Inhibitor specificity of recombinant and endogenous caspase-9

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Apoptosis triggered through the intrinsic pathway by radiation and anti-neoplastic drugs is initiated by the activation of caspase-9. To elucidate control mechanisms in this pathway we used a range of synthetic and natural reagents. The inhibitory potency of acetyl-Asp-Glu-Val-Asp-aldehyde ('Ac-DEVD-CHO'), benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone ('Z-VAD-FMK') and the endogenous caspase inhibitor X-chromosome-linked inhibitor of apoptosis protein ('XIAP') against recombinant caspase-9 were predictive of the efficacy of these compounds in a cell-free system. However, the viral proteins CrmA and p35, although potent inhibitors of recombinant caspase-9, had almost no ability to block caspase-9 in this system. These findings were

also mirrored in cell expression studies. We hypothesize that the viral inhibitors CrmA and p35 are excluded from reacting productively with the natural form of active caspase-9 *in vivo*, making the potency of inhibitors highly context-dependent. This is supported by survival data from a mouse model of apoptosis driven by Sindbis virus expressing either p35 or a catalytic mutant of caspase-9. These results consolidate previous findings that CrmA is a potent inhibitor of caspase-9 *in vitro*, yet fails to block caspase-9-mediated cell death.

Key words: apoptosis, inhibitor of apoptosis protein (IAP), protease, serpin.

INTRODUCTION

Both the initiation and execution phases of apoptosis depend on caspases, cytosolic cysteine proteases that cleave their target substrates after aspartate residues [1]. Caspase-8 initiates the extrinsic apoptotic pathway in response to apoptotic signals transduced by cell-surface death receptors [2]. Caspase-9 initiates the intrinsic apoptotic pathway to remove excess cells of the developing mammalian nervous system, and also as a response to cellular damage [3]. The initiator caspases converge on the activation of the executioner caspase-3 and -7, which together cleave the many protein substrates that define the effector pathway of cell death [3]. The critical role of these proteases in apoptosis is highlighted by the findings of gene-ablation experiments in mice where the phenotypes are malformed, anti-apoptotic and vary from early embryonic lethality (caspase-7 and -8) to perinatal lethality (caspase-3 and -9) [4–8].

The activity of caspases is under the control of natural inhibitors. Indeed, viruses have adopted a successful strategy to ablate the apoptotic response of the host to viral infection by evolving proteins that directly inhibit caspases. For example, the poxvirus serpin CrmA rapidly inactivates caspase-1 and -8, whereas the baculovirus protein p35 inhibits an even wider spectrum of caspases (reviewed in [9]). Similarly, animals have embraced another protein family to regulate caspase activity, namely the inhibitor of apoptosis proteins (IAPs). Human X-chromosome-linked IAP (XIAP) [10], cIAP1 and cIAP2 [11,12] are capable of inhibiting caspase-3, -7 and -9, but are ineffective against caspase-1, -6 or -8. Melanoma ('ML') IAP/Livin demonstrates inhibition of caspase-9, but insubstantial inhibition of caspase-3 [13,14].

In general, analysis of caspase inhibition *in vitro* faithfully predicts the regulation by these inhibitors *in vivo*. For example, caspase-8 is tightly inhibited by CrmA *in vitro* [15], and the

extrinsic pathway is blocked at the level of caspase-8 in cells ectopically expressing CrmA [16]. However, caspase-9 is also inhibited well by CrmA *in vitro* [17], yet there is ample evidence that CrmA does not block apoptosis engaged through the intrinsic pathway [12,18,19]. This discrepancy could be associated with the form of caspase-9 utilized or the absence of the required cofactor [apoptosis protease-activating factor 1 (Apaf-1)], which plays a key role in the activity/activation of caspase-9 [20,21]. We therefore aimed to test the hypothesis that the inhibitor specificity of caspase-9 is context-dependent and consequently may rely on the presence of the apoptotic initiator complex.

EXPERIMENTAL

Materials

Caspase-9 and derivatives thereof were expressed and purified as described previously [21]. The concentration of caspase-9 constructs was determined by active-site titration using benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) from Enzyme System Products (Dublin, CA, U.S.A.). Acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin (Ac-LEHD-AFC) and acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) were purchased from Enzyme System Products. Dithiothreitol was from Diagnostic Chemicals Limited (Oxford, CT, U.S.A.). SDS/PAGE was performed using 8–18% (w/v) acrylamide gels in the 2-amino-2-methylpropan-1,3-diol/glycine discontinuous buffer system described by Bury [22]. After electrophoresis, the gels were either stained using Gel-Code (Pierce, Rockford, IL, U.S.A.) according to the manufacturer's protocol or the proteins were transferred on to Immobilon-P (Millipore, Bedford, MA, U.S.A.) as described by Matsudaira [23]. Western-blot analysis were performed using the Enhanced Chemiluminescence (ECL[®]) Kit (Amersham Biosciences, Piscataway, NJ, U.S.A.) or Super-signal (Pierce) according to the manufacturers' protocol using

Abbreviations used: Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; Ac-LEHD-AFC, acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin; Apaf-1, apoptosis protease-activating factor 1; IAP, inhibitor of apoptosis protein; BIR, baculovirus IAP repeat; CARD, caspase recruitment and activation domain; TRAIL, tumour necrosis factor-related apoptosis-inducing ligand; XIAP, X-chromosome-linked IAP; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

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Figure 1 Determination of inhibitory constants for purified recombinant Δ CARD caspase-9

Ac-LEHD-AFC hydrolysis by caspase-9 in the presence of inhibitors was expressed as $[(v_0/v_i) - 1]$ and plotted against inhibitor concentration, as described in the Experimental section. The upper panel shows the entire inhibitor range tested, and the lower panel is a magnification of the initial linear portions of the inhibition profile. The slope of the lines gives the reciprocal of the initial K_i (app). CrmA (●), p35 (○) and Ac-DEVD-CHO (■) were the inhibitors used.

rabbit anti-(caspase-3) from in-house stocks. Anti-Crma antisera was a gift from Dr David Pickup (Department of Microbiology, Duke University, Durham, NC, U.S.A.), the anti-p35 antibody was from in-house stocks and the anti-XIAP antibody was purchased from BD Biosciences (Lexington, KY, U.S.A.). The proteinaceous caspase inhibitors were obtained as a recombinant proteins purified following expression in *Escherichia coli* as described previously for p35 [24], CrmA [15], XIAP [10] and the baculovirus IAP repeat (BIR) 2 [25] and BIR3 [26] domains of XIAP. Plasmids for the caspase inhibitors CrmA, p35 and XIAP were cloned into pcDNA3. Control plasmid pcDNA3 was obtained from Invitrogen (San Diego, CA, U.S.A.).

Preparation of cytosolic extracts

For preparation of cell-free lysates, we used nitrogen cavitation in the absence of exogenous protease inhibitors. Briefly, HEK-293 cells from 12 10-cm-diameter Petri dishes were harvested by gentle scraping into PBS at 4 °C, pelleted by centrifugation for 5 min at 200 *g*, and subsequently washed twice in the same buffer. The cell pellet was resuspended in 20 mM Pipes, 10 mM KCl, 5 mM EDTA, 2 mM $MgCl_2$ and 1 mM dithiothreitol (pH 7.4) at 4 °C and placed in a nitrogen cavitator at 4 °C (Parr

Table 1 Inhibition of caspase-9 by select caspase inhibitors

Inhibition constants (K_i) of Ac-LEHD-AFC hydrolysis by recombinant Δ CARD caspase-9 were determined. CrmA and p35 are irreversible inhibitors, so the K_i (app) values correspond to the early phase of inhibition (see Figure 1). Caspase-9 C285A is not a direct caspase inhibitor, but is included here for comparative purposes with Figure 2. n.a., not applicable; n.d., not determined.

Inhibitor	K_i (app) (nM)	
	Present study	Other studies
CrmA	2	< 2*
p35	8	n.d.
BIR2	> 10000	n.d.
BIR3	230	22†
Ac-DEVD-CHO	25	60*
Caspase-9 C285A	> 10000	n.a.

* Data from [17].

† Data from [26] and reported at pH 6.5 as IC_{50} .

Instruments, Moline, IL, U.S.A.). The chamber pressure was adjusted to 420 lbf/in² (1 lbf/in² = 6.9 kPa) for 10 min. The sample was collected and the extract cleared by centrifugation at 16000 *g* for 30 min, and the supernatant (cytosolic extract) was recovered. The quality of the lysates was determined by the ability to support rapid generation of caspase activity by addition of cytochrome *c* and dATP as well as the inability to generate caspase activity upon addition of dATP alone.

Preparation of total cell lysates

Cells were collected, washed and resuspended in lysis buffer containing 10 mM Tris/HCl (pH 7.2), 150 mM NaCl, 1 mM EDTA and 1 % (v/v) Triton X-100. The cell suspension was incubated on ice for 15 min and centrifuged at 16000 *g* for 1 min at 4 °C. Before separation by SDS/PAGE, cell extracts were normalized for protein concentration using the Pierce Coomassie Plus Protein assay.

Determination of inhibitory constants

Recombinant caspase-9, Δ CARD [in which the caspase recruitment and activation domain (CARD) has been deleted], was preactivated in salt-free caspase buffer [20 mM Pipes, 10 mM EDTA, 0.1 % CHAPS and 10 % (w/v) sucrose (pH 7.2)] for 10 min at 37 °C. Following this, a range of inhibitors were preincubated with the enzyme for 15 min at 37 °C. The assay was started by the addition of Ac-LEHD-AFC (100 μ M) and measured kinetically for 30 min using an f_{MAX} Fluorescence Plate Reader (Molecular Devices, Sunnyvale, CA, U.S.A.) at an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Reaction mixtures were thermostatically controlled at 37 °C. The individual K_i values for the inhibitors [I] were determined from the uninhibited substrate hydrolysis rate (v_0) and the inhibited rates (v_i), so that a plot of $(v_0/v_i) - 1$ against [I] gives K_i (app), the equilibrium inhibition constant in the presence of substrate [27].

Evaluation of apoptotic response in HEK-293 cells expressing CrmA, p35 or XIAP

Tissue-culture plates (six-well plates) were seeded with HEK-293 cells in complete culture medium [Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 20 units/ml penicillin, 20 mg/ml streptomycin and 10 % (v/v) heat-inactivated fetal bovine serum]. Cells were maintained at 37 °C in 5 % CO₂.

Figure 2 Inhibition of endogenous caspase-9 in HEK-293 cytosolic extracts by caspase inhibitors

The activity of caspase-9 was determined from the level of conversion of pro-caspase-3 (35 kDa) into the large subunit derivatives (17–20 kDa). Cytosolic extracts were preincubated for 15 min in the absence or presence of the indicated inhibitors at the concentrations detailed in the box. Sample 1 is a control without activation, and samples 2–6 were activated using a cytochrome *c*/dATP mixture for 30 min at 37 °C. Samples were separated by SDS/PAGE, electroblotted and probed for caspase-3 using a rabbit polyclonal anti-(caspase-3) antiserum. This antiserum detects the processed forms less efficiently than the pro-form, and therefore it was necessary to overexpose the blots to visualize the small subunit derivatives. This invariably increases non-specific background. Caspase-9 activity is observed by processing of the 35 kDa pro-caspase-3 to the 20 kDa derivative, and subsequent processing to the smaller derivatives results from caspase-3 autoprocessing.

Table 2 Cell survival following inhibitor transfection

HEK-293 cells were transfected with an empty vector control or one of the caspase inhibitors, followed by treatment in the absence (control) or presence of etoposide (20 μ M) or TRAIL (200 ng/ml) to induce apoptosis. Cell survival was measured by counting adherent non-apoptotic cells and results are expressed as a percentage of those for untreated cells (control column). Values are means $\pm$ S.D. of three fields of view.

Construct	Cell survival		
	Control	Etoposide	TRAIL
Vector	95.6 $\pm$ 2.5	28.3 $\pm$ 3.0	22.0 $\pm$ 2.6
CrmA	92.3 $\pm$ 2.9	31.1 $\pm$ 4.1	71.2 $\pm$ 3.1
p35	94.6 $\pm$ 2.1	30.2 $\pm$ 1.7	41.2 $\pm$ 2.3
XIAP	97.0 $\pm$ 1.2	92.5 $\pm$ 2.1	62.4 $\pm$ 0.9

When monolayers reached approx. 60% confluency, cells were incubated for a minimum of 12 h in antibiotic-free medium, followed by transient transfection using LIPOFECTAMINETM 2000 (Invitrogen). A portion (4 μ g) of plasmid DNA encoding p35, CrmA or XIAP and 1 μ g of pEGFP vector (Clontech) (transfection-efficiency indicator) were preincubated with 8 μ l of LIPOFECTAMINETM 2000 in accordance with the manufacturer's instructions. Transfection mixture in serum-free culture medium was added to the cell monolayer and incubated for 4 h. Following incubation, the media was replaced with complete culture medium and cells were incubated for a further 24 h. Transfected cells were incubated under control conditions or in the presence of 20 μ M etoposide (Alexis Corporation, San Diego, CA, U.S.A.) or 200 ng/ml human recombinant tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and observed using fluorescence microscopy. Each field of view contained an average of 65 green-fluorescent-protein-labelled cells, and surviving cells were scored as those remaining on the plate that did not show evident apoptotic body formation.

Recombinant Sindbis virus infection of mice

Recombinant Sindbis viruses expressing p35 or mutant caspase-9 were constructed in the Sindbis virus vector dsTE12Q and infectious viruses were generated as described previously [28]. Virally encoded p35 contained an internal haemagglutinin ('HA') tag (obtained from Professor Paul Friesen, Department of Molecular Virology and Biochemistry, University of Wisconsin, Madison, WI, U.S.A.) or a premature stop codon inserted after codon 37. Essentially identical results were obtained with tagged or untagged p35 (results not shown). Caspase-9 with an active-site mutation [Cys²⁸⁵ $\rightarrow$ Ala (C285A)] was obtained from Dr Yuri Lazebnik (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.) [20]. Mice (3 days old) were injected intracranially with 5000 plaque-forming units of recombinant virus and mortality was monitored for 20 days.

RESULTS

Inhibitory constants for purified recombinant caspase-9

We generated constructs encoding full-length caspase-9 and a form with the first 138 residues deleted (Δ CARD caspase-9). Each was obtained in quantities of 1–2 mg/l following expression in *E. coli* and purified using His₆-affinity tags added to the C-terminus of each construct [21]. The catalytically active portion of each preparation was determined by titration with the covalent inhibitor Z-VAD-FMK [29]. In each case, the amount of catalytically active material was low, with only approx. 1% of full-length caspase-9 and approx. 5% of Δ CARD caspase-9 being in a catalytic configuration. Due to the extremely low activity of full-length caspase-9, we used Δ CARD caspase-9 to determine the kinetic parameters for protease inhibitor interactions *in vitro* (Figure 1 and Table 1). Because CrmA and p35 are tight binding and the binding is probably essentially irreversible, determination of classic equilibrium constants (K_i) is problematic. Therefore we

Figure 3 Expression of caspase inhibitors in HEK-293 cells following transient transfection

Cells were transfected with either empty pcDNA3 vector (EV) or constructs encoding CrmA (**a**), p35 (**b**) and XIAP (**c**) in pcDNA3. After 24 h post-transfection, cells were lysed and these lysates were separated by SDS/PAGE and immunoblotted using specific antisera to CrmA, p35 and XIAP. Lanes labelled U indicate untransfected cells. Molecular masses (in kDa) are indicated on the right.

describe the efficiency of inhibition as a function of the residual activity after 15 min incubation under the specified conditions (see Experimental section). From this we determined initial K_i values, similar to procedures used previously to determine the K_i values of CrmA and p35 with caspase-3, -6, -7 and -8 [15,17,24].

There is a formal possibility that the remaining approx. 95 % of Δ CARD caspase-9 that is not in the active configuration may effect the determination of inhibitor kinetics. However, this is unlikely to be the case with CrmA and p35 because both require an active enzyme for cleavage of the reactive-site loop, leading to inhibition. It is more likely to be a confounding issue for the BIR3 domain of XIAP [30], which may explain the higher K_i value observed using this assay (Table 1).

Interestingly, the response of Δ CARD caspase-9 to inhibition by CrmA, p35 and Ac-DEVD-CHO (Figure 1) and BIR3 was only linear at low inhibitor concentrations. Substrate cleavage declined more slowly than expected for a simple one-step inhibition reaction as inhibitor was increased. This indicates the existence of two distinct equilibria: a tight one, corresponding to the initial K_i , and a weaker one, corresponding to a secondary reaction. This second equilibrium is most probably due to inhibitor-enforced stabilization of a transiently active form of Δ CARD caspase-9. In support of this, the majority of caspase-9 in solution exists as a monomer, but dimer formation is driven by inhibitor binding [31]. More importantly, the initial K_i describes the efficiency of inhibition of active Δ CARD caspase-9 in the preparation.

The K_i values determined in the present study agree well with those determined previously for CrmA and Ac-DEVD-CHO [17], but the value for BIR3 is higher than reported previously [26] (Table 1). We cannot explain the discrepancy. Significantly, however, it does not seem to matter whether the Δ CARD caspase-9 is prepared from a single-chain precursor (present study) or individual subunits [17] in terms of its inhibition by CrmA and Ac-DEVD-CHO.

The lack of detectable inhibition of caspase-9 by BIR2 is consistent with previous reports [10,12,32], and the catalytic mutant of caspase-9 would not be expected to act as a direct inhibitor in a purified system. It was included in the direct inhibition assays as a control for other experiments described below.

Inhibition of cytochrome *c*/dATP-mediated caspase-3 processing

Caspase-9 shows little activity on synthetic peptide substrates, making it difficult to reliably measure its activity in cellular

extracts. However, its activity can reliably be determined by observing the cleavage of its main substrate pro-caspase-3 [20,21,33]. To determine the effect of the inhibitors on the activity of caspase-9 in a more natural setting, we initiated caspase-9-dependent processing of pro-caspase-3 in cytosolic extracts by adding 10 μ M cytochrome *c* and 1 mM dATP. We used inhibitors at final concentrations ranging from 1 nM to 1 μ M. The use of a concentration range in this type of experiments is pivotal for understanding inhibition, as it is not only the affinity of the inhibitors that is of importance, but also the absolute enzyme/inhibitor ratio. Thus even a poor inhibitor may suppress the activity of an enzyme when present in large excess, whereas even the best inhibitor will fail to block an enzyme when the enzyme is present in excess. We also chose to use cytosolic extracts for our analysis rather than whole cells, as the uptake of the synthetic inhibitors or ectopic expression of the recombinant inhibitors is currently impossible to measure accurately.

Since the concentration of endogenous pro-caspase-9 in the extracts is 20 nM, we expected no inhibition by the inhibitors at a concentration of 10 nM or below. The assay we chose to analyse the activity of caspase-9 was based on the cleavage of 35 kDa pro-caspase-3 by cytochrome *c*/dATP-programmed lysates. This assay can also be used to observe the activity of the activated caspase-3, since conversion of the 20 kDa derivative into the 17 kDa derivative is autocatalytic [34]. Interestingly, with the exception of caspase-9 C285A (see below), only BIR3 completely prevented pro-caspase-3 processing at 100 nM (Figure 2). Z-VAD-FMK and p35 showed significant inhibition of caspase-9 activity only at 1 μ M, whereas CrmA and Ac-DEVD-CHO were almost completely ineffective even at this concentration. These data are partly at odds with the kinetic data using recombinant caspase-9 (Table 1). In particular, CrmA and p35 would be expected to be much better inhibitors of caspase-9 than BIR3. That the efficiency of inhibition is switched in the more natural setting of a cell cytosol is due mostly to worse than expected inhibition of caspase-9 by CrmA and p35.

Although BIR2 and p35 did not prevent caspase-9 activity, they were both effective at preventing caspase-3 autoprocessing above 100 nM. This is expected since both are effective inhibitors of recombinant caspase-3 *in vitro* [24,35]. A caveat to the interpretation of caspase-3 inhibition by BIR2 is the intracellular caspase-3 concentration, which is in the region of 200 nM in these cell lysates [36]. Presumably BIR2 was not effective at concentrations below 100 nM, because caspase-3 must first be saturated. Potential inhibition of caspase-3 autoprocessing by BIR3 is masked by the efficient inhibition of the initiating event

Figure 4 Survival of mice injected with Sindbis-virus constructs encoding either p35 or caspase-9 C285A

The expression of Sindbis-virus constructs encoding p35 and caspase-9 C285A was evaluated using immunoblot techniques. Cultured mammalian indicator cells [dorsal root ganglion (A) and baby hamster kidney cells (B)] were mock-infected (mock) or infected with the recombinant Sindbis virus expressing either haemagglutinin-tagged p35 (A) or the caspase-9 C285A (B). Cell lysates were made 26 h post-infection, lysates were separated by SDS/PAGE and immunoblotted with the indicated antibodies, revealing expression of the appropriate proteins. (C) The survival of mice infected with Sindbis virus encoding either full-length p35 ($n = 100$; $\square$), p35 with a premature stop codon ($n = 100$; $\blacksquare$) or caspase-9 C285A ($n = 20$; $\blacktriangle$) was monitored over a period of 20 days. The percentage survival was calculated for each group and illustrated as a function of time. The p35 construct encoding a premature stop codon functioned as a negative control and, at 20 days post-infection, 100% of the animals in this group were dead. Full-length p35 afforded between 35–40% protection, whereas animals infected with Sindbis virus encoding caspase-9 C285A had in excess of 85% survival rates.

in this cascade. However, the lack of inhibition of caspase-3 autoprocessing by CrmA, and apparently by BIR3, coupled with the weak inhibition by Z-VAD-FMK, is consistent with the poor reactivity of these inhibitors with recombinant caspase-3 [15,17,32]. Consequently, the potency of inhibitors towards caspase-3 *in vivo* is faithfully predicted by their *in vitro* efficiency, but this is clearly not the case for caspase-9. Indeed, the most effective inhibitor of caspase-9 in the cytosolic extracts was the full-length catalytic C285A mutant (Figure 2), which was able to completely abrogate caspase-9 activity at concentrations above 10 nM. This is presumably because the mutant acts as a dominant negative for the recruitment of caspase-9 to its activation complex

and can therefore operate at lower concentrations than the protease inhibitors.

Effect of transient expression of the caspase inhibitors CrmA, p35 and XIAP on susceptibility of HEK-293 cells to extrinsic and intrinsic apoptotic stimuli

To determine the relative efficiency of caspase inhibitors in cultured cells, HEK-293 cells were transfected with constructs encoding either CrmA, p35 or XIAP, and subsequently incubated in the absence or presence of initiators of the extrinsic (TRAIL) or intrinsic (etoposide) apoptosis pathways. Cells transfected with an empty pcDNA3 vector were susceptible to death induced via either pathway (Table 2). All three proteins substantially ameliorated death triggered by TRAIL, although CrmA and XIAP were more effective than p35. However, neither CrmA nor p35 were able to rescue etoposide-triggered death, although XIAP was extremely potent. These data indicate that the intrinsic pathway mediated by caspase-9 is resistant to rescue by ectopic expression of CrmA and p35, substantiating the inability of the inhibitors to block caspase-9 in cytosolic extracts. Expression of the transgenes was confirmed by Western-blot analysis (Figure 3).

Effect of p35 and caspase-9 C285A on survival of mice infected with recombinant forms of Sindbis virus

The significance of these findings was pursued *in vivo* by analysing p35 and caspase-9 C285A in a mouse model. Sindbis virus infects neurons of the central nervous system with a lethal outcome in young mice, apparently due to neuronal apoptosis. Lethality can be rescued by insertion of anti-apoptotic genes, such as Bcl-2, into the Sindbis-virus genome [37]. Mice were monitored for 20 days following infection with recombinant Sindbis viruses expressing p35 or caspase-9 C285A (Figure 4). At this stage, no mice expressing a control construct in which a premature stop codon was inserted into the p35 coding sequence survived. Mutant caspase-9 protected 86% of the mice, whereas p35 protected only 35% of the mice. Similar results were obtained previously with CrmA [38], which protected approx. 30% of the mice relative to controls. Therefore the catalytic mutant of caspase-9, operating as a dominant negative, is more effective *in vivo* than p35. Interestingly, expression of p35 conferred a small amount of resistance, and this is probably due to inhibition of caspases (caspase-3, -6 and -7) downstream of caspase-9.

DISCUSSION

It is generally assumed that protease inhibitors effective on purified proteases in a defined system will recapitulate potency when delivered to the active protease *in vivo*. Although our data support this assumption for caspase-3 [15], they refute it for caspase-9. What is different or unique about caspase-9? The activation of caspase-9 *in vivo* occurs upon recruitment to the apoptosome, an oligomeric assembly containing several units of the cofactor Apaf-1 and caspase-9 [20,30,39–41]. This event depends on the presence of the caspase-9 N-terminal peptide containing the CARD domain required for homophilic interactions with Apaf-1 [20,21]. However, in contrast with other caspases, the N-terminal peptide of caspase-9 is not removed upon activation. Despite this observation, it has been customary to generate truncated forms lacking the N-terminal peptide (Δ CARD caspase-9) to facilitate *in vitro* studies [17,21,26,42]. This, as we [21] and others [17,26,42] have observed, is probably due to the extremely low activity of all recombinant forms of caspase-9, particularly the full-length protein. Although

Δ CARD caspase-9 is tightly inhibited by CrmA and p35 (Table 1), the natural form, or at least the form in natural cytosolic extracts and transfected HEK-293 cells, is resistant to inhibition by these proteinaceous inhibitors. These inconsistencies lead us to conclude that apoptosome-bound caspase-9 is resistant to inhibition by these proven caspase inhibitors.

In contrast with the clear structural features of caspase-3 and -7 which may prevent binding of CrmA [43], the structure of caspase-9 does not reveal any reason why CrmA should not bind; the active site in caspase-9 is open and resembles that of caspase-8 [31] more than those of caspases-3 and -7. This is in agreement with the observation that Δ CARD caspase-9 is inhibited well by CrmA as demonstrated in the present study and in a previous publication [17]. The inability of CrmA to inhibit caspase-9 in a more natural setting is probably due to the presence of the CARD domain or steric hindrance imposed within the confines of the apoptosome. However, as the structures of full-length caspase-9 within the apoptosome are not available, it is difficult to predict exactly how the CARD or apoptosome would interfere with the binding of CrmA to caspase-9. Among the most obvious options is that the CARD domain prevents inhibition either by sterically preventing the docking of CrmA in the active site or, alternatively, prevents the structural rearrangement normally associated with the inhibitory mechanism of the serpins [44] and p35 [45]. In contrast, caspase-9 inhibition by XIAP is not thought to require major conformational transitions [30], and XIAP inhibits caspase-9 well *in vitro* and *in vivo*.

Baculovirus p35 also fails to efficiently inhibit the caspase-9-mediated pro-caspase-3 processing in a cell extract system, an *in vitro* cell expression system or the whole-mouse Sindbis-virus system, even though it is a quite potent inhibitor of the recombinant form. Indeed, our data support a previous study [46], which demonstrated that cell extracts from Jurkat cells expressing p35 maintained caspase-9 activity upon the addition of cytochrome *c*. This is somewhat surprising, as p35 is considered to be a pan-caspase inhibitor that binds tightly to most caspases in the apoptotic pathway [24]. Presumably the same steric and/or mechanistic exclusions that prevent CrmA from inhibiting caspase-9 apply to p35.

The inability of p35 to inhibit caspase-9 in the cytosolic extracts drew our attention to other systems in which p35 has been tested. Interestingly, p35 also failed to inhibit the *Drosophila* initiator caspases DRONC [47]. Since DRONC seems to play an equivalent role in *Drosophila* as caspase-9 in vertebrates, namely as the apical caspase of the mitochondrial apoptosis pathway [48], it is tempting to speculate that an equivalent steric/mechanistic exclusion may apply. The unusual inhibitory mechanism of the BIR3 domain of XIAP involves binding to the N-terminus of the small subunit of caspase-9 [30]. Consequently, even monomeric (catalytically inactive) caspase-9, the majority of the material in the recombinant preparations, may deplete the amount of available inhibitor. This could explain why more BIR3 is required to inhibit recombinant caspase-9 (Table 1) than endogenous caspase-9 activated within the apoptosome (Figure 2).

The proteinaceous inhibitors CrmA and p35 are unable to efficiently inhibit caspase-9 *in vivo*, but the synthetic inhibitors are likely to be more reliable when recombinant and natural materials are compared. Although Ac-DEVD-CHO was unable to prevent pro-caspase-3 processing, Z-VAD-FMK was partly effective, as predicted from studies with recombinant material [17]. However, since none of the readily available caspase-specific aldehydes are very good inhibitors of caspase-9 [17], this point is currently difficult to address clearly. On a more positive note, perhaps the most pleasing implication of our results is that the

discrepancy between the good *in vitro* inhibition of Δ CARD caspase-9 and the poor protection of intrinsic pathway apoptosis by ectopic expression of CrmA and p35 can now be reconciled. Clearly CrmA does not protect cells from apoptotic triggers that initiate the intrinsic (post-mitochondrial) pathway by activation of caspase-9 [12,18,19]. Therefore future studies of caspase-9 inhibition may take into account the rather restricted ability of the natural, apoptosome-bound, form from reacting with inhibitors.

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