Granzyme B (GzmB) is a neutral serine protease found in cytolytic lymphocytes; this enzyme is critically involved in delivering the rapid apoptotic signal to susceptible target cells. GzmB has been difficult to study and has not yet been produced in non-mammalian systems because of the complex processing events that are thought to be required for its activation. In this report, we have successfully produced fully active, soluble recombinant GzmB (rGzmB) in a yeast-based system by fusing GzmB cDNA in frame with yeast α-factor cDNA, using the yeast KEX2 signal peptidase to release the processed enzyme into the supernatant of yeast culture. We expressed the proenzyme form of GzmB as well and determined that pro-GzmB is efficiently converted to its active form by the cysteine proteinase signal peptidase, leaving a prodipeptide that must be removed to produce the active enzyme (20). The enzyme thought to be responsible for the specific cleavage of the prodipeptide is the signal peptidase (21). These leader peptides are thought to be cleaved by a signal peptidase to release the native GzmB. In addition, the recombinant enzyme hydrolyzes Boc-Ala-Ala-AlaMet thiobenzyl ester with a $k_{cat}$ of 7 s$^{-1}$ and catalytic efficiency $k_{cat}/K_{m}$ of 181,237 M$^{-1}$ s$^{-1}$; the recombinant enzyme is therefore at least twice as active as purified native GzmB. In addition, the recombinant enzyme hydrolyzes Boc-Ala-Ala-AlaMet thiobenzyl ester with a $k_{cat}$ of 3.2 s$^{-1}$ and a catalytic efficiency $k_{cat}/K_{m}$ of 65,306 M$^{-1}$ s$^{-1}$. Purified rGzmB can also cleave the putative substrate caspase-3 into its signature p20/p10 forms. Unlike caspases, rGzmB is not sensitive to inhibition by several peptide-based inhibitors, including Ac-DEVD-CHO, Ac-YVAD-CMK, and ZIETD-FMK, as well as Zn$^{2+}$ (a known inhibitor of caspase-3). Structural studies of rGzmB may allow us to better understand the substrate specificity of this enzyme and to design better inhibitors.

GzmB$^1$ belongs to a family of serine proteases specifically expressed in cytolytic T lymphocytes (CTL), natural killer (NK) cells, and lymphokine-activated killer (LAK) cells. The importance of GzmB for inducing target cell death has been demonstrated via several lines of evidence (reviewed in Refs. 1 and 2 and confirmed with GzmB null mutant mice (3). CTL and NK cells derived from these mice have a severe defect in their ability to induce early DNA fragmentation and apoptosis in susceptible target cells. Furthermore, in vivo data suggest that GzmB plays a significant role in the development of acute graft-versus-host disease (4, 5). Therefore, a specific inhibitor of GzmB may potentially prove to be an important tool in the modulation of immune responses.

Among all the known granzymes, human and murine GzmB possess an unusual preference for aspartic acid at the P1 site of its substrates (6). This specificity is shared by only one other group of eukaryotic proteases, the caspases (reviewed in Ref. 7). Experiments with cell-free systems and caspase inhibitors have strongly implicated these enzymes in apoptotic cell death (reviewed in Refs. 8 and 9). Caspases are synthesized as proenzymes that generally require processing at aspartic acid residues for activation. Although the enzymes responsible for the activation of these caspases are unknown, there is evidence that these enzymes can autoactivate or cross-activate other family members (10–13). In vitro studies have also shown that GzmB can cleave and activate caspase-3 (CPP32) (14), caspase-8 (FLICE) (15), and other caspases; in addition, GzbM-deficient CTL fail to cleave caspase-3 in target cells during CTL-induced apoptosis (16). However, these data have not determined whether GzmB induces apoptosis through the activation of caspases or whether it acts directly to induce apoptosis. Several studies have shown that peptide-based, selective caspase inhibitors (which do not inhibit GzmB) can block apoptotic cell death and target cell lysis induced by the Fas-FasL pathway, but not the CTL granule exocytosis (perforin/granzyme-dependent) pathway (17, 18).

GzmB is the prototype of a family of serine proteases (A–K) that are synthesized as preproenzymes with an 18-residue leader peptide (23 residues for granzyme A) (reviewed in Ref. 19). These leader peptides are thought to be cleaved by a signal peptidase, leaving a prodipeptide that must be removed to produce the active enzyme (20). The enzyme thought to be responsible for the specific cleavage of the prodipeptide is the lysosomal cysteine protease DPPI (21–23). Previous studies in COS cells have shown that co-transfection of a full-length (prepro) human GzmB cDNA along with a rat DPPI cDNA resulted in the generation of an enzymatically active GzmB, whereas transfection of full-length GzmB cDNA alone led to the production of a catalytically inactive enzyme (22). In addition, recombinant granzyme A (rGzmA) can be converted into a proteolytically active enzyme by incubation with bovine DPPI (23).

Although native GzmB (nGzmB) has been purified, only limited amounts of relatively pure enzyme have been obtained.
Previously, small amounts of rGzmB have been successfully prepared from a mammalian COS cell-based system (22, 24). However, the inability to purify sufficient amounts of rGzmB (without any other contaminating cellular enzymes) has limited the ability to study substrate specificities and to design specific inhibitors. rGzmA has been successfully expressed in *Escherichia coli* (25) and vaccinia virus (23); however, we have repeatedly failed to generate soluble active rGzmB using a variety of expression systems.

In this study, we describe the production of a yeast-based, soluble, secreted form of active rGzmB. This recombinant protein has the same specificities as nGzmB purified from an NK tumor cell line. We showed that pro-GzmB (which contains the propeptide Gly-Glu) can be converted to a fully active form by bovine DPPI. The active mature rGzmB can cleave caspase-3 into its signature p20/p10 forms. We also determined that several peptide-based caspase inhibitors do not significantly inhibit the Asp'ase activity of rGzmB; furthermore, Zn2+, a potentiator of caspase-3 (26), fails to inhibit rGzmB. These results underscore the fundamental differences in substrate specificities between the caspases and GzmB.

**EXPERIMENTAL PROCEDURES**

**Production of Mature and Pro-GzmB in *Pichia pastoris*—**The cDNAs encoding the pro and mature forms of murine GzmB were amplified by the forward primers 5'--GGGGGAGATCCGCGGGGAGC-3' (pro) and 5'-ATCATCGGGGGAGATGAAGC-3' (mature) and the reverse primer 5'-TTAGGTTTCTATTGTTTTACCGGGATAAG-AAACCTGGAAC-3'. A 5' XhoI site and a 3' EcoRI site were introduced into the cDNAs and the products subcloned into the expression vector pPIC5 (Invitrogen, Carlsbad, CA). Both inserts were completely sequenced and found to be identical (except for the propeptide Gly-Glu). Both vectors were electroporated into GS115 (Invitrogen) at 1.5 kV, 50 μF, and 186 ohms. His+ transformants were selected and induced with methanol for 4 days according to a protocol suggested by the manufacturer (Invitrogen). Daily aliquots of induced supernatants from His+ clones were assayed for Asp'ase activity and probed (using Western blotting) for the presence of GzmB using a polyclonal rabbit anti-mouse GzmB antibody (27). The clones expressing pro-GzmB and mature rGzmB were selected and further expanded. A liter of supernatant from each clone was precipitated using 85% ammonium sulfate, resuspended in 1 M NaCl, and sonicated. The gels were either stained directly with the rapid Coomassie Blue stain or Coomassie Blue-stained gels were destained and immunoblotted. Fifty μl of the reaction products were used to assay for Asp'ase activity with Boc-Ala-Ala-Asp-4-methylcoumarin (Bachem, Torrance, CA), and the *Km* and *Vmax* for each substrate using the Michaelis-Menten equation. Correlation coefficients for all plots were >0.99.

**Endoglycosidase H (Endo H) Digestion—**Endo H treatment of proteins to remove N-linked oligosaccharides was performed in 100-μl reactions using 5 μl (0.5 μg) of pro-GzmB, rGzmB, or nGzmB in an assay buffer containing 75 mM sodium citrate, pH 5.5, and 5 μl of 1 unit/ml Endo H (Sigma). Negative controls were performed using 5 μl of MEB buffer with or without Endo H. The reactions were allowed to incubate overnight at 37 °C. Ten μl of the reaction products was analyzed on SDS-PAGE gels and immunoblotted. Fifty μl of the reaction products were used to assay for Asp'ase activity with Boc-Ala-Ala-Asp-S-benzyl as the substrate.

**CPP32 Activation by DPPI—**5 μl (0.5 μg) of pro-GzmB was incubated with 0.1–0.4 unit of purified bovine spleen DPPI (Sigma) in 50 μl of assay buffer containing 50 mM sodium acetate, pH 5.0, and 5 μl of 1 unit/ml Endo H (Sigma). Negative controls were performed using 5 μl of MEB buffer with or without Endo H. The reactions were allowed to incubate overnight at 37 °C. Ten μl of the reaction products was analyzed on SDS-PAGE gels and immunoblotted. Fifty μl of the reaction products were used to assay for Asp'ase activity with Boc-Ala-Ala-Asp-S-benzyl as the substrate.

**CPP32 in Vitro Translation and Activation—**mCPP32 cDNA was generated by reverse transcription-polymerase chain reaction using RNA derived from GzmB-deficient CTL effectors (3). RNA was prepared as described (30). Approximately 1 μg of RNA was incubated with 200 ng of oligo(dT)12, 50 ng of random hexamers (Perkin-Elmer) for 10 min at 68 °C. After 2 min on ice, 10 μl dNTPs, 100 μM dithiothreitol, 0.5 μl of SuperScript reverse transcriptase (Life Technologies, Inc.) was added and incubated at 37 °C for 1 h. After adjusting the total volume to 100 μl with diethyl pyrocarbonate water, 1 μl of the first strand cDNA was used as template. The 833-base pair cDNA was then amplified using the forward primer 5'-GATCTCTAGAGAAGCCCTAGAAAGTGACCATGG-3' (containing an XbaI site) and the reverse primer 5'-GATCAAGCTTCCTCTAGTGATAAAAGTACAGTTCTTTC-G-3' (containing an HindIII site) and subcloned into XbaI-HindIII cleaved PBSKS (Stratagene, La Jolla, CA).

3S-Labeled mCPP32 was obtained using T7 quick coupled transcription/translation system (Promega, Madison, WI). One μg of the plasmid DNA was transcribed and translated in the presence of 20 μCi of Translabel amino acids (ICN, Costa Mesa, CA, 40 μCi of the T7 Master Mix for 90 min at 30 °C. Two μl of the reaction mixture was either incubated alone, in the presence of equivalent amounts of rGzmB or pro-GzmB (as determined by Western) or in the presence of 25 μg of total protein from an NK-like tumor cell line extract (which contains native GzmB), or from an extract prepared from GzmB−/− CTL effectors. These extracts were generated and prepared as described previously (27). After 2 h at 37 °C, the samples were analyzed on 2. D. MacIvor and T. J. Levy, submitted for publication.
RESULTS AND DISCUSSION

*P. pastoris* is a methanotrophic yeast that has been successfully used to produce large quantities of foreign proteins (reviewed in Refs. 31 and 32). A major advantage of the *P. pastoris* expression system is that the foreign protein can be secreted (along with very low levels of native proteins) as the first step of purification. In addition, the secreted proteins are frequently correctly folded and not hyperglycosylated (33). We designed two GzmB expression vectors for expression in Pichia: the first form included the codons for the activation dipetide Gly-Glu (pro-GzmB); the second form starts with the Ile codon at the N terminus of the processed enzyme (rGzmB) (see Fig 1A). These cDNAs were cloned in frame with the α-factor signal peptide in pPIC9, which targets them to the secretory pathway. The expressed proteins also contain the KEX2 recognition sequence Glu-Lys-Arg directly preceding Gly-Glu (pro-GzmB) or Ile-Ile (rGzmB), which allows signal peptide cleavage by the endogenous yeast peptidase KEX2 (34) (Fig. 1A). The supernatant from clones that expressed cleaved pro-GzmB and rGzmB were purified on a Mono S cation exchange column according to the "Experimental Procedures." The major species detected on silver-stained gels (Fig. 1B) and in Western analysis (Fig. 1C) (using reducing conditions) had apparent molecular masses of 30 and 32 kDa. These two bands represent different glycosylated isoforms of the proteins, since Endo H treatment altered the migration of pro-GzmB and rGzmB (as well as nGzmB purified from NK-like cell lines) to approximately 25 kDa (Fig. 1C), the predicted molecular mass of the mature protein. The deglycosylated forms of rGzmB and nGzmB retained full activity toward thiobenzyl ester substrates (data not shown). The final yield of purified rGzmB from 1 liter of yeast culture was approximately 1 mg.

The activity of each recombinant protein was determined using thiobenzyl ester substrates with the same amount of test enzyme, as demonstrated by silver stain and Western analysis (Fig. 1, B and C). An equivalent amount of partially purified nGzmB served as positive control. Pro-GzmB had no measurable Asp'ase activity (Table I and Fig. 4); in contrast, rGzmB was fully active. To further quantitate the catalytic efficiency of our active recombinant enzyme, we determined Michaelis-Menten parameters for two known synthetic thiobenzyl ester substrates of nGzmB. rGzmB hydrolyzed Boc-Ala-Ala-Asp-S-benzyl with an apparent $k_{cat}$ of 17 s$^{-1}$ and a $K_m$ of 181.237 M$^{-1}$ s$^{-1}$ (Table I). The catalytic efficiency of rGzmB is approximately twice that of nGzmB; these values are similar to those reported in the literature by Odake et al. (6) and Poe et al. (35). Similarly, rGzmA is more active than native GzmA purified from granules (25).

Previous studies could not determine whether the Met'ase activity detected in purified GzmB preparations was due to GzmB itself or to another unidentified granzyme that coeluted with GzmB (6). We definitively established that rGzmB hydrolyzes Boc-Ala-Ala-Met-S-benzyl with a $k_{cat}$ of 3.2 s$^{-1}$ and a $K_m$ of 65,306 M$^{-1}$ s$^{-1}$. Again, the catalytic efficiency of rGzmB as a Met'ase is twice that of nGzmB. The cleavage of Boc-Ala-Ala-Met-S-benzyl suggests that there may be GzmB substrates with Met at the P1 position instead of an Asp. rGzmB has no detectable trypatase or chymase activity (Table I), consistent with previous reports (6).

To our knowledge, caspase-3 is the only caspase that has been shown to be a substrate of GzmB both in vitro as well as in vivo (12, 14). Caspase-3 presumably exists in all cells in its inactive, 32-kDa proenzyme form. During CTL-mediated attack on susceptible target cells, nGzmB processes caspase-3 to its active p20/p10 heterodimeric form (12, 36). For this reason, we wished to demonstrate that rGzmB is capable of recognizing and cleaving this physiological substrate. $^{35}$S-Labeled caspase-3 was cleaved to its signature p20 form by both nGzmB and rGzmB (Fig. 2, lanes 3 and 4), whereas neither pro-GzmB nor extracts from CTL effectors deficient in GzmB

![Figure 1: Production of recombinant GzmB.](http://www.jbc.org/content/1631/35/1631/F1)

**FIG. 1.** Production of recombinant GzmB. A, the sites of cleavage by the signal peptidase (SP) to generate pro-GzmB and by DPpi (to generate GzmB) for the native prepro-GzmB are shown. The plasmids for recombinant pro-GzmB and GzmB contain the yeast α-factor signal sequence followed by the sequence Glu-Lys-Arg, which is recognized by the endogenous yeast enzyme KEX2. B, silver-stained gel showing the two different glycosylated isoforms of recombinant pro-GzmB and rGzmB, compared with purified nGzmB. Five µl of pro-GzmB and rGzmB, versus 10 µl of nGzmB purified from a Mono S column, were loaded per lane. C, Western blot analysis of the same three GzmB forms, probed with rabbit anti-mouse GzmB antibody. D, Western blot analysis of Endo H treatment of pro-GzmB, rGzmB, and nGzmB. Control samples were treated identically except that no Endo H was added.

### Table I

**Kinetic properties of native versus recombinant granzyme B**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Kinetic constants</th>
<th>Enzymes</th>
<th>Odake (6)</th>
<th>Poe (35)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$</td>
<td>$K_m$</td>
<td>$k_{cat}/K_m$</td>
<td></td>
</tr>
<tr>
<td>c-Ala-Ala-Asp-S-Bzl</td>
<td>17 s$^{-1}$</td>
<td>93.8 µM</td>
<td>3.2</td>
<td>11 s$^{-1}$</td>
</tr>
<tr>
<td>c-Ala-Ala-Met-S-Bzl</td>
<td>181,327</td>
<td>113,365</td>
<td>500 µM</td>
<td>230,000</td>
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<tr>
<td>c-Ala-Ala-Phe-S-Bzl</td>
<td>3.2</td>
<td>1.5</td>
<td>49</td>
<td>24</td>
</tr>
<tr>
<td>Lys-S-Bzl</td>
<td>65,306</td>
<td>20,270</td>
<td>74</td>
<td>550</td>
</tr>
</tbody>
</table>

*a* Bzl, benzyl.

*b* Units of measure are as follows: $k_{cat} = s^{-1}$; $K_m = \mu M$; $k_{cat}/K_m = M^{-1} s^{-1}$.

*c* NH, no hydrolysis; ND, not done.
were capable of processing caspase-3 (Fig. 2, lanes 2 and 5). Previous studies have shown that two peptides (Ac-DEVD-CHO (37) and Ac-YVAD-CHO or Ac-YVAD-CMK (38, 39)) are potent inhibitors of caspase-3 and caspase-1, respectively. A recent study, using a combinatorial approach, defined the tetrapeptide IEPD as an optimal substrate sequence for GzmB (40). We therefore tested the ability of Ac-DEVD-CHO, Ac-YVAD-CMK, and ZIETD-FMK (a fluoromethyl ketone that closely matches the optimal substrate for GzmB) to inhibit rGzmB. We found that these peptides have essentially no inhibitory effect on rGzmB (or nGzmB) at concentrations up to 800 \( \mu M \) (Fig. 3). It should be noted that the methyl side chains added to ZIETD-FMK to enhance cell permeability might have affected the ability of this peptide to inhibit GzmB in vitro. Prolonged preincubation of the peptides with rGzmB (up to 4 h) did not increase the inhibition (data not shown). 3,4-Dichloroisocoumarin (DCI), a general serine protease inhibitor (4), inhibits rGzmB with an IC\(_{50}\) value of approximately 5 \( \mu M \) (Fig. 3). We also tested the inhibitory effect of Zn\(^{2+}\) (a potent inhibitor of caspase-3 with an IC\(_{50}\) of 0.1 \( \mu M \) (26)) and found that Zn\(^{2+}\) has no effect on rGzmB at concentrations up to 300 \( \mu M \). Other cations such as Cu\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\), Fe\(^{3+}\), and Ca\(^{2+}\) also had no effect on the activity of rGzmB (data not shown).

The ability of GzmB to cleave caspases, and the similarities between the specificities of GzmB and caspases (especially the requirements for Asp in the P1 position and Glu in the P4 position (40)), have suggested that GzmB is an integral part of the caspase cascade leading to cell death. However, more recent reports using specific caspase inhibitors suggest that GzmB may lead to target cell death through a caspase-independent pathway (16). These reports, and our results with peptide and cation inhibitors, underscore the differences between the two classes of enzymes. Although the sequence IEPD may be an optimal substrate for GzmB in vitro, specific inhibition of GzmB may require additional residues beyond the P4-P1 positions.

GzmB, along with the other cytotoxic lymphocyte granzyme family members, is synthesized as a preproenzyme with an 18 residue signal peptide followed by an activation dipeptide Gly-

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**FIG. 2.** mCPP32 is processed to its signature p20 form by rGzmB in vitro. In vitro translated, \(^{35}S\)-mCPP32 was incubated for 2 h at 37 °C alone (lane 1), with equivalent concentrations of pro-GzmB or mature, rGzmB (lanes 2 and 3, respectively), with 25 \( \mu g \) of NK cell line-derived, total protein containing nGzmB (lane 4), or with 25 \( \mu g \) of total protein extract from GzmB/CTL effectors (lane 5). Samples were run on a 10% SDS-PAGE gel, fixed, dried, and autoradiographed for 2 days.

**FIG. 3.** Effects of peptide-based inhibitors on the activity of rGzmB. 0.5 \( \mu g \) of rGzmB was added to buffer containing the indicated concentrations of inhibitors and incubated for 30 min at room temperature. Reactions were then initiated by adding the substrate Boc-Ala-Ala-Asp-S-benzyl to a final concentration of 100 \( \mu M \) and allowed to proceed for an additional 30 min at room temperature. Asp'ase activity was measured at \( A_{405} \); the activity measured in the absence of inhibitor was set at 100%. M, dichloroisocoumarin; DEVD, IETD; YVAD.

**FIG. 4.** Activation of pro-GzmB by bovine DPPI. A, equivalent amounts of pro-GzmB and rGzmB were incubated with the indicated amounts of bovine DPPI or with buffer alone. Asp'ase activity was measured after overnight incubation at 37 °C. DPPI alone has no Asp'ase activity. B, DPPI was preincubated with 0.6–6 \( \mu M \) G-F-CHN\(_2\) or with buffer alone for 2 h. Pro-GzmB was then added and the reactions allowed to proceed overnight. Asp'ase activity was then measured as described. G-F-CHN\(_2\) alone had no effect on pro-GzmB, rGzmB, or the Asp'ase assay itself.
Glu. Activation requires processing of the signal peptide and the activation dipeptide. This dual proteolytic processing pathway appears to apply to the other granzymes, as well as structurally related serine proteases expressed in other hematopoietic cells such as neutrophil elastase, cathepsin G (21), and mast cell chymases (29). The enzyme involved in the proteolytic processing of the prodipeptide is thought to be the lysosomal cysteine protease DPPI (21–23, 29). Previous reports on the processing of rGzmB by purified bovine DPPI demonstrated specific (but low level) conversion to the active form of GzmB.

Possible explanations include cross-species differences in subproteolytic cleavage by DPPI itself has no effect on the Aspase assay, nor does it affect the activity of mature rGzmB (Fig. 4A). This suggests that once the prodipeptide is removed, mature GzmB is resistant to further proteolytic cleavage by DPPI. The activation process is inhibited by the highly specific DPPI inhibitor Gly-Phe-CHN₂ (29), at concentrations as low as 0.6 μM (Fig. 4B). These results, and previous reports (22–23), indicate that DPPI alone is sufficient for the activation of pro-GzmB. A null mutation of DPPI created by homologous recombination in the mouse should confirm the importance or redundancy of this enzyme for the activation of the granzymes and related proteins.

In summary, this study is the first to demonstrate expression of active rGzmB in a non-mammalian system. The enzyme expressed has the same substrate specificities as the purified native enzyme. Inhibition studies also show that the current peptide-based inhibitors of caspases are ineffective at blocking the activity of GzmB. The Pichia system will allow us to produce large amounts of pure recombinant GzmB for structural studies that may allow us to better define substrate specificities and requirements for GzmB inhibition.

Acknowledgments—We thank Dr. Linda Kurz and Marion Riley (Department of Biochemistry, Washington University) for their expertise and assistance in the determination of rGzmB kinetic constants. We also thank Drs. Tom Daly and Mark Sands for preparation of graphs included in the manuscript and Nancy Reidelberger for preparing the manuscript. We thank Dr. Steve Smeenk for suggesting that we try the Pichia system for the production of granzymes.

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