Caspase-1 (interleukin-1 β -converting enzyme) is inhibited by the human serpin analogue proteinase inhibitor 9

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The regulation of caspases, cysteine proteinases that cleave their substrates after aspartic residues, is poorly understood, even though they are involved in tightly regulated cellular processes. The recently discovered serpin analogue proteinase inhibitor 9 (PI9) is unique among human serpin analogues in that it has an acidic residue in the putative specificity-determining position of the reactive-site loop. We measured the ability of PI9 to inhibit the amidolytic activity of several caspases. The hydrolysis of peptide substrates by caspase-1 (interleukin-1 β -converting enzyme), caspase-4 and caspase-8 is inhibited by PI9 in a time-dependent manner. The rate of reaction of caspase-1 with PI9, as well as the rate of substrate hydrolysis of the initial caspase-PI9 complex, shows a hyperbolic dependence on the concentration of PI9, indicative of a two-step kinetic mechanism for inhibition

with an apparent second-order rate constant of $7 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$. The hydrolysis of a tetrapeptide substrate by caspase-3 is not inhibited by PI9. The complexes of caspase-1 and caspase-4 with PI9 can be immunoprecipitated but no complex with caspase-3 can be detected. No complex can be immunoprecipitated if the active site of the caspase is blocked with a covalent inhibitor. These results show that PI9 is an inhibitor of caspase-1 and to a smaller extent caspase-4 and caspase-8, but not of the more distantly related caspase-3. PI9 is the first example of a human serpin analogue that inhibits members of this class of cysteine proteinases.

Key words: apoptosis, cysteine protease, cytokine, protease inhibition.

INTRODUCTION

Caspases [1] are a family of cysteine proteinases that are unusual in that they cleave after aspartic residues [2,3]. Caspase-1 (interleukin-1 β -converting enzyme; ICE) (EC 3.4.22.36) cleaves the Asp¹¹⁵-Ala¹¹⁶ bond in the inactive 31 kDa precursor pre-interleukin 1 β to generate the active 17 kDa cytokine [4,5]. The cDNA for caspase-1 has been cloned; the derived amino acid sequence indicates that it is unrelated to other well-characterized serine or cysteine proteinases [6,7]. Mature caspase-1 is composed of 20 kDa (p20) and 10 kDa (p10) subunits, which are derived from a common 45 kDa precursor [7,8]. The precursor can be cleaved by mature caspase-1 *in vitro* [9]; the proteinase that cleaves the precursor *in vivo* is unknown, although there is evidence to suggest that the processing is autocatalytic [10,11]. The three-dimensional structure of caspase-1 has been solved; it also differs from that of other known proteinases [12,13].

Caspase-1 possesses a high degree of similarity to the product of the *Caenorhabditis elegans* cell death gene *ced-3*, whose product is involved in apoptosis [14]. Several other human homologues have recently been identified on the basis of their sequence similarity with caspase-1 [15,16]. All caspases are apparently expressed as large precursors, which are then processed by one or more unknown proteinases into subunits of approx. 20 and 10 kDa. As implied by the sequence around the cleavage sites, all procaspases can be activated by cleavage with one of the caspases

in vitro; the proteinase that activates the procaspases *in vivo* is not known for many of the caspase family members. The three-dimensional structure of caspase-3 has been solved; with the exception of a few surface loops it is very similar to that of caspase-1 [17,18]. Studies with peptide substrates have indicated that all caspases require an aspartic residue in the P1 position; however, different caspases have a varied, but overlapping, specificity pattern in the P2 to P4 positions [19–21]. However, with the exception of caspase-1, which cleaves preinterleukin 1 β , and caspase-3, which cleaves the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) and the regulatory subunit of protein phosphatase 2A [22–24], information about the substrate *in vivo* for most caspases is not available.

Apparently, all catalytically active caspases, if overexpressed, induce cells to undergo apoptosis [16]. There is also strong evidence for the role of specific caspases at natural abundance. Inhibitors selective for caspase-3 will block apoptosis at concentrations at which inhibitors selective for caspase-1 are ineffective [23]. Caspase-3 is converted from its precursor to active 17 and 12 kDa subunits early in the course of apoptosis; PARP is also cleaved early in the apoptotic process, which is consistent with the activation of caspase-3 [25]. Caspase-3 knock-out mice do not survive to adulthood; post-mortem examination reveals lesions in the brain of these mice that are consistent with defective apoptosis [26]. However, other organs from these mice are less affected by defects in apoptosis, indicating that caspase-

Abbreviations used: AcDEVD-pNA, Ac-Asp-Glu-Val-Asp *p*-nitroanilide; AcYVAD-pNA, Ac-Tyr-Val-Ala-Asp *p*-nitroanilide; CrmA, cytokine response modifier A; ICE, interleukin-1 β -converting enzyme (caspase-1); p10, 10 kDa subunit of caspases; p20, 20 kDa subunit of caspases; PARP, poly(ADP-ribose) polymerase; PI9, proteinase inhibitor 9.

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3 activity is duplicated by other proteinases in some tissues. The recently discovered family members caspase-8 (FLICE) and caspase-10 (Mch4) contain in their prodomains 'death effector' domains that bind to the corresponding domain in the adapter molecule fas-associated death domain, which in turn binds to the death receptor CD95 [27,28]. This directly links caspases to the CD95/Fas apoptotic pathway and places caspase-8 and caspase-10 at the top of a proposed caspase cascade, arranged on the basis of the procaspase activation experiments *in vitro* [15]. In this model, procaspase-8 and procaspase-10 bind to the CD95 complex and are activated by ligand binding to the receptor. Caspase-8 and caspase-10 then activate other caspases, such as caspase-6 and caspase-7, which in turn activate lower caspases, such as caspase-2 and caspase-3. These latter caspases then cleave proteins such as PARP that are cleaved during apoptosis. Studies with caspase-1 knock-out mice have shown that they are deficient in the production of mature interleukin 1 β but they are not deficient in apoptosis, as judged by a variety of model apoptotic systems [29,30]. Thus, whereas caspase-1 is involved primarily in cytokine maturation, other caspases are intimately involved in the apoptotic process.

Serine proteinase inhibitors, or serpins, are a family of 40–50 kDa proteins that are involved primarily in the regulation of physiological processes that are activated by proteinases, such as prohormone activation and haemostasis [31]. Serpins bind at the active site of their cognate proteinases to form an inhibitory complex with 1:1 stoichiometry that is resistant to denaturants [32]. Although most serpins are mammalian in origin, a few viral serpin analogues have been described [33]. In this regard, cytokine response modifier A (CrmA), produced by the cowpox virus, inhibits caspase-1 and thereby modulates the host inflammatory response [34]. CrmA and a recently described serpin analogue from bovine adrenal medulla that inhibits a proteinase involved in enkephalin processing [35] are novel in that they interact with cysteine proteinases; other known serpins inhibit serine proteinases.

Proteinase inhibitor 9 (PI9), which was cloned from a placental cDNA library on the basis of its similarity to the cytoplasmic antiproteinase PI6, is unique among human serpin analogues so far reported in that it contains an acidic residue (Glu³⁴⁰) in the specificity-determining position of the active loop [36]. The reactive loop region of PI9 (Val-Val-Ala-Glu³⁴⁰-Cys) is similar to that of CrmA (Leu-Val-Ala-Asp-Cys) and to the cleaved sequence in preinterleukin 1 β (Tyr-Val-His-Asp-Ala), suggesting that PI9 might have a similar proteinase-binding specificity to that of CrmA. In addition, PI9 contains no signal sequence for export from the cell and it is most similar to intracellular serpins, suggesting that it might inhibit cytosolic, rather than secreted, proteinases [36]. PI9 was recently shown to rapidly inhibit *in vitro* the serine proteinase granzyme B [37], which can process procaspase-3 into its active form [38,39]. Here we report the interaction of PI9 with caspase-1, caspase-4 and caspase-8, but not with caspase-3. The tight inhibitory complex forms slowly, and it can be isolated by immunoprecipitation. This is the first example of a human cytosolic serpin analogue that inhibits this important new class of cysteine proteinases.

EXPERIMENTAL

Materials

Ac-Tyr-Val-Ala-Asp *p*-nitroanilide (AcYVAD-pNA) and carbobenzyloxylglycine *p*-nitrophenyl ester were from Bachem Bioscience (King of Prussia, PA, U.S.A.). Ac-Asp-Glu-Val-Asp-*p*-nitroanilide (AcDEVDPNA) was from California Peptide Research (Napa, CA, U.S.A.). Ac-Asp-Glu-Val-Asp-(7-amino-

methyl)coumarin was obtained from Alexis Corp. (San Diego, CA, U.S.A.). PBS was from Digene Diagnostics (Beltsville, MD, U.S.A.). Cathepsin B, its substrate carbobenzyloxy-Arg-Arg-(7-aminomethyl)coumarin and papain were obtained from Sigma (St. Louis, MO, U.S.A.). Biotin-Tyr-Val-Ala-Asp-acyloxymethyl ketone (ICE Inhibitor III, biotin conjugate) was purchased from Calbiochem (La Jolla, CA, U.S.A.). Mouse anti-(caspase-3) monoclonal antibody was obtained from Transduction Laboratories (Lexington, KY, U.S.A.). Rabbit polyclonal antibodies against caspase-1 p10 and caspase-1 p20 were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Rabbit anti-(caspase-4) IgG was a gift from D. Banach (BASF Bioresearch Corporation, Worcester, MA, U.S.A.). Horseradish-peroxidase-conjugated donkey anti-rabbit IgG was from Amersham (Arlington Heights, IL, U.S.A.). Goat anti-rabbit IgG and horseradish-peroxidase-conjugated goat anti-mouse IgG were from Oncogene Science (Cambridge, MA, U.S.A.).

Preparation of caspases

Recombinant caspase-1 was prepared from *Escherichia coli* BL21 (DE3)/pLysS bearing a plasmid containing the full-length cDNA for caspase-1 by affinity chromatography on aldehyde-inhibitor-linked resin as described [7]. This caspase-1 contained glutathione covalently bound to the active-site thiol to stabilize the enzyme for storage. Dithiothreitol was included in assays and incubations to remove this ligand. Histidine-tagged caspase-3, caspase-4 and caspase-8 were expressed in *E. coli* and purified by immobilized-metal-affinity chromatography, as described [40]. Results comparing histidine-tagged caspase-1 with material purified without the histidine tag indicate that there is no difference in the Michaelis–Menten parameters between the two enzymes (D. Giegel, unpublished work). Therefore the histidine tag was not removed from caspase-3, caspase-4 or caspase-8.

Preparation of PI9

Recombinant PI9 was purified from transformed yeast lysates by heparin–agarose and ion-exchange chromatographies, as described [36]. The Mono-Q-purified PI9 was dialysed at 4 °C against 25 mM sodium citrate/1 mM dithiothreitol (pH 5.1) and subsequently applied to a Mono S FPLC HR5/5 column equilibrated at room temperature with the same buffer. PI9 was eluted from the column in a linear gradient of NaCl (0–1 M) in equilibrating buffer. Fractions from the Mono S column were analysed by SDS/PAGE; fractions containing purified PI9 were pooled and stored at –80 °C.

Anti-PI9 antibodies

Antibodies against pure recombinant PI9 were generated in rabbits [41]; the IgG fraction was purified by Protein A–Sephacrose column chromatography.

Synthesis of Ac-Tyr-Val-Lys(fluorescein)-aspartic α -aldehyde

A solution (1 ml) containing 200 mM H₃BO₃, pH 8.5, and 10 mg (14.5 μ mol) of Ac-Tyr-Val-Lys-aspartic α -aldehyde (Bachem Bioscience) in water was mixed with 1 ml of dimethylformamide containing 13.8 mg (29 μ mol) of fluorescein 5,6-succinimidyl ester (Molecular Probes, Eugene, OR, U.S.A.) and incubated at room temperature for 30 min. The product was purified by HPLC on a Vydac reverse-phase C₁₈ column and a linear gradient of acetonitrile/water with 0.1% (v/v) trifluoroacetic acid. Freeze-drying yielded 7.0 mg (7.7 μ mol; 53%) of orange

powder. Electrospray MS ($M + H^+ = 908.1$) confirmed the identity of the product.

Kinetic characterization of proteinase inhibition by PI9: inhibition in the presence of substrate

The hydrolysis of tetrapeptide *p*-nitroanilides by caspases was measured with a Cary-4 UV spectrophotometer (Varian Instruments, Palo Alto, CA, U.S.A.). The substrates were AcYVAD-pNA for caspase-1 and caspase-4, and AcDEVD-pNA for caspase-3 and caspase-8. The reactions were performed in HGE buffer [100 mM Hepes/20% (v/v) glycerol/0.5 mM EDTA (pH 7.4)] containing 0.5 mg/ml BSA, substrate and enzyme (1.5–5 nM caspase-1, 0.15 nM caspase-3, 50 nM caspase-4 or 10 nM caspase-8). Dithiothreitol (5 mM) was added to the caspase-1, caspase-4 and caspase-8 assays to stabilize the enzyme. A control experiment showed no effect of dithiothreitol on the inhibition of caspase-3 by PI9; because dithiothreitol destabilized this enzyme it was omitted from reactions containing caspase-3. The release of *p*-nitroaniline from pNA substrates at 30 °C was followed spectrophotometrically at 380 nm for 10 min; an aliquot of PI9 was then added. The reaction was followed for a further 140 min and the amount of *p*-nitroaniline released was calculated from an ϵ_{380} of 14 600 M⁻¹·cm⁻¹, which was determined by using authentic *p*-nitroaniline in the same buffer.

Inhibition of caspases by PI9 in the absence of substrate

To measure the time course of inactivation in the absence of substrate, caspase-1 (50 nM) and PI9 (0 to 5 μ M) were incubated at 30 °C in a 90 μ l reaction containing HGE buffer, 0.5 mg/ml BSA and 5 mM dithiothreitol. At different times, a 15 μ l aliquot of the reaction was diluted 1:10 into an assay mixture containing 5 mM dithiothreitol, 0.5 mg/ml BSA and 200 μ M AcYVAD-pNA (final concentrations) in HGE buffer that had been equilibrated at 30 °C. The release of *p*-nitroaniline was followed spectrophotometrically for 10 min at 380 nm with a Cary 4 spectrometer.

To measure the inactivation rates of caspase-3 and caspase-4 in the absence of substrate, a slightly different experiment was performed with a 96-well plate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). Solutions (200 μ l final volume) containing HGE buffer plus 0.5 mg/ml BSA, 5 mM dithiothreitol and 0–2 μ M PI9 (final concentrations) were incubated at 30 °C in one column of wells. Proteinase was added simultaneously to all these wells with a multichannel pipette to a final concentration of 0.25 μ M to start the reaction; 20 μ l aliquots were transferred at intervals to wells containing 80 μ l of HGE buffer plus 0.5 mg/ml BSA, 5 mM dithiothreitol and the appropriate *p*-nitroanilide substrate. With caspase-3, the substrate was 200 μ M AcDEVD-pNA; with caspase-4 it was 1 mM AcYVAD-pNA. The release of product was followed spectrophotometrically for 5 min at 405 nm.

To measure the inactivation rate of caspase-8 in the absence of substrate, an experiment was performed with a 96-well fluorescence plate reader (Molecular Devices). Solutions (100 μ l final volume) containing HGE buffer plus 0.5 mg/ml BSA, 5 mM dithiothreitol and 0–10 μ M PI9 (final concentrations) were incubated at 30 °C in one column of wells. Caspase-8 was added simultaneously to all these wells with a multichannel pipette to a final concentration of 0.1–0.2 μ M to start the reaction; 10 μ l aliquots were transferred at intervals to wells containing 190 μ l of HGE buffer plus 0.5 mg/ml BSA, 5 mM dithiothreitol and 150 μ M Ac-Asp-Glu-Val-Asp-(7-aminomethyl)coumarin (final concentrations). The release of product was followed for 5 min

by fluorescence. The excitation and emission filters were 390 and 460 nm respectively.

Breakdown of the caspase-1–PI9 complex

To measure the rate of dissociation of the caspase-1–PI9 complex, a gel-filtration chromatography procedure was used. The caspase-1–PI9 complex was formed by incubating 2.5 μ M caspase-1 and 2.5 μ M PI9 for 1 h in the presence of 5 mM dithiothreitol in HGE buffer containing 0.5 mg/ml RNase A as a non-specific protein. On the basis of the inactivation data, this duration would result in less than 20% residual caspase-1 activity. The dissociation reaction was initiated by diluting the complex 1:5 into HGE buffer containing 5 mM dithiothreitol, 0.5 mg/ml RNase A, 0.5% DMSO (from fluorescent peptide stock) and 10 μ M Ac-Tyr-Val-Lys(fluorescein)-aspartic α -aldehyde (final concentration), and the reaction was incubated at 30 °C. At intervals, 35 μ l aliquots were separated on a Sephadex-75 column (3.2 mm $\times$ 300 mm; Pharmacia Biotechnology, Piscataway, NJ, U.S.A.) with a SMART chromatography system (Pharmacia). The eluent buffer was 50 mM sodium phosphate/500 mM NaCl/0.1% (w/v) CHAPS (pH 7.4), flowing at 50 μ l/min; the fluorescein was detected at 490 nm and quantified with SMART software.

Other proteinases

The inhibition of cathepsin B was measured by preincubating 0.5 μ M enzyme with 1 μ M PI9 in HGE buffer containing 2 mM dithiothreitol. Cathepsin B activity was assayed with the fluorescent substrate carbobenzyloxy-Arg-Arg-(7-aminomethyl)-coumarin, as described [42]. The inhibition of papain by PI9 was measured with the 96-well plate assay as described above for caspase-1. Papain was activated at 4 °C for 20 min in 50 mM potassium phosphate buffer/0.5 mM EDTA/20 mM cysteine (pH 7.0); the cysteine was removed with a BioGel P-6 spin column (Bio-Rad Laboratories, Hercules, CA, U.S.A.) pre-equilibrated with 50 mM potassium phosphate/0.5 mM EDTA (pH 7.0). Activated papain was added to the preincubation solution containing 50 mM potassium phosphate, 0.5 mM EDTA, pH 7.0, and 0 or 1 μ M PI9 (final concentration) to give a final concentration of 0.5 μ M to start the reaction. Aliquots of the preincubation mixture were added at intervals to an assay mixture containing 625 μ M carbobenzyloxyglycine *p*-nitrophenyl ester in 50 mM potassium phosphate buffer/0.5 mM EDTA (pH 7.0); the release of *p*-nitrophenol was followed spectrophotometrically at 405 nm [43].

SDS/PAGE of caspase-1–PI9 complex

A solution of 670 nM caspase-1 and 1.33 μ M PI9 in 50 μ l of 100 mM Hepes/10% (w/v) sucrose/0.1% (w/v) CHAPS/5 mM dithiothreitol (pH 7.4) was allowed to react with 30 °C. At intervals a 10 μ l aliquot was quenched by the addition of 12 μ l of 2 $\times$ gel loading buffer (Integrated Separation Systems, Hyde Park, MA, U.S.A.) containing 10% (v/v) 2-mercaptoethanol and heating at 100 °C for 10 min. Duplicate 10–20% (w/v) gradient polyacrylamide gels (Novex Experimental Technologies, San Diego, CA, U.S.A.) were loaded with 10 μ l samples and run in a Tris/Tricine buffer system in accordance with the manufacturer's instructions. One gel was silver-stained with the Novex kit; the other was blotted to nitrocellulose in Novex blotting buffer at 215 mA for 1 h. The nitrocellulose was blocked with 2% (w/v) BSA/2% (w/v) non-fat dried milk/0.3% (v/v) Tween 20 in PBS and analysed by Western blotting with an enhanced

chemiluminescence kit (ECL[®], Amersham). The primary antibody, anti-PI9 (5 μ l, 4.6 mg/ml), was added directly to the blocking solution (25 ml). The secondary antibody, horseradish-peroxidase-conjugated donkey anti-rabbit IgG, was used at 1:5000 dilution. After exposure the blot was stripped in accordance with the ECL kit instructions and reprobed with anti-(caspase-1) antibodies in an analogous manner.

Immunoprecipitation of caspase-1-PI9 complex

Solutions of caspase-1 (1.5 μ M) and caspase-4 (6 μ M) were treated with a 10-fold excess of the caspase-1 inhibitor biotin-Tyr-Val-Ala-Asp-acyloxymethyl ketone in HGE buffer for 1 h at 30 °C to block the active sites covalently [44]. Blocked or untreated caspases were incubated with or without PI9 (each protein was at 1.0 μ M) in 20 μ l of HGE buffer with 0.5 mg/ml BSA and 5 mM dithiothreitol at 30 °C for 1 h; 500 μ l of ice-cold TBS-T buffer [50 mM Tris/HCl (pH 7.4)/150 mM NaCl/1 mM EDTA/1% (v/v) Triton X-100] containing 10 mg/ml BSA was added to stop the reaction. Anti-PI9 antibodies (2 μ l, 4.6 mg/ml) were added to each reaction; the solutions were mixed at 4 °C for 1 h. Protein A-agarose (20 μ l) was added and the suspensions were mixed for a further 1 h at 4 °C and centrifuged. The pellets were washed twice with TBS-T buffer containing BSA and once with TBS-T buffer. The pellets were suspended in approx. 20 μ l of the last wash, then 20 μ l of 2 $\times$ gel loading buffer containing 10% (v/v) 2-mercaptoethanol was added. The samples were heated to 100 °C for 10 min; 20 μ l of each sample was run on a 14% (w/v) polyacrylamide gel with a Tris/glycine buffer or on a 10–20% (w/v) gradient polyacrylamide gel with a Tris/Tricine buffer, along with a sample of the blocked enzymes as a control. The gel was transferred to nitrocellulose, which was blocked with milk and BSA as described above. Interaction of the secondary antibody with the anti-PI9 used for immunoprecipitation was decreased by treating the blot with 2 μ g/ml goat anti-rabbit IgG in blocking buffer at room temperature for 1 h. The blot was then probed with anti-(caspase-4) (1:1000 dilution in blocking buffer), anti-(caspase-1 p20) and anti-(caspase-1 p10) antibodies, or anti-(caspase-3), as described above.

RESULTS

Isolation of the caspase-PI9 complexes

To isolate the serpin-caspase complex, immunoprecipitation was performed *in vitro*. As a control to verify that the interaction between caspase and PI9 required a free active site in the caspase, the active site cysteine residues of caspase-1 or caspase-4 were alkylated by using the irreversible caspase-1 inhibitor biotin-Tyr-Val-Ala-Asp-acyloxymethyl ketone [44]. An assay with 200 μ M AcYVAD-pNA showed that 10% of the original caspase-1 activity and 7% of the original caspase-4 activity remained. By using Protein A-agarose, a mixture of PI9 and active-site-blocked or untreated caspase-1 or caspase-4 was precipitated with anti-PI9 antibodies. After SDS/PAGE [10–20% (w/v) gradient gel] with a Tris/Tricine buffer system and immunoblotting, a band that was immunoreactive with anti-p10 could be detected in only the caspase-1 (Figure 1a) or caspase-4 (Figure 1b) reactions that contained untreated caspase. No band was seen in control reactions without added PI9. A faint band was detectable from the reactions with blocked enzyme with an intensity approximately corresponding to the amount of activity remaining in the blocked enzyme samples. The p20 subunits comigrated in this gel system with a minor immunoreactive component of the anti-PI9 antibodies used in the immunoprecipitation. Therefore a small amount of p20 would be obscured

Figure 1 Immunoprecipitation of caspase-PI9 complexes

The complex between caspase-1 (a) or caspase-4 (b) and PI9 was formed *in vitro*, precipitated with anti-PI9 antiserum and separated on a 10–20% (w/v) gradient polyacrylamide gel with a Tris/Tricine/SDS buffer system. The gels were immunoblotted and probed with anti-(caspase-1) p10 antibodies. Lanes contained a blocked caspase standard (S) or the immunoprecipitated complex from a reaction of blocked (B) or untreated (U) caspase incubated with (+) or without (–) PI9, as indicated below each gel picture.

by this impurity. The immunoprecipitates were separated on a 14% (w/v) polyacrylamide gel by using a Tris/glycine buffer system; p20 was more clearly resolved from this impurity to give the same result as that seen with p10 (results not shown). An analogous experiment with caspase-3 showed no evidence for the precipitation of a caspase-3-PI9 complex (results not shown). To ensure that blocking the active site did not interfere with recognition by the anti-(caspase-1) or anti-(caspase-4) antibodies, a standard sample of blocked enzyme was loaded directly on the gel. These results show that a tightly bound complex between caspase-1 or caspase-4 and PI9 formed when the two proteins were incubated together *in vitro* and that this was precipitated with anti-PI9 antibodies. In addition, blocking the active site of the enzyme with a covalent inactivator greatly diminished the formation of the complex.

Because serpins are known to form SDS-stable complexes with serine proteinases [31], we attempted to detect complex formation between caspase-1 and PI9 by SDS/PAGE. Electrophoresis followed by silver-staining or immunoblotting showed no evidence for the formation of a high-molecular-mass complex (results not shown). The behaviour of PI9 in this system was similar to that of CrmA with caspase-1 in a similar experiment [45]. Non-denaturing gel electrophoresis of the caspase-1-PI9 complex was not entirely successful with recombinant proteins. This was similar to the results obtained with caspase-1 and CrmA [45]. Support for a stoichiometric association between caspase-1 and PI9 comes from monitoring the release of caspase-1 from a preformed, fully inhibited complex of PI9 and caspase-1 (see below).

Figure 2 Effect of PI9 on hydrolysis by caspases

Zero time corresponds to the addition of PI9. **(a)** Progress curves for the hydrolysis of 20 μ M AcYVAD-pNA by 1.5 nM caspase-1 in the absence (solid line) or presence (broken line) of 500 nM PI9. **(b)** Progress curves for the hydrolysis of 20 μ M AcDEVD-pNA by 0.15 nM caspase-3 in the absence (solid line) or presence (broken line) of 500 nM PI9. **(c)** Progress curves for the hydrolysis of 20 μ M AcYVAD-pNA by 50 nM caspase-4 in the absence (solid line) or presence (broken line) of 100 nM PI9. **(d)** Progress curves for the hydrolysis of 20 μ M AcDEVD-pNA by 10 nM caspase-8 in the absence (solid line) or presence (broken line) of 500 nM PI9.

Mechanism A:

Mechanism B:

Scheme 1

Figure 3 Effect of PI9 on initial rate of hydrolysis by caspase-1

Inhibition of caspases by PI9

To determine whether the caspase-PI9 complex was inhibitory, we examined the effect of adding PI9 directly to a caspase assay system. Progress curves show that the reaction between caspase-1 (Figure 2a), caspase-4 (Figure 2c) or caspase-8 (Figure 2d) and 500 nM PI9 was consistent with a slow-binding inhibitory pattern. Caspase-3, in contrast, was not inhibited by 500 nM PI9 (Figure 2b) during the course of this experiment.

Models for slow-binding inhibitors

Two kinetic mechanisms for the slow-binding inhibition of enzymes are commonly encountered [46]. These mechanisms are summarized in Scheme 1. Slow-binding inhibition could arise from a simple second-order interaction between enzyme (E) and inhibitor (I) in which the rate of complex formation is slow

(mechanism A). Alternatively, slow-binding behaviour can arise when enzyme and inhibitor rapidly form an initial complex $E \cdot I$, which isomerizes slowly to form the final, tight complex $E \cdot I^*$ (mechanism B). These mechanisms can be differentiated kinetically by examining the behaviour of the enzyme-inhibitor system at differing concentrations of inhibitor. In the initial stage of the reaction, the slow steps have not progressed significantly. In the presence of substrate, mechanism A would predict that the initial rate of substrate hydrolysis would be independent of inhibitor concentration because $E \cdot I^*$ has not built up to a significant extent. In contrast, mechanism B would predict that initially I would be a classical competitive inhibitor of E, and increasing the concentration of inhibitor would decrease the initial rate of substrate hydrolysis hyperbolically. If the reaction between enzyme and inhibitor were followed for a long period, an apparent

Figure 4 Effect of 0 μM (●), 0.25 μM (○) or 1 μM PI9 (×) on the activity of caspases in the absence of substrate

(a) Caspase-1 activity; (b) caspase-3 activity; (c) caspase-4 activity; (d) caspase-8 activity.

rate constant, k_{app} , for the formation of $\text{E} \cdot \text{I}^*$ can be determined. Mechanism A predicts that the rate of the slow step in the reaction between E and I would depend on the concentration of I; k_{app} would show a linear dependence on [I]. In contrast, mechanism B predicts that the rate of the slow step in the reaction between E and I would depend on the concentration of $\text{E} \cdot \text{I}$; k_{app} would show a hyperbolic dependence on [I], limited ultimately by the concentration of enzyme.

Effect of varying PI9 concentration on initial rate of hydrolysis

To determine which mechanism of inhibition applies to the inhibition of caspase-1 by PI9, an analysis of the effects of varying the PI9 concentration was performed. Progress curves for the hydrolysis of substrate in the presence of a slow binding inhibitor can be fitted to the following integrated rate equation:

$$P = v_{\text{ss}}t + (v_i - v_{\text{ss}})(1 - e^{-k_{\text{app}}t})/k_{\text{app}} \quad (1)$$

where P is the amount of product produced at time t , to determine the initial and final rates of hydrolysis (v_i and v_{ss} respectively) as well as the apparent rate of inactivation, k_{app} [46]. The data for inhibition of caspase-1 with various amounts of PI9 were fitted to eqn. (1); in all cases the best fit to the experimental data was obtained when v_{ss} was fixed at 0. The variation of v_0/v_i , where v_0 is the initial rate in the absence of inhibitor, with varying PI9 concentration was examined in more detail. If the inhibition follows mechanism A, no variation of v_i with [PI9] would be expected and v_0/v_i would always be 1. For mechanism B, in which PI9 would function as a competitive inhibitor, v_0/v_i would follow the following equation:

$$v_0/v_i = 1 + [K_m/(K_m + [S])][[I]/K_i] \quad (2)$$

which can be readily derived from the standard equation for

competitive inhibition. Here, K_m is the Michaelis constant for hydrolysis of substrate with concentration [S] and K_i is the dissociation constant for the binding of PI9 of concentration [I] in the rapid equilibrium step (i.e. $K_i = k_{-1}/k_1$ in mechanism B). A plot of v_0/v_i against PI9 concentration would reveal a linearly increasing relationship with intercept 1.

Figure 3 shows the results of such an analysis with caspase-1 and PI9. This plot shows that v_0/v_i increases with increasing PI9 concentration, suggesting that the interaction follows mechanism B. An analysis of the slope of this line by using the relationship described in eqn. (2) and known values for K_m and substrate concentration gives a value for K_i of $2.7 \pm 0.13 \mu\text{M}$ for the inhibition of caspase-1 by PI9. Inhibition of caspase-4 was not analysable by this method because the best value of v_{ss} was not 0 and the inhibition of caspase-8 was too slow.

Effect of varying PI9 concentration on apparent inactivation rate

To determine the effect of varying the PI9 concentration on the apparent rate of inactivation, PI9 and caspase were incubated in the absence of substrate; at intervals an aliquot was removed to assay with a saturating amount of substrate. Because the volume of incubation was small, this experiment allowed the use of PI9 concentrations above the K_i measured in the preceding experiment. Figure 4 shows the results of this experiment for caspase-1, caspase-3, caspase-4 and caspase-8. For caspase-1 (Figure 4a), the addition of PI9 caused a more rapid disappearance of hydrolytic activity than in the control reaction. With caspase-3 (Figure 4b), there was no difference in the rate of disappearance of enzymic activity in the presence and the absence of PI9. Caspase-4 showed a more complicated behaviour (Figure 4c): in the absence of PI9, the disappearance of caspase activity showed biphasic characteristics; although a number of different

Mechanism A': **Mechanism B':** **Scheme 2**

E_{inact} , inactive enzyme.

buffer conditions were tried, no conditions could be found under which the enzyme was more well-behaved. The addition of PI9 to the enzyme resulted in inactivation that was qualitatively more rapid but was difficult to analyse quantitatively. Caspase-8 (Figure 4d) was fairly stable in the absence of PI9, showing little disappearance of activity. The addition of PI9 caused an apparent first-order decrease in activity. These experiments show that PI9 inhibits caspase-1, caspase-4 and caspase-8, but not caspase-3, in a time-dependent manner.

Models for slow-binding inhibitors accounting for slow autoinactivation of the enzyme

To define more closely the interaction between caspase-1 and PI9, the models depicted in Scheme 1 were modified to take into account the inactivation of the enzyme in the absence of any inhibitor (Scheme 2). Mechanisms A' and B' predict identical behaviour to mechanisms A and B respectively when the initial rate of hydrolysis is considered, because the autoinactivation reaction governed by the rate constant k_{inact} is slow and no significant amounts of E_{inact} have built up. Therefore the conclusions of the previous experiment, based on the initial rates of hydrolysis of substrate, would not change when considering mechanisms A' and B'.

When the effects of the inactivation step on the behaviour of the apparent rate constant for disappearance of activity in mechanism B' is examined, some changes from the predictions of mechanism B are found. We shall assume that E, I and $E \bullet I$ are in rapid equilibrium, i.e. that k_1 and k_{-1} are fast relative to the time necessary to assay enzyme activity, or k_{cat} in Michaelis-Menten terminology. The inactivation rate constant k_{inact} and the forward inhibition rate constant k_2 are low enough for us to be able to measure an initial rate of hydrolytic activity when an $E + I$ reaction is diluted into an assay, i.e. $k_1, k_{-1} \gg k_{\text{cat}} \gg k_{\text{inact}}, k_2$. The results in Figures 3 and 4 suggest that the slow step proceeds substantially to completion, and the reaction can be considered irreversible, i.e. $k_{-2} \ll k_2$.

We can define an equilibrium dissociation constant for the rapid part of the reaction, K_i :

$$K_i = k_{-1}/k_1 = [E][I]/[E \bullet I] \quad (3)$$

Defining $R \equiv [E] + [E \bullet I]$, assuming that $[I] \gg [E \bullet I]$, and substituting and rearranging, we get:

$$[E \bullet I] = R[I]/(K_i + [I]) \quad (4)$$

and

$$[E] = RK_i/(K_i + [I]) \quad (5)$$

R also represents the concentration of enzyme that is available to react with substrate in an assay, because competitive inhibition with I in the assay, owing to the rapid phase of the mechanism, is minimal because the inhibitor is diluted well below K_i and the substrate concentration is well above K_m . The observed disappearance of enzymic activity, dR/dt , is a combination of that due to inactivation and that due to inhibition:

$$dR/dt = -k_{\text{inact}}[E] - k_2[E \bullet I] = -k_{\text{inact}}RK_i/(K_i + [I]) - k_2R[I]/(K_i + [I]) \quad (6)$$

$$dR/dt = -[(k_{\text{inact}}K_i + k_2[I])/(K_i + [I])]R \quad (7)$$

which solves to:

$$R = E_0 e^{-k_{\text{app}}t} \quad (8)$$

where E_0 is the amount of assayable enzyme at time zero and

$$k_{\text{app}} = (k_{\text{inact}}K_i + k_2[I])/(K_i + [I]) \quad (9)$$

Note that if k_{inact} is 0, eqn. (9) simplifies to that predicted by mechanism B [46].

Figure 5(a) shows the variation in k_{app} for caspase-1 inactivation with changing PI9 concentration. It shows clearly that the relationship between k_{app} and PI9 concentration is non-linear. The curve represents a fit to eqn. (9), showing that the binding of PI9 to caspase-1 is consistent with mechanism B'. This plot also shows that k_{inact} calculated from the fit $[(1.0 \pm 0.28) \times 10^{-4} \text{ s}^{-1}]$ agrees with that actually measured with no PI9 $[(1.2 \pm 0.20) \times 10^{-4} \text{ s}^{-1}, n = 2]$, suggesting that the assumption that $k_{-2} \ll k_{\text{inact}}, k_2$ is valid. This plot yields estimates of $K_i = k_{-1}/k_1 = 3.2 \pm 0.56 \mu\text{M}$ and $k_2 = (2.3 \pm 0.17) \times 10^{-3} \text{ s}^{-1}$. (All errors are given as S.D.). This estimate of K_i agrees well with that obtained from examining the effect of PI9 on caspase hydrolysis rate. Figure 5(b) shows the results of a similar experiment with caspase-8. In this case, too, the plot is non-linear, suggesting that a two-step inhibitory mechanism also governs caspase-8 inhibition by PI9. The data were fitted to a simplified version of eqn. (9) in which k_{inact} was set to 0, providing estimates for K_i of $16.0 \pm 3.7 \mu\text{M}$ and for k_2 of $(2.1 \pm 0.32) \times 10^{-3} \text{ s}^{-1}$. These values suggest that the differences in apparent rate of inactivation of caspase-1 and caspase-8 are due to weaker binding in the first step ($E \bullet I$), rather than slower formation of the tight complex ($E \bullet I^*$). These results also show that the initial complex $E \bullet I$ does not form with a high enough affinity to measure K_i by changes in the initial rate of hydrolysis of substrate in the presence of PI9 (as in the experiment in Figure 3).

Measuring the 'off' rate constant, k_{-2}

To measure the rate of breakdown of the $E \bullet I^*$ complex, the complex was preformed by incubating $2.5 \mu\text{M}$ caspase-1 with

Figure 5 Effect of PI9 on apparent rate on inactivation of caspases

(a) Caspase-1; (b) caspase-8.

Figure 6 Separation of Ac-Tyr-Val-Lys(fluorescein)-aspartic α -aldehyde bound to caspase-1, which was released from the caspase-PI9 complex, from free peptide by gel-filtration chromatography

The labelled peptide was detected by absorbance at 490 nm. The chromatograms show the separation of caspase-labelled peptide complex from unincorporated peptide (which was eluted in a large peak at approx. 40 min). Shown are the chromatograms of 18 pmol of caspase-1 complexed with $10 \mu\text{M}$ (350 pmol) labelled peptide at 10 min, 3 h and 23 h (from bottom to top) after the addition of peptide to the preformed caspase-PI9 complex. The positions of molecular-mass standards are indicated. $\text{OD}_{490 \text{ nm}} = A_{490}$.

$2.5 \mu\text{M}$ PI9; the reaction mix was diluted 1:5 into a 'quench' solution containing $10 \mu\text{M}$ of the fluorescently labelled peptide inhibitor Ac-Tyr-Val-Lys(fluorescein)-aspartic α -aldehyde, which binds rapidly and tightly to active caspase-1 (R. V. Talanian, unpublished work). At this concentration of PI-9, the residual enzyme activity was less than 20%. The caspase-labelled peptide complex was separated from unincorporated peptide by gel-filtration chromatography. Figure 6 shows chromatographs obtained 10 min, 3 h and 23 h after dilution into the quench solution. The caspase-labelled peptide complex was eluted at an apparent molecular mass of 45 kDa, which is consistent with the elution pattern of caspase-1 itself [47]. The amount of caspase-peptide complex detected 23 h after dilution corresponded to 90–100% of the amount of caspase-peptide detected when caspase was allowed to react directly with peptide in the absence of PI9; further incubation of the diluted complex, up to 72 h, did not result in the formation of more labelled caspase (results not shown). The amount of reactive caspase was estimated from

Figure 7 Recovery of reactive caspase-1 from caspase-PI9 complex

Chromatograms at various times after quenching were integrated to determine the amount of Ac-Tyr-Val-Lys(fluorescein)-aspartic α -aldehyde bound to caspase, expressed relative to the maximal amount of active caspase. Circles and squares show the results of two independent experiments. The line represents an exponential fit to the data.

integrating the chromatogram; the results of two independent experiments are summarized in Figure 7, which shows that reactive caspase was released from the complex as an apparent first-order process with a rate constant of $7.3 \times 10^{-5} \text{ s}^{-1}$. Combined with the values for K_1 and k_2 , this allows an estimate of the overall inhibition constant $K_i^* [= K_1 k_{-2} / (k_2 + k_{-2})]$ of 95 nM. These results further support the suggestion that PI-9 and caspase-1 form a stoichiometric complex. Almost full inactivation of caspase-1 was observed at a stoichiometric concentration of PI-9. Additionally, quantitative recovery of the initial amount of caspase-1 in the reaction was observed only after the caspase-1-PI-9 complex was allowed to decay.

DISCUSSION

In the present study we have demonstrated that the human serpin analogue PI9 inhibits the amidolytic activity of three members of the caspase family of cysteine proteinases. Furthermore, we have isolated the complex between caspase-1 or caspase-4 and PI9 by immunoprecipitation. PI9 is the first instance of a human serpin analogue that inhibits these proteinases. Based on the results in this work, a model for the kinetic mechanism of caspase-1

Mechanism A: **Mechanism B:** **Scheme 3**

inhibition by PI9 can be proposed (Scheme 3). This model is analogous to mechanism B proposed by Morrison and Walsh [46], in which the caspase and PI9 form an initial, rapid complex ($\text{E} \cdot \text{I}$), followed by slow isomerization to a tight complex ($\text{E} \cdot \text{I}^*$). Our model also accounts for the autoinactivation of caspase during prolonged incubation. The present study allows us to estimate the rate constants associated with each step; these results are summarized in Scheme 3, mechanism A. The autoinactivation step apparently affects only the free enzyme (E), rather than the enzyme complexed with PI9 ($\text{E} \cdot \text{I}$, $\text{E} \cdot \text{I}^*$) or with substrate ($\text{E} \cdot \text{S}$), as almost all of the released caspase during the reactivation experiment is able to bind labelled peptide inhibitor, and saturating concentrations of substrate greatly decrease autoinactivation. In addition, the enzyme as prepared contains glutathione disulphide bonded to the active-site cysteine residue; this enzyme is highly stable compared with enzyme that has been activated with dithiothreitol, even if the dithiothreitol is subsequently removed by a rapid desalting column (D. Giegel, unpublished work).

An alternative explanation for the observed reversal of the caspase-1–PI9 complex is that the inhibitor is slowly cleaved by the enzyme to release free enzyme and proteolysed inhibitor. In this case, the slow rate of reversal of the $\text{E} \cdot \text{I}^*$ in Scheme 3, mechanism A (k_{-2}), could actually be the rate of cleavage of PI-9 by caspase 1. The modified reaction pathway can be seen in Scheme 3, mechanism B. However, conclusive proof could not be found for the presence of cleaved PI9 after a prolonged reaction in the presence of caspase-1 (results not shown).

Caspases are involved in at least two highly regulated processes in cells. Caspase-1 is the enzyme that cleaves preinterleukin 1β to the mature cytokine, which is involved in a variety of in-

flammatory processes [48,49]. Serpin analogues have been proposed to be involved in regulating the activity of caspase-1 *in vivo* [48]. All caspases described so far seem to be translated as precursors that contain a prodomain of various lengths plus both the large and small subunits of the mature enzyme, all in a single polypeptide chain. The amino acid sequence around the cleavage sites suggests that the precursors can be cleaved either autocatalytically or by other caspases to form the active proteinase. Recent results suggest that intermediates in the processing pathway for converting procaspase-1 to active p20 and p10 might themselves cleave procaspase-1 but do not have pre-interleukin 1β cleavage activity [11]. In activated monocytic cells, which secrete interleukin 1β , only procaspase-1, not the p20 and p10 subunits of the active caspase, is detectable, even though preinterleukin 1β cleavage activity is present [9]. As caspase-1 can cleave its 45 kDa precursor to form active heterodimers [9,10], the lack of detectable active proteinase suggests that something must be modulating the amount of active caspase in cells such that all the proenzyme is not converted into the active form. Other caspases are involved in apoptosis. A cascade mechanism for proteinase activation in apoptosis has been proposed [50] that is reminiscent of the proteinase cascade in complement activation and blood clotting. Serpins inhibit a number of the proteinases involved in the latter cascade, presumably regulating the activation of clotting [31]. Although little is currently known about the concentration and distribution of active and inactive caspases in apoptotic or pro-apoptotic cells, regulatory factors analogous to serpins might be involved in attenuating apoptosis as well.

PI9 has been shown to inhibit the apoptotic serine proteinase granzyme B *in vitro* [37]. However, PI9 is found in the cytosol,

whereas granzyme B is found in the secretory granules of the cells examined; in addition, PI9 mRNA was detected in a number of cell lines that do not express granzyme B [37]. We have confirmed the ability of PI9 to inhibit granzyme B *in vitro* and we have also noted that different cultures of the NK target cell line K562, a human myelogenous leukaemia line, contain variable amounts of PI9 that do not correlate with the NK cytolytic rate for these cells (J. R. Dahlen and W. Kisiel, unpublished work). Accordingly, the physiological significance of the granzyme B–PI9 interaction remains unclear.

Interestingly, CrmA binds much more rapidly to caspase-1 than PI9 apparently does, with an apparent second-order rate constant of $2 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ [45]. In addition, the interaction of PI9 and granzyme B is also rapid, with an apparent second-order rate constant of $2 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ [37]. The apparent second-order rate constant, k_2/K_1 , for the interaction of caspase-1 and PI9 is approx. $700 \text{ M}^{-1} \cdot \text{s}^{-1}$. The slow inactivation of caspase-1 by PI9 might have several causes. The interaction of CrmA with caspase-1 or granzyme B, or of PI9 with granzyme B, could have a different purpose from the interaction of caspases and PI9. CrmA serves to completely abrogate the interleukin-1 β -mediated inflammatory response generated when the cowpox virus infects its host organism; PI9 could serve to completely inactivate the granzyme B in the cell that might escape from the secretory vesicles [37]. In contrast, mammalian inhibitors of caspases would serve to attenuate their activity in a more controlled manner and would therefore not require rapid binding to enzyme. In addition, nothing is known about the intracellular concentration of PI9, which might be high enough for the interaction between caspase and PI9 to occur relatively rapidly. Alternatively, caspase-1 might not be the 'target' for PI9 *in vivo*; instead, PI9 might interact with much higher affinity with one of the other caspases in the cell. It is also possible that PI9 might interfere with the processing of caspases by binding tightly to procaspases, one of the caspase-processing intermediates, or other caspase 'maturases', such as granzyme B, and thereby regulate the amount of active enzyme in the cell.

The pattern of inhibition specificity by PI9 revealed by this study is remarkable. Caspases can be grouped into subfamilies by a phylogenetic tree analysis [1,23], which reveals that caspase-1 and caspase-4 are closely related (55% sequence identity), whereas caspase-3 and caspase-8 are more distantly related to the other caspases (30–35% identity to each other or to caspase-1 and caspase-4). Substrate specificity studies with tetrapeptides [20,21] suggest that caspases can be grouped into three groups based on preferences in the P4 position of substrates: group I caspases (including caspase-1 and caspase-4) prefer aromatic residues in the P4 position of substrates; group II caspases (including caspase-3) prefer aspartate; group III caspases (including caspase-8) prefer aliphatic non-aromatic residues. On the basis of these results alone, caspase-3 would be predicted to be resistant to PI9 inhibition, which is consistent with the results found. Caspase-8 would be predicted to be the caspase most readily inhibited by PI9, which contains a valine residue in P4. However, the results of this study indicate that PI9 in fact inhibits caspase-1 and qualitatively caspase-4 preferably to caspase-8, suggesting that other factors, in addition to the residues in the P4 position, must be regulating the rate at which PI9 inhibits caspases. Other studies have shown that CrmA similarly inhibits caspase-1 strongly [34] but interacts much more weakly with caspase-3 [23], suggesting that the differential inhibition of different caspases by serpin analogues might be a general phenomenon.

We describe here the first evidence for the interaction of any caspase with a human serpin analogue. The interaction between

caspase-1 or caspase-8 and PI9 is consistent with a two-step inhibitory mechanism. Our results provide evidence that PI9 and other, as yet undiscovered, serpin analogues might attenuate the activity of caspases *in vivo*, thereby regulating the cellular processes in which they are involved. Further work is under way to analyse the interaction of PI9 with caspases *in vivo* and to search for other serpin analogues that might interact with these interesting cysteine proteinases.

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