A Cytosolic Granzyme B Inhibitor Related to the Viral Apoptotic Regulator Cytokine Response Modifier A Is Present in Cytotoxic Lymphocytes

Using a polymerase chain reaction strategy we identified a serine proteinase inhibitor (serpin) in human bone marrow that is related to the cellular serpin proteinase inhibitor 6 (PI-6) and the viral serpin cytokine response modifier A (CrmA). This serpin, proteinase inhibitor 9 (PI-9), has an unusual reactive center P1(PGlU)-P1′(Cys), which suggests that it inhibits serine proteinases that cleave after acidic residues. The only known serine proteinase with this specificity is granzyme B, a granule cytotoxin produced by cytotoxic lymphocytes. To test the interaction of PI-9 with granzyme B we prepared recombinant hexa-histidine tagged PI-9 in a yeast expression system. Addition of the recombinant protein to native granzyme B resulted in an SDS-resistant complex typical of serpin-serine proteinase interactions. Further analysis showed that complex formation followed bimolecular kinetics with a second order rate constant of 1.7 ± 0.3 × 10^6 M^-1 s^-1, which is in the range for a physiologically significant serpin-proteinase interaction. Recombinant PI-9 also completely abrogated granzyme B and perforin-mediated cytotoxicity in vitro. Examination of PI-9 mRNA distribution demonstrated that it is expressed in immune tissue, primarily in lymphocytes. The highest levels of PI-9 mRNA and protein were observed in natural killer cell leukemia cell lines and in interleukin-2 stimulated peripheral blood mononuclear cells, which also produce granzyme B. Like PI-6, PI-9 was shown to be a cytosolic protein that is not secreted. Fractionation of natural killer cells and stimulated peripheral blood mononuclear cells demonstrated that PI-9 is in a separate subcellular compartment to granzyme B. These results suggest that PI-9 serves to inactivate misdirected granzyme B following cytotoxic cell degranulation. This may explain why cytotoxic cells are not damaged by their own granzyme B during destruction of abnormal cells.

Natural killer cells and cytotoxic T lymphocytes destroy virus-infected and malignant cells by inducing apoptosis, and each killer cell can sequentially destroy many targets without being damaged by its own cytotoxins. Key components of the cytotoxic apparatus are the serine proteinases, granzymes A and B, and the pore-forming protein, perforin (1). Following cell to cell contact these proteins are released from killer cell granules into the intercellular space, and death of the target cell follows. The events leading from granule exocytosis to apoptosis are poorly understood. Granzyme B promotes DNA degradation in the target cell possibly by activating the apoptotic cysteine proteinase CPP32 (2, 3), and killing is likely to involve intracellular proteolytic pathways requiring both serine and cysteine proteinases. Target cells resist killing if they contain synthetic serine or cysteine proteinase inhibitors (1), or if they express the viral apoptotic proteinase inhibitor, CrmA (4). Serine proteinases function in many processes dependent on proteolysis, such as blood coagulation, fibrinolysis, cell-mediated cytotoxicity, complement fixation, and tissue remodeling (5). Regulation of serine proteinase activity is generally achieved by complex formation with cognate serine proteinase inhibitors (serpins) that act as pseudo- or suicide substrates. Proteinase binding to the serpin pseudo-substrate region, which consists of an exposed loop near the carboxyl terminus, induces a conformational change in the serpin that stabilizes the complex so that it is essentially irreversible. Cleavage of the serpin by the proteinase occurs at the “reactive center” between two amino acids designated P1 and P1′. The P1 residue largely dictates the specificity of serpin-proteinase binding although those residues immediately surrounding the cleavage site also contribute to the affinity and fidelity of the interaction (5).

Until recently serpins appeared to be confined to regulating extracellular serine proteinases, but an unusual viral serpin, cytokine response modifier A (CrmA) (6), is now known to inhibit intracellular cysteine proteinases such as those involved in apoptosis (ICE, CPP32, Nedd-2, and ICH-2) and the maturation of interleukin-1β (7). CrmA also inhibits granzyme B, which is essential for the rapid induction of DNA fragmentation in cytotoxic lymphocyte (CL)-induced apoptosis (8). Expression of CrmA renders cells resistant to apoptosis (9, 10) and to killing by CLs (4). The unique features of CrmA are partly explained by the nature of the cysteine and serine proteinases it inhibits: all cleave at acidic residues, particularly aspartic acid, and this preference is reflected by the presence of aspartic acid at the P1 position in CrmA. Thus the properties of...
CrmA show that serpins can function intracellularly, they can inhibit more than one type of proteinase (cross-class inhibition), and they can regulate CL killing and apoptosis.

In addition to CrmA, there are two endogenous serpins that may control intracellular proteolysis. These are plasminogen activator inhibitor 2, a monocyte protein thought to regulate tumor necrosis factor-induced apoptosis in addition to its role in regulating extracellular urokinase (11); and proteinase inhibitor 6 (PI-6), a non-secreted epithelial cell serpin that inhibits more than one type of proteinase (cross-class inhibition) (12). Although they have different P1 residues, these endogenous serpins and CrmA have several similarities. In particular, they lack NH$_2$- and COOH-terminal sequences present in most other serpins (5, 6), and they display a high degree of homology in the regions flanking the reactive center.

The possibility that endogenous serpins are involved in the regulation of intracellular processes such as apoptosis prompted us to search for serpins related to PI-6 and PAI-2. Using a PCR-based strategy we have independently identified and characterized a new human serpin (proteinase inhibitor 9) recently described as a serpin of unknown function (13). Here we show that PI-9 is an intracellular serpin present in lymphocytes, and that it is an efficient granzyme B inhibitor which protects cells against perforin and granzyme B mediated killing.

**Experimental Procedures**

**Cloning and Characterization of a New Serpin—Degenerate primers**

PB117 and PB118 (Table I) were designed to conserve sequences flanking the PI-6 and ovalbumin serpin reactive centers (14, 15). Total RNA was isolated from fresh human bone marrow aspirate (16). cDNA was synthesized from the RNA using avian myeloblastosis virus reverse transcriptase and oligo(dT) (17, and PCR was carried out at four Mg$^{2+}$ concentrations (0.5, 1.0, 1.5, and 2.0 mm), using 20 pmol of each primer and 100 ng of template in 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100 containing 200 µM dNTP and 2 units of TaqI (Promega).

35 cycles of 95°C for 90 s, 45°C for 60 s, and 72°C for 90 s were performed. Amplified 100-base pair products separated by 2% agarose gel electrophoresis, purified from the gels, and cloned into pCRII (Invitrogen) for sequence analysis. A clone with an in-frame fusion of the His-tag and no second site mutations was chosen for the subsequent steps.

The modified PI-9 cDNA was released from pCRII by EcoRI digestion, cloned into the vector pHL-D2, and expressed as an intracellular protein in Pichia pastoris as described previously for PI-6 (18). Prepararation of PI-6 and PI-9 His-tagged protein was as described (18), except that PI-9 was eluted from the nickel affinity column in 20 mM imidazole following washing in 4 mM imidazole.

**PI-9 and Granzyme B Kinetics**

Preparation of active granzyme B from YT cell granules has been described (19). For stoichiometric determinations, 10 pmol of granzyme B was incubated with different concentrations of PI-9 at 37°C in 20 mM Hepes pH 7.4, 100 mM NaCl, 0.05% (v/v) Nonidet P-40 (20). Residual enzyme activity was determined after 15 min by a two-stage assay using Boc-Ala-Ala-Asp-$\beta$-naphtylamide, with varying concentrations of granzyme B (21). The rate of inhibition of granzyme B by PI-9 was determined by incubating equimolar enzyme and inhibitor at 37°C, and determining residual activity periodically (18, 22). The second order rate constant was calculated as described (18).

Trypsin, chymotrypsin, plasmin, pancreatic elastase, neutrophil elastase, and cathepsin G were obtained from Sigma. Preparation of thrombin and native gel electrophoresis were purified from the gels and cloned into the vector pCRII (Invitrogen) for analysis. Clones containing known serpin sequences were screened out by Southern or colony hybridization to specific oligonucleotides (PB116 and PB126–129; Table I). The remaining clones were sequenced and candidate serpin sequences were identified by the presence of the hinge region motif (Thr-Ala-Ala-Ala(Thr/Ser)) (5) and a conserved Phe-Cys-Ala-Asp sequence not encoded by the primers.

An oligonucleotide probe PB148 (Table I) was designed to span the putative reactive center of a new serpin sequence amplified from bone marrow RNA. Using this probe a truncated cDNA clone lacking the first 20 residues of the coding sequence was isolated from a human placental cDNA agt11 library (Clontech).

**TABLE I**

| Oligo | 5'-3' sequence | Template | S/SAS | Position |
|-------|----------------|----------|-------|----------|
| PB163 | AGACCTCTGAGGGCCTGAG | agt11 | S | 1107 |
| PB116 | ATGCGGTGTCGCAGA | PI-6 | S | 803 |
| PB117 | AA/GA/AAAAGA/GAAIGGA/GTG | Ovalbumin serpin | A | 1184 |
| PB118 | GA/GTG/TATG/TG/C/AAATG/GAAG/GAG | Ovalbumin serpin | S | 1090 |
| PB126 | GAACATGACATCAGGAG | PI-2 | S | 1132 |
| PB127 | CGTCTCATGTCGCC | MNEI | S | 505 |
| PB128 | GGATCATCACCTGCT | SCCA | S | 356 |
| PB129 | ATTCCTGACAGCAG | Maspin | S | 963 |
| PB130 | GACACCTGAGGGCCTGAG | PI-9 | S | 1184 |
| PB131 | GTTCTTCGTCTTCTCCTGC | PI-9 | S | 828 |
| PB132 | AAAATTGAAGAGTTGTTG | PI-9 | A | 766 |
| PB133 | TGGCCTAGGCGAGCT | PI-9 | S | 558 |
| PB134 | GTGTCTGATCAGTGC | PI-9 | S | 828 |
| PB135 | AAAATTGAAGAGTTGTTG | PI-9 | A | 828 |
| PB136 | AGACCTCTGAGGGCCTGAG | PI-9 | S | 1132 |

**Preparation of PI-6 and PI-9 His-tagged protein** was as described (18). Thrombin and native gel electrophoresis were as described (18). The second order rate constant was calculated as described (18).
RNA Analysis—Leukocyte cell line RNA was prepared as described (16). PI-9 mRNA and cDNA for reverse transcription PCR analysis was as described (24). PCR was carried out using 20 pmol each of PB161 and PB173 in 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100 containing 200 μM dNTP and 2 units of Tth (Promega). 35 cycles of 95°C for 90 s, 50°C for 60 s, and 72°C for 90 s were carried out. Amplified products were separated on a 1.5% agarose gel, visualized by ethidium bromide staining, transferred to nylon, and hybridized to a [³²P]cDNA-labeled PI-9 cDNA probe (17).

For Northern analysis, total RNA from the indicated lines was prepared (16), separated on a 1% formaldehyde-agarose gel, and transferred to Nitran. The membrane was hybridized to a [³²P]cDNA-labeled PI-9 cDNA probe (17).

Cell Fractionation—Leukocyte cell lines used for PCR and Northern analysis were obtained from the ATCC and cultured according to the ATCC's specifications. FDC-P1 cells were grown in Dulbecco's modified Eagle's medium containing 10% heat inactivated fetal bovine serum, 10−4 M asparagine, and 50 units/ml IL-3. PI-6 and PI-9 cDNAs were placed under the control of the cytomegalovirus promoter in expression vectors based on pCMV-2 (26). COS-1 cells were transfected, permeabilized, and subjected to indirect immunofluorescence using the appropriate antibodies.

Antibodies, Cells, Plasmids, and Transfections—Rabbit antibodies to recombinant PI-6 were raised by standard procedures (25). Rabbit antisera have been described for PI-6 (12), and a monoclonal antibody (2C5) has been raised against granzyme B (19). Rabbit antisera and 2C5 ascites fluid were used at 1:2000 for immunoblotting, and blots were developed using an enhanced chemiluminescence detection kit (DuPont).

Antibodies, Cells, Plasmids, and Transfections—Leukocyte cell lines were transfected, permeabilized, and subjected to indirect immunofluorescence using the appropriate antibodies as described (12). COS cells producing PI-9 were metabolically labeled (12) with [³⁵S]methionine for 1 h at 37°C, then chased for 3 h in complete medium containing excess cold methionine. Lactate dehydrogenase assays (27) estimated nonspecific cell lysis at less than 1% during this procedure. Media and lysates from transfected and mock-transfected cells were immunoprecipitated using PI-9 antibodies coupled to protein A-Sepharose, and analyzed by 10% SDS-polyacrylamide gel electrophoresis and fluorography.

Cell Fractionation—PBMC were isolated from normal human peripheral blood by isopaque-Ficoll centrifugation and incubated for 7 days in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 100 units/ml IL-2. Stimulated cells were collected, disrupted by nitrogen cavitation, and fractionated into cytosolic and granule components by Percoll density gradient centrifugation exactly as described (28). Fractions containing granule components were identified by N-benzoyloxycarbonyl-L-lysine thiopeptidase esterase activity (29) and the presence of marker contaminants were identified by antibody (30). PI-9 in fractions was followed by 12% SDS-polyacrylamide gel electrophoresis followed by immunoblotting and ECL using rabbit antibodies raised against the recombinant protein (25). Granzyme B on the same membrane was detected by immunoblotting with monoclonal antibody 2C5.

RESULTS
Identification of a New Serpin using a PCR Strategy—We have previously identified and characterized an intracellular serpin (PI-6) that bears structural similarity to the viral serpin, CrmA, and the endogenous serpin, PAI-2 (12, 14, 30). The restricted tissue distribution of PI-6 and PAI-2 (12, 31), together with the emerging role of CrmA and PAI-2 as inhibitors of apoptosis (9–11), suggested the existence of other serpins that regulate intracellular proteolysis.

To search for related serpins we designed a PCR-based strategy. Alignment of PI-6 with the closely related ovalbumin serpins (15) allowed us to design degenerate oligonucleotide primers based on conserved sequences flanking the variable reactive center (Fig. 1 and Table 1). On a suitable serpin template these primers amplify the reactive center loop, and new candidate sequences can be identified by conserved serpin sequences inside the primer binding sites and the presence of novel sequences between them. Amplification and analysis of sequences from human bone marrow RNA by PCR using these primers identified a novel serpin sequence with a reactive site different from PI-6. Sequence analysis of cDNA clones subsequently isolated from human bone marrow libraries confirmed that the new serpin is closely related to PI-6 (63% amino acid identity), and that it shows significant homology to CrmA (37%), with the highest degree around the reactive center (Fig. 1). This molecule has recently been independently described as a serpin of unknown function designated as PI-9 (13).

Comparison of the PI-9 sequence with other serpins, particularly PI-6 and CrmA, suggested that its P1 and P1′ residues are glutamic acid and cysteine, respectively (Fig. 1). Moreover the sequence preceding the P1 residue contained the hinge motif (P₁-Gly-Thr-Glu-Ala-Ala-Ala-Ala(Ser/Thr)-P₇), which is the CL granule protease, granzyme B, although it cleaves only known serine proteinase to have this substrate specificity. The molecule is a functional serpin, and that it would inhibit a serine proteinase cleaving after glutamic acid residues. The only known serine proteinase to have this substrate specificity is the CL granule protease, granzyme B, although itcleaves more efficiently after aspartic acid (21).

PI-9 Inhibits Granzyme B—To test the interaction of PI-9 with granzyme B, hexa-histidine-tagged recombinant PI-9 was produced in a yeast expression system and purified by nickel affinity chromatography. As predicted from the cDNA sequence, the purified recombinant protein had a molecular mass of 42 kDa (Fig. 2a).

A feature of serpin-serine proteinase interactions is the formation of a stable complex that is not dissociated by SDS (5). Addition of recombinant PI-9 to purified native granzyme B resulted in the formation of a 67-kDa complex that was apparent following reduction, boiling, and electrophoresis in SDS (Fig. 2a). The complex was recognized by antibodies to both granzyme B (Fig. 2b) and PI-9 (data not shown). By contrast, complex formation between PI-6 and granzyme B did not occur (Fig. 2, a and b).

Using similar assays the interaction of PI-9 with other serine proteinases was also examined. No SDS-resistant binding of PI-9 to trypsin, chymotrypsin, thrombin, plasmin, pancreatic elastase, neutrophil elastase, or cathepsin G was observed, and complexes were not detected in the absence of SDS by native gel electrophoresis (data not shown).

FIG. 1. Comparison of the new serpin to PI-6 and CrmA. Identical residues are boxed and the vertical arrow indicates the reactive center. Horizontal arrows show the sequences used to design degenerate PCR primers.
Complex formation between a serpin and proteinase can be described by second order kinetics, and association rate constants of $10^5 - 10^7 \text{ M}^{-1} \text{s}^{-1}$ are observed in physiologically significant interactions (32). The stoichiometry of the reaction is usually equimolar and initial formation of a Michaelis complex is followed by the formation of a kinetically stable (locked) tetrahedral complex (5, 32). To examine the interaction of PI-9 and granzyme B in more detail, we first established the stoichiometry of the reaction by titrating a fixed amount of granzyme B against varying amounts of inhibitor and measuring residual proteolytic activity (Fig. 2c). The 1:1 ratio observed is typical of a serine proteinase-serpin interaction (5). We then analyzed the rate of inhibition of granzyme B by PI-9. The reaction followed standard bimolecular kinetics as indicated by linearity over three or more reaction half-lives (Fig. 2d). The association rate constant ($k_a$) for complex formation was calculated as $1.7 \pm 0.3 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$. This is well within the range for physiologically important serpin-proteinase interactions (5, 32), and is approximately 10-fold greater than the constant for the granzyme B and CrmA interaction ($2.9 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ (20)), indicating that inhibition of granzyme B by PI-9 occurs more rapidly.

PI-9 Inhibits Granzyme B-mediated Cytotoxicity—These results suggested that PI-9 is a specific and physiologically relevant inhibitor of granzyme B. If so, we predicted that it should be able to inhibit biological effects of granzyme B. To test this we examined the ability of PI-9 to prevent granzyme B/perforin-mediated cytotoxicity in vitro (23, 33). In this system, death by apoptosis and DNA degradation is induced in susceptible cells by the synergistic action of purified granzyme B and sublytic amounts of perforin, and is indistinguishable from death induced by exposure to intact CLs.

PI-9 mRNA Is Expressed in Immune Tissue and Lymphocytes—If PI-9 is a physiological regulator of granzyme B, it is likely to function by protecting cells against misdirected or inappropriately activated granzyme B. This role would demand that PI-9 is directed to or synthesized in a physiological compartment likely to contain or encounter the proteinase. Surveys of the tissue distribution of PI-9 mRNA by Northern analysis showed that it is present in low levels as 4.4- and 2.7-kb transcripts in spleen, thymus, peripheral blood leukocytes, testis, and placenta (Fig. 4A). An additional 1.5-kb mRNA was observed in testis.

Taken with the fact that it was originally identified in bone
marrow, this pattern of expression suggested that PI-9 is produced primarily by cells of the immune system. This was supported by a preliminary examination of leukocyte cell lines by reverse transcriptase-PCR (data not shown). Visible PCR products following agarose gel electrophoresis and ethidium bromide staining indicated significant PI-9 expression in the T cell line PEER-1; the B cell lines REH, NALM-1, and RAJI; and the stem cell line K562. Expression in other lines HSB-2, MOLT-4 (T cell), and LIL-1 (B cell) was detected following Southern analysis of the PCR products. By similar analysis, little or no expression was detected in the megakaryocyte line, MEG-01, or in the monocye/myeloid lines THP-1, U937, and HL60. Three T cell lines (DU528, JURKAT, and JM1), one B cell line (BALL-1), and a stem cell line (K5-1) produced little or no PI-9 mRNA.

As shown in Fig. 4B, Northern analysis of other lymphocyte and myelocyte cell line RNA demonstrated three transcripts of approximately 4, 2.5, and 1.5 kb, correlating with those seen in the tissue RNA analysis (Fig. 4A). However, as in the tissue RNA, the 1.5-kb transcript was not present in every cell type. PI-9 appeared to be more highly expressed in the leukocyte lines because it was easily detected in 20 μg of total RNA (containing 1% mRNA) from most of these cells, whereas only low levels were detectable in 2.5 μg of mRNA prepared from the tissues.

The PI-9 transcripts were observed primarily in two natural killer cell leukemia lines, Lopez (34) and YT, and in the Epstein-Barr virus-transformed B cell lines (MANN, MSAB, LEIF-T, DBB, RAJI, and DAUDI). They were also present in interleukin-2 (IL-2) activated cytolytic lymphocytes from PBMC and in the T cell line HPB-ALL. No transcripts were detected in the myelomonocytic lines, HL60 (Fig. 4B) and U937 (not shown). In most of the positive lines, the 2.5-kb transcript predominated and the highest levels of PI-9 mRNA were observed in cells that also express granzyme B (YT, Lopez T, and IL-2 stimulated PBMCs). Taken together these results suggest that PI-9 is primarily produced by lymphocytes, particularly CLs and B cells.

At present, the nature of the different PI-9 transcripts is not understood but they do not represent cross-hybridization of the probe to related serpin sequences (e.g. PI-6 or PAI-2) because an identical pattern was obtained on hybridization to a 32P-labeled 1.6-kb PI-9 cDNA. The final wash was in 0.1 × SSC at 65°C and the membrane was exposed to x-ray film for 2 weeks. B, total RNA (20 μg) was isolated from the indicated leukocyte cell lines, fractionated, and transferred to a nylon membrane. The membrane was first hybridized to the PI-9 cDNA probe, and following this experiment it was stripped and hybridized to a 32P-labeled granzyme B cDNA (34). The final wash following each hybridization was in 0.1 × SSC at 65°C, and the membrane was exposed to x-ray film for 48 h. The positions of ribosomal RNAs on the membranes are indicated by black dots.

**Fig. 4.** Distribution of PI-9 mRNA. A, a multitissue Northern blot containing 2.5 μg of mRNA per lane was hybridized to 32P-labeled 1.6-kb PI-9 cDNA. The final wash was in 0.1 × SSC at 65°C and the membrane was exposed to x-ray film for 2 weeks. B, total RNA (20 μg) was isolated from the indicated leukocyte cell lines, fractionated, and transferred to a nylon membrane. The membrane was first hybridized to the PI-9 cDNA probe, and following this experiment it was stripped and hybridized to a 32P-labeled granzyme B cDNA (34). The final wash following each hybridization was in 0.1 × SSC at 65°C, and the membrane was exposed to x-ray film for 48 h. The positions of ribosomal RNAs on the membranes are indicated by black dots.
glycoforms (Fig. 5B).

**PI-9 and Granzyme B Are in Separate Subcellular Compartments in CLs**—The highest level of PI-9 mRNA and protein was observed in YT cells which also produce large amounts of granzyme B (19). Taken with the observation that PI-9 is present in IL-2 activated PBMCs, this is consistent with a protective role for PI-9 in cytotoxic cells synthesizing granzyme B. There are two ways in which PI-9 could exhibit a protective effect: (i) it is packaged with granzyme B in granules to maintain the protease in a quiescent state until degranulation; or (ii) it is in a separate (cytosolic) compartment and serves to inactivate granzyme B that leaks from granules before or during degranulation. Although the first possibility is less likely given the essentially irreversible interaction between PI-9 and granzyme B in vitro, it predicts that the serpin and protease will co-localize in granules. By contrast, the second possibility predicts that they will be in separate subcellular compartments.

Fractionation of YT cells into granule and cytosolic compartments followed by electrophoresis and immunoblotting demonstrated that granzyme B and PI-9 are in different cellular compartments, with granzyme B separating with granule proteins and PI-9 with the cytosolic marker lactate dehydrogenase (data not shown). To confirm that granzyme B and PI-9 are in different subcellular compartments in a more physiological setting, human PBMCs activated with IL-2 were analyzed. The stimulated PBMC consisted of 80% T cells (50% CD3+ CD8+ and 30% CD3+ CD4+) and 20% NK cells (CD3− CD56+ CD2+) by cytofluorography (data not shown). Cells were collected, disrupted, and the subcellular components fractionated by density gradient centrifugation. Immunoblotting then demonstrated that PI-9 was mainly in the cytosolic fractions, while granzyme B was associated with denser fractions containing granule-associated proteins (Fig. 6).

**DISCUSSION**

PI-9 is a member of a branch of the serpin superfamily exemplified by ovalbumin (13, 15). These ovalbumin serpins are grouped on the basis of amino acid sequence similarity, and the lack of NH2- and COOH-terminal sequences present in the serpin prototype, α-proteinase inhibitor. At present, they also include plasminogen activator inhibitor (PAI-2 (39)), monocyte/neutrophil elastase inhibitor (40), squamous cell carcinoma...
antigens 1 and 2 (41, 42), maspin (43), proteinase inhibitor 6 (PI-6 (14, 30, 44)), proteinase inhibitor 8 (PI-8 (13)), and proteinase inhibitor 10 (PI-10/bomapin (45)). Most of the ovalbumin serpins are secreted to participate in extracellular processes, however, at least one (PI-6) is an intracellular protein (12), and another (PAI-2) appears to regulate both intra- and extracellular proteolysis (31).

The physiological roles of most of these ovalbumin serpins are unknown but monocyte/neutrophil elastase inhibitor, maspin, PAI-2, and squamous cell carcinoma antigens may function in tumorigenesis and inflammation. For example, monocyte/neutrophil elastase inhibitor has been postulated to control serine proteinases found at inflammatory sites to prevent damage to surrounding tissue (46), and maspin is a candidate tumor suppressor (43). Increases in squamous cell carcinoma antigens levels and release are associated with squamous cell carcinoma (41), while extracellular and intracellular PAI-2 expression is increased in monocytes during inflammation (37).

Our studies define a role for PI-9 in the regulation of the CL granule proteinase, granzyme B. It is well known that CLs can sequentially kill many target cells and that CLs are resistant to the effects of their own cytotoxins, however, the protective mechanisms involved have remained obscure. Our demonstration of cytosolic PI-9 in CLs synthesizing granzyme B begins to explain how CLs may resist their own cytotoxins. We propose that by inactivating granzyme B that is misdirected into the CL cytoplasm during degranulation and target cell killing, intracellular PI-9 is part of a mechanism that protects CLs from autolysis. The presence of PI-9 in B cells suggests that this serpin is also produced by other cells of the immune system likely to be in the vicinity of activated CLs, thus reducing the possibility of granzyme B-mediated damage to bystander cells.

Previous evidence has suggested that some ovalbumin serpins regulate intracellular proteolysis (11, 12, 15). This evidence includes structural and functional similarities to the cowpox virus serpin CrmA, an intracellular protein that regulates cytokine processing and can prevent apoptosis in cultured cells by inhibiting cytosolic cysteine proteinases related to interleukin-1β-converting enzyme (9, 20, 47, 48). Although it has the unusual property of interacting with cysteine proteinases, CrmA is clearly a serpin in structure as indicated by its ability to inhibit the serine proteinase granzyme B (20). Examples of ovalbumin serpins that resemble CrmA include PAI-2, which appears to regulate a proteinase involved in apoptosis in addition to its extracellular role as an inhibitor of urokinase (11, 31), and PI-6, PI-8, and PI-9 which resemble CrmA in the reactive center loop particularly in possessing a P1′ cysteine.

The existence of a viral serpin (CrmA) that efficiently inhibits cysteine proteinases involved in apoptosis, and the observation that intracellular serine proteinases are also activated during cell death (49, 50), suggests that endogenous intracellular serpins may function as apoptotic regulators. For example, PAI-2 may represent an endogenous regulator of an apoptotic serine proteinase although its intracellular target has yet to be identified (11). Our studies suggest that PI-9 may be a cellular CrmA homologue because it is very similar in the reactive center loop, and is also a granzyme B inhibitor. It is therefore interesting to speculate that CrmA has evolved from the bovine equivalent of PI-9, and that PI-9 is a regulator of apoptosis.

A key similarity between PI-9 and CrmA is an acidic P1 residue. Current dogma suggests that the identity of P1 residue in a particular serpin reflects the substrate preference of its target proteinase (5). Since granzyme B cleaves after aspartic acid much more efficiently than glutamic acid (21), a physiological granzyme B inhibitor might be expected to possess a P1 aspartic acid. This view is contradicted by the fact that CrmA (which has a P1 aspartic acid) is a much poorer granzyme B inhibitor than PI-9, and suggests that although the nature of the P1-P1′ residues is important other factors contribute to the specificity and affinity of the serpin-proteinase interaction.

Given these considerations, the nature of the PI-9 P1 residue does not immediately indicate whether or not PI-9 is likely to inhibit the apoptotic cysteine proteinases, which cleave after aspartic acid (7). One view is that PI-9 might also inhibit the cysteine proteinase(s) on the apoptotic pathway triggered by granzyme B thereby offering additional protection to the CL. Since granzyme B can activate the central apoptotic proteinase CPP32 in vitro (2, 3), PI-9 could inhibit CPP32. Alternatively, it could be argued that the P1 glutamic acid makes PI-9 a selective granzyme B inhibitor which cannot efficiently interact with cysteine proteinases. This would allow CLs to be killed by granzyme B–independent apoptosis and facilitate their removal from the immune system following the normal processes of differentiation, maturation, and proliferation. These possibilities may eventually be distinguished by examining the interaction of a PI-9 P1′(Glu) to P1′(Asp) mutant with granzyme B and key cysteine proteinases, and the effect of PI-9 on non-granzyme B–mediated apoptosis.

In conclusion, we have demonstrated that a new serpin present in lymphocytes is an efficient inhibitor of granzyme B and of granzyme B-mediated cell killing. The existence of this granzyme B inhibitor may explain why cytotoxic lymphocytes are not killed by exposure to the potent cytotoxins they release to destroy target cells.

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