The Human Cytotoxic T Cell Granule Serine Protease Granzyme H Has Chymotrypsin-like (Chymase) Activity and Is Taken Up into Cytoplasmic Vesicles Reminiscent of Granzyme B-containing Endosomes*

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Serine proteases (granzymes) contained within the cytoplasmic granules of cytotoxic T cells and natural killer cells play a variety of roles including the induction of target cell apoptosis, breakdown of extracellular matrix proteins and induction of cytokine secretion by bystander leukocytes. Different granzymes display proteolytic specificities that mimic the activities of trypsin or chymotrypsin, or may cleave substrates at acidic (“Asp-ase”) or at long unbranched amino acids such as Met (“Met-ase”). Here, we report that recombinant granzyme H has chymotrypsin-like (chymase) activity, the first report of a human granzyme with this proteolytic specificity. Recombinant 32-kDa granzyme H expressed in the baculovirus vector pBacPAK8 was secreted from SF21 cells and recovered by Ni-affinity chromatography, using a poly-His tag encoded at the predicted carboxyl terminus of full-length granzyme H cDNA. The granzyme H efficiently cleaved Suc-Phe-Leu-Phe-SBzl (v = 185 nM/s at [S] = 0.217 mM) and also hydrolyzed Boc-Ala-Ala-X-SBzl (X = Phe, Tyr, Met, Nle, or Nva) with slower rates but had little trypsinase or Asp-ase activity. Enzymatic activity was inhibited completely by 0.1 mM 3,4-dichloroisocoumarin and 84% by 1.0 mM phenylmethylsulfonyl fluoride. Fluoresceinated granzyme H was internalized in a temperature-dependent manner by Jurkat cells into endosome-like vesicles, suggesting that it can bind to cell surface receptors similar to those that bind granzyme B. This suggests a hitherto unsuspected intracellular function for granzyme H.

Granzymes are serine proteases expressed exclusively by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells, and stored with a pore-forming protein, perforin, in lysosome-like secretory granules (1). Humans express five granzymes: granzyme B (grB) which cleaves after Asp residues (Asp-ase) (2, 3); grA and tryptase-2 which are trypsin-like (cleavage after basic residues) (4); grM which cleaves after Met and other long, unbranched hydrophobic residues and is expressed only in NK cells (5); and grH, whose substrate specificity is unknown (6, 7). Because of its ability to activate pro-apoptotic caspases and mimic the cleavage of their downstream substrates, grB has been strongly implicated in inducing perforin-dependent target cell apoptosis (8, 9), but this function can also be carried out with lesser efficiency by grA and tryptase-2 (10). Putative non-apoptotic functions have been described for grA, including B lymphocyte mitogenesis, thrombin activation, induction of cytokine secretion by monocytes, and cleavage of extracellular matrix proteins (proteoglycans, type IV collagens, lamin, and fibronectin), thus potentially facilitating T and NK cell migration through the subendothelial matrix (4, 11–13).

Mice express at least nine granzymes, several of which including granzymes D, E, F, G, and possibly grC display chymotrypsin-like (chymase) activity. This type of proteolytic activity has been postulated to be important for enhancing the membranolytic properties of perforin (14), although this view has remained controversial. Human CTL/NK cell granules have high levels of chymase activity, but the enzyme responsible for this activity has yet to be identified, and to date, no human granzyme with chymase activity has been found (1). As grH has no direct rodent counterpart and is the only human granzyme whose enzyme activity remains unknown, it has been postulated to be responsible for the chymase activity seen in human granule extracts. GrH is a close structural relative of grB, and shares 71% amino acid identity with it (6). The genes encoding the two molecules map within 30 kilobases on chromosome 14, and form part of the serine protease gene cluster that also contains a number of cathepsin genes expressed in mast cells and myeloid cells (15). Indeed, it has been proposed that interlocus recombination between the ancestral grB and grH genes led to substitution of exon 3, intron 3 and part of exons 4 of grH by grB sequences (16). GrB is expressed by both CTL and NK cells, but it has recently been demonstrated that the 5' noncoding regions of the grH gene can confer NK-specific expression of a reporter gene expressed in mice (17). The high degree of conservation between grB and grH has made detecting and purifying these two granzymes and production of monospecific reagents problematic, highlighting the need for caution when interpreting results obtained with antibody or even nucleic acid probes (18). Thus, it still remains to be determined whether grH is expressed solely in human NK cells or in a broader array of lymphocytes.
to infect SF21 cells according to standard protocols. Final optimized expression involved infection of SF21 cells at a multiplicity of infection of 0.1 to 100 ml of culture medium 3 days prior to harvest. Culture supernatant was cleared of cellular debris by centrifugation and then recombinant protein was purified by Ni-affinity chromatography. The nickel resin (1 ml bed volume per 200-ml culture supernatant) with bound product was washed extensively in buffer containing 20 mM imidazole, and then serial 0.5-ml elutions were performed using an imidazole step gradient commencing at 100 mM. Recombinant product reproducibly eluted at 250 mM imidazole. The grH was then dialyzed against phosphate-buffered saline or TE (150 mM NaCl, 1 mM EDTA) and stored at 4 °C or −20 °C. The yield was calculated spectrophotometrically at 280 nm, using the theoretical extinction coefficient of 1.0, according to Gill and von Hippel (20), and confirmed by SDS-PAGE and silver staining in comparison with known quantities of protein standards.

**Enzyme Assays**—The enzymatic hydrolysis of peptide thioester substrates was measured in 0.1 M HEPES, 0.5 M NaCl, pH 7.5 buffer containing 9% Me₃SO and at 23 °C in the presence of DTNB (3). The initial rates were measured at 405 nm (ε₄05 = 13,260 M⁻¹ cm⁻¹) on a Molecular Devices microplate reader after 10 μl of enzyme stock (grH, 285 μg/ml; mutant, 290 μg/ml) was added to a well containing 0.2 ml buffer, 10 μl of DTNB (5 mM), and 10 μl of substrate (2.5–5.6 mM). The hydrolysis of AMC substrates was monitored by fluorescence change (λₑx = 360 nm, λₑm = 465 nm) at 23 °C using a Tecan Spectrofluor microplate reader.

Percent inhibition was measured by incubating the enzyme with an inhibitor (1 or 10 mM) in a buffer for 10 or 20 min at 23 °C. The substrate, DTNB, and buffer were added to assay the residual enzyme activity using a microplate reader. The substrate Suc-Phe-Leu-Phe-SBzl was purchased from Enzyme Systems Products, Livermore, CA. Other substrates and various inhibitors were synthesized in the Powers Laboratory at the Georgia Institute of Technology, School of Chemistry and Biochemistry.

**Western Blotting**—A polyclonal antiserum raised in rabbits against human grB and found to cross-react with grH was used to detect grH and grB in immunoblots, which were performed from 10 or 12.5% gels using an electroblotter (Bio-Rad) and standard protocols.

**Confocal Microscopy**—GrB was purified from the lysates of YT cells as described previously (9). GrH and grB were labeled with fluorescein isothiocyanate as described previously (9). For each granzyme, conjugation with fluorescein resulted in the loss of less than 20% of proteolytic activity. Jurkat cells were centrifuged onto microscope slides using a cytospin centrifuge and viewed by confocal microscopy as described (9).

**RESULTS**

**Expression of Recombinant grH in Baculovirus-infected Cells**—Only in recent times has it become possible to express and purify granzymes from recombinant sources, thereby facilitating the study of their biochemical and biological functions. Ley and colleagues were the first to report the expression of enzymatically active grB in yeast cells (21), and pro-grA expressed and purified from bacteria then activated with entekinase was used by Lieberman and colleagues to identify potential cell membrane-associated substrates/ligands for this granzyme (22). Having had no success expressing soluble grH

| Table I | Yields of recombinant grH from a typical purification |
|---------|---------------------------------------------------|
| grH     | Protein elution | Protein concentration |
| 250 mM imidazole |
| Inactive | 1 | 327 μg/ml |
|          | 2 | 290 μg/ml |
|          | 3 | 185 μg/ml |
|          | 4 | 99 μg/ml  |
| Yield of purified protein per litre of culture s/n: 3.8 mg |
| Active  | 1 | 206 μg/ml |
|          | 2 | 153 μg/ml |
|          | 3 | 55 μg/ml  |
| Yield of purified protein per litre of culture s/n: 1.7 mg |

**Fig. 1.** A, expression of 32-kDa recombinant grH in SF21 culture supernatants. Western blot analysis of culture supernatants of SF21 cells 3 days after infection with baculovirus constructs expressing Ser-to-Ala mutated (“inactive”) grH or wild type enzyme (“active”), probed with anti-grB polyclonal antiserum. The supernatants from three independent clones derived with each construct are shown for comparison. Immunostaining of native 32-kDa grB (and possibly grH) in the lysate of the human NK leukemia cell line YT is shown for comparison. Culture medium from Sf21 cells expressing an irrelevant baculovirus of the human NK leukemia cell line YT is shown for comparison. Western blot analysis of culture supernatants of Sf21 cells expressing an irrelevant baculovirus of the human NK leukemia cell line YT is shown for comparison. Immunostaining of native 32-kDa grB (and possibly grH) in the lysate of the human NK leukemia cell line YT is shown for comparison.

**MATERIALS AND METHODS**

**Cell Culture**—The human T cell leukemia cell line Jurkat and the NK leukemia cell line YT were cultured in RPMI medium supplemented with 10% bovine serum at 37 °C in air containing 5% carbon dioxide. Insect serum-free adapted Sf21 cells were cultured in Sf-900 II medium (Life Technologies, Inc.) at 27 °C in room air, either in static cell culture or in 1 liter shaker flasks.

**Expression and Purification of Recombinant grH**—The cDNA encoding the full-length mature wild type grH protein was spliced by overlap extension polymerase chain reaction onto a heterologous leader sequence known to support secretion of recombinant product when expressed in serum-free adapted SF21 cells. Two codons encoding the activation dipeptide present at the aminoterminal of all granzymes were deleted to facilitate proteolytic activity of the secreted product (19). A mutated form of the protein was also engineered by introducing a mutation that altered the active site Ser residue to Ala. A hexa-His “tag” was also encoded at the predicted carboxyl terminus of both polypeptides for ease of purification. Both cDNA constructs were inserted into the EcoRI sites of pBacPAK8 vector (CLONTECH) and used...
in bacterial or yeast expression systems, we attempted expression of this granzyme in baculovirus-infected cells. The full-length cDNA encoding grH (6) was expressed following deletion of two codons encoding the amino-terminal activation dipeptide (Glu-Glu) (19), to allow accurate folding of the nascent polypeptide chain following processing of the heterologous leader peptide (see “Materials and Methods”). To assist with assigning the proteolytic specificity of the recombinant protease, a mutated version of the enzyme in which the active site Ser182 was replaced with Ala was expressed in parallel cultures. A hexahis tag was also engineered at the carboxyl terminus of both proteins to permit easy purification and concentration. Following the initial screening of culture supernatants from a panel of recombinant baculovirus-infected Sf21 cells, several clones were identified, which secreted 32-kDa proteins corresponding to Ser-to-Ala mutated or wild type grH, that reacted in immunoblots with a polyclonal anti-grB antiserum (Fig. 1A). This antiserum was previously shown to cross-react with epitopes expressed on fusion proteins of grH with bacterial glutathione S-transferase (grH-GST), but not grA-GST, or grM-GST (data not shown). Expression levels of the mutated grH were consistently approximately 1.5–2.5-fold higher than for wild type enzyme, as estimated by densitometric measurements of the immunoblot signals (see below). The expressed proteins consisted of the 26-kDa polypeptide backbone to which 6 kDa of mannose-rich carbohydrate had been added, as judged by in vitro cleavage with endoglycosidase H (data not shown).

GrH Is a Chymase—Clones expressing the highest amounts of wild type or mutated grH were selected and expanded in culture, and the secreted recombinant granzyme protein was purified using Ni-affinity chromatography. For both the wild type and mutated enzymes, elution with 250 mM imidazole resulted in highly purified protein, as judged by SDS-PAGE and silver staining (Fig. 1B). Optimized yields were in the range of 2–4 mg/liter of harvested supernatant for the mutated grH and typically about one-half this amount for wild type grH. For example, the final yields obtained from the four successive 250 mM imidazole fractions shown in Fig. 1B were 3.8 mg/liter culture supernatant for mutated enzyme and 1.7 mg/liter for wild type enzyme (Table I). Sequencing of the amino termini of both proteins indicated efficient removal of the leader peptides, with >90% of the final product judged to have the predicted mature amino terminus of grH (Ile-Ile-Gly-Gly, data not shown). We next screened the recombinant proteins against a small panel of peptide thiobenzyl ester substrates to ascertain whether proteolytic activity was present in either protein (Fig. 2). The wild type grH demonstrated strong cleavage of Suc-Phe-Leu-Phe-SBzl, less efficient but reproducible cleavage of Boc-Ala-Ala-Met-SBzl, but no tryptase (cleavage of Z-Lys-SBzl) or Asp-ase activities. In contrast, the Ser-to-Ala mutated grH showed no proteolytic

**Table II**

| Substrates | [S] (mM) | rate (nM/s) |
|------------|---------|------------|
| Z-Lys-SBzl | 0.217   | 0          |
| Z-Arg-SBzl | 0.217   | 1.80       |
| Boc-Trp-SBzl | 0.043 | 0          |
| Z-Trp-Arg-SBzl | 0.109 | 0.63       |
| Boc-Ala-Ala-Asp-SBzl | 0.217 | 0          |
| Boc-Ala-Ala-Gly-SBzl | 0.217 | 5.03       |
| Boc-Ala-Ala-Val-SBzl | 0.217 | 0          |
| Boc-Ala-Ala-Nva-SBzl | 0.217 | 28.4       |
| Boc-Ala-Ala-Leu-SBzl | 0.243 | 15.1       |
| Boc-Ala-Ala-Nle-SBzl | 0.243 | 26.4       |
| Boc-Ala-Ala-Ser-SBzl | 0.217 | 7.54       |
| Boc-Ala-Ala-Pro-SBzl | 0.217 | 0          |
| Boc-Ala-Ala-Phe-SBzl | 0.109 | 73.1       |
| Boc-Ala-Ala-Tyr-SBzl | 0.217 | 56.1       |
| Boc-Ala-Ala-Trp-SBzl | 0.043 | 0          |
| Suc-Ala-Ala-Met-SBzl | 0.217 | 73.8       |
| Boc-Ala-Ala-Met-SBzl | 0.217 | 46.9       |
| Boc-Ala-Ala-Arg-SBzl | 0.217 | 3.41       |
| Suc-Phe-Leu-Phe-SBzl | 0.217 | 185        |
| MeO-Suc-Ala-Ala-Pro-Val-SBzl | 0.109 | 121        |
| Suc-Ala-Ala-Pro-Phe-SBzl | 0.217 | 1.26       |
| Suc-Ala-Ala-Pro-Phe-AMC | 0.227 | 19.2       |
| MeO-Suc-Ala-Ala-Pro-Val-AMC | 0.227 | 0          |
| Suc-Ala-Phe-Leu-AMC | 0.227 | 0          |
| Tos-Gly-Pro-Arg-AMC | 0.227 | 0          |
| Suc-Leu-Tyr-AMC | 0.227 | 0          |
activity against any of the substrates. Together, these data strongly suggested that grH has chymotrypsin-like activity and that the proteolytic activity purified from the baculovirus-infected cultures was indeed due to grH.

Kinetic and Inhibitor Studies—To extend these observations, we then undertook a kinetic analysis of grH hydrolytic activity against a more comprehensive panel of synthetic oligopeptide thiobenzyl esters or aminomethylcoumarin (AMC) substrates (Table II). The results indicate that grH is a chymotrypsin-like enzyme which prefers a hydrophobic amino acid residue (e.g. Phe or Tyr) at the P1 site with Suc-Phe-Leu-Phe-SBzl being the best substrate. GrH can also tolerate an aliphatic residue such as Met, Nva, or Nle at P1 with slower rates. However, there is very little activity toward substrates which contain Asp, Arg, or Val at the P1 site. No activity has been detected with the AMC substrates. The mutant grH does not show any activity toward Suc-Phe-Leu-Phe-SBzl and Suc-Ala-Ala-Met-SBzl.

Various synthetic inhibitors such as isocoumarins, peptide chloromethyl ketones, and peptide phosphonates have been previously tested with other granzymes (23, 24), and the same inhibitors were used to test recombinant grH (Table III). The most effective inhibitor was the general serine protease inhibitor DCI which completely inhibited grH at 0.1 mM after 10 min of pre-incubation. PMSF also showed some inhibition, but at a higher inhibitor concentration (1 mM). Peptide phosphonates and peptide chloromethyl ketones which contain the Phe-Leu-Phe sequence were also moderate inhibitors of grH at 0.1 mM concentration. Interestingly, the most effective phosphonate inhibitor was FTC-Aca-Phe-Leu-Phe(P)(OPh)2. This indicates that grH may have a remote subsite for very hydrophobic structures.

**Fluorescinated grH Can Be Internalized into Endosome-like Vesicles in Jurkat Cells**—It has recently been shown that the binding of radioiodinated grB to putative target cell surface receptors is saturable and can be blocked by an excess of unlabeled grB (25). Furthermore, cell surface binding of grB is followed by temperature-dependent uptake into Rab4-positive early endocytic vesicles (26). To determine whether recombinant grH could be internalized in a manner analogous to grB, we exposed healthy Jurkat cells to recombinant, enzymatically active grH which had been labeled with fluorescein (FITC-grH), and examined cellular binding and uptake of grH using confocal microscopy (Fig. 3). After incubation at 4 °C for 10 min, we observed cell surface binding of FITC-grH that was similar in appearance to that seen with FITC-grB. We observed punctate cell surface fluorescence with both enzymes, however the staining seen with FITC-grB was slightly more coarse than that seen with FITC-grH. When the cells were warmed to 37 °C, uptake of both grB and grH was seen in well defined cytoplasmic vesicles highly suggestive of endosomes. Very similar patterns of staining were also observed with FITC-transferrin, which is taken up via clathrin-mediated endocytosis following association with its cognate receptor; however, there was no uptake of FITC-labeled 20-kDa dextran at either tem-

| Inhibitors                      | % Inhibition (10-min incubation) | % Inhibition (20-min incubation) |
|--------------------------------|----------------------------------|----------------------------------|
| DCI                            | 94                               | 100                              |
| PMSF                           | 5                                | 84                               |
| Z-Phe-Leu-Phe(OPh)2            |                                   |                                   |
| 7-(Biotin-Aca-Aca)-NH-4-Cl-3-(OCH2) Ph-IC | NI                               |                                   |
| Z-Gly-Leu-Phe-CH2Cl            | NI                               | NI                               |
| FTC-Aca-Phe-Leu-Phe(OPh)2      | 76                               | 57                               |
| Suc-Phe-Leu-Phe-CH2Cl          | 32                               | 30                               |
| Boc-Phe-Leu-Phe-CH2Cl          | NI                               | NI                               |

*NI, no inhibition.

**Fig. 3.** Uptake of grH into Jurkat cells mimics the endocytic uptake of grB. Confocal images of small clusters of Jurkat cells incubated for 10 min with FITC-grB or FITC-grH at either 4 °C or 37 °C. Cells incubated with FITC-labeled 20-kDa dextran showed no membrane binding or intracellular uptake of fluorescent label (data not shown). The experiment shown is representative of four similar experiments.

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**Chymase Activity of Recombinant Granzyme H**

**TABLE III**

*Inhibition of grH by various inhibitors*

GrH was incubated with inhibitor in pH 7.5 buffer for 10 or 20 min at 23 °C, and then the substrates Suc-Phe-Leu-Phe-SBzl and DTNB were added to assay the residual enzyme activity in the microplate wells.
perature (Ref. 9, and data not shown). Incubation of Jurkat cells with Ser-to-Ala-mutated grH resulted in a reduction, but only incomplete inhibition of uptake (data not shown). As seen previously with grB in the absence of perforin, the uptake of grH into endosomes was not associated with any apparent toxicity of the Jurkat cells, which remained indefinitely viable under the conditions of study (data not shown). Overall, our data suggested that grB and grH are both capable of uptake into target cells by similar mechanisms, but that neither granzyme is capable of inflicting cell death when applied to the surface of cells without other pro-apoptotic mediators such as perforin.

**DISCUSSION**

Although granzymes were first isolated many years ago (27), the inability to express them in large quantities and to assess the effects of site-specific mutations has hindered the study of their complex and varied biological functions. Granzymes are conventionally purified from cultures of cytolytic lymphocytes or NK leukemia cell lines that co-express many proteolytic enzymes with different specificities (2, 3, 5, 18). Given the close physico-chemical similarities between various granzymes (especially grB and grH), it has been difficult to ensure that apparently pure granzyme preparations are not contaminated with minuscule quantities of other proteases. Thus, it has been argued that small amounts of rat RNK-chymase-1 co-purified with perforin or grB might be responsible for amplifying the pro-apoptotic synergy observed with these two molecules, perhaps by modifying the structure of perforin (14). The availability of large quantities of recombinant granzymes should enable such issues to be resolved and, additionally, should provide sufficient purified enzyme to attempt formal structural analysis by crystallographic or other means. Previous studies based on x-ray crystallographic studies have demonstrated that Gly226 of chymotrypsin A is important for conferring its chymase activity, and this observation has been used to design grM mutants that lose their ability to cleave at Met but acquire mase activity, and this observation has been used to design predicted using computer modeling that Arg208 occupies the substrate pocket of chymotrypsin A, and the small size of Gly is insufficient to permit bulky hydrophobic side chains to be accommodated and cleaved. In an analogous manner, it has been predicted using computer modeling that Arg208 occupies the corresponding position in grB (29). This residue has indeed recently been shown to be important for conferring the substrate specificity of grB, in that it is capable of electrostatically attracting the negatively charged acidic side chains of Asp or Glu (29). It is interesting to note that position 208 of the structurally related grH is Gly, in keeping with the observed chymotrypsin-like activity of this granzyme, as described in the present study.

Like many chymases, grH is capable of cleaving polypeptides at a variety of different P1 amino acids. While our study demonstrated a clear preference for Phe or Tyr but not Trp, a number of smaller, unbranched hydrophobic or even polar residues were cleaved with some efficiency. Interestingly, the rat RNK-chymase-1 has specificity for Phe and Trp, but does not cleave efficiently after Tyr residues (14). If grH and RNK-chymase-1 have analogous functions and thus cleave common substrates in their respective species, this variation in cleavage preference may be useful in delineating candidate cleavage sites of substrate proteins. By contrast with these two chymases, grB and grA cleave target proteins almost exclusively at acidic (Asp, Glu) or basic (Lys, Arg) residues, respectively (2, 3). Another possibility consequence of the broad spectrum of cleavage for grH and RNK-chymase-1 is that their physiological substrates might possibly be targeted at a number of different sites, but this prediction awaits verification when cellular substrates of these two granzymes are identified and the cleavage sites defined.

Our studies have also demonstrated for the first time that like grB (9, 30) and grA (31), grH is potentially capable of uptake into the target cell cytoplasm in vesicles highly reminiscent of those containing grB. This finding raises many issues related to the intracellular trafficking and ultimately the functions of granzymes. First, what is the nature of cell surface granzyme receptors, and do specific receptors exist for granzymes of different proteolytic specificity? Second, is there a requirement for proteolytic activity of a granzyme for its cellular uptake and trafficking between intracellular compartments? Third, can different granzymes be taken up into the same endosome? And finally, what are the physiological function of granzymes other than grB inside target cells? We have recently shown that the cytoplasmic granules of CTL/NK cells contain multiple different pathways for achieving target cell apoptosis. Some of these pathways rely on the Asp-ase activity of grB and/or the activation of caspases; however, cell death can still proceed with remarkable efficiency independently of the Asp-ase activity of grB, as indicated both by our own work with purified pro-apoptotic molecules and findings in vivo with grB-deficient mice (32). Intriguingly, apoptosis occurring independently of grB can occur through a non-nuclear pathway, suggesting that cytoplasmic or cell membrane substrates of alternative granzymes might be instrumental in mediating this form of cell death (33, 34). The ability to produce large amounts of granzyme of various specificity by recombinant means should facilitate the study of this and related questions.

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Chymase Activity of Recombinant Granzyme H