reacting against human PARP (Lamarre *et al.*, 1988) was a generous gift of Guy Poirier, Laval University, Quebec, Canada.

Mutagenesis of Bcl-2 and transfection into R6 cells

Substitution of Asp31, Asp34 and Asp36 in murine Bcl-2 (mBcl-2) by glutamic acids [Bcl-2 (D31E, D34E, D36E)] was carried out by PCR-based site-directed mutagenesis using the 5′ primer GACCCAAGCTTGGTACCGAGCTCGG encompassing the *Hin*dIII and *Kpn*I multiple cloning site of the pcDNA3 vector upstream of the mBcl-2 cDNA and the 3′ primer CAGGGGCGCCGCCTCCGCTTCTCCAGCTTCCCACTCGTA (mutated nucleotides are underlined) corresponding to Tyr28 to Leu40 of mBcl-2. The 5′ primer contained a *Hin*dIII and the 3′ primer a *Nar*I site in order to subclone the PCR-amplified product into mBcl-2pcDNA3. The resulting Bcl-2(D31E, D34E, D36E) cDNA was purified by CsCl and verified for the correct sequence by dideoxynucleotide sequencing (Microsynth, Switzerland). 2 μg of the mutant Bcl-2 cDNA were transfected into subconfluent R6 cells using 5 μg Superfect™ as described by the manufacturer. G418-selected mixed cell populations and isolated cell clones were analysed for mutant Bcl-2 protein expression by anti-Bcl-2 immunoblotting and high Bcl-2 expressers were used to study Bcl-2 cleavage and death protection in response to alphavirus infection.

Cell lines and culture conditions

Rat 6 embryo fibroblasts (R6) were grown as monolayers in Dulbecco's modified Eagles medium (DMEM) supplemented with 5% inactivated (30 min, 56°C) fetal calf serum (FCS) (Sera Lab, UK). R6 cells constitutively expressing Bcl-2 (R6-Bcl-2), the Bcl-2 mutant devoid of BH4 (R6-Bcl-2ΔBH4) or the Bcl-2 (D31E, D34E, D36E) mutant were grown in the same medium containing 50 μg/ml of hygromycin B (Juro Supply, Switzerland) or G418 (Life Technologies, Switzerland). Vector control L929pMV12 mouse fibroblasts and U937pMEP human premonocytic cells as well as their Bcl-2-overexpressing counterparts (L929-Bcl-2 and U937-Bcl-2) were cultured in RPMI 1640 medium containing 50 μg/ml hygromycin B and supplemented with 5% FCS (Borner, 1996). The cells were infected with SFV or SIN at m.o.i. ranging from 1 to 30, or treated with the apoptotic agents NH_4Cl (50 mM) or staurosporine (1 μM).

Viability assays and virus production

The various R6, U937 and L929 cell derivatives were plated in 6 cm diameter Petri dishes and infected at a m.o.i. of 30. Their viability was determined by trypan blue exclusion at serial time points after infection. With increasing time following infection, R6, R6-Bcl-2, L929pMV12 and L929-Bcl-2 cells gradually detached from the monolayers. Viability was determined by pooling the floating cells with the adherent cells. All experiments were repeated at least three times with similar results. For virus production, Vero cells were plated in 96-well plates and virus titres were calculated according to Kärber (1931).

DNA extraction and electrophoresis

The same number of R6 and R6-Bcl-2 cells were plated on 10 cm diameter culture plates and infected with SFV at a m.o.i. of 30. At several time points following infection, adherent and floating cells were pooled, washed in ice-cold phosphate-buffered saline (PBS) and lysed by incubation on ice for 10 min in 200 μl lysis buffer (5 mM Tris–Cl, pH 8, 10 mM EDTA, 0.5% Triton X-100). Proteins were extracted twice with 200 μl Sevag (phenol:chloroform:isoamylalcohol, 25:24:1). The aqueous phase containing nucleic acids was treated with 0.1 mg/ml RNase at 37°C for 30 min and then loaded onto a 1% agarose gel containing ethidium bromide to visualize DNA fragmentation by UV illumination. NH_4Cl-treated cells were subjected to the same procedure.

Phase-contrast microscopy

Microscopy was performed with a Nikon microscope and photographs were taken using a 35 mm film (Ilford HP5 plus 400 ASA).

Immunoblot analysis

Western blot analyses were performed under denaturing conditions. Samples for SDS–PAGE analysis were prepared by the addition of Laemmli buffer and boiling for 5 min. Equal amounts of protein were subjected to electrophoresis in 12.5% SDS–PAGE, transferred onto Immobilon-P polyvinylidenedifluoride (PVDF) membrane (Millipore) and reacted with the respective antibodies in 1% non-fat dry milk in TTBS (20 mM Tris–Cl, pH 7.5, 500 mM NaCl, 0.05% Tween-20). Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse sera (Sigma, Switzerland), using the Supersignal™ substrate kit (Pierce, Socochim) as detection reagent.

Subcellular fractionation

For total protein extracts, detached and adherent cells were pooled and directly lysed in Laemmli buffer followed by boiling for 5 min. To prepare nuclear and cytosolic fractions the following protocol was used. After infection with SFV (m.o.i. 30) of ~8×10^6 R6-Bcl-2 cells for 48 h, cells were scraped and centrifuged at 1200 *g* for 8 min at room temperature. The pelleted cells were resuspended in PBS and recentrifuged. The washed cells were resuspended in RSB buffer (100 mM Tris–Cl, pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$), containing the protease inhibitors PMSF (200 μM), pepstatin (1 μg/ml) and aprotinin (10 μg/ml), and kept on crushed ice for 30 min. The cells were then passed 10 times through a 26G needle and the cytoplasm was separated from the nuclei by centrifugation at 4000 *g* at 4°C for 10 min in a Heraeus table-top centrifuge. The isolated nuclei were aliquoted and resuspended either in RSB alone or RSB containing 1% NP-40. The samples were kept on ice on a rocking platform for 30 min. The untreated and NP-40-treated nuclei were centrifuged at 4000 *g* at 4°C for 10 min and the nuclear pellets as well as their supernatants were subjected to anti-Bcl-2 (27-6) immunoblotting as described above.

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Note added in proof

During the reviewing process a paper by Cheng *et al*. (1997) was published, showing that Bcl-2 was cleaved at Asp34 in response to Fas-induced apoptosis.