Inhibition of distant caspase homologues by natural caspase inhibitors

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Caspases play an important role in the ability of animal cells to kill themselves by apoptosis. Caspase activity is regulated *in vivo* by members of three distinct protease inhibitor families, two of which, baculovirus p35 and members of the inhibitor of apoptosis (IAP) family, are thought to be caspase specific. However, caspases are members of the clan of cysteine proteases designated CD, which also includes animal and plant legumains, and the bacterial proteases clostripain, gingipain-R and gingipain-K. Since these proteases have been proposed to have a common mechanism and evolutionary origin, we hypothesized that the caspase inhibitors may also regulate these other proteases. We tested this hypothesis by examining the effect of the natural caspase inhibitors on other members of protease clan CD. The IAP family proteins were found to have only a slight inhibitory effect on gingipain-R. The cowpox viral cytokine-response modifier A (CrmA) serpin had no effect on any of the proteases tested but a single point mutation of CrmA (Asp → Lys) resulted in strong inhibition of gingipain-K. More substantial, with respect to the hypothesis, was the strong inhibition of gingipain-K by wild-type p35. The site in p35, required for inhibition of gingipain-K, was mapped to Lys^{94}, seven residues C-terminal to the caspase inhibitory site. Our data indicate that the virally encoded caspase inhibitors have adopted a mechanism that allows them to regulate disparate members of clan CD proteases.

Key words: apoptosis, gingipain, inhibitory mechanism, proteases.

INTRODUCTION

The protease clan CD contains homologous cysteine proteases, including the caspases (family C14), gingipains (family C25), legumains (family C13), and clostripain (family C11). Each of these is thought to share a catalytic-site motif and a common scaffold to their catalytic domains, implying shared ancestry [1]. Indeed, this has been demonstrated directly for caspases and gingipain-R by structural elucidation [2]. The substrate specificities of the proteases in clan CD are dominated by the interactions of the primary substrate (S1) subsites. Caspases prefer aspartate in their S1 pocket [3,4], legumains prefer asparagine [5], gingipains-K prefers lysine, and gingipain-K and clostripain prefer arginine [6,7]. Thus the clan-CD scaffold has the facility to adopt very distinctive protease specificities. Apart from the major differences in S1, caspases and gingipain-R share a similar fundamental mechanism for binding extended peptide chains [2], and this can be expected to apply to all members of the clan.

In general, natural protease inhibitors tend to be specific for individual scaffolds and catalytic mechanisms. For example, members of the kazal or kunitz family of serine protease inhibitors are specific for serine proteases of the chymotrypsin (clan SA) and subtilisin (clan SB) families [8], and tissue inhibitors of metalloproteases preferentially inhibit matrix metalloproteases [9]. Cystatins prefer cysteine proteases from clan CA [10], though they can also inhibit legumain, albeit via a second reactive site [11]. Therefore, given the conservation in mechanism and mode of substrate binding, one may predict that natural inhibitors of one of the families within clan CD should inhibit members of the other families, providing that the S1 specificity was satisfied.

Presently, most known natural inhibitors of clan CD proteases are directed against caspases. These include the baculovirus anti-apoptotic protein p35, which can potently inhibit most known caspases (including those from humans, *Caenorhabditis elegans* and the insect *Spodoptera frugiperda* [12–14]. The cowpox viral cytokine-response modifier A (CrmA) is more selective, being a strong inhibitor of caspases 1 and 8 [15]. In contrast to these viral caspase inhibitors, endogenous caspase regulation is achieved by members of the inhibitor of apoptosis (IAP) family [16].

To test the hypothesis that caspase inhibitors should inhibit other members of clan CD, in the present study we compared the effect of p35, CrmA and the human IAP family member X-linked IAP (XIAP) on gingipains, clostripain and human legumain.

EXPERIMENTAL

Proteins

The cDNA encoding *Autographa californica* nuclear polyhedrosis virus p35 plus a C-terminal His_6 purification tag was cloned into the expression vector pET21b(+) (Novagen, Madison, WI, U.S.A.), expressed in *Escherichia coli* strain BL21(DE3)pLysS, and purified by a Ni-chelate affinity method [14]. Upon storage of p35 at 4 °C over a week, the inhibitory capacity declined, therefore all assays were conducted with material prepared within one week.

Overlap PCR mutagenesis was employed to substitute Lys^{94}, Lys^{225} and Lys^{257} of p35 with an arginine residue, or Cys^{2} with an alanine residue, and to generate P1 mutants, with arginine, lysine or asparagine replacing the natural aspartic acid of CrmA. The

Abbreviations used: AMC, 7-(4-methyl)coumarylamide; CrmA, cytokine-response modifier A; DTT, dithiothreitol; pNa, *p*-nitroanilide; D-VLK-pNa, D-Val-Leu-Lys-pNa; IAP, inhibitor of apoptosis; XIAP, X-linked IAP.

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full sequence of each construct was checked by cycle-sequencing, using an ABI BigDye terminator kit on an ABI 377 sequencer (PE Applied Biosystems, Foster City, CA, U.S.A.), to verify the success of the mutagenesis and the absence of any other mutations. For p35 mutants, the appropriate expression construct was then transformed into *E. coli* strain BL21(DE3)pLysS, expressed and purified as for the wild type. The purity was judged to be greater than 95 %, similar to that of the wild type. For CrmA mutants, the appropriate expression plasmid was then transformed into *E. coli* strain TG1.

For expression of CrmA in *E. coli*, the full-length *crmA* gene was ligated to a linker that encoded an N-terminal His_6 fusion to facilitate purification. The coding sequence started with the initiator methionine residue, followed by His_6, a serine residue and then the entire coding region of CrmA, and expression in *E. coli* was performed as described previously [17]. XIAP was provided by Quinn Deveraux (Novartis, La Jolla, CA, U.S.A.).

Recombinant caspase-3 was obtained following expression in *E. coli* [18]. Active-site concentrations of caspase-3 were determined [19] by titration with the irreversible caspase inhibitor benzoxycarbonyl-Val-Ala-Asp-CH_2F (Enzyme System Products, Dublin, CA, U.S.A.). Clostripain was purchased from Sigma (St. Louis, MO, U.S.A.). Pig legumain [20] and gingipains [7] were purified as described previously, and the active sites were titrated [21] using D-Phe-Phe-Arg-CH_2Cl (Enzyme System Products).

Enzyme assays

The caspase substrate acetyl-Asp-Glu-Val-Asp-pNa [where pNa is *p*-nitroanilide], the clostripain/gingipain-R substrate D-Pro-Phe-Arg-AMC [where AMC is 7-(4-methyl)coumarylamide], and the gingipain-K substrate D-Val-Leu-Lys-pNa (D-VLK-pNa) were purchased from Enzyme System Products. The legumain substrate benzoxycarbonyl-Ala-Ala-Asn-AMC was purchased from Graham Knight (Department of Biochemistry, University of Cambridge, Cambridge, U.K.).

Assays using colorimetric pNa substrates (absorbance at 405 nm) were performed on a SpectraMAX 340 plate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). Assays using fluorogenic AMC substrates (excitation wavelength 380 nm and emission wavelength 460 nm) were carried out on a fMax Fluorescence Microplate reader (Molecular Devices).

The buffer used for the legumain assays was 50 mM Mes, 400 mM NaCl, 0.01 % (v/v) CHAPS, 10 % (w/v) sucrose, 20 mM 2-mercaptoethanol, pH 5.75. The buffer used for the caspase assays was 20 mM Pipes, 10 % sucrose, 100 mM NaCl, 0.1 % CHAPS, 1 mM EDTA, 10 mM dithiothreitol (DTT), pH 7.2. The buffer used for the gingipain-R and clostripain assays was the same as that used for caspase assays except that EDTA was replaced with 1 mM $CaCl_2$. Reactions of 100 μl contained enzyme, inhibitors and substrate (final concentration 100–200 μM) in the appropriate buffer. All reagents were pre-equilibrated at 37 °C for at least 5 min before mixing, and the caspases were preactivated for 5 min with 10 mM DTT. The lowest inhibitor concentration was at least 10-fold that of the enzyme. A control without inhibitor was used for all progress-curve experiments to ensure that no substrate depletion, product inhibition or spontaneous enzyme inactivation occurred.

Inhibition rates and equilibria were determined by plotting the progress of the reaction, when a reaction was initiated by the addition of enzyme to a mixture of substrate and inhibitor [22]. Inhibition reactions too fast for examination by pseudo-first-order conditions were analysed by direct second-order replotting of the progress-curve data [22].

SDS/PAGE and N-terminal sequencing

An 8–18 % linear acrylamide gradient SDS gel run in a 2-amino-2-methyl-1,3-propanediol/glycine/HCl system was used for resolving proteins [23]. Samples were boiled in SDS sample buffer containing 50 mM DTT for 5 min and then loaded on to the stacking gel. For N-terminal sequencing, protein samples were resolved by SDS/PAGE and transferred to an Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.) by electroblotting [24]. The membrane was briefly stained with Coomassie Brilliant Blue R-250, destained and washed with water. Appropriate bands were excised and sequenced by Edman degradation on a 476A protein sequencer (Applied Biosystems).

RESULTS

Initial inhibitor screening

Initial screening was conducted by incubating proteases in the range 1–10 nM with a large excess (1 μM) each of p35, CrmA and CrmA P1 mutants (Asp → Lys, Asp → Arg or Asp → Asn) or XIAP. Samples were allowed to incubate at 37 °C for 30 min, followed by addition of an appropriate substrate. For caspases, gingipains and clostripain, a pH of 7.2 was used, which was optimal for the inhibitors [14,15,25], but for legumain a lower pH was needed, because of the relatively low optimum of this enzyme. Therefore it was first determined that the inhibitors were competent at a pH compatible with legumain activity. Since it was necessary to verify inhibitor competence by titration against caspases, a compromise was made between the activity of caspases at a non-optimal pH [18] and the activity of legumain at a non-optimal pH. A pH of 5.75 was selected, since caspases 3 and 8 were sufficiently active at this pH to allow determination of inhibitor potency in the range where legumain was also active. CrmA, p35 and XIAP were all determined to be fully active at pH 5.75 by titration against caspase 3 (for p35 and XIAP) or caspase 8 (for CrmA). The arginine-specific gingipains are encoded by two genes in *Porphyromonas gingivalis*, referred to as *rgpA* and *rgpB*, the former of which encodes a substantial C-terminal extension of tandemly repeated haemagglutinin domains [26]. The catalytic domains and activities of the two products, gingipain-R/A and gingipain-R/B respectively, are very similar, and were used interchangeably during this study. Nevertheless, some experiments (see Table 1) were performed with both forms.

As expected, all three wild-type inhibitors completely inhibited the activity of their target caspases. In assessing inhibition against other proteases, further investigation was conducted only if more than 10 % inhibition was detected in the initial screening. This would equate to a K_i of greater than 10 μM, or a second-order rate constant of less than $10^2\ M^{-1} \cdot s^{-1}$, beyond which it would be difficult to distinguish true inhibition from competitive substrate binding, and therefore impractical to investigate any

Table 1 Initial inhibition data

Inhibition (+) was defined as a decrease in enzyme activity of more than 10% in the presence of 1 μM of each inhibitor. nd = not determined.

Enzyme	p35	CrmA	CrmA (Asp → Lys)	CrmA (Asp → Asn)	XIAP
Gingipain-R/A	−	−	nd	nd	−
Gingipain-R/B	−	−	−	−	nd
Gingipain-K	+	−	+	−	−
Legumain	−	−	−	−	−
Clostripain	−	−	−	−	−
Caspase 3	+	+	−	−	+

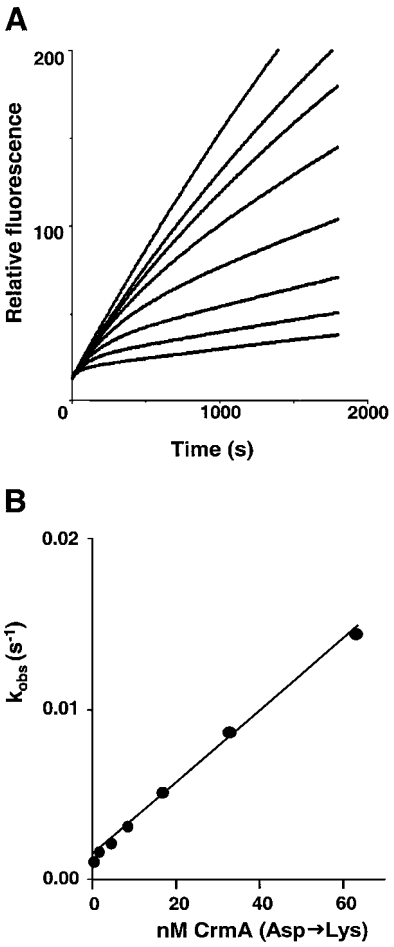

Figure 1 Inhibition of gingipain-K by CrmA

Gingipain-K was added to a final concentration of 1 nM to prewarmed 37 °C mixtures of CrmA in the range 0–64 nM containing 100 μM D-Val-Leu-Lys-pNa to monitor the reaction. Product formation, expressed as relative fluorescence, was recorded over the time course of the reactions (**A**). The uninhibited rate (v_0) is given by the curve of upper slope. The pseudo-first-order rates of inhibition (k_{obs}) were plotted against CrmA concentration (**B**) to allow calculation of the apparent rate of inhibition (k_{ass}).

mechanistic significance. As shown in Table 1, clostripain, legumain and gingipain-R were not inhibited by any of the proteins, gingipain-K was inhibited by the Asp → Lys P1 mutant of CrmA and, perhaps more suprisingly, also by the allegedly caspase-specific p35.

Inhibition by CrmA

Since wild-type CrmA failed to inhibit any of the non-caspases, the natural P1 aspartate residue was substituted in order to redirect specificity. The Asp → Arg mutant was targeted towards the arginine-specific clostripain and gingipains; the Asp → Lys mutant was targeted towards the lysine-specific gingipain-K; and the Asp → Asn mutant was targeted against the asparagine-specific legumain. Of these, only the Asp → Lys mutant was effective and kinetic analysis revealed that (CrmA)Asp → Lys inhibited gingipain-K. We analysed inhibition by progress curves, and the interaction was characterized by an apparent second-order rate constant (k_{ass}) of $(2.1 \pm 0.08) \times 10^5$ $M^{-1} \cdot s^{-1}$ (Figure 1). The interaction was very tight, since recovery in activity was not detected over prolonged periods or following dilution of pre-formed complexes to an inhibitor concentration of 2 nM. Thus the interaction had a K_i of < 0.2 nM, or was kinetically irreversible. The inhibition of gingipain-K by CrmA(Asp → Lys) was similar, from a mechanistic viewpoint, to that of caspase 8 by wild-type CrmA [15]. Both appeared to operate in a single kinetic step to form a very tight interaction, but there was no evidence of SDS-stable complexes (results not shown). However, the absence of such a complex may be because of the inherent lability of a presumptive *S*-ester under the conditions required to visualize it.

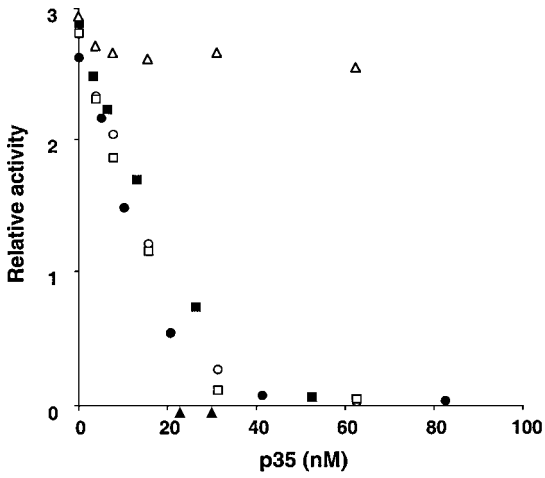

Figure 2 Specificity of gingipain-K inhibition by p35

The active concentration of gingipain-K used in this assay was 3.75 nM by titration with D-Phe-Phe-Arg-CH_2Cl. This was used to determine the respective concentrations of wild-type p35 and four mutants. Reactants were incubated for 30 min at 37 °C, after which D-Val-Leu-Lys-pNa was added to determine residual protease activity. Total protein concentrations of p35 derivatives are shown on the x-axis. ●, wild type; □, Lys^{225} mutant; ■, Lys^{257} mutant; ○, Lys^{225}/Lys^{257} mutant; △, Lys^{94} mutant. The arrowheads represent the low and high values of the titration intercepts on the x-axis.

Inhibition by p35

Wild-type p35 inhibited gingipain-K but not the other non-caspase members of clan CD. Analysis of inhibition was attempted with progress curves and the interaction was found to be very tight, since no recovery in activity over prolonged periods or following dilution of pre-formed complexes to an inhibitor concentration of 2 nM was detected. Thus the interaction had a K_i of < 0.2 nM or was kinetically irreversible, similarly to the interaction of gingipain-K with CrmA(Asp → Lys).

Analysis of the interaction of p35 with gingipain-K by SDS/PAGE revealed that the inhibitor was cleaved at three positions. These were determined, by Edman degradation, to be at Lys^{94}, Lys^{225} and Lys^{257}. On the grounds that one of these cleavage sites would represent the inhibitory site, each lysine residue was substituted in turn or in combination, by site-directed mutagenesis, to arginine. With the exception of the $Lys^{94} \rightarrow Arg$ mutation, all single substitutions had minimal effect on inhibition (Figure 2). The $Lys^{94} \rightarrow Arg$ mutation completely abolished inhibitory activity, whereas the combined $Lys^{225}/Lys^{257} \rightarrow Arg$ mutations were fully inhibitory. However, these interactions were characterized by partition ratios of 6–8, since an average of 6–8 molecules of p35 were required to inhibit one molecule of gingipain-K (Figure 2). This is in contrast with the interaction of gingipain-K with CrmA(Asp → Lys), which showed no evidence of partitioning. The rate of inhibition was too rapid to be determined under pseudo-first-order conditions ($I \gg E$),

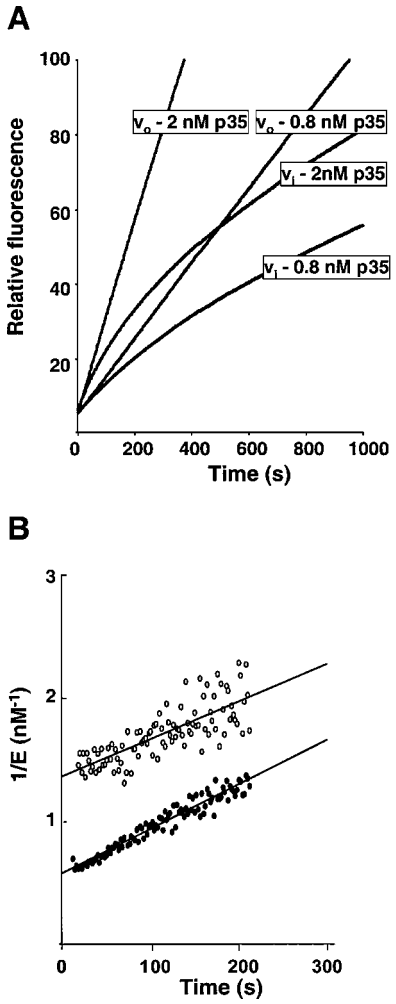

Figure 3 Inhibition rate of gingipain-K by the combined Lys^{225}/Lys^{257} → Arg mutant p35

Gingipain-K, substrate and p35 were allowed to equilibrate for 5 min at 37 °C then rapidly mixed to yield the indicated final concentrations in 100 μl wells of a microplate. Substrate hydrolysis was measured continuously during the time course of the experiment. (**A**) Progress curves obtained with 0.8 nM or 2 nM gingipain-K in the absence (v_0) or presence (v_i) of an equal concentration of p35, based on pre-titration. (**B**) Replot of the data obtained when residual enzyme activity (E) was calculated by subtracting the v_i values from the v_0 values at each time point during the initial decay phase (first 300 s).

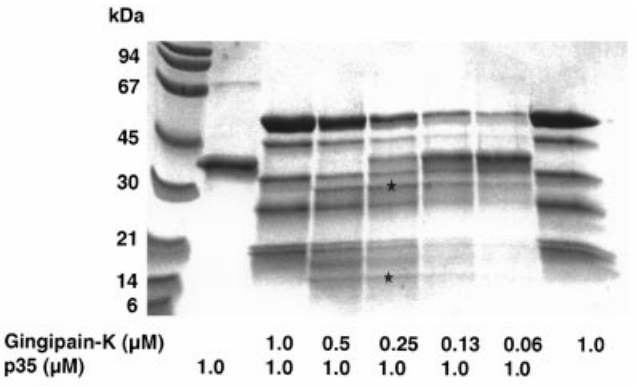

Figure 4 Proteolysis of p35 during its reaction with gingipain-K

Reactions (30 μl final volume) containing 1 μM p35 Lys$^{225/257}$ → Arg double mutant and the indicated final concentrations of gingipain-K were incubated for 30 min at 37 °C, followed by boiling and analysis by SDS/PAGE. The multiple bands in the gingipain-K preparation originate from the several haemagglutinin domains that populate the isolated enzyme [7], and the catalytic domain is the major at approx. 55 kDa. Cleavage of p35 to fragments of 25 kDa and 10 kDa (black stars) was essentially complete at a inhibitor/enzyme ratio of approx. 4.0. Molecular-mass markers are shown on the left.

therefore second-order conditions were applied (I = E) yielding an apparent second-order rate constant (k_{ass}) of $(3.3 \pm 0.3) \times 10^6\ M^{-1} \cdot s^{-1}$ for the combined Lys^{225}/Lys^{257} → Arg mutant (Figure 3).

Because of the complexity of gingipain-K and the complexity of the fragments produced when wild-type p35 was allowed to react with the enzyme, the interaction of gingipain-K with the combined Lys^{225}/Lys^{257} → Arg mutant of p35 was analysed by SDS/PAGE (Figure 4). Cleavage of p35 was observed with the concomitant production of fragments at 25 kDa and 10 kDa, consistent with proteolysis at position 94 in the reactive-site loop of the inhibitor. No SDS-stable complex was observed under the conditions used for sample preparation and analysis, and the partition ratio determined by this method was approx. 4. This was lower than the ratio of 6.7 defined by enzymic titration (Figure 2), and can probably be accounted for by the less precise quantification of band analysis with SDS/PAGE.

The recently elucidated mechanism of inhibition of caspase 8 by p35 demonstrates a crucial role for the side-chain thiol of Cys^2 of the inhibitor [27], and therefore the reaction of gingipain-K with a Cys → Ala mutation at position 2 was investigated. As shown in Figure 5, mutation at this position abrogated the inhibitory activity of p35 and converted it to a gingipain-K substrate. This indicates a vital role for Cys^2 in both caspase and gingipain inactivation, which provides evidence for a common mechanism of inhibition.

DISCUSSION

CrmA was previously known to inhibit diverse proteases from distinct clans, including the serine proteases granzyme B [17] and glutamyl endopeptidase [28], as well as the cysteine proteases caspase 1 and 8 [15,29]. These proteases, although representing different catalytic mechanisms, have in common the ability to readily accept an aspartic acid side chain into their primary substrate pockets. This, although not always sufficient, seems to be required for inhibition by CrmA. On the basis of primary sequence [30] and structure [31], CrmA is a member of the serpin family and members of this family display extraordinary adaptability for inhibiting diverse proteases. Simply mutating the primary interaction site, the P1 residue, can switch the specificity of serpins, such as α_1-proteinase inhibitor, from elastase-like to thrombin-like [32]. Serpins are considered to be mechanism-based inactivators, in other words they require the catalytic mechanism of their target proteases to generate a tight complex [33]. The ultimate complex of trypsin and α_1-proteinase inhibitor contains an ester between the catalytic serine of the enzyme and the carbonyl carbon of the scissile bond of the inhibitor [34], and complex formation forced a substantial disruption of the structure of the enzyme. It was assumed that CrmA, given its structural similarity to α_1-proteinase inhibitor, would operate by the same mechanism and therefore present a substrate-like sequence to an attacking protease. In the case of CrmA, the simple Asp → Lys P1 mutation rendered it a good gingipain-K inhibitor. If CrmA were to follow the suggested serpin inhibitory pathway the result would be an *S*-ester between its scissile carbonyl and the catalytic cysteine of gingipain-K. The fact that

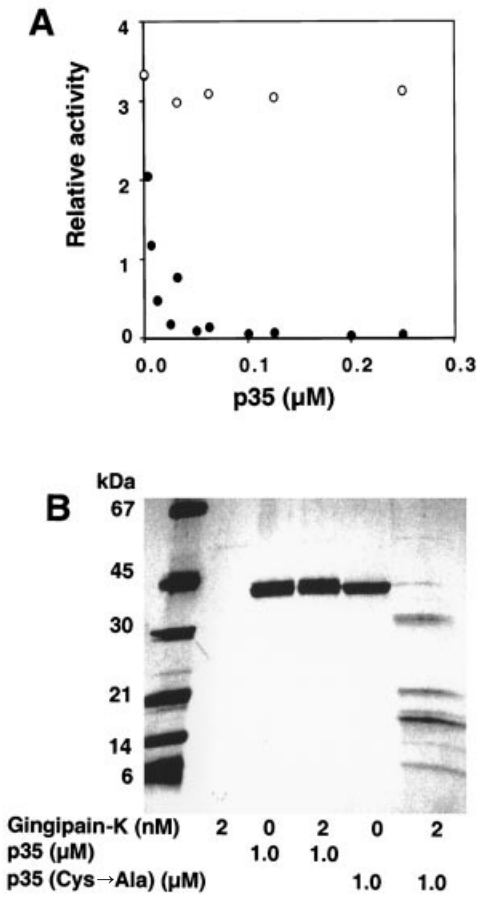

Figure 5 Titration of gingipain-K by p35 derivatives

The active concentration of gingipain-K used in this assay was 2 nM by titration with D-Phe-Phe-Arg-CH_2Cl. This was used to determine the respective concentrations of wild-type p35 and the p35 $Cys^2 \rightarrow$ Ala mutant (**A**). Reactants were incubated for 30 min at 37 °C, after which D-Val-Leu-Lys-pNa was added to determine residual protease activity. The p35 total protein concentrations are shown on the *x*-axis; (●) wild type, (○) $Cys^2 \rightarrow$ Ala mutant. A sample was withdrawn from each incubation mixture and incubated for 30 min at 37 °C, followed by boiling and analysis by SDS/PAGE (**B**). The multiple bands in the far right lane originate from gingipain-K-mediated proteolytic destruction of the p35 $Cys^2 \rightarrow$ Ala mutant.

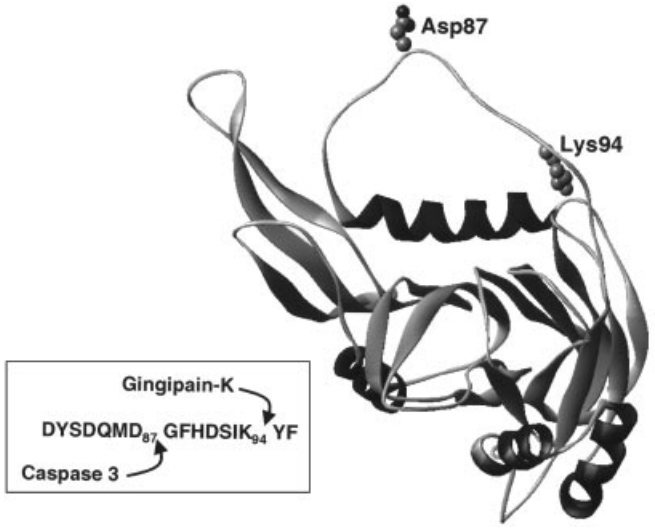

Figure 6 Primary interaction sites in the p35 molecule

The p35 molecule is shown as a ribbon representation [38], with the side-chains of Asp^{87} and Lys^{94} highlighted in CPK. These represent the P1 residues for caspases and gingipain-K respectively, and are contained in a mobile loop that constitutes the protease targeting region of p35 (sequence shown in the inset). The Figure was rendered with WebLab Viewer (MSI, San Diego, CA, U.S.A.).

a covalent complex between CrmA and gingipain-K was not observed, using SDS/PAGE (results not shown), is most likely because of the relative instability of thiol esters compared with oxygen esters (that form with serine proteases), but is fully consistent with the lack of an observable thiol ester following SDS/PAGE of caspase/CrmA complexes [15].

Two of the three families of inhibitors that were analysed, p35 and the IAPs, have previously been considered to be caspase-specific [12–14,16]. Although this could not be refuted for the IAPs, at least by using the human member of the family XIAP, it was clearly disproved for p35. Previously a number of serine proteases from distinct clans had been used, including the caspase analogue granzyme B, as well as representatives of the papain family (clan CA), but inhibition by p35 was not detected [14]. In the present study, however, the data on the inhibition of gingipain-K contradict the hypothesis that p35 is caspase-specific. Perhaps, more surprising, is the shift of the reactive centre to position 94, seven residues downstream of the reactive centre for caspase inhibition at Asp^{87}, indicating that the reactive site loop of p35 is unusually plastic in its ability to radically switch interaction sites (Figure 6). The inhibitory pathway used to inhibit caspase 8 involves proteolysis in the reactive site loop of p35, resulting in a radical transition of the buried N-terminus to a position where it abuts the catalytic histidine residue of the protease [27]. Mutation of Cys^2 abrogates caspase inhibition [27]. Since mutation of Cys^2 also abrogated inhibition of gingipain-K, we conclude that the same pathway is in operation, despite the large shift in the scissile bond relative to the caspase scissile bond. Presumably there is something in the structure or mechanism of p35 that restricts inhibition to clan CD proteases, which includes caspases and the gingipains. Interestingly, we were unable to detect inhibition of gingipain-R by the $Lys^{94} \rightarrow$ Arg mutant of p35. This is probably because of excessive partitioning, since this mutant was a substrate for gingipain-R. However, the rapid and tight inhibition of gingipain-K, a close homologue of gingipain-R, was clear-cut, despite an evident partition ratio.

The rather large partition ratio observed for gingipain-K indicated that the inhibition was not particularly efficient. Since cleavage of p35 was observed only at Lys^{94} (when the combined $Lys^{225}/Lys^{257} \rightarrow$ Arg mutant was used), partitioning was most likely because of competition between a substrate pathway and an inhibitory pathway at this site. The substrate pathway predominates, but 1 in 6–8 molecules of p35 were able to trap gingipain-K. Our previous studies on the inhibition of caspases by p35 demonstrated a slight partitioning of 1.3 during the reaction of p35 with caspase 3 [14], and it appears therefore that the large partitioning observed with gingipain-K represents a similar mechanism but with a lower yield of the productive (inhibitory) pathway. This behaviour is typical of mechanism-based inactivators [35] and, as far as protein inhibitors go, had been observed previously only with serpins [33]. The reason for partitioning in serpins pertains to the nature of the inhibitory pathway, which results from a large translocation of the cleaved reactive-site loop across the serpin surface [34]. If the protease has time to detach (hydrolyse the ester) before adopting the inhibited location, then partitioning occurs. We presume that a

similar conformational transition must take place during inhibition of gingipain-K by p35, as demonstrated recently for caspase 8 inhibition based on the crystal structure of the p35/caspase 8 complex [27].

Given our finding that p35 is selective for two disparate clan CD proteases, but non-inhibitory towards other protease scaffolds, there is presumably something unique about the mechanism of members of the clan. The most singular property of caspases and gingipains is the unusual placement of the catalytic residues. The nucleophilic cysteine residue is more distant from the general base histidine than in other protease clans, and these two residues bracket the scissile bond [36]. This situation is so far peculiar to clan CD, and is likely to be at least one of the properties confining inhibition by p35 to this protease fold. Finally, though *A. californica* nuclear polyhedrosis virus p35 is almost certainly designed to target caspases *in vivo*, we note that p35 from two other baculovirus that target *Leucania separata* and *Heliothis armigera* do not contain consensus caspase-recognition motifs in their reactive-site loops. Therefore we propose that p35 may be capable of targeting other non-caspase members of clan CD in certain infected hosts. Such members may include the recently described clan member separase, which is required for sister chromatid separation during anaphase [37].

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