Importance of the P4' Residue in Human Granzyme B Inhibitors and Substrates Revealed by Scanning Mutagenesis of the Proteinase Inhibitor 9 Reactive Center Loop*

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The cytotoxic lymphocyte serine proteinase granzyme B induces apoptosis of abnormal cells by cleaving intracellular proteins at sites similar to those cleaved by caspases. Understanding the substrate specificity of granzyme B will help to identify natural targets and develop better inhibitors or substrates. Here we have used the interaction of human granzyme B with a cognate serpin, proteinase inhibitor 9 (PI-9), to examine its substrate sequence requirements. Cleavage and sequencing experiments demonstrated that Glu340 is the P1 residue in the PI-9 RCL, consistent with the preference of granzyme B for acidic P1 residues. Ala-scanning mutagenesis demonstrated that the P4' region of the PI-9 RCL is important for interaction with granzyme B, and that the P4' residue (Glu344) is required for efficient serpin-proteinase binding. Peptide substrates based on the P4-P4' PI-9 RCL sequence and containing either P1 Glu or P1 Asp were cleaved by granzyme B (k_{cat}/K_m 9.5 × 10^3 and 1.2 × 10^4 s^{-1} M^{-1}, respectively) but were not recognized by caspases. A substrate containing P1 Asp but lacking P4' Glu was cleaved less efficiently (k_{cat}/K_m 5.3 × 10^4 s^{-1} M^{-1}). An idealized substrate comprising the previously described optimal P4-P1 sequence (Ile-Glu-Pro-Asp) fused to the PI-9 P1'-P4' sequence was efficiently cleaved by granzyme B (k_{cat}/K_m 7.5 × 10^5 s^{-1} M^{-1}) and was also recognized by caspases. This contrasts with the literature value for a tetrapeptide comprising the same P4-P1 sequence (k_{cat}/K_m 6.7 × 10^4 s^{-1} M^{-1}) and confirms that P' residues promote efficient interaction of granzyme B with substrates. Finally, molecular modeling predicted that PI-9 Glu344 forms a salt bridge with Lys27 of granzyme B, and we showed that a K27A mutant of granzyme B binds less efficiently to PI-9 and to substrates containing a P4' Glu. We conclude that granzyme B requires an extended substrate sequence for specific and efficient binding and propose that an acidic P4' substrate residue allows discrimination between early (high affinity) and late (lower affinity) targets during the induction of apoptosis.

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Cytotoxic lymphocytes (CLs) play a key role in cell-mediated immunity, destroying foreign, virus-infected, or tumor cells (1). Contact between a CL and a target cell leads to the release of cytotoxins such as perforin, serine proteinases (granzymes), and death ligands (FasL and TRAIL) from specialized CL secretory granules into the intercellular space. Target cell death then proceeds by the induction of apoptosis using either of two pathways: activation of death receptors on the target cell surface coupled to caspase activation within the cell or uptake of granzymes in a perforin-dependent manner that leads to caspase activation and intracellular proteolysis (reviewed in Refs. 1 and 2).

The molecular basis of granzyme-induced cell death is not fully understood. The current model is that perforin and granzymes are endocytosed by the target cell and that perforin eventually disrupts the endocytic vesicle, thus releasing granzymes into the cytoplasm (3, 4). Caspase activation, loss of mitochondrial membrane potential, proteolysis of key housekeeping proteins, DNA degradation, and the disintegration of cellular structures follows rapidly. A key granule cytotoxin in this process is the serine proteinase, granzyme B. It is an unusual proteinase with a preference for cleaving after Asp, a property that it shares with caspases. On entering the cytoplasm of a cell, granzyme B induces death by at least two pathways. One pathway is caspase dependent and results in rapid DNA degradation and death. The other pathway is caspase independent and may involve direct degradation of essential proteins such as DNA-PKcs, NuMA, and PARP (5). Both pathways involve loss of mitochondrial membrane potential. The pivotal role of granzyme B in granule-mediated cell death is illustrated by mice lacking granzyme B. These animals produce CLs that are unable to induce rapid DNA degradation and death of target cells, although slower killing occurs, possibly mediated by other granzymes (6).

Because caspases themselves are activated by cleavage after Asp, granzyme B is able to proteolytically activate several caspases in vitro (7), and there is evidence that this also occurs in cells killed by CLs (8, 9). Of the other known granzyme B substrates within cells, most appear to be cleaved after Asp but not necessarily at sites recognized by caspases. Granzyme B has also been implicated in the cleavage of the BCL-2 family member BID (10), in components of the DNA repair machinery, and...
and in the generation of autoimmune antigens (11). Thus, identification of noncaspase substrates and elucidation of the cleavage specificity of granzyme B is attracting increasing interest. Screening of combinatorial tetra-peptide substrate libraries has indicated that granzyme B has an optimal P4-P1 recognition motif of Ile-Glu-Pro-Asp (12), resembling the activation sites of procaspace 3 and procaspace 7, both of which are activated by granzyme B (7). It also resembles the optimal recognition motif of the group III or initiator caspases, implying that granzyme B functions as an initiator of the caspase proteolytic cascade. This motif has recently been confirmed using combinatorial methods and extended using phage display to suggest that the preferred P2’ residue is Gly and that the consensus recognition motif for granzyme B is Ile-Glu-Xaa-(Asp/Xaa)-Gly (13). However, synthesis of a peptide comprising an optimized P4-P2’ sequence resulted in a molecule that was not cleaved efficiently by granzyme B (13), suggesting that residues outside this motif contribute to substrate binding.

A different approach to understanding the interaction of granzyme B with substrates is to study its interaction with a natural inhibitor, such as a serpin. Serpins belong to a large metazoan and virus protein superfamily (14) and have a common structure and mode of action; each contains a variable C-terminal reactive center loop (RCL) resembling the substrate of its cognate proteinase that is flanked by two highly conserved hinge domains. On proteinase binding, the serpin is cleaved and undergoes a conformational change leading to the irreversible locking of the serpin-proteinase complex. Cleavage of the serpin by the proteinase occurs between two residues in the loop designated P1 and P1’. The P1 residue is crucial and largely dictates the specificity of the serpin-proteinase interaction, whereas residues surrounding the cleavage site contribute to the affinity of the interaction (15).

The human serpin, proteinase inhibitor 9 (PI-9), is an efficient inhibitor of granzyme B that prevents granzyme B-mediated apoptosis in certain cell types and is thought to protect C1s from autolysis (16). It follows that the PI-9 RCL resembles a natural substrate of granzyme B and that residues within the RCL important for inhibitory function interact with the sub-

EXPERIMENTAL PROCEDURES

Recombinant Protein Production and Mutagenesis—Recombinant PI-9 was produced using a Pichia pastoris expression system and purified as described (16). Recombinant human granzyme B zymogen was produced and purified using a similar system and then activated by recombinant bovine enterokinase cleavage (17). Recombinant human caspases were produced as described (16). Site-directed mutagenesis of recombinant bovine enterokinase cleavage (18) was achieved by annealing of the appropriate substrate for 1 h.

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Kinetic Analysis—The interaction of granzyme B with PI-9 and derivatives was analyzed using procedures described in Ref. 19. Protein concentrations were determined by Bradford assay, and as active site titrants of granzyme B were not available, protease activity was routinely determined by assessing complex formation with a 2-fold excess of highly purified wild type PI-9. Only batches of granzyme B in which greater than 95% of protease showed complex forming activity with PI-9 were used in the kinetic experiments. Briefly, under pseudo-first order conditions a constant amount of enzyme (0.2 nm) was mixed with different concentrations of inhibitor and excess substrate (100 μm substrate 2) in 500 μl. Progress curves were monitored at 37 °C for 2 h in a Perkin-Elmer LS50B spectrophurometer (excitation, 320 nm; emission, 420 nm). The stoichiometry of inhibition (SI) and rate constant (k_on) for the interaction of granzyme B with PI-9 and each mutant were derived as described (19, 20). Experiments were performed at three times, and the weighted means of the determinations are reported.

Cleavage of quenched fluorescence and spectrophotometric granzyme B substrates and derivation of k_on values was as described (17). The substrate IETD-pNA was purchased from Calbiochem. Cleavage of commercial substrates was performed as previously (16) in 100 mM HEPES, pH 7.5, 10% sucrose, 0.1 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol containing 50 μM of the appropriate substrate for 1 h at 37 °C. An arbitrary activity ratio was used to compare caspase activity on the various substrates and is defined as maximal fluorescence in the presence of caspase divided by maximal fluorescence in the absence of caspase.

Substrate Synthesis—The various internally quenched substrates were prepared on a 25-μmol scale on a Rink AM amide polystyrene-based resin (resin particles are functionalized with 4-[2,4-dimethoxy-phenyl-Fmoc-aminoethyl]-phenoxy linker), using standard solid phase Fmoc protocols (21) on a Perkin Elmer Biosciences Synergy instrument. The ϵ-dinitrophenyl (dnp) lysine residue was introduced into the sequence as its N-α-Fmoc derivative, employing 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate and aminobenzoyl (abz) residue was introduced as its t-butyl carbonyl-protected pentafluorophenyl ester (23). On completion of the synthesis, the peptides were obtained as their C-terminal amides by cleavage from the support by treatment with a solution of trifluoroacetic acid in 50% (v/v) dichloromethane containing a carboxylation scavenger mixture (ethanedithiol, water, thioanisole 1:1:2).

When samples of the PI-As-containing peptides were analyzed by reverse-phase HPLC, two major components were detected in every instance. This is probably due to the formation of β-aspartyl-linked sequences arising out of a transpeptidation reaction of the Asp-Ser peptide bond, which is a common problem in the synthesis of such peptides (24). However, the reaction can be reversed by treatment with dilute ammonia. When applied to our peptides, this protocol resulted in the generation of products containing one major component. The purity and identity of each peptide were confirmed using capillary electrophoresis and matrix-assisted laser desorption ionization-time of flight mass spectroscopy.

Molecular Modeling of the PI-9 RCL-Granzyme B Interaction—Human granzyme B is 69% identical to rat granzyme B. The x-ray crystal structure of rat granzyme B complexed to a variant of the small serine proteinase inhibitor ecotin (Ref. 25; Protein Data Bank identifier 1FI8) was used as a template to model human granzyme B using the program MODELLER (26). The structure of rat granzyme B in complex with ecotin enabled us to model the P7-P1 region of PI-9 into the active site of human granzyme B using techniques similar to those previously described (27). Specifically, we superposed our model of human granzyme B onto the x-ray crystal structure of rat granzyme B-ecotin by using the program by Arthur et al. (28). We then generated a model of rat granzyme B residues 6–77 of ecotin (residues 77–84 representing the P7-P1 region) to leave the model of human granzyme B with the P7-P1 region of ecotin. We converted the P7-P1 sequence to IETD of ecotin to that of PI-9 (SCFVVAE) using the “mutate” facility in the program Quanta (MSI Inc., San Diego, CA). Because the P' region of the inhibitor is missing in the structure of the rat granzyme B-ecotin...
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RESULTS AND DISCUSSION

Identification of the P1 Residue in the PI-9 RCL—The identity of the PI-9 P1 residue as Glu340 has been inferred previously by serpin sequence comparisons and by the established preference of granzyme B for acidic P1 residues (31, 32). This assignment is supported by mutagenesis experiments substituting Glu340 with Ala or Asp, which resulted in serpins with insignificant or very low inhibitory activity against granzyme B (16). However, sequence comparisons are unreliable for identifying P1 residues because the RCL length varies from serpin to serpin, and different residues may be utilized in interactions with different proteinases. The latter point is illustrated by the PI-9-related serpin, PI-6, which uses different P1 residues for interactions with chymotrypsin and thrombin (33). In the case of PI-9, there is another acidic residue (Glu340) in the RCL, and granzyme B also cleaves after Met (32), so it is formally possible that either Glu340 or Met343 acts as the P1 residue and that Glu340 contributes only to initial inhibitor binding.

To confirm the assignment of Glu340 as the P1 residue, cleavage and peptide sequencing experiments were undertaken. To avoid rapid inhibition of the enzyme by PI-9 and promote cleavage of the serpin by granzyme B, a variant of PI-9 was used. This carries a mutation in the proximal hinge (Thr326 to Arg) previously shown to abrogate inhibitory activity and convert the serpin into a granzyme B substrate (16). The recombinant serpin was incubated with native granzyme B, the resulting 38- and 4-kDa cleavage products were alkylated and separated by HPLC (Fig. 1), and then the smaller fragment was subjected to amino acid sequence analysis (data not shown).

Because of low level sequence contamination, the residues in the first and fifth positions were equivocal, but the sequence amino terminus-Xaa-Cys-Met-Glu-Xaa-Gly-Pro-Arg was otherwise unambiguous and corresponded to the PI-9 RCL sequence from Cys341 to Arg348. This indicated that granzyme B cleavage had occurred between Glu340 and Cys341, proving that for this protease Glu340 is the P1 residue in the PI-9 RCL.

Scanning Mutagenesis of the PI-9 RCL—The serpin-proteinase interaction involves the reversible formation of an initial complex, followed by conversion to a locked complex by cleavage of the serpin between the P1 and P1′ residues, which triggers rapid insertion of the RCL into the serpin A β-sheet before decylation is completed (15, 34, 35). Distortion, inactivation, and translocation of the trapped proteinase to the opposite pole of the serpin results from RCL insertion (36). Alternatively, catalysis is completed before RCL insertion, the proteinase escapes, and the complex dissociates, releasing cleaved serpin and active enzyme (34). In other words, following initial complex formation the pathway bifurcates and can follow an inhibitory or substrate route depending on the rate of RCL insertion. Kinetic analysis of the interaction can indicate whether formation of the locked complex is favored (the inhibitory pathway) or whether cleavage and dissociation is favored (the substrate pathway). For example, if the SI is unitary the inhibitory pathway is favored, whereas higher SI values indicate that the serpin is seen more as a substrate by the enzyme. The second order association constant (kassoc) is a measure of the rate of RCL insertion and complex formation. The higher the kassoc, the faster the reaction and the less likely it is to follow the substrate pathway. Multiplication of the kassoc by the SI yields a value for the rate of formation of the nonreversible intermediate that precedes the inhibitory/substrate pathway branch point (37, 38).

It is generally accepted that the specificity and affinity of the serpin-proteinase interaction is dictated primarily by the P1 residue (39). This is consistent with the inhibition of degradative proteinases that have a broad substrate range and high catalytic rate (pancreatic, neutrophil, and lysosomal protein-
ases) and do not have stringent sequence motifs around the P1 that influence substrate binding. It is also classically illustrated by the Pittsburgh mutation of α1-antitrypsin, which retargets the serpin from elastase to thrombin by conversion of its P1 residue from Met to Arg (39, 40). However, granzyme B differs from the degradative proteinases in that its catalytic and biological properties suggest that it is a regulatory enzyme that acts on a limited set of proteins. In other words, it is highly selective, and it therefore follows that substrate specificity is also likely to be influenced by residues around the P1. This is supported by work showing that the P4 and P3 residues are important in granzyme B substrates (12) and that, unlike pancreatic serine proteinases, it is not capable of hydrolyzing amidate substrates of less than three residues (13). Thus, binding and inhibition of granzyme B by PI-9 is likely to depend on the P4 and P3 residues and probably others surrounding the P1.

We have previously shown that alteration of the PI-9 P1 residue to Asp results in an inhibitor that is 100-fold less effective and has properties of a granzyme B substrate (Fig. 2 and Ref. 16). We have also shown that the PI-9 granzyme B interaction requires a mobile RCL, because mutation of the proximal hinge abrogates inhibitory activity and converts PI-9 into a substrate (Fig. 2). To identify other residues in the P1-9 RCL loop important for granzyme B inhibitory function, we carried out site-directed mutagenesis, substituting the P7 to P5 residues in turn with Ala (except the P2 Ala, which was replaced with Gly). Mutated serpins were produced in the P. pastoris system and prepared to greater than 90% purity using immobilized metal affinity chromatography (for example, see Fig. 2).

Mutants were tested for the ability to form SDS-stable complexes with granzyme B (illustrated in Fig. 2) and then were subjected to kinetic analysis. SI and $k_{\text{ass}}$ values were derived for the interaction of each serpin with granzyme B. With the exception of the variant with a mutation in the proximal hinge, all mutants retained inhibitory activity as indicated by their ability to complex with granzyme B (data not shown), but there were marked differences noted in the stoichiometry and kinetics of the interactions. As shown in Table I and discussed below, pronounced effects on the inhibitory capacity of PI-9 were observed for mutations at P7, P4, P3, P1, P3’, and P4’.

**Properties of the P7-P1 Mutants**—Molecular modeling shows that P7 probably does not interact with the active site of granzyme B (see below). Mutating this residue increased the SI of the PI-9-granzyme B interaction and decreased the $k_{\text{ass}}$ 3-fold, indicating that inhibitory function is affected. Because the SI × $k_{\text{ass}}$ value for the interaction is close to that of wild type, indicating little effect on intermediate formation, it is unlikely that this mutation affects proteinase docking. It is more likely that it slows or prevents complete insertion of the RCL into the A β-sheet, increasing the probability that the reaction will follow the substrate pathway.

By contrast, mutating the PI-9 P4-P1 residues apparently affects proteinase binding to the serpin prior to RCL insertion, and the results are consistent with the known substrate characteristics and preferences of granzyme B (12, 13). Combinatorial and deletion analysis has shown that a minimal peptide substrate for granzyme B must contain a P4 residue and that the optimal sequence is Ile-Glu-(Pro/Thr)-Asp (12). However, the relative contribution of each residue in this sequence to substrate binding has not been determined. Granzyme B will cleave peptide substrates having either Ile or Val at the P4 position (although Ile is slightly preferred), and most natural substrates have either P4 Ile or Val (see Table III). Thus, the presence of a P4 Val in the wild type PI-9 RCL is consistent with this residue playing an important role in granzyme B binding. Our analysis showed that substitution of the P4 Val for Ala in the PI-9 RCL results in decreased inhibitory function as indicated by a 4-fold decrease in the $k_{\text{ass}}$ emphasizing the importance of Val in this position for the binding of granzyme B to substrates and inhibitors.

Peptide substrate analysis has indicated a preference for Glu at P3 (12, 13), but examination of natural substrates show that human granzyme B tolerates other residues at this position (Table III). At present it is not clear how important the P3 residue is for granzyme B binding. Tetrapeptide substrates with Val at this position are cleaved by rat granzyme B at 20% of the efficiency of substrates containing Glu (13), whereas substrates containing P3 Ala are cleaved at 60% efficiency. Interestingly human granzyme B does not cleave substrates containing P3 Ala or Val as efficiently (<20%) as rat granzyme B (12, 13), indicating subtle differences in the substrate binding characteristics of granzyme B from different species. Our analysis shows that converting the P1/P3 P3 from Val to Ala has a pronounced negative effect on inhibition of human granzyme B (approximately a 10-fold decrease in the $k_{\text{ass}}$ without markedly affecting the SI, which illustrates that P3 is important for enzyme binding and suggests that it may be more important than the P4 residue.

At P2 there is reported to be a slight preference for proline (12, 13), but substrates containing Ala or Gly are also cleaved. Interestingly, peptide substrates with P2 Gly are cleaved less efficiently than those containing P2 Ala by both rat and human granzyme B, and this is reflected in the poorer inhibitory capacity we observed for the PI-9 mutant carrying a P2 Ala to Gly substitution (Table I). Nevertheless, our data support the previous conclusion that the P2 residue does not contribute significantly to substrate binding.

Finally, it is known that Asp is preferred at P1 for substrate cleavage by granzyme B (32, 41). As we have previously shown (illustrated in Fig. 2 and Table I), substitution of the P1 Glu with Asp results in a much poorer inhibitor that is cleaved efficiently by granzyme B (16). Substitution with Ala resulted in an essentially inactive inhibitor, confirming the importance of the P1 in substrate and inhibitory function. Overall, our results clearly show that efficient substrate or inhibitor binding to human granzyme B requires an extended sequence upstream of the cleavage site and that the relative importance of the various residues is $P1 > P3 > P4 > P2 > P5$. 

**Properties of the P’ Mutants**—Substrate phage display anal-
Directed mutagenesis of the PI-9 RCL

Using a two-sided probability (p) test, the mean $k_{cat}$ values for the mutants varied significantly from wild type (p < 0.01) in each case except C341S (p < 0.1).

| Mutant | RCL sequence | $k_{cat}$ | $k_{cat}$ | $k_{cat}$ |
|--------|--------------|----------|----------|----------|
| Wild type | ... | ... | ... |
| T327R (P14) | ... | ... | ... |
| S334A (P7) | ... | ... | ... |
| C335A (P6) | ... | ... | ... |
| F336A (P5) | ... | ... | ... |
| V337A (P4) | ... | ... | ... |
| V338A (P3) | ... | ... | ... |
| A339G (P2) | ... | ... | ... |
| E340A (P1) | ... | ... | ... |
| E340D (P4) | ... | ... | ... |
| C341A (P14) | ... | ... | ... |
| C342A (P2) | ... | ... | ... |
| M343A (P14) | ... | ... | ... |
| E344A (P4) | ... | ... | ... |
| E344D (P4) | ... | ... | ... |
| C345A (P5) | ... | ... | ... |

Synthesis of Granzyme B Substrates Based on the PI-9 RCL—To further test the idea that the P4-P4' region of PI-9 mimics a substrate of granzyme B, we synthesized quenched fluorescence substrates based on this sequence. In the first instance we synthesized two peptides: abz-Val-Val-Ala-Glu-Ser-Met-Glu-Lys-dnp (substrate 1) and abz-Val-Val-Ala-Glu-Ser-Met-Glu-Lys-dnp (substrate 2). For ease of synthesis and peptide stability, we used Ser at the P1' and P2' positions instead of Cys. This was suggested by mutagenesis experiments demonstrating that substitution of the P1' Cys with either Ser or Ala had no significant effect on inhibitory function (Table I).

The only difference between substrates 1 and 2 is the identity of the P1 residue. Given the preference of granzyme B for Asp, and the fact that the PI-9 E340D mutation resulted in a molecule with characteristics of a substrate rather than inhibitor (Table I), we predicted that substrate 2 would be cleaved more efficiently. As shown in Table II, both substrates were cleaved by granzyme B, but substrate 2 was cleaved three times more efficiently than substrate 1. The kinetics compared favorably with published values for the cleavage of tetrapeptides by granzyme B; the best tetrapeptide (Ac-Ile-Glu-Pro-Asp-p-nitroanilide (Ref1 in Table II)) yields a $k_{cat}$ of 5.7 M$^{-1}$ s$^{-1}$ and $k_{cat}$ of 4.16 s$^{-1}$ (13), compared with 13 M$^{-1}$ and 1.5 s$^{-1}$, respectively, for substrate 2. In terms of the specificity constant ($K_m/k_{cat}$) substrate 2 is 1.8-fold better than the reference substrate.

Although substrate 2 binds more tightly to granzyme B than the best tetrapeptide substrate, it is cleaved at a slower rate. This lower cleavage efficiency may be due to a suboptimal P4-P1 sequence. To test this idea, we synthesized an idealized granzyme B substrate comprising the best reported P4-P1 sequence linked to the P1'-P4' sequence of PI-9 (substrate 3). This substrate bound 10 times more tightly to granzyme B than the tetrapeptide substrate and was turned over at an equivalent rate, yielding a 11-fold higher specificity constant (Table II). Interestingly, the sequence of substrate 3 resembles the known activation site for granzyme B on caspase 3 (Table III), which includes an acidic P4' residue and is a known in vivo target of granzyme B (8, 44).

To test the importance of the P4' residue in the interaction between granzyme B and substrates, we also synthesized a substrate identical to substrate 2 except that the P4' Gly is replaced by Ala (substrate 4). As shown in Table II, substrate 4 did not bind as tightly as substrate 2 to granzyme B (5-fold increase in $K_m$) and was turned over more efficiently (2.5-fold increase in $k_{cat}$). These results are consistent with an important role for the P4' residue in a direct interaction with granzyme B.
gyrome B rather than in maintenance of PI-9 RCL conformation.

**Modeling of the Granzyme B-PI-9 RCL Interaction**—To put our results in a structural context, we modeled human granzyme B using the structure of rat granzyme B (25) as a template (the two proteins are 69% identical). We then docked the P7-P5 portion of the PI-9 RCL into the active site of the model using the three-dimensional structures of a rat granzyme B-ecotin complex (25) and a trypsin-ecotin complex (29) as guides. Using the three-dimensional structures of a rat granzyme B, we noted that differences in the substrate profiles and binding characteristics in rat but not mouse granzyme B, suggesting there may be variations that the P1 Asp for Ala. This would have the effect of stabilizing side chain conformations of the model the granzymes from various species. We also noted that Lys24 in granzyme B is close to the P4′ Glu (5.5 Å) but is too distant to form a salt bridge.

**Site-directed Mutagenesis of Granzyme B**—To test the idea that Lys27 in granzyme B interacts with the P4′ Glu of PI-9, we produced two mutants of granzyme B: Lys27 to Ala, and Lys24 to Ala. We predicted that the first mutant would bind less efficiently to PI-9 and to substrates containing P′ residues because the salt bridge can no longer form. By contrast, the second mutant should bind as efficiently wild type granzyme B to inhibitors or substrates because Lys24 is not predicted to participate in intermolecular interactions.

As shown in Table IV, both mutants cleaved a tetrapeptide substrate lacking P′ residues to the same extent as wild type, which demonstrates that neither mutation perturbs the catalytic function of the enzyme. By contrast, when compared with wild type or the K24A mutant, the K27A mutant showed a marked decrease in the ability to bind and cleave a substrate (substrate 3) containing P′ residues. The decrease in $k_{cat}$ for the interaction suggests that the P′ residues play a role in positioning the substrate in the active site cleft. Furthermore, the K27A mutant showed a marked decrease in its ability to interact with PI-9 (a 4-fold increase in the SI and a 20-fold decrease in $k_{cat}$). Taken together, these results strongly support the prediction that the P′ Glu of PI-9 is involved in a salt bridge with Lys27 of granzyme B and suggest that high affinity interactions of granzyme B with substrates and inhibitors require the participation of P′ residues particularly an acidic P′.

**Caspase Recognition of the Granzyme B Substrates**—Caspases and granzyme B show similarities in substrate recognition and biological function. Combinatorial analyses suggest that granzyme B and the group III (activator) caspases prefer substrates with similar P4-P1 sequences, consistent with their roles as upstream activators of the caspase cascade and apoptosis (12). To see how far this similarity extends, we tested the ability of three group III caspases (caspases 6, 8, and 10) to cleave our substrates 1–3 and compared this to caspase 5 (group I) and caspase 3 (group II). As shown in Fig. 4, none of the caspases effectively cleaved substrates 1 or 2, whereas two of the group III caspases (caspase 6 and 10) cleaved substrate 3. The failure of caspases to cleave substrates 1 and 2 probably reflect stricter P4-P1 requirements compared with granzyme B. For example, group I caspases prefer His at P2 (and caspase 5 also prefers Glu at P3); group II caspases prefer Asp at P4; and group III caspases prefer Glu at P2 (12). The ability of caspase 6 and caspase 10 to cleave substrate 3 is not surprising, given that its P4-P1 sequence is close to optimal. However, the failure of caspase 8 to cleave substrate 4 is unexpected and indicates that the P′ residues in this substrate prevent efficient interaction with the binding site of the enzyme. Taken together these results show that substrates based on the PI-9 RCL (substrates 1 and 2) can be used to discriminate caspases and granzyeme B in situations where both are active.

**The Role of the P′ Residues in Substrate and Inhibitor Binding**—Overall, our results indicate that the P1′-P4′ residues of granzyme B substrates are important for substrate binding,
the optimal P1–P4 sequence for granzyme B recognition remains to be determined, perhaps by combinatorial or phage display methods. Our work also suggests that efficient and selective granzyme B inhibitors require P residues and may explain why effective tetrapeptide or other synthetic granzyme B inhibitors have not been reported to date (45).

**FIG. 3. Model of the complex between human granzyme B and the PI-9 RCL.** A, a stereo view of the region around the active site of human granzyme B (gray) and the RCL of PI-9 (cyan). The PI-9 P4 residue Glu344 (labeled red ball and stick) makes a salt bridge (dashed line) with Lys27 of granzyme B (dark blue ball and stick). The PI-9 P1 residue (Glu341) is labeled and shown in magenta ball and stick. The granzyme B catalytic triad (His44, Asp88, and Ser183) is in green ball and stick. The distance between the Oe2 of P4 Glu and the Nz of Lys27 is 2.6 Angstroms, the angle Ce-Nz-Oe2 is 145° and the angle Nz-Oe2-Cα is 98.8°. Distance and angle measurements were performed using Quanta (MSI Inc.). B, details of the interaction between the PI-9 RCL peptide and human granzyme B. The peptide backbone of the PI-9 RCL is shown in cyan, the P4 residue (Glu344) in red stick, and the P1 residue (Glu341) in magenta stick. Other side chains are shown in light gray stick. The Ca trace of the proteinase is in black, and Lys27 is shown in dark blue stick. The active site triad (His44, Asp88, and Ser183) is in green stick. Additional side chains and backbone atoms that form hydrogen bonds (dashed lines) to the RCL of PI-9 are shown in black and are labeled. For clarity, hydrophobic contacts are not shown. The figures were produced using MOLSCRIPT (47).

**TABLE IV**

| Enzyme | Interaction with substrates | Interaction with PI-9 |
|--------|-----------------------------|----------------------|
|        | IKTD-pNA                    | abs-IEPDSSMEK-dnp     |               |
|        | $K_m$ $k_{cat}$             | $K_m$ $k_{cat}$       | SI $k_{cat}$ |
|        | $\mu M$ $s^{-1}$           | $\mu M$ $s^{-1}$     | $M^{-1} s^{-1}$ |
| Wild type | 106 ± 6 2.4 | 5.8 ± 0.2 4.4 ± 0.2 | 1.0 1.7 ± 0.2 $10^6$ |
| K27A    | 118 ± 9 1.8 | 38.7 ± 6.3 0.7 ± 0.1 | 4.4 8.2 ± 2.1 $10^4$ |
| K24A    | 114 ± 7 2.3 | 6.8 ± 1.0 4.2 ± 0.6 | 1.2 1.2 ± 0.1 $10^6$ |

but the optimal P1′-P4′ sequence for granzyme B recognition remains to be determined, perhaps by combinatorial or phage display methods. Our work also suggests that efficient and selective granzyme B inhibitors require P′ residues and may explain why effective tetrapeptide or other synthetic granzyme B inhibitors have not been reported to date (45).
Acidic P4\(^{-}\) residue in the PI-9 RCL is consistent with the idea that this inhibitor has evolved to maximize granzyme B binding at the expense of cleavage, a point that is reinforced by the presence of the nonpreferred Glu at the P1 position. Examination of the known granzyme B cleavage sites on protein substrates reveals that some like those in caspase 3 and PARP include acidic P4\(^{-}\) residues (Table III). However, many others do not have acidic P4\(^{-}\) residues in their characterized granzyme B cleavage sites. This suggests that granzyme B has two sub-classes that are defined by the presence or absence of acidic P4\(^{-}\) residues: one set that binds with high affinity but cleaves slowly, and another set that binds with lower affinity but cleaves more rapidly. It also implies a hierarchy of targets for granzyme B in the cell, which is consistent with its role as an initiator of apoptosis; it binds preferentially to and inactivates proteins that act early in apoptotic pathways and then selectively degrades a secondary set of targets. However, it is not possible to group known natural substrates into sub-categories to predict which are early (low \(K_m\), low \(k_{cat}\)) or late (higher \(K_m\) and \(k_{cat}\)) targets because binding and catalytic constants have not been reported for their interactions with granzyme B (Table III).

In closing, our results demonstrate that P4-P4\(^{-}\) sequences in natural substrates and inhibitors are required for efficient binding to human granzyme B and that the highest affinity is observed when an acidic P4\(^{-}\) residue is present. This is consonant with the narrow substrate range of granzyme B and the regulatory role it plays in cytotoxic lymphocyte-mediated apoptosis, in contrast to the degradative serine proteinases that have a large range of substrates and low sequence selectivity. It is interesting to note that so far only one other serine proteinase has been shown to have such an extended sequence requirement for substrate or inhibitor binding. Interaction of tissue-type plasminogen activator with its cognate inhibitor plasminogen activator inhibitor 1 has been shown to require the P1’-P5’ residues of the serpin (46). This is entirely consistent with the regulatory role of tPA in plasminogen activation and its restricted range of substrates.

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Importance of the P4′ Residue in Human Granzyme B Inhibitors and Substrates Revealed by Scanning Mutagenesis of the Proteinase Inhibitor 9 Reactive Center Loop

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