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Inhibition of apoptosis and clonogenic survival of cells expressing crmA variants: optimal caspase substrates are not necessarily optimal inhibitors

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To study the role of various caspases during apoptosis, we have designed a series of caspase inhibitors based on the cowpox virus cytokine response modifier A (crmA) protein. Wild-type crmA inhibits caspases 1 and 8 and thereby protects cells from apoptosis triggered by ligation of CD95 or tumour necrosis factor (TNF) receptors, but it does not protect against death mediated by other caspases. By replacing the tetrapeptide pseudosubstrate region of crmA (LVAD) with tetrapeptides that are optimal substrates for the different families of caspases, or with the four residues from the cleavage site of the baculovirus protein p35 (DQMD), we have generated a family of caspase inhibitors that show altered ability to protect against cell death. Although DEVD is the optimal substrate for caspase 3, crmA DEVD was degraded rapidly and was a weaker inhibitor than crmA DQMD, which was not degraded. Unlike wild-type crmA and crmA DEVD, crmA DQMD was able to inhibit apoptosis caused by direct activation of caspase 3 and protected lymphoid cells from death induced by radiation and dexamethasone. Significantly, the protected cells were capable of sustained growth.

Keywords: apoptosis/caspase/crmA/serpin

Introduction

Apoptosis is the physiological mechanism that removes unwanted and potentially harmful cells (Jacobson *et al*., 1997). The key effector proteins of apoptosis are a family of cysteine proteases termed caspases (Alnemri *et al*., 1996) which cleave their substrates at specific sites defined by a four amino acid sequence (P_4–P_1) where the P_1 is an obligatory aspartate residue (Nicholson and Thornberry, 1997).

While the *Caenorhabditis elegans* caspase Ced-3 is required for all programmed cell deaths in *C.elegans* (Ellis and Horvitz, 1986), mammals have many different caspases, some of which are necessary for apoptosis during development (Kuida *et al*., 1996, 1998; Hakem *et al*., 1998). To date, 13 mammalian caspases have been identified that can be grouped according to their primary structure, the nature of their pro-domains or by their substrate specificity (Table I).

To become activated, the caspase precursor polypeptides must themselves be cleaved at particular aspartate residues and assembled into the mature form. Cleavage of the caspase precursors can be autocatalytic or can be mediated by other mature caspases, leading to a cascade of caspase activation. For example, caspase 8 and caspase 9 can cleave and activate pro-caspase 3 (Schlegel *et al*., 1996).

As apoptosis can be used as a defensive strategy to prevent virus replication, it is not surprising that many viruses encode inhibitors of apoptosis (Vaux, 1993). The cytokine response modifier A (crmA) protein from cowpox virus (Ray *et al*., 1992) is able to bind to and inhibit caspases 1 and 8 and so inhibit production of interleukin-1β as well as apoptosis triggered by ligation of CD95 (Fas/APO-1) or tumour necrosis factor (TNF) receptors (Muzio *et al*., 1996; Zhou *et al*., 1997).

Structurally, crmA resembles members of the serine protease inhibitor (serpin) family, and like them inhibits proteases by acting as a pseudosubstrate (Ray *et al*., 1992). The pseudosubstrate site of crmA contains the residues LVAD. Most serpins are cleaved by their target proteases, undergo a change in conformation and form a stable complex (Whisstock *et al*., 1998). *In vitro*, wild-type crmA is cleaved by caspase 1 (Komiyama *et al*., 1994; Xue and Horvitz, 1995) but not by the *C.elegans* caspase Ced-3 (Xue and Horvitz, 1995). Mutation of the LVAD site of crmA to a Ced-3 cleavage site allowed crmA to be cleaved by Ced-3 and inhibited some developmental cell death in *C.elegans* (Xue and Horvitz, 1995).

The baculovirus *Autographa californica* nuclear polyhedrosis virus encodes the gene for another caspase inhibitor, p35 (Clem *et al*., 1991). p35 can block a broader range of caspases than crmA, and can inhibit caspases from insects, nematodes and mammals. Like crmA, p35 also acts as a competitive caspase inhibitor but it is not structurally a serpin. The pseudosubstrate region for p35 has the residues DQMD (Bump *et al*., 1995; Xue and Horvitz, 1995).

While crmA is a potent inhibitor of apoptosis induced by the TNF family of receptors, it does not inhibit efficiently apoptosis triggered in other ways, such as by serum withdrawal, γ-irradiation or treatment with chemotherapeutic drugs (Smith *et al*., 1996; Newton *et al*., 1998). Apoptosis induced by such stimuli can be inhibited by Bcl-2 and presumably proceeds by a pathways not involving caspase 1 or caspase 8 (Strasser *et al*., 1995).

Increased apoptosis has been implicated as a major contributor to the pathophysiology of several human diseases, including neoplasia, autoimmune disease, infectious disease, hypoxic–ischaemic injury and neurodegenerative disorders (Strasser *et al*., 1991, 1997; Barr and Tomei, 1994). Specific caspase inhibitors may help determine whether anti-apoptotic drugs would be useful in treating such diseases. However, for inhibition of caspases to be a useful therapeutic strategy, inhibition of cell death should allow the long-term survival of functional cells.

Here we have replaced the pseudosubstrate region of crmA with residues designed to be optimal substrates for each class of caspase (Thornberry *et al*., 1997). Two such constructs, crmA DQMD and crmA LEAD, showed altered specificity and afforded significantly greater protection against certain death stimuli than wild-type crmA. Although DEVD is the optimal peptide substrate for caspase 3, crmA DEVD was degraded rapidly in cells undergoing apoptosis, suggesting that caspase-inhibitory drugs based on the preferred substrates may not necessarily be the most effective. Moreover, cells which were rescued by inhibition of caspase activity were able to proliferate, suggesting that cell death was not merely delayed but was prevented.

Results

Generation of crmA variants

To generate caspase-specific inhibitory genes, the pseudosubstrate region of crmA (LVAD) was replaced with the preferred cleavage sites for each class of caspase (Thornberry *et al*., 1997) (Figure 1). For experiments in *Schizosaccharomyces pombe*, the variants were inserted into pURAS vector.

Table I. Mammalian caspases

Caspase	Phylogenetic group	Prodomain	Optimal peptide substrates
caspase 5	ICE-like	CARD	WEHD
caspase 4	ICE-like	CARD	WEHD
caspase 1	ICE-like	CARD	WEHD
caspase 7	CPP32-like	short	DEVD
caspase 3	CPP32-like	short	DEVD
caspase 6	CPP32-like	CARD	VE(H/V)D
caspase 8	FLICE-like	DED	(L/D)E(T/V)D
caspase 10	FLICE-like	DED	
caspase 2	Ced-3 like	CARD	DEHD
caspase 9	Ced-3 like	CARD	LEHD

Phylogenetic classification (Alnemri *et al*., 1996) is based on similarities in peptide sequence. Prodomains may contain motifs such as the caspase recruitment domain (CARD) (Hofmann *et al*., 1997) or the death effector domain (DED) which allow association with adaptor molecules such as Apaf-1 and FADD (Chinnaiyan *et al*., 1995; Zou *et al*., 1997). The optimal peptide substrates were determined by Thornberry *et al.* (1997).

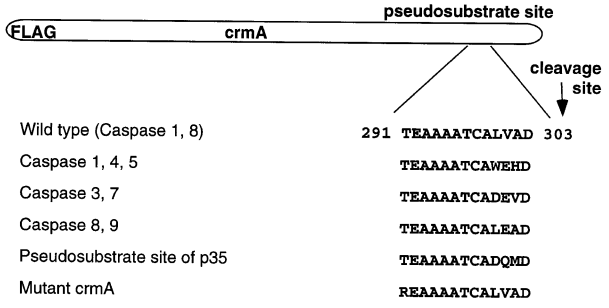

Fig. 1. CrmA constructs and their predicted targets. CrmA was cloned into a vector in-frame with an N-terminal FLAG epitope tag. Site-directed mutagenesis was used to replace the pseudosubstrate region of crmA (residues 299–303, LVAD) with the optimal substrates (underlined) for each class of caspase. A loss-of-function crmA (291T→R) was used as negative control. All constructs were validated by sequencing.

Production of an auto-activating caspase 3 gene

Ectopic overexpression of pro-caspase 3 in mammalian cells does not induce apoptosis because it is unable to undergo autocatalytic activation (Srinivasula *et al*., 1998). However, when the caspase 3 cDNA was joined in-frame to the coding regions for *Escherichia coli* β-galactosidase (LacZ), the fusion protein was able to autoactivate when expressed in mammalian and yeast cells (Figures 2 and 3) probably by inducing spontaneous multimerization and autoactivation. Consistent with its ability to activate in the absence of other caspases, caspase 3–LacZ was toxic to the yeast *S.pombe* whereas caspase 3 alone, and a catalytically inactive mutant (C163G) caspase 3–LacZ, was not (Figure 2). Further, these data show that caspase 3–LacZ kills *S.pombe* in a caspase-dependent manner.

CrmA DQMD can inhibit caspase 3-induced cell death

The ability of the crmA variants to inhibit caspase 3-induced death was tested in both yeast and mammalian cells. Killing of yeast by active caspase 3 was inhibited by the crmA DQMD variant more efficiently than by any other crmA variant or by wild-type crmA (Figure 2). As a negative control, a catalytically inactive caspase 3–LacZ fusion protein allowed normal growth, observed in six independent clones. Only the crmA DQMD variant was able to block caspase 3-induced apoptosis in mammalian cells (Figure 3C). While cells expressing caspase 3–LacZ and wild-type crmA exhibited apoptotic morphology, cells transfected with caspase 3–LacZ and crmA DQMD stained blue and had normal morphology (Figure 3A and B). Furthermore, mutant caspase 3–LacZ did not induce apoptosis in transiently transfected mammalian cells, either alone or transfected with the crmA variants (data not shown).

Unlike crmA DQMD, Bcl-2 could not inhibit apoptosis caused by activated caspase 3 (Figure 3C, lane 5), suggesting that Bcl-2 functions at a step(s) upstream of caspase 3 activation.

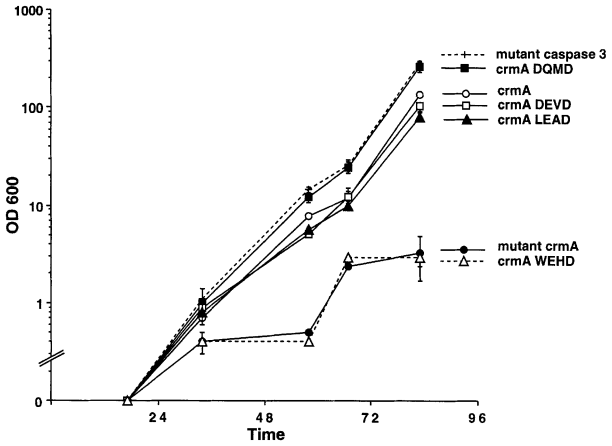

Fig. 2. Expression of caspase 3–LacZ in *S.pombe* is toxic, but can be inhibited by crmA DQMD. Yeast clones stably expressing caspase 3–LacZ or a catalytically inactive (C163G) mutant under a thiamine-repressible promoter were transfected with constructs encoding each of the crmA variants. Removal of thiamine from the growth media induced caspase 3 expression. Yeast growth was inhibited as the caspase levels increased. Cell number was determined by measuring OD_{600}. Mean growth ± SD of six independent clones expressing each of the crmA variants as well as mutant caspase 3–LacZ is shown.

Inhibition of DEVD cleavage activity in stably transfected cells

The crmA variants were transfected into WEHI-7 cells, a murine thymoma cell line which undergoes apoptosis in response to treatment with dexamethasone or γ-irradiation (Flomerfelt and Miesfeld, 1994), and at least three independent clones stably expressing high levels of these proteins were selected. Typical examples of protein expression in clones of each crmA variant as determined by flow cytometry and by Western blotting are shown (Figure 4A and B).

To determine the effect of the crmA variants on caspase activity in stable cell lines, WEHI-7 clones expressing either wild-type crmA, crmA variants or Bcl-2 were treated with 1 μM dexamethasone and then incubated with Phiphilux, a rhodamine-based substrate with a DEVD peptide backbone which fluoresces when cleaved (Oncoimmunin). After 24 h, dexamethasone induced an increase in fluorescence, indicating induction of DEVD cleavage activity. This experiment was repeated several times using three independent clones of each construct. A typical experiment is shown (Figure 5). Induction of fluorescence was reduced in clones expressing Bcl-2, crmA DQMD and partially in crmA LEAD. Presumably Bcl-2 inhibited DEVD cleavage activity by blocking events upstream of caspase activation, whereas the crmA DQMD variant acted as a direct inhibitor of DEVD cleavage activity. CrmA LEAD also significantly suppressed DEVD cleavage activity at 24 h, but death was not significantly reduced in these lines after 48 h of exposure to dexamethasone (Figure 6A).

Protection of cells stably expressing crmA variants

Parental WEHI-7 cells and those expressing wild-type crmA were sensitive to dexamethasone and γ-irradiation, whereas lines expressing Bcl-2 were protected (Figure 6A and B). CrmA DQMD provided partial protection against apoptosis caused by both stimuli. CrmA LEAD afforded partial protection against radiation-induced apoptosis in WEHI-7 cells but did not block dexamethasone-induced apoptosis (Figure 6A and B).

Wild-type crmA is known to inhibit CD95-mediated cell death (Strasser *et al*., 1995; Tewari and Dixit, 1995). To test whether the crmA variants were also able to do so, they were stably expressed in the CD95-sensitive lymphoid line SKW-6. Both crmA DQMD and crmA LEAD were able to block CD95-mediated cell death at least as well as wild-type crmA (Figure 6C), whereas crmA DEVD and mutant crmA no longer protected.

Western blot analysis of the clones showed that all clones expressed similar levels of the crmA variant proteins, and that treatment with dexamethasone caused degradation of the protein concomitant with the induction of apoptosis. Only the crmA DQMD protein, the most efficient inhibitor of dexamethasone-induced apoptosis, remained intact (Figure 7).

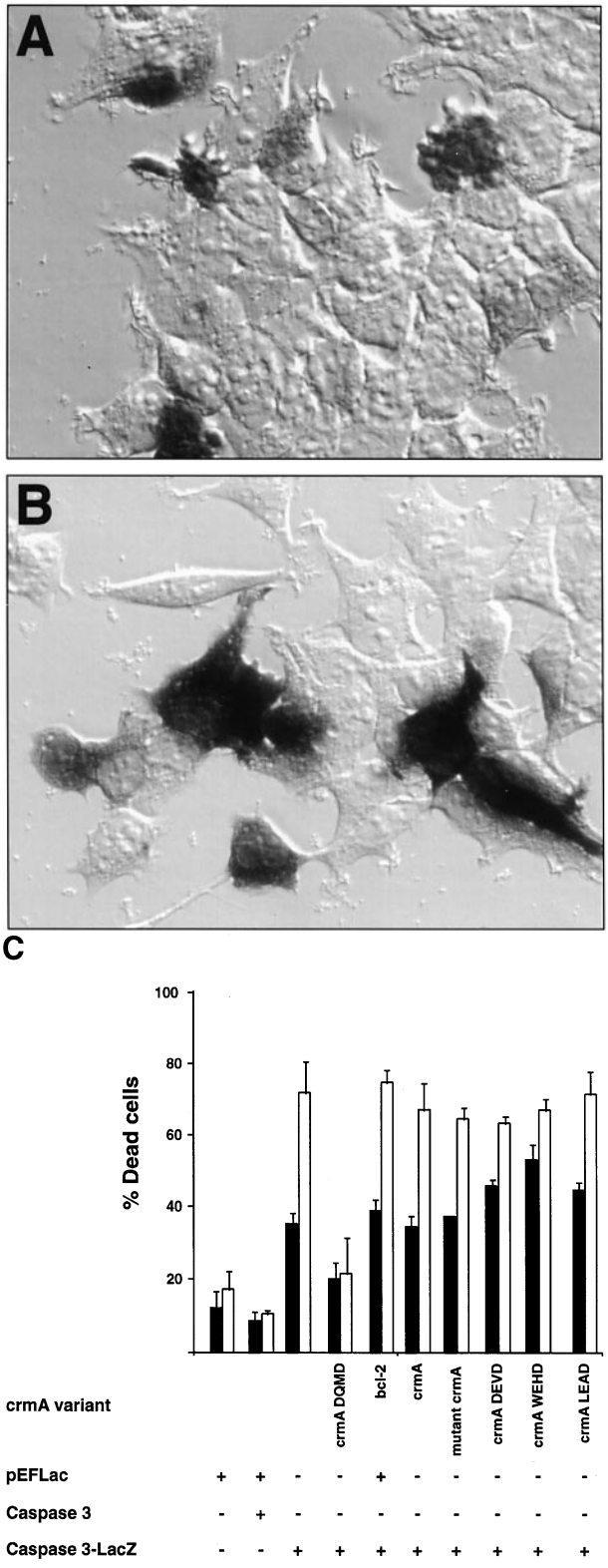

Fig. 3. Caspase 3–LacZ fusion protein expressed in mammalian cells is able to autoactivate and induce apoptosis, which can be inhibited strongly by crmA DQMD. The caspase 3–LacZ fusion gene was transiently expressed in 293T cells in the absence (**A**) or presence (**B**) of plasmid encoding crmA DQMD plasmid. The cells were stained with X-gal to indicate cells that expressed the caspase 3–LacZ fusion gene, and the proportion of apoptotic blue cells was determined by their morphology by a blinded observer. The results of similar experiments using other crmA variants assayed at 24 h (filled bars) and 48 h (empty bars) were quantitated and are shown in (**C**). Error bars indicate ± 2 SEM. Each transfection was done in triplicate and scored blinded. The results show a typical experiment. This experiment has been repeated on several occasions.

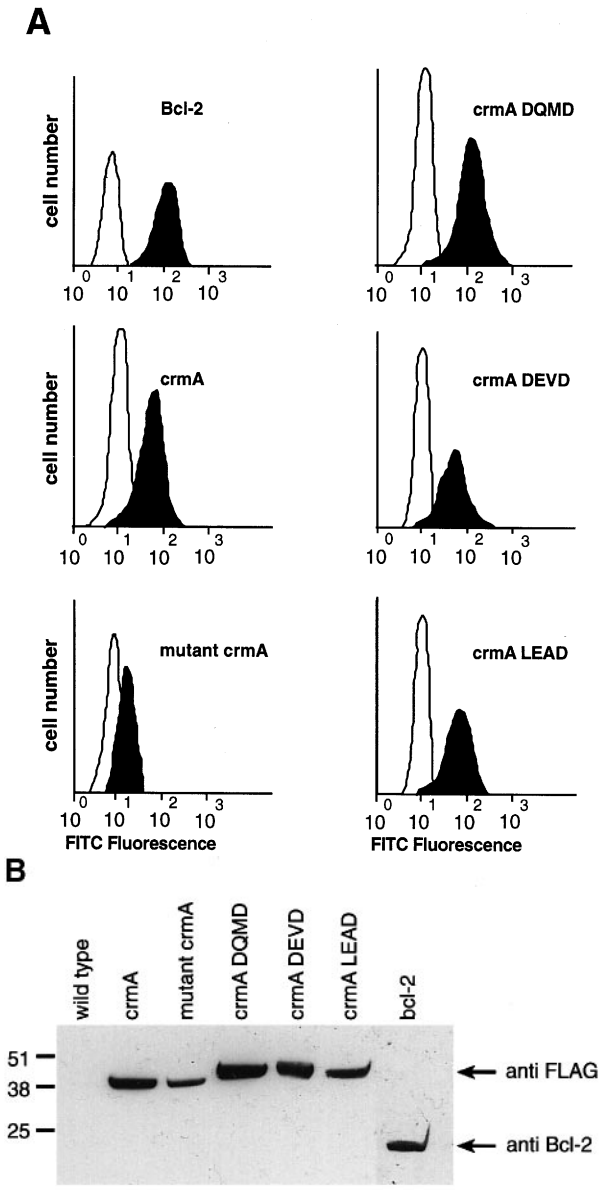

Fig. 4. Stable expression of crmA variants in WEHI-7 cell lines. (**A**) Flow cytometric analysis of cytoplasmic staining using anti-FLAG antibodies on WEHI-7 cells bearing crmA variant plasmids (black histograms). Four independent clones were analysed for each group, and a typical one is depicted in each case. White histograms show staining of parental cells. (**B**) Western blot analysis of WEHI-7 cells expressing crmA variant plasmids.

Clonogenic survival of cells protected by caspase inhibitory genes

As some models of apoptosis suggest activation of caspase 3 occurs following rupture of the mitochondria (Vanderheiden *et al.*, 1997), we wished to determine whether inhibition of caspase 3 was able to rescue cells fully, or whether it merely delayed the death of cells that would eventually succumb from loss of mitochondrial activity. To do this, we performed clonogenic assays.

WEHI-7 lines expressing the crmA variants or Bcl-2 were irradiated, and then cultured in soft agar to determine their ability to form colonies (Figure 8). Bcl-2 and crmA DQMD, and to a lesser degree crmA LEAD, were not only able to inhibit apoptosis, but the rescued cells retained the ability to proliferate.

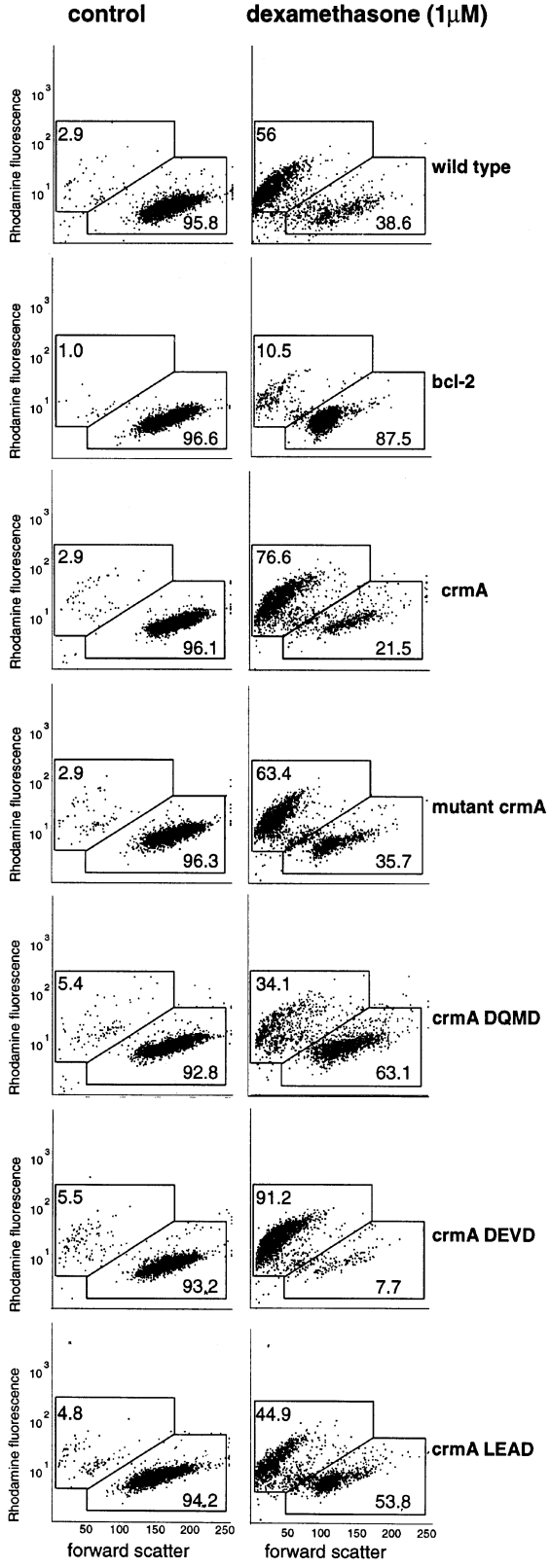

Fig. 5. Inhibition of DEVD cleavage activity by crmA variants. WEHI-7 lines were incubated with 1 μM dexamethasone for 24 h and analysed on the basis of size (forward scatter, *y*-axis) and caspase activity (Phiphilux fluorescence, *x*-axis). The percentage of apoptotic cells/cells with DEVD cleavage activity (upper boxed region) and non-apoptotic cells (lower boxed region) are shown. Results show a typical experiment which has been repeated with three independent clones for each construct.

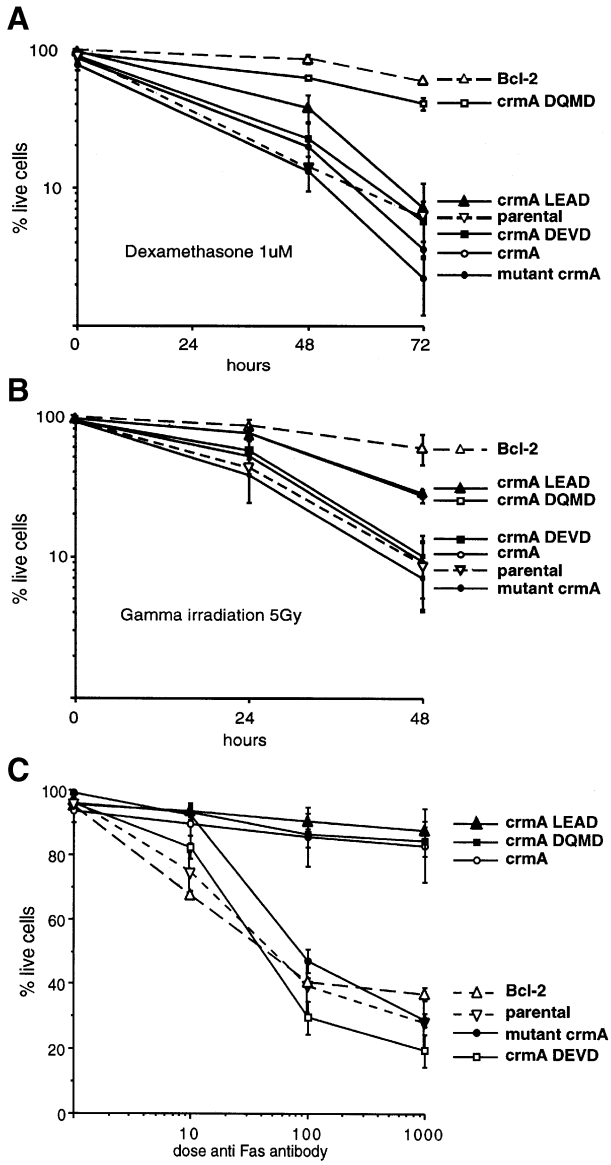

Fig. 6. CrmA DQMD protects cells against apoptosis induced by dexamethasone (**A**), and CrmA DQMD and crmA LEAD protect against γ-irradiation (**B**), but wild-type crmA does not. WEHI-7 cell lines (three or four independent lines for each construct) expressing the crmA variants or Bcl-2 were exposed to 1 μM dexamethasone or γ-irradiation. Cells were analysed for plasma membrane integrity by exclusion of PI (live cells). Wild-type crmA, crmA DQMD and crmA LEAD protect cells against death induced by ligation of CD95, but crmA DEVD does not (**C**). Cells stably expressing wild-type crmA, crmA variants or Bcl-2 (three or more independent clones for each construct) were treated with antibodies to CD95. Cells were analysed for membrane integrity by exclusion of PI (live cells) at 48 h. Values shown in (A), (B) and (C) represent the means of four separate experiments. Error bars show ± 2 SEM.

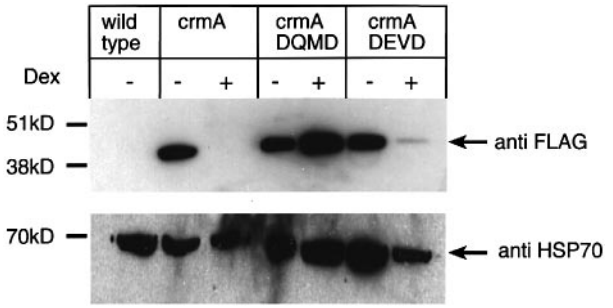

Fig. 7. Wild-type crmA and crmA DEVD are degraded rapidly in cells undergoing apoptosis, whereas crmA DQMD resists cleavage. Total cell lysates from WEHI-7 lines treated for 24 h with 1 μM dexamethasone were separated on SDS–PAGE and probed with anti-FLAG antibody. The same blot was stripped and reprobed with an antibody to HSP-70 as a loading control.

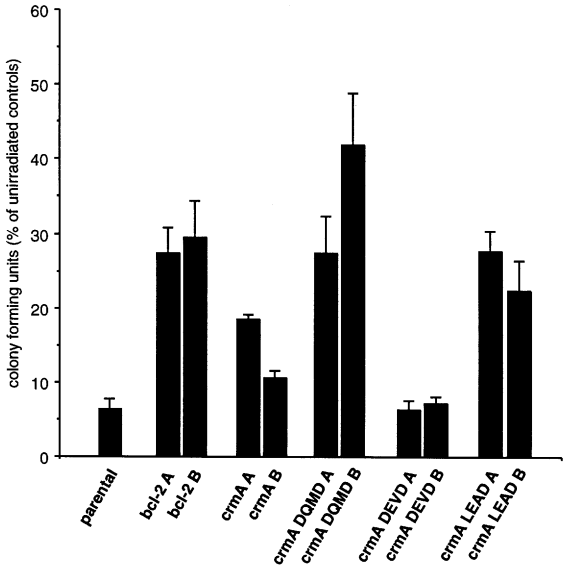

Fig. 8. CrmA DQMD protects cells from γ-irradiation and allows clonal survival. Two independent clones of each construct were either exposed to 2.5 Gy of γ-irradiation or were not irradiated (control), and were then plated in soft agar. Colonies were counted on day 12. The number of colony-forming cells is expressed as a percentage of non-irradiated controls. Error bars represent ± 1 SEM.

Discussion

We have used the sequence of the preferred substrates for the different caspase family members to design specific caspase inhibitors (Thornberry *et al.*, 1997). The crmA variants produced showed altered ability to protect against different stimuli when compared with wild-type crmA, with some variants showing increased activity and others showing decreased activity. Wild-type crmA, unlike p35, cannot be cleaved by Ced-3, nor can it block Ced-3; and wild-type crmA is poor at blocking cell death caused by cellular stresses such as serum withdrawal or γ-irradiation (Smith *et al.*, 1996; Newton *et al.*, 1998). When the pseudosubstrate site of p35 (DQMD) was introduced into crmA replacing the wild-type sequence (LVAD), this protein could now be cleaved by Ced-3, and blocked Ced-3-dependent cell death (Xue and Horvitz, 1995; Xue *et al.*, 1996). Here we show that crmA DQMD is also capable of inhibiting cell death in mammalian cells caused by a variety of stimuli, including that caused directly by activated caspase 3 (Figure 3).

An unexpected finding was that the crmA variants containing optimal substrate sequences in their pseudo-substrate region were not the optimal caspase inhibitors. For example, although the preferred substrate of caspase 3 is DEVD, crmA DEVD was unable to block any of the death stimuli tested, showed no enhanced function against caspase 3 and did not block CD95-induced killing, whereas crmA DQMD gave greater protection against caspase 3-mediated killing in both yeast and mammalian

cells (Figures 2 and 3) and retained the ability to protect against death induced by CD95 (Figure 6C).

Structurally, crmA is a serpin. Most serpins, e.g. α-1 anti-trypsin or anti-thrombin III, bind to their target enzyme, are cleaved and undergo a stressed to relaxed transformation, to form a stable configuration complexed with the protease (Whisstock *et al*., 1998). CrmA is cleaved by caspase 1 *in vitro*, and alteration to crmA DQMD allowed cleavage by Ced-3 and blocked some developmental cell death in *C.elegans* (Komiyama *et al*., 1994; Xue and Horvitz, 1995). We observed stable levels of crmA DQMD in cells protected from dexamethasone whereas wild-type crmA and other crmA variants offered no protection and were degraded. This suggests that only crmA DQMD could form stable inhibitory complexes with target caspases in addition to caspase 1 (Figure 7). Presumably, proteins may not be able to inhibit caspases either because they do not bind efficiently to a target caspase, cannot form a stable complex or are cleaved by caspases and subsequently degraded.

There are two well-characterized pathways to apoptosis (Strasser *et al*., 1995). Death induced by ligation of CD95 proceeds by the recruitment of FADD, activation of caspase 8 and then activation of downstream caspases such as caspase 3 (Chinnaiyan *et al*., 1995; Boldin *et al*., 1996; Muzio *et al*., 1996; Medema *et al*., 1997). This pathway can be blocked by crmA but not by Bcl-2 (Strasser *et al*., 1995; Smith *et al*., 1996; Huang *et al*., 1997). The second death pathway involves adaptor molecules such as Apaf-1 which activate caspase 9 and then downstream caspases such as caspase 3 (Zou *et al*., 1997; Hakem *et al*., 1998; Kuida *et al*., 1998). This pathway can be blocked by Bcl-2 family members (Hu *et al*., 1998). Unlike wild-type crmA, crmA DQMD and to a lesser extent crmA LEAD were able to inhibit the same cell death pathways as Bcl-2. This indicates that these variants could inhibit caspases other than caspase 8, but does not define which caspases are blocked, or at which point(s) in the pathway they are working. The experiments in *S.pombe* show that crmA DQMD can directly inhibit caspase 3, but it is likely that other caspases were also blocked in WEHI-7 cells since thymocytes in caspase 3-deficient animals retain normal sensitivity to radiation and dexamethasone (Kuida *et al*., 1996; Woo *et al*., 1998).

CrmA LEAD showed activity in several different systems. It was able to inhibit the DEVD-cleaving activity induced by dexamethasone in WEHI-7 cells (Figure 5), it reduced apoptosis in WEHI-7 cells caused by γ-irradiation (Figure 6B) and blocked the death of SKW-6 cells exposed to anti-CD95 antibodies (Figure 6C). While this latter activity probably results from inhibition of caspase 8, neither this nor inhibition of caspase 3 explain the reduction in cell death caused by γ-irradiation since caspase $3^{-/-}$ and caspase $8^{-/-}$ lymphocytes are normally sensitive to dexamethasone- and radiation-induced apoptosis (Strasser *et al*., 1995; Kuida *et al*., 1996; Woo *et al*., 1998). Since crmA LEAD is a good substrate for caspase 9, it is possible that inhibition of this caspase may be responsible. Caspase $9^{-/-}$ thymocytes and splenocytes are resistant to γ-irradiation, suggesting that this caspase is required for apoptosis in response to irradiation (Hakem *et al*., 1998; Kuida *et al*., 1998). CrmA LEAD may block another caspase in the pathway of radiation-induced apoptosis, but this caspase is unlikely to be caspase 8 since both wild-type crmA and crmA LEAD block CD95-induced apoptosis, but only crmA LEAD can inhibit radiation-induced apoptosis.

The expression of caspases in yeast provided a convenient method for examining the function of individual caspases in isolation. Caspase 3 expression perturbed yeast morphology and prevented their growth. This was dependent on normal caspase function, since a catalytically inactive mutant of caspase 3 was not toxic, and the crmA DQMD variant was able to protect them. As there are no endogenous caspases in *S.pombe*, the effects of caspase expression cannot be due to activation of secondary caspases.

Analysis of caspase 3 activity has been difficult because procaspase 3 does not autoactivate in mammalian cells. Serendipitously, when caspase 3 was expressed as a fusion with LacZ, it became active, and was an effective death stimulus. The most likely explanation is that the β-galactosidase polypeptides encoded by LacZ caused the fusion proteins to multimerize, thereby allowing caspase 3 to autoactivate. Consistent with this notion, dimerization of FKBP–caspase 3 fusions by FK1012 also caused caspase 3 activation (Maccorkle *et al*., 1998). In neither case was Bcl-2 able to inhibit apoptosis, indicating that Bcl-2 acts at a step prior to caspase activation. This is consistent with the genetic evidence indicating that the *C.elegans* Bcl-2 homologue, Ced-9, acts on Ced-4 and upstream of the *C.elegans* caspase Ced-3 (Shaham and Horvitz, 1996).

As inappropriate apoptosis is implicated in a number of human diseases, cell death inhibitors might have therapeutic value. Drugs that block caspase activity would be the most direct way of inhibiting cell death. However, if caspase activation occurs after irreversible commitment to cell death, then inhibition of these enzymes would have negligible utility. Peptide inhibitors of caspases can block caspase activation efficiently and inhibit cell death in response to a variety of stimuli but, in some cases, while inhibition of caspases delayed the morphological changes of apoptosis it did not alter the eventual fate of the cell (Xiang *et al*., 1996; Mccarthy *et al*., 1997; Amarantemendes *et al*., 1998). Bcl-2, in contrast, permits not only cell survival, but rescued cells retain clonogenic activity (Vaux *et al*., 1988; Flomerfelt and Miesfeld, 1994). *In vitro*, the peptide caspase inhibitors are highly efficient, inhibiting markers of caspase activation in the nanomolar range (Nicholson *et al*., 1995), but require many fold higher doses to inhibit caspases directly in cells and may not completely abolish enzymatic activity *in vivo* (Thornberry *et al*., 1992; Nicholson *et al*., 1995; Nicholson, 1996). Genetic inhibitors of caspases may be a better way to test the validity of caspase inhibition as a therapeutic goal. The finding that cells rescued from radiation-induced apoptosis by caspase inhibition were able to proliferate indicates that even though apoptosis was blocked, the cells were not doomed to die a necrotic death, and at least a proportion retained clonogenic activity similar to Bcl-2-overexpressing cells (Figure 8). These results in mammalian cells strengthen the conclusion in an insect system that caspase inhibition can rescue functional cells (Davidson and Steller, 1998).

Materials and methods

Cell lines and culture

293T (human embryonic kidney) and WEHI-7 (murine T lymphoblast) cells were cultured in RPMI media supplemented with 10% fetal calf serum (FCS) and added antibiotics. SKW-6 (human B lymphoblast) cells were cultured in RPMI supplemented with 10% FCS, L-asparagine (100 μM) and 2-mercaptoethanol (50 μM). SKW-6 cells stably expressing a neomycin-resistant Bcl-2 plasmid were used (Huang *et al*., 1997). Dexamethasone was used at a concentration of 10^{-6} M. WEHI-7 cells were suspended at 1×10^5 cells/ml before irradiation at 2.5, 5.0 and 10.0 Gy. For CD95-mediated killing, SKW-6 cells were cultured at 1×10^5 cells/ml and incubated with a mouse monoclonal IgM antibody to human CD95 (CH11, Upstate Biotechnology) at 10, 100 and 1000 ng/ml for 48 h.

Transfection

293T cells were transfected using either Lipofectamine® (Gibco-BRL) or polyethyleneimine (Fluka) as described (Boussif *et al*., 1995). A total of 1×10^5 cells were plated in 12- or 24-well plates, washed the following day and then transfected using a total of 1 μg of DNA and 5 μl of lipofectamine diluted in serum-free RPMI (as per the manufacturer's protocol) or 4 μl of polyethyleneimine working solution diluted in normal saline (Boussif *et al*., 1995) in serum-free medium for 4 h. Cells were then washed and returned to medium with serum. At 24 or 48 h following transfection, cells were washed in cold phosphate-buffered saline (PBS) then fixed in 0.5% formaldehyde/0.1% glutaraldehyde in PBS at 37°C for 10 min. Cells were then washed in PBS and stained for β-galactosidase activity as previously described (Hawkins *et al*., 1996).

Stable cell lines were made by electroporating 1×10^7 WEHI-7 or SKW-6 cells with the pEF vectors containing the N-terminally FLAG-tagged crmA constructs or Bcl-2 linearized with *Fsp*I. Cells from each transfection were split into four separate cultures to ensure that independent lines were established. Cells were selected using 4–8 μg/ml puromycin (Sigma). Single cell cloning was performed on the single cell deposition unit on the FACstar+ (Becton Dickinson). Selected clones were tested for expression of the construct by staining with anti-FLAG antibody (Sigma) (Strasser *et al*., 1995) and analysed by flow cytometry or Western blotting.

Plasmids and mutagenesis

Inserts were cloned into the mammalian expression vector pEF FLAGpGKpuro (Huang *et al*., 1997) which allowed expression of N-terminally epitope-tagged proteins using the same Kozak initiation sequences. The crmA variants were constructed using a PCR mutagenesis strategy and *Pfu*I as previously described (Picard *et al*., 1994). The oligonucleotides encoding the preferred pseudosubstrate sequence (Thornberry *et al*., 1997) and the 18 bases either side of the crmA pseudosubstrate site were as follows: DQMD, GCAGCAACTTGTGCGGATCAAATGGATTGTGCATCAACAGTT; DEVD, GCAGCAACTTGTGCGGACGAGGTCGACTGTGCATCAACAGTT; LEAD, GCAGCAACTTGTGCGCTCGAGGCCGACTGTGCATCAACAGTT; and WEHD, GCAGCAACTTGTGCGTGGGAGCACGACTGTGCATCAACAGTT. A loss-of-function mutant crmA (T291R) (Tewari and Dixit, 1995) subcloned into the same vector was used as a negative control. The mutagenized constructs were verified by sequencing.

Yeast plasmids

The plasmid pURAS is a pFL20 derivative containing a pBR322 backbone, a *URA3* gene for selection and *S.pombe* ars1 and stb elements (Losson and Lacroute, 1983). Transcription is driven from a constitutive *adh* promoter. The plasmid pNeu is a pREP derivative and contains a pUC backbone and a *LEU2* selectable marker. Transcription is driven from an *nmt1* full strength promoter (Maundrell, 1993). pURAS was modified by insertion of an oligonucleotide containing a consensus Kozak and *Asc*I site and recreating a unique *Bam*HI to create pURAS K. The same Kozak oligonucleotide was introduced into pNeu, and downstream of this a multiple cloning site oligonucleotide, Adaptor(A), was introduced [Kozak(K), 5′-GATCGCCACCATGGCGCGCCG-3′, 5′-CGGTGGTACCGCGCGGCCTAG-3′; Adaptor(A), 5′-ATCCCATATGCTAGCGTCGACAAGGCGCGCCATTAATCTAGATAGT-3′; and 5′-TCGAACTATCTAGATTAATGGCGCGCCTTGTCGACGCTAGCCATATGG-3′] to create pNEU KA. CrmA variants were amplified by *Pfu*I PCR using the following primers which introduce an N-terminal FLAG tag: 5′-CGGGCGCGCCGAGACTACAAGGACGACGATGACAAGCATATGGATATCTTCAGGGAAATC-3′ and 5′-GCGCTAGCCCCGGGTTAATTAGTTGTTGGAGAGCAAT-3′, and cloned *Asc*I–*Sma*I into *Asc*I–*Pvu*II-digested pURAS K. pNEU KA LacZ was created by *Pfu*I PCR and inserting a LacZ cassette into *Nhe*I–*Sal*I sites and was tested for functionality. pNEU KA LacZ was digested with *Bam*HI–*Nhe*I and the *Pfu*I PCR-amplified caspase 3, 5′-CGGGATCCTCATGGAGAACACTGAAAACTCA-3′, 5′-CGCGCTAGCGTGATAAAAATAGAGTTCTTTTG-3′, was inserted via *Bam*HI–*Nhe*I. The caspase 3 mutant was generated with *Pfu*I PCR using the previous 5′ and 3′ oligonucleotides and 5′-CGGATGCTCTTCCGGCCGTGGTACAGAACTG-3′, 5′-CGGATGCTCTTCGGCCGGCCTGAATAATGAAAAGT-3′. The caspase 3 cassette was then placed into pEF KA LacZ using *Bam*HI–*Nhe*I. All constructs were verified by restriction digest and sequencing.

Yeast transformation

Transformation was performed with a standard lithium acetate protocol and plating on selective medium (see http://www.bio.uva.nl/pombe/handbook). All clones were always maintained on selective medium.

Death assay

Apoptosis of transiently transfected 293T cells was determined by morphology of cells that stained blue with X-gal (Diagnostic Chemicals) (Hawkins *et al*., 1996). The viability of stable cell lines was determined by propidium iodide (PI, Sigma) exclusion assessed by flow cytometry (Becton Dickinson). PI was added to cultures at 5 μg/ml. Dead cells lose plasma membrane integrity and allow entry of PI, detectable as increased fluorescence on the FL-2 or FL-3 channel.

Yeast growth assays

Yeast growth was assayed in both liquid culture and by serial dilution on minimal supplemented agar. In liquid culture, the OD_{600} was measured by taking six independent measurements for each culture at each time point. As cells reached the end of exponential growth, they were diluted further to allow continued growth and measurement. On minimal agar plates, yeast were plated in serial 10-fold dilutions; 4 μl of each dilution were plated. The results of the serial plating were always in agreement with the results of the liquid culture assays. Data from agar plates are shown elsewhere.

DEVD cleavage activity

To measure caspase activity, 5×10^5 cells were suspended in 50 μl of Phiphilux G_2D_2 substrate (Oncoimmunin) for 1 h at 37°C following the manufacturer's protocol. Cleavage of the peptide linker of Phiphilux (sequence DEVD) separates the rhodamine moieties and results in fluorescence detectable by flow cytometry (Becton Dickinson) on the FL-2 channel.

Western blotting

Lysates from 1×10^6 WEHI-7 cells were lysed in 100 μl of lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 40 μg/ml aprotinin, 20 μg/ml leupeptin] at 4°C. Then 10 μl of sample were run on either 10 or 8–16% linear gradient polyacrylamide gels (Bio-Rad). Gels were transferred to nitrocellulose membrane (Hybond-C extra, Amersham). Membranes were blocked in 5% milk powder in PBS, and probed with either anti-FLAG antibody (M2, Sigma) or an anti-HSP-70 antibody (rabbit polyclonal antibody, gift from Robyn Anderson), both at dilutions of 1:1000. The secondary antibody was goat anti-mouse IgG horseradish peroxidase-conjugated antibody (Southern Biotechnology) used at a dilution of 1:1000.

Clonal assay

Two independent clones of WEHI-7 cells stably expressing wild-type crmA, crmA variants or Bcl-2 were tested for clonogenicity. A total of 1×10^5/ml WEHI-7 clones were suspended in 1 ml of culture medium and then irradiated at 2.5 Gy or not exposed (control). Varying dilutions of each original sample were then cultured in soft agar (RPMI with 20% FCS and 0.3% agar at 37°C). Colonies were counted after 10 days in culture. The numbers of colony-forming units in irradiated cultures were expressed as a percentage of unirradiated controls.

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