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Granzyme M Is a Regulatory Protease That Inactivates Proteinase Inhibitor 9, an Endogenous Inhibitor of Granzyme B*

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Granzyme M is a trypsin-fold serine protease that is specifically found in the granules of natural killer cells. This enzyme has been implicated recently in the induction of target cell death by cytotoxic lymphocytes, but unlike granzymes A and B, the molecular mechanism of action of granzyme M is unknown. We have characterized the extended substrate specificity of human granzyme M by using purified recombinant enzyme, several positional scanning libraries of coumarin substrates, and a panel of individual *p*-nitroanilide and coumarin substrates. In contrast to previous studies conducted using thiobenzyl ester substrates (Smyth, M. J., O'Connor, M. D., Trapani, J. A., Kershaw, M. H., and Brinkworth, R. I. (1996) *J. Immunol.* 156, 4174–4181), a strong preference for leucine at P1 over methionine was demonstrated. The extended substrate specificity was determined to be lysine = norleucine at P4, broad at P3, proline > alanine at P2, and leucine > norleucine > methionine at P1. The enzyme activity was found to be highly dependent on the length and sequence of substrates, indicative of a regulatory function for human granzyme M. Finally, the interaction between granzyme M and the serpins α_1-antichymotrypsin, α_1-proteinase inhibitor, and proteinase inhibitor 9 was characterized by using a candidate-based approach to identify potential endogenous inhibitors. Proteinase inhibitor 9 was effectively hydrolyzed and inactivated by human granzyme M, raising the possibility that this orphan granzyme bypasses proteinase inhibitor 9 inhibition of granzyme B.

Cytotoxic lymphocytes, which include cytotoxic T cells and natural killer (NK)[1] cells, recognize and kill host cells infected with intracellular pathogens such as viruses and certain types of bacteria. Death of target cells is predominantly mediated through granule exocytosis. In this process lysosome-like vesicles whose principal components are perforin and a family of serine proteases known as the granzymes are vectorially secreted toward the target cell. Perforin then facilitates entry of granzymes into the target cell, whereupon key protein substrates such as caspases are cleaved to induce death (2). In addition to being important mediators of immunity, some granzymes may also represent potential drug targets for treatment of autoimmune disorders (3).

The five known human granzymes have varied primary substrate specificities. Granzymes A and K cleave after basic residues (4, 5); granzyme B cleaves after aspartic acid (6); granzyme H cleaves after aromatic residues (7), and granzyme M cleaves after long aliphatic residues such as methionine, norleucine, and leucine (1). Granzyme M is unique among the family because it is specifically expressed in NK cells and thus may have evolved to serve a specialized function in innate immunity (8). Several studies have demonstrated that granzymes A and B serve as important effectors of lymphocyte cytotoxicity by inducing nuclear and non-nuclear damage in target cells (2). In contrast, far less is known about the orphan human granzymes H, K, and M. Evidence has been presented for granzyme K and a chymase-like serine protease from human NK cells, presumably granzyme H, contributing to induction of target cell damage (9, 10). It has also been demonstrated recently (11) that treatment of target cells with purified granzyme M and sublytic quantities of perforin leads to lysis.

Toward gaining a better understanding of the molecular function of granzyme M and its NK cell specificity, biochemical characterization of this enzyme is presented here with respect to its substrate specificity and interaction with three serpin macromolecular inhibitors. To obtain protease that is free from contaminating proteolytic activity, human granzyme M was recombinantly expressed in the yeast *Pichia pastoris* and purified to homogeneity. The primary (P1)[2] and extended (P4–P2) substrate specificity of the recombinant enzyme was first characterized by using positional scanning libraries of fluorogenic tetrapeptide coumarin substrates (12). Single substrates were then used for validation of library results and a more detailed kinetic characterization. Finally, in an attempt to explore how the activity of human granzyme M is controlled under physiological conditions, interaction of the enzyme with the serpins α_1-antichymotrypsin (ACT), α_1-proteinase inhibitor (α_1PI), and proteinase inhibitor 9 (PI9) was characterized. These three serpins are known to inhibit other leukocyte proteases that display hydrophobic primary specificity such as neutrophil elastase, cathepsin G, proteinase 3, and chymase (13, 14) and are thus good candidate physiological inhibitors of human granzyme M.

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[1] The abbreviations used are: NK, natural killer; ACT, α_1-antichymotrypsin; DTT, dithiothreitol; PI, proteinase inhibitor; ACC, 7-amino-4-carbamoylmethylcoumarin; PNGase F, *N*-glycosidase F; AMC, 7-amino-4-methylcoumarin; *p*NA, *p*-nitroanilide; Suc, succinyl; MES, 2-(*N*-morpholino)ethanesulfonic acid; HPLC, high pressure liquid chromatography; SI, stoichiometry of inhibition; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

[2] Nomenclature for the substrate amino acid preference is Pn, Pn-1, . . ., P2, P1, P1′, P2′, . . ., Pm-1′, Pm′, with amide bond hydrolysis occurring between P1 and P1′. The corresponding enzyme binding sites are denoted by Sn, Sn-1, . . ., S2, S1, S1′, S2′, . . ., Sm-1′, Sm′ (34).

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise stated, all chemicals were purchased from Sigma. Oligonucleotide primers were synthesized on an Applied Biosystems Expedite DNA synthesizer (Foster City, CA). Molecular weight markers for gel electrophoresis, restriction enzymes, and *N*-glycosidase F (PNGase F) were purchased from New England Biolabs (Beverly, MA) and used according to the manufacturer's instructions. Human granzyme B was a generous gift from Dr. Nancy Thornberry (Merck). Human granzyme A was recombinantly expressed and purified as described previously (15). Human PI9 and anti-PI9 antibodies were prepared and purified as described previously (16, 17). Human ACT and α_1PI were purchased from Calbiochem. The *p*-nitroanilide (*p*NA) substrates Suc-AAPL-*p*NA, Suc-AAPn-*p*NA, Suc-AAPM-*p*NA, and Suc-AAPK-*p*NA, and the 7-amino-4-methylcoumarin (AMC) substrates Ac-AAPA-AMC, Suc-AAPF-AMC, and Ac-IEPD-AMC were purchased from Bachem (Torrance, CA).

Heterologous Expression of Human Granzyme M—The cDNA encoding mature human granzyme M was amplified from I.M.A.G.E. clone 112558 and subcloned into the yeast expression vector pPICZα A (Invitrogen). The resulting construct permitted the sequence of mature human granzyme M to immediately follow the Kex2 signal cleavage site of the *Saccharomyces cerevisiae* α-factor secretion signal. The vector was linearized with SacI and transformed into the X33 strain of *Pichia pastoris*. Clones with the integrated human granzyme M cDNA were selected by resistance to Zeocin™ (Invitrogen) and were used to inoculate 1-liter shaker flask cultures.

Purification of Recombinant Human Granzyme M—After 3 days of induction with methanol, the conditioned media from the shaker flask cultures was isolated and loaded onto an SP-Sepharose cation exchange column (Amersham Biosciences). The column was washed with 4 column volumes of 50 mM MES, pH 6.0, 50 mM NaCl, and bound protein was eluted with 4 column volumes of 50 mM MES, pH 6.0, 1 M NaCl. Eluted protein was concentrated, exchanged into 50 mM MES, pH 6.0, 50 mM NaCl, and loaded onto a Mono-S cation exchange fast protein liquid chromatography column (Amersham Biosciences). The column was washed with 4 column volumes of 50 mM MES, pH 6.0, 50 mM NaCl, and bound protein was eluted with 50 column volumes of a linear gradient of 0.32 M NaCl to 0.48 M NaCl in 50 mM MES, pH 6.0. Fractions containing human granzyme M eluted between 0.38 and 0.45 M NaCl. These were pooled, concentrated, and exchanged into 50 mM MES, pH 6.0, 50 mM NaCl. Purity of the preparation was assessed by SDS-PAGE and Coomassie Brilliant Blue staining, followed by densitometric analysis with a MultiImage™ Light Cabinet (Alpha Innotech, San Leandro, CA).

Preparation of Anti-human Granzyme M Antibodies—Surface loops of human granzyme M with low similarity to those of the other four human granzymes were identified, and the sequence analysis software package MacVector (Oxford Molecular, Madison, WI) was used to rank their antigenic potential. Peptides corresponding to the amino acid sequence of two loops were synthesized using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry on an Applied Biosystems automated peptide synthesizer (Foster City, CA), purified by reversed-phase HPLC, and characterized by MALDI-TOF mass spectrometry. Each peptide was conjugated to keyhole limpet hemocyanin and used to immunize rabbits for polyclonal antiserum production (Covance Corp., Richmond, CA). Antibodies raised against the peptide corresponding to residues 98–109 of human granzyme M demonstrated good reactivity and selectivity against the recombinant enzyme and were used for subsequent studies.

Immunoblot Analysis—Protein samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked in TBST (Tris-buffered saline with 0.1% Triton X-100) containing 5% nonfat dry milk, washed with TBST, incubated in a dilution of anti-human granzyme M antibodies or anti-human PI9 antibodies in TBST containing 5% nonfat dry milk, washed with TBST, incubated in a dilution of horseradish peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad) in TBST containing 5% nonfat dry milk, and washed with TBST once again. Antibody-bound protein bands were then detected by enhanced chemiluminescence (Amersham Biosciences).

Active Site Titration of Human Granzyme M and Other Proteases—Bovine trypsin was active site-titrated with 4-methylumbelliferyl *p*-guanidinobenzoate and was then used to determine the concentration of a solution of the macromolecular serine protease inhibitor ecotin using Suc-AAPK-*p*NA as the substrate. Ecotin was then used to active site-titrate human granzyme M and bovine chymotrypsin using Suc-AAPL-*p*NA as the substrate, human neutrophil elastase and porcine pancreatic elastase using Ac-AAPA-AMC as the substrate, and human neutrophil cathepsin G using Suc-AAPF-AMC as the substrate.

Positional Scanning Synthetic Combinatorial Libraries—The preparation and characterization of the P1-diverse, P1-Leu, and P1-Met libraries of 7-amino-4-carbamoylmethylcoumarin (ACC) substrates used in this study are described elsewhere (12, 18). Screening of these libraries was also carried out as described previously. Briefly, $\sim 10^{-9}$ mol of each well of the P1-Leu or P1-Met stock libraries was added to 57 or 60 wells, respectively, of a 96-well Microfluor plate (Dynex Technologies, Chantilly, VA), and 10^{-10} mol of each well of the P1-diverse stock library was added to 20 wells of a 96-well Microfluor plate. Final concentration of each substrate in the assay was ~ 0.1 μM for the P1-Leu and P1-Met libraries and ~ 0.01 μM for the P1-diverse library. Assays were initiated by addition of ~ 500 nM enzyme and were conducted at 30 °C in assay buffer containing 100 mM HEPES, pH 7.4, 200 mM NaCl, 0.01% Tween 20, and 1% Me_2SO. Hydrolysis of substrates was monitored fluorimetrically with an excitation wavelength of 380 nm and an emission wavelength of 460 nm on a Spectramax Gemini microtiter plate reader (Molecular Devices, Sunnyvale, CA).

Synthesis of Single Coumarin Substrates—Single ACC substrates were prepared as described previously (12), purified by reversed-phase HPLC, and characterized by MALDI-TOF mass spectrometry.

Single Substrate Kinetics—Enzyme activity was monitored at 25 °C in assay buffer containing 100 mM HEPES, pH 7.4, 200 mM NaCl, and 0.01% Tween 20. Human granzyme M concentration in assays ranged from 10 nM to 2 μM and substrate concentration ranged from 2 μM to 3 mM. Substrate stock solutions were prepared in Me_2SO, and final Me_2SO concentrations in assays never exceeded 2% because higher concentrations were found to be detrimental to enzyme activity. Hydrolysis of ACC substrates was monitored as described for library assays, and hydrolysis of *p*NA substrates was monitored spectrophotometrically at 405 nm on a Molecular Devices UVmax microtiter plate reader (Molecular Devices, Sunnyvale, CA).

Analysis of Serpin-Granzyme M Complex Formation by SDS-PAGE—The concentration of human ACT and human α_1PI were calculated using $\epsilon_{280} = 3.9 \times 10^4$ and 2.8×10^4 M^{-1} cm^{-1}, respectively. The concentration of human PI9 was determined by the Bradford assay (Bio-Rad). ACT, α_1PI, or PI9 were incubated at 25 °C with 1 μM human granzyme M at a 2:1 serpin to protease molar ratio in 50 μl of assay buffer for 24 h. Assay buffer was the same as described above for substrate kinetics, but 1 mM DTT was added for reactions between PI9 and granzyme M to prevent inactivation of the serpin following oxidation of cysteine 342 in the reactive center loop. After the incubation, all samples were deglycosylated using PNGase F and analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining.

Titration of Granzyme M with Serpins—200 nM human granzyme M was incubated at 25 °C with 0–200 nM ACT in assay buffer for 24 h, 0–200 nM α_1PI in assay buffer for 96 h, and with 0–8 μM PI9 in assay buffer supplemented with 1 mM DTT for 24 h. The remaining enzymatic activity after these incubation times was determined using 100 μM Ac-KVPL-ACC. Substrate hydrolysis was monitored as described for library assays.

Serpin Kinetics—The inhibition of human granzyme M by ACT, α_1PI, and PI9 was characterized by monitoring the hydrolysis of 1 mM Ac-KVPL-ACC by 2 nM protease in the presence of varying serpin concentrations in assay buffer or assay buffer supplemented with 1 mM DTT for reactions between PI9 and granzyme M. Substrate hydrolysis was monitored as described for library assays over the course of 2 h. Serpin concentrations ranged from 0.48 to 7.6 μM for ACT, 0.45 to 7.2 μM for α_1PI, and 2.3 to 37.2 μM for PI9. Data from substrate hydrolysis progress curves were then fit to Equation 1 by using the program KaleidaGraph™ (Synergy Software, Reading, PA),

$$P = v_s + \frac{(v_i - v_s)(1 - e^{-k't})}{k'} \quad \text{(Eq. 1)}$$

where P represents arbitrary fluorescence units at time t; v_i is the initial velocity; v_s is the steady-state velocity, and k' is an apparent first-order rate constant (19). Along with inhibitor concentrations, these apparent rate constants were then fit to Equation 2,

$$k' = k_{off}\left[1 + \frac{1}{K_i(1 + [S]/K_m)}\right] \quad \text{(Eq. 2)}$$

where k' is the apparent first-order rate constant; [S] is the substrate concentration; K_m is the Michaelis constant for interaction of the substrate with the protease, and $K_i = k_{off}/k_{on}$ is the overall inhibition constant (19). The second-order association rate constants $k_{on} = k_a$ for inhibition of human granzyme M by serpins were derived from the K_i

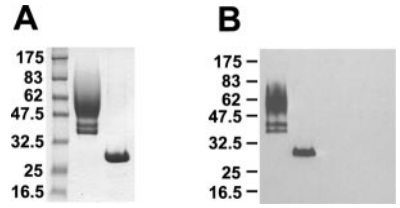

FIG. 1. **Purified recombinant human granzyme M.** *A*, Coomassie Brilliant Blue-stained gel. Molecular weight markers (*lane 1*), 40 μg of human granzyme M in glycosylated form as isolated after purification (*lane 2*), and 8 μg of deglycosylated human granzyme M (*lane 3*). *B*, immunoblot analysis using anti-human granzyme M polyclonal antibody. 200 ng of human granzyme M in glycosylated form as isolated after purification (*lane 1*), 40 ng of deglycosylated human granzyme M (*lane 2*), 40 ng of deglycosylated human granzyme A (*lane 3*), and 40 ng of deglycosylated human granzyme B (*lane 4*).

and k_{off} values obtained from this secondary fit.

PI9 Inactivation—All reactions were carried out at 25 °C in 10 μl of activity buffer supplemented with 1 mM DTT. 0.5 μM human granzyme B was incubated for 1 h with 1 μM PI9 or 1 μM PI9 that had been preincubated for 1 h at a concentration of 2 μM with 1 μM human granzyme M. These two samples and controls for each individual protein were then diluted hundredfold into water, and 7.5 μl of these diluted samples were used for SDS-PAGE and subsequent immunoblot analysis with anti-human PI9 antibodies. PI9 inactivation was also verified by using 200 μM Ac-IEPD-AMC to measure the relative activity of 10 nM human granzyme B following a 30-min incubation with 2 μM PI9, 2 μM PI9 and 10 nM human granzyme M, or 2 μM PI9 and 10 nM human granzyme M that had been preincubated for 30 min before addition of human granzyme B. Substrate hydrolysis was monitored as described for library assays in assay buffer supplemented with 1 mM DTT.

RESULTS

Expression and Purification of Human Granzyme M—The gene for mature human granzyme M was subcloned from I.M.A.G.E. clone 112558 into pPICzα A for expression in the methylotropic yeast *P. pastoris*. The nucleic acid sequence of this gene was found to be identical to that first reported for human granzyme M (20). The protein was expressed as a C-terminal fusion to the *S. cerevisiae* α-factor signal sequence, allowing for purification of the mature enzyme from the media following secretion and cleavage of the signal sequence by Kex2. Following induction with methanol, purification of mature human granzyme M from conditioned media was carried out using SP-Sepharose and Mono-S cation exchange chromatography. Typical yields of purified protein were between 0.2 and 1.0 mg/liter of culture.

SDS-PAGE followed by Coomassie Brilliant Blue staining indicated the recombinant enzyme was differentially glycosylated, migrating as a doublet between 33 and 48 kDa, and as a broad smear of hyperglycosylated enzyme between 48 and 83 kDa (Fig. 1*A*, *lane 2*). Glycosylation of human granzyme M in *P. pastoris* is expected because the enzyme contains three potential *N*-linked glycosylation consensus sites. Treatment with PNGase F caused all bands to decrease in apparent molecular weight and to coalesce at 26 kDa (Fig. 1*A*, *lane 3*). Densitometric analysis of this deglycosylated band indicated that the enzyme was greater than 98% pure. A polyclonal antibody raised against a peptide corresponding to residues 98–109 of human granzyme M recognized all glycosylated species and the single deglycosylated species of the recombinant enzyme (Fig. 1*B*, *lanes 1* and *2*).

P1 Substrate Specificity—The primary specificity of purified recombinant human granzyme M was profiled using a positional scanning synthetic combinatorial library of tetrapeptide coumarin substrates referred to as the P1-diverse library (12). As described previously, this library is of general structure Ac-*XXX*P_1-ACC and is composed of 20 wells in which the P1 position is sequentially fixed as 1 of 20 different amino acids (excluding cysteine and including the methionine isostere norleucine), although P4, P3, and P2 are randomized in all wells, resulting in 8,000 substrates/well. Human granzyme M only displayed activity with P1-Leu, P1-Nle, and P1-Met in the P1-diverse library, in agreement with previous studies conducted with thiobenzyl ester substrates, purified native rat enzyme from RNK-16 cells (21), and supernatant from COS cells transiently transfected with human and mouse granzyme M (1, 22). In contrast to prior studies, human granzyme M displayed an extremely pronounced primary specificity for leucine, with relative activities of 100% for P1-Leu, 46% for P1-Nle, and 16% for P1-Met (Fig. 2*A*). When purified recombinant human granzyme M was assayed with *p*-nitroanilide substrates containing Leu, Nle, or Met at P1, activity was observed with all three substrates, but a clear preference for P1-Leu was apparent once again (Fig. 2*B*). In relative terms, the enzyme displayed 100% activity with Suc-AAPL-*p*NA, 52% activity with Suc-AAPn-*p*NA, and 37% activity with Suc-AAPM-*p*NA.

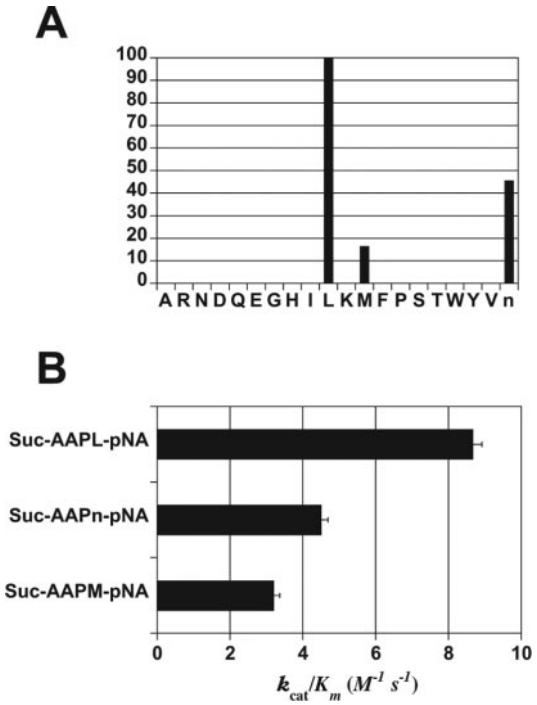

FIG. 2. **P1 substrate specificity of human granzyme M.** *A*, profile with the P1-diverse library, where the *y* axis represents activity relative to the P1 leucine well, and the *x* axis represents the positioned P1 amino acid (with norleucine represented by *n*). Data represents the average from three separate experiments. *B*, second-order rate constants for the hydrolysis of *p*-nitroanilide substrates with leucine, norleucine, or methionine at P1 by human granzyme M. Data represent the average, and *error bars* represent the S.D. from two separate experiments.

Extended Substrate Specificity—The P4-P2 substrate specificity of human granzyme M was profiled using two different positional scanning synthetic combinatorial libraries of tetrapeptide coumarin substrates referred to as the P1-Leu library and the P1-Met library (12). The P1-Leu library is composed of three sub-libraries of general structures Ac-*XX*P_2L-ACC, Ac-*X*P_3*X*L-ACC, and Ac-P_4*XX*L-ACC. Each of these is composed of 19 wells in which the P2, P3, or P4 position is sequentially fixed as 1 of 19 different amino acids (excluding cysteine and methionine and including the methionine isostere norleucine), whereas the remaining positions are randomized, resulting in 361 substrates/well. The P1-Met library is set up in an analogous manner, with the only difference being that, in contrast to the P1-Leu library, methionine is also present at randomized and fixed positions, resulting in 400 substrates/

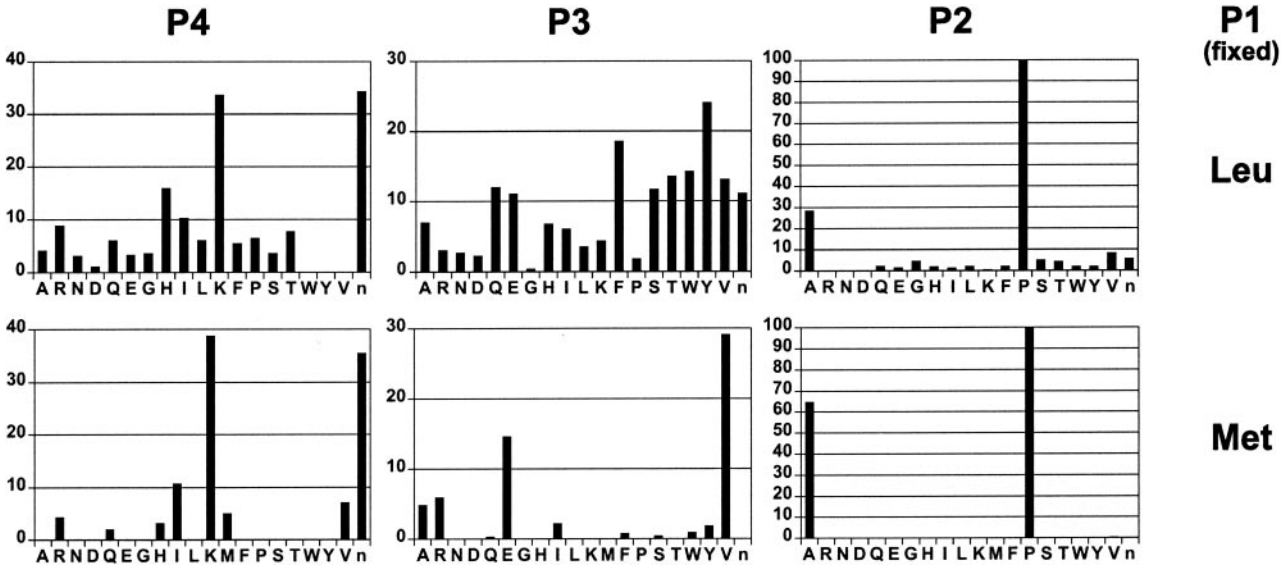

FIG. 3. **P4-P2 substrate specificity of human granzyme M.** Profiles with the P1-Leu and P1-Met libraries, where the *y* axis represents activity relative to the P2 proline well, and the *x* axis represents the positioned P4, P3, or P2 amino acid (with norleucine represented by *n*). All rates can be normalized to the single well exhibiting highest activity in each library because a single experiment simultaneously collects data on all three subsites. Data represent the average from two separate experiments.

well. Profiling of human granzyme M using the P1-Leu library revealed narrow specificity at P2, with a preference for proline over alanine, broad specificity at P3 with a preference for the aromatic residues phenylalanine and tyrosine, and intermediate specificity at P4 with a preference for lysine and the methionine isostere norleucine (Fig. 3). To validate these results and explore whether the specificity at P4-P2 is dependent on the identity of the P1 residue, human granzyme M was also profiled using the P1-Met library. Results at P2 and P4 with the P1-Met library were generally consistent with P1-Leu library results. Notably, specificity at P3 was different, with a preference for valine and glutamate instead of aromatic residues (Fig. 3).

Analysis of Substrate Specificity with Single Substrates—Because the positional scanning libraries described above are composed of pools of substrates, activities reflect the average preferred residues at a particular site. Quantitative kinetic analysis to address issues such as cooperativity can only be carried out with single, purified substrates. To validate the results obtained using the positional scanning libraries and to analyze the extended substrate specificity of human granzyme M in more detail, a series of single ACC substrates was prepared and assayed (Table I). Out of all tested permutations of residues found to be preferred based on the P1-Leu and P1-Met library profiles, the enzyme exhibited the highest preference for Ac-KVPL-ACC, with a k_{cat}/K_m of 1,900 M^{-1} s^{-1}. In contrast, the enzyme showed no detectable activity with Ac-GRLL-ACC, a substrate with sub-optimal P4-P2 residues, indicating the importance of primary sequence recognition for human granzyme M. Shortening the optimal tetrapeptide substrate Ac-KVPL-ACC to Ac-VPL-ACC resulted in a dramatic 560-fold drop in activity to a k_{cat}/K_m of 3.4 $M^{-1}s^{-1}$, indicating the importance of extended substrate interactions for human granzyme M. As observed previously for other serine proteases, the decrease in activity with the shorter substrate can be attributed to a loss in stabilization during the acylation transition state because k_{cat} decreases by 159-fold whereas K_m increases by only 3.5-fold. In addition to being a potent granzyme M substrate, Ac-KVPL-ACC was also very selective for human granzyme M in the context of other serine proteases known to accept leucine at P1 (Fig. 4).

Human granzyme M displayed a preference for leucine in the P1 position of the substrate pairs Ac-KVPL-ACC and Ac-KVPM-ACC, Ac-KYPL-ACC and Ac-KYPM-ACC, and Ac-KEPL-ACC and Ac-KEPM-ACC, with $(k_{cat}/K_m)_{P1\text{-}Leu}/(k_{cat}/K_m)_{P1\text{-}Met}$ ratios of 3.3, 13.3, and 5.7, respectively (Table I). Even though the relative activities observed with the P1-diverse library are averages of activities from mixtures of many other substrates, the average of the three ratios listed above is

TABLE I
Steady-state kinetic parameters for the hydrolysis of single ACC substrates by human granzyme M

Values are expressed as the mean ± S.D. from four or more separate experiments.

Substrate	k_{cat}/K_m	k_{cat}	K_m
	M^{-1} s^{-1}	s^{-1}	μM
Ac-KVPL-ACC	1,900 ± 150	0.70 ± 0.03	370 ± 24
Ac-VPL-ACC	3.4 ± 0.4	0.0044 ± 0.0008	1,300 ± 117
Ac-GRLL-ACC	ND[a]		
Ac-KEPL-ACC	990 ± 96	0.79 ± 0.08	800 ± 125
Ac-KYPL-ACC	760 ± 85	0.77 ± 0.04	1,000 ± 68
Ac-KYAL-ACC	160 ± 20	0.06 ± 0.01	400 ± 126
Ac-RYPL-ACC	147 ± 9	0.22 ± 0.01	1,500 ± 156
Ac-MEPL-ACC	80 ± 3	0.142 ± 0.008	1,800 ± 159
Ac-KVPM-ACC	572 ± 6	0.74 ± 0.04	1,300 ± 69
Ac-KEPM-ACC	175 ± 1	0.42 ± 0.04	2,400 ± 211
Ac-KVAM-ACC	106 ± 7	0.077 ± 0.005	720 ± 63
Ac-KYPM-ACC	57 ± 8	0.115 ± 0.008	2,000 ± 366

[a] Not measurable with up to 2 μM enzyme and 2 mM substrate.

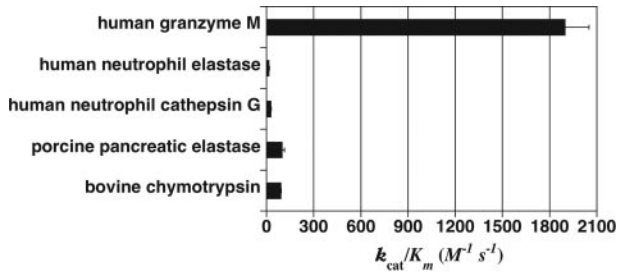

FIG. 4. **Selectivity of the optimal human granzyme M synthetic substrate Ac-KVPL-ACC.** Second-order rate constants for the hydrolysis of Ac-KVPL-ACC by human granzyme M, human neutrophil elastase, human neutrophil cathepsin G, porcine pancreatic elastase, and bovine chymotrypsin. Data represent the average, and *error bars* represent the S.D. from two or more separate experiments.

7.4, which compares favorably with the ratio of 6.3 for P1 leucine to P1 methionine relative activity in the P1-diverse library (Fig. 2*A*). Although the P1-Leu library profile for human granzyme M indicated a preference for aromatic residues at P3, the most preferred substrate among Ac-KVPL-ACC, Ac-KYPL-ACC, and Ac-KEPL-ACC was Ac-KVPL-ACC (Table I), indicating valine is really the optimal P3 residue for P1 leucine substrates. In agreement with the P1-Met library profile, Ac-KVPM-ACC was the most preferred P1 methionine substrate among Ac-KVPM-ACC, Ac-KYPM-ACC, and Ac-KEPM-ACC (Table I), indicating valine is also the optimal P3 residue for P1 methionine substrates.

In agreement with library results, human granzyme M displayed the highest activity with the P2 proline substrates in the pairs Ac-KYPL-ACC and Ac-KYAL-ACC, and Ac-KVPM-ACC and Ac-KVAM-ACC, with $(k_{cat}/K_m)_{P2\text{-}Pro}/(k_{cat}/K_m)_{P2\text{-}Ala}$ ratios of 4.7 and 5.4, respectively (Table I). The corresponding $(k_{cat})_{P2\text{-}Pro}/(k_{cat})_{P2\text{-}Ala}$ ratios for the two pairs of substrates are 12.8 and 9.6, respectively, whereas the $(K_m)_{P2\text{-}Pro}/(K_m)_{P2\text{-}Ala}$ ratios are 2.5 and 1.8, respectively. Thus, although the P2 alanine substrates associate with the enzyme more efficiently to form the ground state Michaelis complex, the P2 proline substrates are turned over far more efficiently, indicating that preference for P2 proline is manifested in the acylation transition state. Binding of the conformationally flexible P2 alanine substrates is more favorable than that of the conformationally constrained P2 proline substrates even though the former are expected to incur a higher loss in entropy upon binding than the latter. A potential explanation is that P2 alanine substrates can access many modes of binding in the ground state, whereas P2 proline substrates are more restricted to the transition state conformation.

The discrepancy in P3 specificity between the P1-Leu library and P1 leucine single substrates may be due to small biases in the library or cooperativity during catalysis. The $(k_{cat}/K_m)_{P1\text{-}Leu}/(k_{cat}/K_m)_{P1\text{-}Met}$ ratios for the three substrate pairs Ac-KVPL-ACC and Ac-KVPM-ACC, Ac-KYPL-ACC and Ac-KYPM-ACC, and Ac-KEPL-ACC and Ac-KEPM-ACC vary significantly from one another, indicating that human granzyme M exhibits some cooperativity between the P3 and P1 sites. These ratios reveal that when P1 changes from methionine to leucine, activity increases approximately twice as much if P3 is glutamate instead of valine and four times as much if P3 is tyrosine instead of valine. Thus, in addition to small biases in the P1-Leu library that may become more apparent in instances of broader specificity such as that observed at P3, discrepancy between the P1-Leu library and single substrates is probably attributable to nonadditivity in protease-substrate interactions.

Library profiles indicated a preference for lysine and the methionine isostere norleucine at P4. In agreement with library results, the $(k_{cat}/K_m)_{P4\text{-}Lys}/(k_{cat}/K_m)_{P4\text{-}Arg}$ ratio of 5.1 for the substrate pair Ac-KYPL-ACC and Ac-RYPL-ACC (Table I) indicates human granzyme M does not accept the two basic residues interchangeably at P4. Because norleucine is isosteric with methionine, it was interesting to find that human granzyme M displayed approximately equal activities with lysine and norleucine but no activity with methionine at P4 in the P1-Met library. To investigate whether the lack of P4 methionine activity in the P1-Met library was due to oxidation of the methionine side chain, the substrate Ac-MEPL-ACC was prepared and judged to be unoxidized by HPLC purification and mass spectrometric analysis. The $(k_{cat}/K_m)_{P4\text{-}Lys}/(k_{cat}/K_m)_{P4\text{-}Met}$ ratio of 5.7 for the substrate pair Ac-KEPL-ACC and Ac-MEPL-ACC (Table I) indicates methionine is in fact not one of the most preferred residues at P4 and that P1-Met library results were not biased because of methionine oxidation.

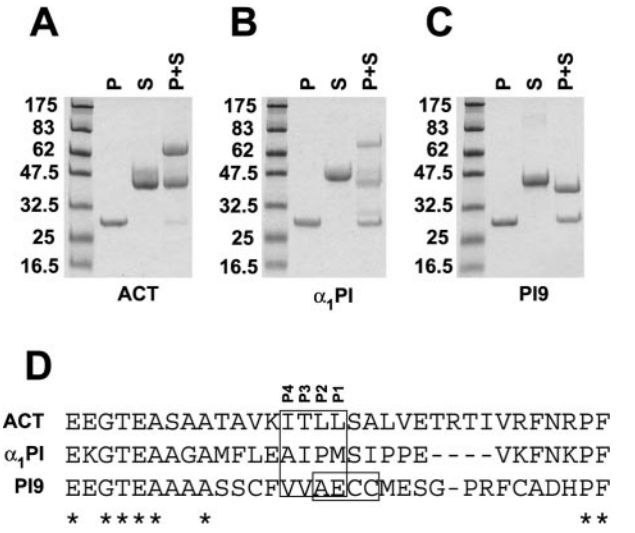

FIG. 5. **Analysis of the interaction between human granzyme M and serpins.** Coomassie Brilliant Blue-stained gels show protease (*lanes P*) and serpins (*lanes S*) incubated separately and together (*lanes P + S*). All samples were deglycosylated prior to SDS-PAGE analysis. *A*, reaction with ACT. Molecular weight markers (*lane 1*), human granzyme M (*lane 2*), ACT (*lane 3*), and human granzyme M with ACT (*lane 4*). *B*, reaction with α_1PI. Molecular weight markers (*lane 1*), human granzyme M (*lane 2*), α_1PI (*lane 3*), and human granzyme M with α_1PI (*lane 4*). *C*, reaction with PI9. Molecular weight markers (*lane 1*), human granzyme M (*lane 2*), PI9 (*lane 3*), and human granzyme M with PI9 (*lane 4*). *D*, alignment of serpin-reactive center loop sequences. Alignment of ACT residues 342–370, α_1PI residues 342–374, and PI9 residues 324–355. Identical residues are designated by *asterisks*. The *larger box* designates the P4 to P1 amino acids of each serpin usually recognized by target proteases, whereas the *smaller box* designates the alternate P4 to P1 amino acids of PI9 recognized by human neutrophil elastase.

Interaction of Human Granzyme M with Serpins—The three serpins ACT, α_1PI, and PI9 were chosen by using a candidate-based approach to be tested as macromolecular inhibitors of human granzyme M because they are known to interact with proteases that, like granzyme M, display hydrophobic specificity. These include neutrophil elastase and proteinase 3, which are also genetically related to granzyme M (13, 14). To determine whether any of these serpins are inhibitors of human granzyme M, each was incubated with the protease at a 2:1 serpin-to-protease molar ratio for 24 h. Samples were then deglycosylated with PNGase F and analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining. Incubation of the enzyme with ACT resulted in a higher molecular weight band corresponding to an SDS-stable ACT-protease complex (Fig. 5*A*). Incubation with α_1PI resulted not only in a higher molecular weight serpin-protease complex, but also in another new band of slightly lower molecular weight than intact α_1-PI corresponding to cleaved serpin (Fig. 5*B*). Incubation with PI9 resulted in complete cleavage of the serpin (Fig. 5*C*). The small decrease in molecular weights compared with intact α_1PI and PI9 is suggestive of cleavage at the reactive center loop.

Human granzyme M was then titrated with the three serpins to determine their stoichiometries of inhibition (SI) (Table II). Very long incubation times were used to allow for complete complexation. ACT inhibited the enzyme with an SI of 1.2, indicating that practically all ACT enters the inhibitory pathway to form stable complexes with human granzyme M (23). α_1PI inhibited the enzyme with the slightly higher SI of 1.6, indicating that approximately a third of all α_1PI reacting with human granzyme M enters the noninhibitory pathway in which the enzyme completes the deacylation step with subsequent release and inactivation of the serpin (23). PI9 inhibited the

TABLE II
Kinetic constants for the interaction of human granzyme M with serpins

k_a values are expressed as the mean ± S.D. from two separate experiments, and SI values are representative values from single experiments.

Serpin	k_a	SI	$k_a \times$ SI
	$M^{-1}\ s^{-1}$		$M^{-1}\ s^{-1}$
ACT	450 ± 18	1.2	540
α_1PI	170 ± 36	1.6	270
PI9	930 ± 71	63	59,000

enzyme with the high SI of 63, indicating that it is a much better substrate than inhibitor of human granzyme M. The stoichiometries of inhibition determined for each of the three serpins are thus in agreement with analysis of reactions between serpins and human granzyme M by SDS-PAGE, demonstrating that the protease forms a stoichiometric and inhibited complex with ACT, is mostly inhibited by α_1PI but also partially cleaves it, and cleaves the vast majority of PI9.

As expected from the long incubation times used in the previous experiments, the second order association rate constant k_a for the formation of the serpin-granzyme M complexes was reduced when compared with other known serpin-protease pairs such as α_1PI-neutrophil elastase (Table II). However, the rate of acylation $k_a \times$ SI of human granzyme M by PI9 was 59,000 $M^{-1}\ s^{-1}$. As an upper bound approximation for k_{cat}/K_m, this rate indicates PI9 may be a relevant macromolecular substrate of human granzyme M. For comparison, granzyme B cleaves the apoptotic substrate poly(ADP-ribose) polymerase with a k_{cat}/K_m of 23,000 $M^{-1}\ s^{-1}$ (24). Although the cleavage sites created by granzyme M in ACT, α_1PI, and PI9 were not identified by sequencing, likely P1 residues can be predicted by alignment (Fig. 5*D*). The observation that the $k_a \times$ SI for acylation of human granzyme M by ACT is faster than the rate of acylation by α_1PI (Table II) is in agreement with the preference displayed by the enzyme for leucine over methionine at P1 because the serpin-reactive center P1 residue is leucine in ACT and methionine in α_1PI. On the other hand, the P2 proline in α_1PI is preferred more than the P2 leucine in ACT, which may partly offset the effect of the P1 residues. In contrast to ACT and α_1PI, no correlation can be made between the reactive center loop of PI9 and the substrate specificity of human granzyme M. Like other ovalbumin-related serpins such as PI6, PI8, and MNEI, PI9 has two reactive sites (14, 25). Granzyme B, which is known to cleave substrates following aspartate residues, cleaves PI9 following glutamate 340, whereas human neutrophil elastase, which is known to cleave substrates following alanine, valine, and isoleucine residues, cleaves PI9 following cysteine 342. Because the substrate specificity of granzyme M is more similar to that of neutrophil elastase, it is possible that it reacts with PI9 at this same alternate site.

To demonstrate that cleavage of PI9 by human granzyme M leads to inactivation of the serpin, human granzyme B was incubated for 1 h with intact PI9 or PI9 that had been preincubated for 1 h with human granzyme M. Western blot analysis indicated that the major species after incubation of human granzyme B with PI9 was the higher molecular weight serpin-protease complex, whereas cleaved PI9 was the major species after incubation with PI9 that had been preincubated with human granzyme M (Fig. 6*A*). Cleavage of PI9 by human granzyme M thus abolished its ability to form a complex with human granzyme B. A direct competition between human granzyme B inactivation by PI9 and human granzyme M inactivation of PI9 revealed that the former occurs more rapidly. Granzyme M only rescues granzyme B activity in the presence of PI9 if the serpin is preincubated with granzyme M prior to the addition of granzyme B (Fig. 6*B*). This is consistent with the fact that the reported k_a for the inhibition of human granzyme B by PI9 is $1.7 \times 10^6\ M^{-1}\ s^{-1}$ (25), 30-fold faster than the $k_a \times$ SI for acylation of human granzyme M by PI9 (Table II).

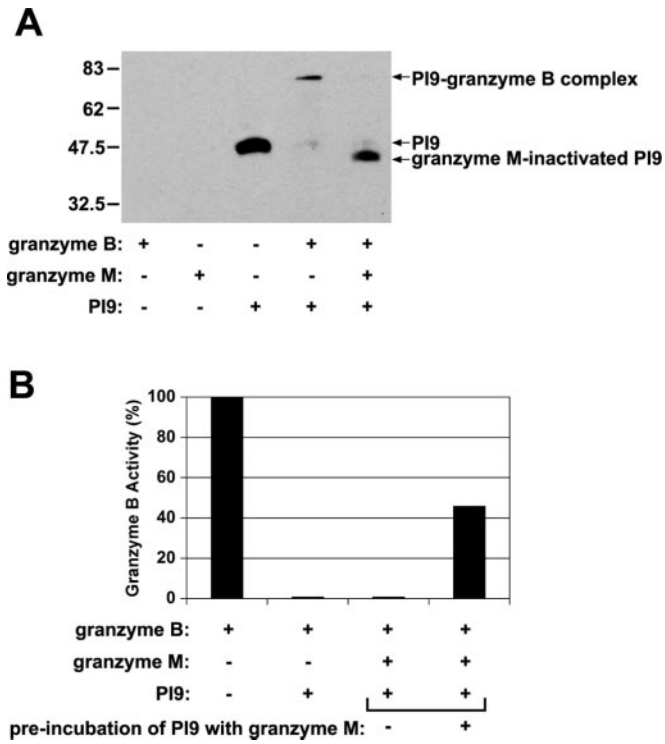

FIG. 6. **Inactivation of PI9 by human granzyme M.** *A*, immunoblot analysis using anti-human PI9 polyclonal antibody. 3 ng of human granzyme B (*lane 1*), 3 ng of human granzyme M (*lane 2*), 10 ng of PI9 (*lane 3*), 3 ng of human granzyme B incubated for 1 h with 10 ng of PI9 (*lane 4*), and 3 ng of human granzyme B incubated for 1 h with 10 ng of PI9 that was preincubated with human granzyme M for 1 h (*lane 5*). *B*, inhibition of granzyme B by PI9 is faster than inactivation of PI9 by granzyme M. Relative activity of 10 nM human granzyme B measured after a 30-min incubation with 2 μM PI9, 2 μM PI9 and 10 nM human granzyme M, or 2 μM PI9 and 10 nM human granzyme M that had been preincubated for 30 min before addition of human granzyme B.

DISCUSSION

The ability of cytotoxic lymphocytes to kill pathogen-infected host cells is largely dependent on perforin and members of the granzyme family of serine proteases. In contrast to better characterized family members such as granzymes A and B, the mode of action of the NK cell-specific granzyme M in cytotoxicity has not yet been determined. Because the biological function of a protease is largely determined by the events following cleavage of its macromolecular substrates, identification of proteins cleaved by an orphan protease such as granzyme M is an important step for elucidation of its function. Examples of other regulatory trypsin-fold serine proteases such as thrombin and plasmin indicate that preferred small molecule substrate specificities often correlate with sites of cleavage in protein substrates (12). Study of protease substrate specificity thus provides valuable information for identification of physiological substrates and their cleavage sites. Additionally, such studies guide the design of synthetic substrates and inhibitors. For these reasons, the extended substrate specificity of human granzyme M was characterized using recombinantly expressed protease that was purified to homogeneity and thus free from contaminating proteolytic activity. The availability of an expression system such as *P. pastoris* that is capable of producing reagent quantities of the enzyme also greatly facilitates future structure and function studies. The substrate specificity of the

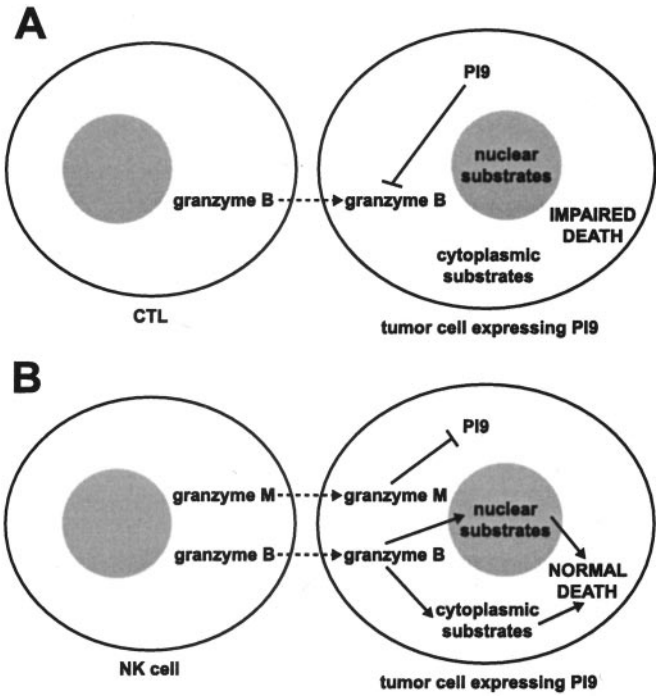

FIG. 7. **A model for granzyme M inactivation of PI9 *in vivo*.** *A*, cytotoxic lymphocyte-mediated death of tumor cells can be blocked by PI9. *B*, NK cells may use granzyme M to overcome PI9 inhibition of granzyme B in PI9-expressing tumor cells.

enzyme was determined to be lysine = norleucine at P4, broad at P3 but preference for valine, proline > alanine at P2, and leucine > norleucine > methionine at P1.

The primary substrate specificity of granzyme M has been characterized previously by using purified native rat enzyme from RNK-16 cells (21) and supernatant from COS cells transiently transfected with human and mouse granzyme M (1, 22). Granzyme M from all three species was characterized as preferentially cleaving peptide bonds following methionine residues but also following norleucine and leucine residues. The results provided here clearly demonstrate that human granzyme M has a far stronger preference for leucine over methionine at P1. The explanation for this discrepancy lies in the fact that previous studies were carried out using thiobenzyl ester substrates for characterization of specificity, which are inherently much less specific than amides because of a more labile scissile bond (26). The results obtained for recombinant human granzyme M with coumarin and *p*-nitroanilide substrates thus highlight the importance of using amide substrates instead of ester substrates in protease specificity studies.

Characterization of the extended substrate specificity of human granzyme M demonstrated the activity of the enzyme to be highly dependent on the sequence and length of synthetic substrates. Furthermore, the kinetic parameters obtained for human granzyme M with the optimal substrate Ac-KVPL-ACC were comparable with those obtained for other regulatory proteases with their own specific substrates (27). These observations suggest human granzyme M is likely to play a regulatory and not digestive role as the number of proteins cleaved by it will be restricted by its specificity. For example, as judged by a Congo Red-conjugated elastin cleavage assay, human granzyme M was unable to cleave elastin,[3] which is an important substrate of the related proteases human neutrophil elastase and proteinase 3 (28). A limited set of macromolecular substrates for human granzyme M is also consistent with what is known about human complement factor D, its closest relative with 40% identity at the amino acid level. The only known natural substrate for complement factor D is complement factor B (29).

The function of a regulatory protease is not only determined by its subset of protein substrates but also by the location and nature of its interaction with endogenous inhibitors. The serpins are a family of structurally conserved proteins, most of which maintain homeostasis by regulating the activity of a variety of trypsin-fold serine proteases. ACT is the physiological inhibitor of neutrophil cathepsin G and is also known to inhibit mast cell chymase. α_1PI is the physiological inhibitor of neutrophil elastase and is also known to inhibit neutrophil proteinase 3 (13, 23). Although the intracellular serpin PI9 is generally thought of as the physiological inhibitor of granzyme B, this serpin was also found to be a potent inhibitor of neutrophil elastase (14). Human granzyme M is related to some of these proteases by sequence and related to all by virtue of also being a leukocyte protease that displays hydrophobic primary specificity. The interaction of human granzyme M with the serpins ACT, α_1PI, and PI9 was thus characterized in an attempt to identify potential physiological inhibitors. The second order association rate constants for the enzyme with all three serpins were low in comparison to those of known serpin-protease pairs (25). On the other hand, PI9 was found to be a much better substrate than inhibitor of human granzyme M, with cleavage of the serpin resulting in loss of its ability to inhibit human granzyme B. It has been postulated that some tumors evade cytotoxic T lymphocyte-mediated killing by blocking the activity of granzyme B through overexpression of PI9 (30). The ability of human granzyme M to clear the way for granzyme B-mediated target cell damage by inactivation of PI9 may thus be a means by which natural killer cells overcome this escape mechanism (Fig. 7).

The serpin-reactive center loop sequence, particularly the identity of the P1 residue, has long been recognized as a major determinant of protease recognition (13). On the other hand, although serpins inhibit proteases by a suicide substrate mechanism, secondary interactions between serpin and protease can sometimes take precedence over interactions at the reactive center loop. Merely swapping the sequence of this loop in ACT with that of a number of different serpins is not sufficient to transfer new specificity to ACT (31). Additionally, there are several cases where a serpin-reactive center loop does not correlate well with the substrate specificity of its target protease. For example, although granzyme B exclusively processes substrates following aspartic acid (32), it reacts efficiently with its endogenous inhibitor PI9 by using glutamic acid 340 as the P1 residue (33). Inefficient inhibition of granzyme M by ACT, even though this serpin has a P1 leucine residue, is thus not out of the ordinary. Cleavage of PI9 by granzyme M following a non-leucine or methionine residue could also be explained by secondary interactions taking precedence over reactive center loop interactions. Alternatively, granzyme M may also have a preference for cysteine at P1 and may cleave PI9 following a cysteine as is the case with neutrophil elastase (14). This redox-active residue was not included in any substrate libraries or single substrates used in the specificity studies for synthetic reasons. The explanation for the high stoichiometry of inhibition that makes PI9 a good granzyme M substrate is unclear. It is likely that the cleaved PI9 reactive center loop still inserts into the serpin β-sheet, but as granzyme M is not kinetically trapped, it is possible that its active site is not well distorted after structural rearrangement of the covalent complex (23).

In summary, the P4 to P1 substrate specificity of recombi-

[3] S. Mahrus, W. Kisiel, and C. S. Craik, unpublished results.

nant human granzyme M was characterized by using several combinatorial libraries of substrates and single substrates. The finding that this orphan member of the granzyme family has a strong dependence on defined and extended interaction with substrates suggests it has a regulatory function. Most significantly and in contrast to previous studies, human granzyme M was found to cleave substrates directly following leucine residues far more efficiently than following methionine residues. A candidate-based approach for identification of a physiological inhibitor resulted instead in identification of PI9 as a macromolecular substrate, albeit an unconventional one because there seems to be no correlation between human granzyme M substrate specificity and a potential cleavage site in this serpin. Investigation of the physiological relevance of PI9 as a human granzyme M substrate and discovery of other protein substrates containing the consensus cleavage site motif defined herein will further define the function of this orphan granzyme.

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REFERENCES

1. Smyth, M. J., O'Connor, M. D., Trapani, J. A., Kershaw, M. H., and Brinkworth, R. I. (1996) *J. Immunol.* **156,** 4174–4181
2. Russell, J. H., and Ley, T. J. (2002) *Annu. Rev. Immunol.* **20,** 323–370
3. Tak, P. P., Spaeny-Dekking, L., Kraan, M. C., Breedveld, F. C., Froelich, C. J., and Hack, C. E. (1999) *Clin. Exp. Immunol.* **116,** 366–370
4. Beresford, P. J., Kam, C. M., Powers, J. C., and Lieberman, J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 9285–9290
5. Wilharm, E., Parry, M. A., Friebel, R., Tschesche, H., Matschiner, G., Sommerhoff, C. P., and Jenne, D. E. (1999) *J. Biol. Chem.* **274,** 27331–27337
6. Poe, M., Blake, J. T., Boulton, D. A., Gammon, M., Sigal, N. H., Wu, J. K., and Zweerink, H. J. (1991) *J. Biol. Chem.* **266,** 98–103
7. Edwards, K. M., Kam, C. M., Powers, J. C., and Trapani, J. A. (1999) *J. Biol. Chem.* **274,** 30468–30473
8. Sayers, T. J., Brooks, A. D., Ward, J. M., Hoshino, T., Bere, W. E., Wiegand, G. W., Kelly, J. M., Smyth, M. J., and Kelley, J. M. (2001) *J. Immunol.* **166,** 765–771
9. Shi, L., Kam, C. M., Powers, J. C., Aebersold, R., and Greenberg, A. H. (1992) *J. Exp. Med.* **176,** 1521–1529
10. Woodard, S. L., Jackson, D. S., Abuelyaman, A. S., Powers, J. C., Winkler, U., and Hudig, D. (1994) *J. Immunol.* **153,** 5016–5025
11. Kelly, J. M., Waterhouse, N. J., Cretney, E., Browne, K. A., Ellis, S., Trapani, J. A., and Smyth, M. J. (2004) *J. Biol. Chem.* **279,** 22236–22242
12. Harris, J. L., Backes, B. J., Leonetti, F., Mahrus, S., Ellman, J. A., and Craik, C. S. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 7754–7759
13. Potempa, J., Korzus, E., and Travis, J. (1994) *J. Biol. Chem.* **269,** 15957–15960
14. Dahlen, J. R., Foster, D. C., and Kisiel, W. (1999) *Biochim. Biophys. Acta* **1451,** 233–241
15. Bell, J. K., Goetz, D. H., Mahrus, S., Harris, J. L., Fletterick, R. J., and Craik, C. S. (2003) *Nat. Struct. Biol.* **10,** 527–534
16. Sprecher, C. A., Morgenstern, K. A., Mathewes, S., Dahlen, J. R., Schrader, S. K., Foster, D. C., and Kisiel, W. (1995) *J. Biol. Chem.* **270,** 29854–29861
17. Annand, R. R., Dahlen, J. R., Sprecher, C. A., De Dreu, P., Foster, D. C., Mankovich, J. A., Talanian, R. V., Kisiel, W., and Giegel, D. A. (1999) *Biochem. J.* **342,** 655–665
18. Dauber, D. S., Ziermann, R., Parkin, N., Maly, D. J., Mahrus, S., Harris, J. L., Ellman, J. A., Petropoulos, C., and Craik, C. S. (2002) *J. Virol.* **76,** 1359–1368
19. Morrison, J. F. (1982) *Trends Biochem. Sci.* **7,** 102–105
20. Smyth, M. J., Sayers, T. J., Wiltrout, T., Powers, J. C., and Trapani, J. A. (1993) *J. Immunol.* **151,** 6195–6205
21. Smyth, M. J., Wiltrout, T., Trapani, J. A., Ottaway, K. S., Sowder, R., Henderson, L. E., Kam, C. M., Powers, J. C., Young, H. A., and Sayers, T. J. (1992) *J. Biol. Chem.* **267,** 24418–24425
22. Kelly, J. M., O'Connor, M. D., Hulett, M. D., Thia, K. Y., and Smyth, M. J. (1996) *Immunogenetics* **44,** 340–350
23. Silverman, G. A., Bird, P. I., Carrell, R. W., Church, F. C., Coughlin, P. B., Gettins, P. G., Irving, J. A., Lomas, D. A., Luke, C. J., Moyer, R. W., Pemberton, P. A., Remold-O'Donnell, E., Salvesen, G. S., Travis, J., and Whisstock, J. C. (2001) *J. Biol. Chem.* **276,** 33293–33296
24. Casciola-Rosen, L., Andrade, F., Ulanet, D., Wong, W. B., and Rosen, A. (1999) *J. Exp. Med.* **190,** 815–826
25. Sun, J., Bird, C. H., Sutton, V., McDonald, L., Coughlin, P. B., De Jong, T. A., Trapani, J. A., and Bird, P. I. (1996) *J. Biol. Chem.* **271,** 27802–27809
26. Hedstrom, L., Szilagyi, L., and Rutter, W. J. (1992) *Science* **255,** 1249–1253
27. Harris, J. L., Peterson, E. P., Hudig, D., Thornberry, N. A., and Craik, C. S. (1998) *J. Biol. Chem.* **273,** 27364–27373
28. Owen, C. A., and Campbell, E. J. (1999) *J. Leukocyte Biol.* **65,** 137–150
29. Volanakis, J. E., and Narayana, S. V. (1996) *Protein Sci.* **5,** 553–564
30. Medema, J. P., de Jong, J., Peltenburg, L. T., Verdegaal, E. M., Gorter, A., Bres, S. A., Franken, K. L., Hahne, M., Albar, J. P., Melief, C. J., and Offringa, R. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 11515–11520
31. Djie, M. Z., Stone, S. R., and Le Bonniec, B. F. (1997) *J. Biol. Chem.* **272,** 16268–16273
32. Thornberry, N. A., Rano, T. A., Peterson, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P., Chapman, K. T., and Nicholson, D. W. (1997) *J. Biol. Chem.* **272,** 17907–17911
33. Sun, J., Whisstock, J. C., Harriott, P., Walker, B., Novak, A., Thompson, P. E., Smith, A. I., and Bird, P. I. (2001) *J. Biol. Chem.* **276,** 15177–15184
34. Schechter, I., and Berger, A. (1968) *Biochem. Biophys. Res. Commun.* **32,** 898–902