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*J Immunol* 2013; 190:1319-1330; Prepublished online 26 December 2012;
doi: 10.4049/jimmunol.1202542
http://www.jimmunol.org/content/190/3/1319

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/01/04/jimmunol.1202542.DC1

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Identification of SERPINB1 As a Physiological Inhibitor of Human Granzyme H

Li Wang,*1 Qian Li,*1 Lianfeng Wu,* Shengwu Liu,* Yong Zhang,† Xuan Yang,* Pingping Zhu,* Honglian Zhang,* Kai Zhang,‡ Jizhong Lou,‡ Pingsheng Liu,‡ Liang Tong,‡ Fei Sun,‡ and Zusen Fan*†

The granzyme/perforin pathway is a major mechanism for cytotoxic lymphocytes to eliminate virus-infected and tumor cells. The balance between activation and inhibition of the proteolytic cascade must be tightly controlled to avoid self damage. Granzyme H (GzmH) is constitutively expressed in NK cells and induces target cell death; however, how GzmH activity is regulated remains elusive. We reported earlier the crystal structures of inactive D102N-GzmH alone and in complex with its synthetic substrate and inhibitor, as well as defined the mechanisms of substrate recognition and enzymatic activation. In this study, we identified SERPINB1 as a potent intracellular inhibitor for GzmH. Upon cleavage of the reactive center loop at Phe343, SERPINB1 forms an SDS-stable covalent complex with GzmH. SERPINB1 overexpression suppresses GzmH- or LAK cell–mediated cytotoxicity. We determined the crystal structures of active GzmH and SERPINB1 (LM-DD mutant) in the native conformation to 3.0- and 2.9-Å resolution, respectively. Molecular modeling reveals the possible conformational changes in GzmH for the suicide inhibition. Our findings provide new insights into the inhibitory mechanism of SERPINB1 against human GzmH.

The Journal of Immunology, 2013, 190: 1319–1330.

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atural killer cells and CTLs play important roles in immune surveillance against virus-infected or transformed tumor cells. Granzyme (Gzm) permeabilizes target cells to destroy their target cells (1, 2). Gzms are members of the serine protease family with highly conserved structural architectures but different substrate specificities. Five human Gzms (A, B, H, K, and M) have been identified in cytosolic granules, which induce cell death to eliminate virus-infected or tumor cells by proteolysis of intracellular target substrates (1). Their response activities must be tightly controlled. Lack of Gzm activity lowers immune responsiveness, leading to infection and cancer. Overactivity of Gzm results in cellular damage and tissue destruction. However, how Gzm activity is modulated remains elusive.

Gzm H (GzmH) is constitutively expressed in NK cells, suggesting that it plays a critical role in NK cell–mediated immunity (3–5). We (6) and Fellows et al. (3) demonstrated that GzmH induces caspase-dependent and -independent cell death with DNA degradation. GzmH can directly proteolyze two adenoviral proteins essential for viral replication: the adenovirus DNA-binding protein and the adenovirus 100K assembly protein for virus assembly (7). GzmH is also able to cleave the multifunctional phosphoprotein La to abrogate hepatitis C virus–internal ribosome entry site–mediated translational activity (8). We recently showed that GzmH participates in the clearance of hepatitis B virus (HBV) via cleavage of the HBx protein (9). Adoptive transfer of GzmH-expressing NK cells into an HBV mouse model augments HBV clearance. Notably, low GzmH expression in cytotoxic lymphocytes is correlated with susceptibility to HBV infection in individuals. We also recently reported the crystal structure of the enzymatically inactive D102N-GzmH mutant and defined the substrate specificity of GzmH (10).

Serine protease inhibitors (serpins) are a class of proteins that is primarily known to irreversibly suppress the proteolytic activities of serine proteases. The native inhibitory serpin fold contains a variable reactive center loop (RCL), which is the primary site of association with target proteases (11). Upon cleavage at the P1 site in the RCL, serpins undergo a conformational change by which the serpin and the target protease form a covalent complex to block the enzymatic activity. SERPINB9 was identified as an intracellular inhibitor of Gzm B (GzmB) (12). SERPINB4 is an inhibitor of Gzm M (GzmM) and suppresses GzmM-mediated cell death (13). However, no physiological intracellular inhibitors have

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Received for publication September 10, 2012. Accepted for publication November 28, 2012.

This work was supported by the National Natural Science Foundation of China (30830030, 31128003, 31174377, 3122062, and 30972616), the 973 Program (2010CB919502), the Innovative program of the Chinese Academy of Sciences (XDA01010407), and the KC Wong Education Foundation (Hong Kong).

L. Wang and Q.L designed and performed research, analyzed data, and wrote the manuscript; L. Wu and P.L. contributed to the gramule isolation; S.L., P.Z., and H.Z. performed cytolyssis assays; Y.Z. and J.L. performed structure modeling; X.Y. and K.Z. contributed to protein purification and crystalization; L.T. and F.S. supervised all crystallographic studies and analyses; and Z.F. initiated the research, analyzed data, and wrote the manuscript.

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The atomic coordinates and structure factors have been deposited in the Protein Data Bank (http://www.rcsb.org) under accession numbers 4GA7 for LM-DD-SERPINB1 and 4GAW for wtGzmH.

The online version of this article contains supplemental material.

Abbreviations used in this article: CMA, conducanmycin A; Gzm, granzyme; GzmB, granzyme B; GzmH, granzyme H; GzmM, granzyme M; HBV, hepatitis B virus; ICAD, inhibitor of caspase-activated Dnase; pAb, polyclonal Ab; PEG3350, polyethylene glycol 3350; RCL, reactive center loop; RT, room temperature; serpin, serine protease inhibitor; SI, stoichiometry of inhibition; siRNA, small interfering RNA; wt, wild-type.

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been defined for human GzmH. In this study, we identified that SERPINB1 is an intracellular inhibitor of human GzmH. GzmH cleaves SERPINB1 at Phe\(^{343}\) in the RCL to mediate suicide inhibition. We also identified the crystal structures of the full-length SERPINB1 molecule and the active form of human GzmH, providing structural insights into the inhibitory mechanism.

**Materials and Methods**

**Cell lines, Abs, and reagents**

The human NK tumor cell line YTS, the human T cell lymphoblast-like cell line Jurkat, and the MHC class I–negative mutant B lymphoblastoid cell line 721.221 (provided by Dr. Baoxue Ge, Institute of Health Science, Chinese Academy of Sciences) were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin. Commercial Abs used included mouse mAb against GzmH, mouse mAb against perforin, rabbit polyclonal Ab (pAb) against EEA1, rabbit pAb against Lamp1 (Abcam), rabbit pAb against La (Cell Signaling Technology), goat pAb against SERPINB1, rabbit antisera against NM23-H1 (Santa Cruz Biotechnology), \&-actin (Sigma-Aldrich), Alexa Fluor 488–conjugated goat anti-rat IgG (BioLegend); Alexa Fluor 594–conjugated donkey anti-mouse IgG, Alexa Fluor 488–conjugated donkey anti-mouse IgG, Alexa Fluor 594–conjugated donkey anti-rabbit IgG (Molecular Probes), and HRP-conjugated secondary Abs (Santa Cruz Biotechnology).

**Granule isolation from YTS cells**

A total of 1 \(\times\) \(10^6\) YTS cells was washed three times in ice-cold PBS and resuspended into 20 ml relaxation buffer (100 mM KCl, 1.25 mM EGTA, 10 mM PIPES, 3.5 mM MgCl\(_2\)). ATP (1 mM) was added just before use. The cells were disrupted on ice using a nitrogen bomb at 450 \(\times\) \(g\) with N\(_2\) for 25 min (Parr Instrument Company Model 4639 Cell Disruption Vessel). The homogenates were separated from pelleted nuclei, mitochondria, and the remaining intact cells by centrifugation at 400 \(\times\) \(g\) at 4 \(\circ\)C for 10 min. The final supernatant was postnuclear supernatant, which was centrifuged at 20,000 \(\times\) \(g\) for 10 min, resulting in a 30% Percoll gradient. The enriched organelles were resuspended in 50 mM Tris buffer and disrupted by at least three freeze–thaw cycles in liquid nitrogen and then centrifuged at 14,800 \(r\)pm for 20 min to separate the soluble proteins and membrane proteins.

**Confocal microscopy**

Cultured YTS cells (10\(^3\)) were washed three times with PBS and spread onto poly-1-lysine–coated glass slides (Sigma-Aldrich) for 5 min. The slides were rinsed twice to remove nonadherent cells. Cells adhering to the slide were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.2% Nonidet P-40 in PBS for 5 to 10 min at room temperature (RT). After 30 min of blocking with 10% donkey serum (Life Technologies), the slides were rinsed with 10% donkey serum (Life Technologies) and incubated with appropriate secondary Abs. Images were acquired with an Olympus LSCM-FV500 laser scanning confocal microscope.

**Protein expression and purification**

The human SERPINB1 cDNA was amplified from the YTS cDNA library using specific primers (forward: 5'–GGTGCCTGGGCGGCGCTTC–3'; reverse: 5'–GGCTTATTTAATAACCTAGT–3'). A nested PCR was performed using templates from first-round PCR with primers (forward: 5'–GAATTCGTAGAGCGAGTCGAGC–3'; reverse: 5'–TATCCGAGTAAAGGAAAATACCTC–3'). The full-length SERPINB1 gene was cloned into the pET-15b vector (Novagen) with a N-terminal His tag. SERPINB1 RCL-mutated plasmids were generated using the QuickChange site-directed mutagenesis kit (Stratagene). All constructs were confirmed by DNA sequencing. His-tagged wild-type wtSERPINB1, F343A–SERPINB1, and LM-DD–SERPINB1 were generated and purified using Ni-NTA resin for 6\(\times\)His-tagged proteins, Resource Q Sepharose anion exchange, and, finally, Superdex 75 gel-filtration chromatography (GE Healthcare). wtGzmH and D102N–GzmH proteins were overexpressed in Escherichia coli Rosetta (DE3) as inclusion bodies and then refolded and purified, as previously described (10).

**Pull-down assay**

For Ni-NTA pull-down assay, 50 \(\mu\)l His Resin was charged by Ni\(^{2+}\), balanced, and incubated with 200 ng wtSERPINB1 in a binding buffer containing 40 mM Tris (pH 8), 200 mM NaCl, 10 mM imidazole, and 10% glycerol. SERPINB1–attached Ni-NTA His beads were incubated with purified D102N–GzmH at 4 \(\circ\)C for 2 h. For Affi-Gel10 conjugated pull-down assay, 200 ng D102N–GzmH was conjugated with Affi-Gel10 beads and then incubated with purified wtSERPINB1 in binding buffer containing 40 mM HEPES (pH 7.5), 200 mM NaCl, and 10% glycerol at RT for 30 min. After washing with a binding buffer, the beads were resolved in loading buffer and boiled for 10 min, and bound proteins were detected by Western blotting.

**Gel-filtration chromatography**

Gel-filtration chromatography was performed on a Superdex 75 column by HPLC (GE Healthcare). The column was equilibrated with two column volumes of buffer A (40 mM Tris [pH 8], 150 mM NaCl). A mixture of purified wtSERPINB1 and D102N–GzmH proteins was incubated at a molar ratio of 1:1 at 4 \(\circ\)C for 30 min, and the individual protein was separately injected into the column running with buffer A. Samples were fractionated with a flow rate of 0.5 ml/min and collected as 0.5 ml fractions after injection into the column. Protein fractions were separated by SDS-PAGE on 12% gels.

**Detection of complex formation**

wtSERPINB1 and GzmH proteins were incubated at 37 \(\circ\)C in 25 mM Tris (pH 8), 150 mM NaCl for the indicated time periods, with or without synthesized GzmH inhibitor (10). YTS cells lysates were incubated at 37 \(\circ\)C for the indicated times. The reactions were stopped by 5 \(\times\) SDS loading buffer, resolved in SDS-PAGE, and detected by Coomassie Brilliant Blue staining or immunoblotting with the indicated Abs.

**Knockdown of GzmH and SERPINB1 in YTS cells**

RNA sequences against GzmH (5‘-GATCCCGGGTGAAGCTCTCC-CTTTTCAGGAAATGGAAGAGACGTTCACACGGG-3’ [sense] and 5‘-GCTTAAAAAGTGTGAACGTCTCTTGTTTTT-3‘ [antisense]) were designed based on pSUPER system instructions (Oligogene) and cloned as previously reported (14). For production of lentivirus, 293T cells were seeded onto a 10-cm dish and transfected with 4 \(\mu\)g transfer vector pSin-EPF-2a-GzmH, 4 \(\mu\)g gag-pol, and 2 \(\mu\)g envelop plasmids using Lipofectamine 2000 (Invitrogen). After 48 h of incubation, culture supernatants were harvested, passed through a 0.45-\(\mu\)m filter, and concentrated by ultracentrifugation at 20,000 \(r\)pm for 2 h. YTS cells were infected by lentivirus in the presence of 8 \(\mu\)g/ml Polybrene. Stable clone pools were selected and maintained in the presence of 1 \(\mu\)g/ml puromycin. For SERPINB1 silencing, SERPINB1 and scrambled control small interfering RNA (siRNA) consisting of 21 bp with a two-base deoxy-nucleotide overhang were purchased from GenePharma (Shanghai, China). The following sequences were used: siSERPINB1, sense: 5‘-GGCGGUGAGUGAGAACCATT-3’; antisense: 5‘-AUUGUUUCUCACUAAACGCCCTT-3’; and control, sense: 5‘-UCUCGGCAAGUCUCCAGUTT-3’; antisense: 5‘-ACGCUGACUCGUCCGAGATT-3’. Transient transfection of YTS cells was performed with Cell Line Nucleofector Kit R (Amaxa). A total of 2 \(\times\) \(10^6\) YTS cells was pelleted at 100 \(\times\) \(g\), resuspended in 100 \(\mu\)l transfection solution, and nucleofected with Amaxa program O-017. The samples were then boiled for 10 min, resolved by SDS-PAGE, and detected by Coomassie Brilliant Blue staining or immunoblotting with the indicated Abs.

**Cleavage assay and detection of stoichiometry of inhibition**

Granule isolation from YTS cells was incubated with 721.221 cell lysates (1 \(\times\) \(10^5\)) were incubated with wtGzmH (0.4 \(\mu\)M) and the indicated amounts of wtSERPINB1 at 37 \(\circ\)C for 2 h in 20 \(\mu\)l cleavage buffer of the following composition: 1 M HEPES (pH 7.5), 200 mM NaCl, 10% glycerol, and 10 \(\mu\)g/ml PMSF. The reactions were terminated and subjected to immunoblotting, as described (10). For stoichiometric determinations, 100 \(\mu\)M GzmH was preincubated separately with different concentrations of wtSERPINB1, as indicated by GzmH to wtSERPINB1 motor ratios in a range from 0 to 2. At 37 \(\circ\)C,
for 1 h in 25 mM Tris (pH 8), 150 mM NaCl, and relevant synthetic substrates (300 μM) were added to the samples and diluted to 100 μl for an additional 30-min incubation at 37˚C. The remaining enzyme activity was determined by measuring absorbance at 405 nm using a Multilabel Counter (Wallac 1420 Victor; PerkinElmer). The corresponding substrates were as follows: Ac-PTSY-pNA for GzmH, Suc-VANR-pNA for Gzm A, Z-IEPD-AFC for GzmB, Ac-YRFK-pNA for Gzm K, and Suc-AAPL-pNA for GzmM.

GzmH loading and LAK cell–mediated cytolysis assay

wtSERPINB1 and F343A-SERPINB1 were cloned into the pSIN-EF2 vector and then lentiviruses were produced as described above. Jurkat cells were infected with the described lentivirus, and stable clone pools were selected and maintained in the presence of 1 μg/ml puromycin. Jurkat cells stably transfected with control vector, wtSERPINB1, and F343A-SERPINB1 were washed twice in serum-free RPMI 1640, and then 1 μM GzmH or 1 μM GzmB was loaded into Jurkat cells with adenovirus at 37˚C for 4 h. Then cells were harvested and stained with FITC-conjugated annexin V and propidium iodide (Bender Med Systems), followed by flow cytometry (FACSCalibur; BD Biosciences). Data were analyzed using FlowJo software (TreeStar). PBMCs from healthy donors were purchased from the Beijing Blood Center and isolated by Ficoll-Hypaque gradient centrifugation. For generation of LAK cells, PBMCs were cultured in RPMI 1640 medium supplemented with 15% FBS (Life Technologies) with 500 U/ml recombinant human IL-2 for 4 d. Jurkat cells stably transfected with control vector, wtSERPINB1, and F343A-SERPINB1 were labeled with 200 μCi 51Cr for 1 h. LAK cells were added to 51Cr-labeled Jurkat cells at an E:T ratio of 1. After 4 h of incubation, the supernatants were harvested, and the radioactivity was measured as described previously (9). For the inhibition assay, LAK cells were pretreated with 500 nM concanamycin A (CMA) for 2 h or with 300 μM GzmH inhibitor for 1 h before incubation with target cells.

Crystallization and data collection

Initial hits were obtained using commercially available sparse matrix screens (Hampton Research) in the hanging drop vapor-diffusion method at 16˚C. Optimization was conducted and diffraction-quality crystals of LM-DD-SERPINB1 were eventually obtained in 0.18 M lithium citrate tribasic tetrahydrate, 18% (w/v) polyethylene glycol 3350 (PEG3350), with a protein concentration near 8 mg/ml. Crystals were cryoprotected in reservoir solution supplemented with 30% (w/v) PEG3350 and then flash frozen in liquid nitrogen. The data set was obtained on the Shanghai Synchrotron Radiation Facility beamline BL17U (λ = 0.98 Å) at 100 K. Crystals of wtGzmH were obtained in a solution containing 0.2 M Li2SO4, 0.1 M Bicine (pH 8.5), 25% (w/v) PEG3350. Data sets were collected at 100 K at the beamline BL5A (λ = 0.9 Å) of the Photon Factory (Tsukuba, Japan). All diffraction data were processed using the HKL-2000 suite of programs (15).

Structure determination and refinement

The structure of SERPINB1 was solved by molecular replacement with Phaser (16), using plasminogen activator inhibitor-2 as the search model (PDB code 1BY7). The structure of wtGzmH was solved by molecular replacement with COMO using the structure of D102N-GzmH as a search model (PDB code 3TK9). Structure refinement was carried out using CNS and REFMAC, and COOT was used for manual model building (17). Final

FIGURE 1. SERPINB1 is fractionated with GzmH from the separation of cytotoxic granules. (A) Diagram of isolation steps of secretory lysosomes from the NK cell line YTS. YTS cells were disrupted by nitrogen decompression, and the secretory lysosomes were separated from other organelles by Percoll-gradient fractionation. The product of each step is abbreviated in parentheses. (B) Soluble components in the isolated secretory lysosomes were identified by mass spectrometry. Secretory lysosomes were disrupted by three freeze–thaw cycles. Supernatants (S2) were subjected to SDS-PAGE and visualized by Coomassie Brilliant Blue staining (upper panel). Arrows denote three major bands. Peptide sequencing was performed as shown in the lower panel. (C) Isolated fractions were detected by immunoblotting. (D) Localization of SERPINB1 in YTS cells was visualized via confocal microscopy. GzmH, perforin, LAMP1, and EEA1 staining red (left panels), SERPINB1 staining green (middle panels), and merged images (right panels). Original magnification ×400. These images are representative of at least four separate experiments.
refinement statistics are given in Table I. Stereochemical parameters were evaluated with PROCHECK (18). Figures showing structural representations were prepared with the program PyMOL (http://www.pymol.org/).

Structure modeling of GzmH with SERPINB1

The initial models were constructed with Modeler software (19), using the serpin–trypsin complex (PDB code 1K9O) (20) as the Michaelis complex model for the SERPINB1–GzmH complex and the α1PI/PPE covalent complex (PDB code 2D26) (21) for the inhibitory complex. The active site was refined by our previously solved GzmH–decapeptide complex (PDB code 3TJV) (10). In the inhibitory complex model, the carbon atom of the carbonyl group of Phe343 in serpin is linked to the oxygen atom of the Ser197 side chain via a single bond. The all-atomic Molecular Dynamics simulations were performed for both models to relax them and eliminate local unreasonable conformation caused by residue mutation or artificial effect (22, 23). The last snapshots of 50-ns Molecular Dynamics trajectories were extracted for structural analysis.

Results

SERPINB1 is fractionated with GzmH from the separation of NK cell granules

The human NK cell line YTS is well established as a main expression cell line of GzmH (3, 10). To identify intracellular inhibitors of GzmH, we isolated secretory lysosomes of YTS cells using self-forming Percoll gradients (Fig. 1A). Based on the size and high density, secretory lysosomes could be enriched after fractionation. To separate the soluble contents from membrane-associated proteins, the pellet granules were subjected to freeze–thaw cycles prior to SDS-PAGE resolution. Three major proteins (∼66, 45, and 35 kDa) were visualized in the supernatants by Coomassie staining (Fig. 1B). Each band was cut and digested by trypsin for peptide sequencing. The first band ∼66 kDa primarily contained HSP70 and perforin. The second band ∼45 kDa included SERPINB1, SERPINB8, and SERPINB9. The bottom band ∼35 kDa contained GzmB (56.7%) and GzmH (14.6%), which is consistent with previous reports (3, 24). Notably, much greater amounts of SERPINB1 (32.4%) were contained in the second band compared with SERPINB9 or SERPINB8. We observed that SERPINB1 was distributed in the cytoplasmic sections and also fractionated with GzmH in the purified granules (F19–F24), as well as in the early endosomes (F10–F13) (Fig. 1C). EEA1 (early endosome marker) was used to distinguish between early endosomes and lysosomes. SERPINB1 was distributed in the cytosol and partially colocalized with GzmH in YTS cells, as observed by confocal microscopy.

FIGURE 2. SERPINB1 associates with GzmH to form an SDS-stable serpin–protease complex. (A) wtSERPINB1 can pull down GzmH. His-tagged wtSERPINB1 attached to Ni-NTA beads was incubated with purified D102N-GzmH at 4˚C for 2 h. Binding proteins were visualized by immunoblotting with anti-GzmH Ab (upper panel) or anti-SERPINB1 Ab (lower panel). The naked beads served as a non-specific control. (B) GzmH binds to SERPINB1. D102N-GzmH conjugated to Affi-Gel10 beads was incubated with purified SERPINB1 at RT for 30 min, followed by immunoblotting. (C) SERPINB1 forms a Michaelis complex with inactive GzmH. A mixture of purified wtSERPINB1 and D102N-GzmH proteins was incubated for 5 h at 4˚C at a molar ratio of 1:2, or the individual protein was injected separately into a Superdex 75 column running with 40 mM Tris (pH 8), 150 mM NaCl. The elution fractions of the mixture (gray line) and individual proteins (D102N-GzmH, dashed line; SERPINB1, black line) were recorded with a flow rate of 0.5 ml/min and collected as 0.5-ml fractions after injection into the column in the upper panel. Proteins in the elution were visualized by Coomassie Brilliant Blue staining after resolution. D102N-GzmH (26 kDa) showed the protein peak at 13.8 ml and SERPINB1 (45 kDa) at 11.65 ml. The mixture showed an earlier elution volume at 10.8 ml, which contained both SERPINB1 and D102N-GzmH proteins. The latter peak of the mixture contained excessive D102N-GzmH. (D) SERPINB1 forms an SDS-stable inhibitory complex with active GzmH. Aliquots of SERPINB1 (0.5 µM) were treated separately with active Gzm (0.5 µM) and then subjected to SDS-PAGE. The black arrow denotes SERPINB1, the white arrow marks Gzm, and the gray arrow points to the SDS-stable complex formed by SERPINB1 and Gzm. (E) SERPINB8 fails to form an SDS-stable serpin–protease complex with active GzmH. Aliquots of SERPINB8 were incubated separately with all five Gzms at 37˚C and visualized by Coomassie Brilliant Blue staining. The black arrow represents the individual Gzm, the open arrow SERPINB1 or SERPINB8, and the gray arrow covalent Gzm-inhibitor complexes.
SERPINB1 localized with a lysosome marker LAMP1, which indicates that SERPINB1 is also localized in the granules known as specialized secretary lysosomes. Because SERPINB1 is a typical clade B serpin molecule, it does have a membrane-spanning domain, suggesting that it is a cytosolic molecule. How SERPINB1 functions in NK cell granules requires further investigation.

SERPINB1 associates with GzmH to form a classical SDS-stable serpin–protease complex

We wanted to determine the interaction between SERPINB1 and GzmH. Soluble His-tagged wtSERPINB1 was expressed and purified from E. coli. We refolded tag-free active human GzmH (wtGzmH) and enzymatically inactive D102N-GzmH from inclusion bodies, as previously described (10). wtGzmH showed proteolytic activity, whereas D102N-GzmH had no activity for the synthetic substrate Ac-PTSY-pNA. D102N-GzmH was incubated with SERPINB1-bound Ni resin or Ni2+ resin alone at 4°C for 2 h. His-SERPINB1 beads efficiently pulled down D102N-GzmH (Fig. 2A). D102N-GzmH–immobilized Affi-Gel 10 beads were also able to bind wtSERPINB1 (Fig. 2B). The naked beads were used as a negative binding control. To further verify the binding affinity of SERPINB1 for GzmH, we mixed wtSERPINB1 with D102N-GzmH at a molar ratio of 1:2 on ice for 5 h, and the mixture or aliquots of individual protein were injected into a Superdex 75 column for size-exclusion chromatography (Fig. 2C, lower panel). Elution fractions were resolved with SDS-PAGE, followed by Coomassie staining (Fig. 2C, lower panel). We observed that wtSERPINB1 and D102N-GzmH both appeared in a higher molecular mass peak of 70 kDa than did wtSERPINB1 molecule alone (45 kDa) or D102N-GzmH alone (26 kDa). The molecular masses were calculated by calibration standards.

Serpins are known as suicide inhibitors; they first bind to a specific target protease to form a Michaelis complex. Then, the serpin forms an SDS-stable covalent complex with the target protease upon cleavage by this protease to block enzymatic activity (20). To test whether SERPINB1 forms an SDS-stable covalent-bound complex with GzmH, we incubated wtSERPINB1 with wtGzmH (Fig. 2D). Notably, an SDS-stable complex band was observed as the result of the incubation of wtSERPINB1 with wtGzmH, whereas weak complex bands were also observed in the incubation with GzmB and GzmM. In contrast, inactive D102N-GzmH had no such activity. However, SERPINB8, another serpin molecule found in the cytotoxic granules, failed to interact with GzmH or other Gzm (Fig. 2E).

SERPINB1 directly blocks the proteolytic activity of GzmH

To determine whether SERPINB1 can inhibit the enzymatic activity of GzmH, we measured the concentrations of wtGzmH and...
wtSERPINB1 and then incubated aliquots of wtGzmH with different doses of wtSERPINB1, followed by examination of enzymatic activity. We found that SERPINB1 inhibited the proteolytic activity of GzmH with a stoichiometry of inhibition (SI) of 1.55 ± 0.27 and an apparent second-order rate constant of 1.10 (± 0.12) × 10^6 M⁻¹ s⁻¹ (Fig. 3A, Supplemental Table I). However, SERPINB1 failed to suppress the enzymatic activities of the other four granzymes (Fig. 3A), which is in agreement with a previous report (25). To assess whether SERPINB1 inhibition is physiologically relevant, we incubated wtGzmH with tumor cell lysates by adding increasing concentrations of wtSERPINB1 (Fig. 3B). As expected, wtSERPINB1 completely blocked the proteolysis of both substrates. The GzmH synthetic inhibitor Ac-PTSY-CMK was able to inhibit the enzymatic activity of GzmH (Fig. 3B), which was used as an inhibition control. Gzm A–activated endonuclease NM23-H1 (25) was used as a good loading control.

To further confirm that SERPINB1 covalently binds to GzmH, we incubated wtGzmH with wtSERPINB1 at different molar ratios. Expectedly, a stable SERPINB1–GzmH inhibitory complex was formed with the predicted molecular mass of ~70 kDa in a dose-dependent fashion (Fig. 3C). Inactive D102N-GzmH mutant failed to form such a complex, suggesting that the formation of the stable SERPINB1–GzmH complex requires the enzymatic activity of GzmH. This stable SERPINB1–GzmH complex also appeared in a time-dependent manner (Fig. 3D). The Ac-PTSY-CMK inhibitor abrogated the complex formation by inhibition of the activity of wtGzmH. Taken together, these results indicate that SERPINB1 disables the enzymatic activity of GzmH through the covalent complex formation.

**GzmH cleaves SERPINB1 at Phe343 in the RCL**

SERPINB1 was reported to inhibit elastase- and chymotrypsin-like proteases through degradation of its RCL (25). GzmH is a chymotrypsin-like protease with preference for bulky residues, such as Tyr and Phe, at the P1 position (27). As shown in Fig. 4A, only one Phe residue, Phe343, is present in the RCL. To determine the cleavage site of SERPINB1 by GzmH, we mutated Phe343 to Ala (F343A-SERPINB1) and generated its recombinant protein. Interestingly, F343A-SERPINB1 lost its inhibitory action against GzmH (Fig. 4B, middle panel), whereas wtSERPINB1 exhibited strong inhibition activity (Fig. 4B, left panel). Additionally, F343A-SERPINB1 failed to form the stable SERPINB1–GzmH complex (Fig. 4C), whereas wtSERPINB1 formed the complex with wtGzmH.

We (10) and other investigators (8) demonstrated that acidic residues in the P3’ and P4’ sites of physiological substrates are preferred for human GzmH. wtSERPINB1 bears Leu346 and Met347 at the P3’ and P4’ sites, respectively. Thus, we mutated both Leu346 and Met347 to Asp (LM-DD-SERPINB1), as shown in Fig. 4A. LM-DD-SERPINB1 exhibited a stronger inhibitory effect on proteolysis of GzmH than did wtSERPINB1 (Fig. 4B, right panel). In addition, the SI for LM-DD-SERPINB1 to GzmH was close to 1, indicating that LM-DD-SERPINB1 augments the efficiency of inhibition. We also calculated the association rate constant for GzmH inhibition in the presence of wtSERPINB1 or LM-DD-SERPINB1.

**FIGURE 4.** Inhibition of GzmH by SERPINB1 occurs through cleavage of the RCL. (A) Schematic diagram showing the recognition modes of GzmH against SERPINB1. The P1 residue for GzmH was predicted to be Phe343. The putative acting P1 residue Phe343 was mutated to Ala as F343A-SERPINB1, whereas the P2’ P3’ residues Leu346–Met347 were mutated into Asp–Asp as LM-DD-SERPINB1. (B) Phe343A mutation abolishes its inhibitory action to GzmH. Increasing concentrations of SERPINB1 variants were incubated with wtGzmH (100 nM) at 37°C for 2 h. Then synthetic substrates were added, and remaining activity was measured. The SI was calculated by linear regression analysis. (C) F343A-SERPINB1 mutant fails to form the covalent complex. F343A-SERPINB1 and wtSERPINB1 were incubated with wtGzmH at 37°C for 1 h, followed by Coomassie brilliant blue staining (upper panel) and immunoblotting against SERPINB1 (middle panel) or GzmH (lower panel). (D) The LM-DD-SERPINB1 variant promotes the stable serpin–protease complex formation. LM-DD-SERPINB1 and wtSERPINB1 were incubated with wtGzmH at 37°C for 1 h, followed by Coomassie staining. The black arrows denote SERPINB1, the white arrows mark GzmH, and the gray arrows mark the SDS-stable complex. WB, Immunoblotting.
We found that LM-DD-SERPINB1 enhanced the inhibition efficiency by 5-fold, with an apparent second-order rate constant of $5.37 \pm 0.60 \times 10^7 \text{M}^{-1}\text{s}^{-1}$. LM-DD-SERPINB1 also strengthened the formation of the stable SERPINB1–GzmH complex (Fig. 4D). Collectively, these results identify that Phe$^{343}$ in the RCL is the P1 site for GzmH.

**Overexpression of SERPINB1 suppresses GzmH- or LAK cell–mediated cell death**

Given that SERPINB1 was initially isolated from YTS cells, we explored whether native SERPINB1 can inhibit native GzmH when they encounter each other in cells. Because GzmH is stored in the acidic granules, we disrupted all of the membrane compartments using a buffer containing 0.5% Nonidet P-40. YTS cell lysates were incubated at 4 or 37˚C for the indicated times. As shown in Fig. 5A, the stable covalent complex was generated at 37˚C under which conditions GzmH recovered its enzymatic activity. The Ac-PTSY-CMK inhibitor abolished the formation of the stable covalent complex (Fig. 5B). Addition of wtGzmH produced a stable covalent complex band that was lower than that of the native GzmH, largely due to glycosylation of native GzmH in YTS cells (4). Knockdown of SERPINB1 by siRNA (Fig. 5C) or knockdown of GzmH by short hairpin RNA (Fig. 5D) attenuated the formation of the stable complex. Notably, the classical SDS-stable protease inhibitor complex was observed in primary NK cells (Fig. 5E). However, SERPINB1 could not form a covalent complex with inactive GzmH in the lysates of YTS and primary NK cells at 4˚C (Fig. 5A, 5E), indicating that...

**FIGURE 5.** SERPINB1 overexpression inhibits GzmH- and LAK cell–mediated cell death. (A) The SDS-stable SERPINB1–GzmH complex is formed in lysates of YTS cells. Lysates of YTS cells were incubated for different times at 37˚C or at 4˚C for 2 h, followed by immunoblotting. The SERPINB1–GzmH complex (∼70 kDa) was formed only when cell lysates were incubated at 37˚C. (B) GzmH inhibitor abolishes the complex formation. wtGzmH or the synthetic GzmH inhibitor (Ac-PTSY-CMK, inh) was added to YTS cell lysates and detected as in (A). Silencing of SERPINB1 (C) or GzmH (D) reduces the complex formation. SERPINB1 and GzmH were knocked down separately in YTS cells by siRNA or short hairpin RNA, and treated as above. In lanes 1 and 4, lysates were incubated at 4˚C for 2 h; in lanes 2 and 5, lysates were incubated at 4˚C for 1 h and at 37˚C for 1 h; and in lanes 3 and 6, lysates were incubated at 37˚C for 2 h. (E) The complex exists in primary NK cells. Primary NK cell lysates were incubated for the indicated times at 37 or 4˚C and treated as above. The asterisk marks a nonspecific band of GzmH. (F) Jurkat cell lines with wtSERPINB1 and F343A-SERPINB1 expression were established. Jurkat cells were infected with lentivirus encoding wtSERPINB1 or F343A-SERPINB1, and stable cell lines were selected by puromycin. wtSERPINB1 and F343A-SERPINB1 were examined by Western blot. β-actin served as a loading control. (G) Overexpression of wtSERPINB1 represses GzmH-induced cell death. Jurkat cells stably overexpressing empty vector, wtSERPINB1, or F343A-SERPINB1 were treated with 1 μM GzmH (left panel) or 1 μM GzmB (right panel) plus adenovirus (Ad) at 37˚C for 4 h, followed by flow cytometry. Total dead cells, including Annexin V and propidium iodide single-positive and double-positive cells, are shown as mean ± SD. (H) Overexpression of wtSERPINB1 protects Jurkat cells from LAK cell–mediated cytotoxicity. Jurkat cells overexpressing empty vector, wtSERPINB1, or F343A-SERPINB1 were labeled with $^{51}$Cr and incubated with IL-2–activated LAK cells at an E:T ratio of 1. For a specific inhibition assay, LAK cells were pretreated with 500 nM CMA for 2 h or with 300 μM GzmH inhibitor for 1 h before incubation with target cells. **p < 0.01. The gray arrows mark the SDS-stable complex formed by endogenous SERPINB1 and GzmH; the black arrow denotes the complex formed by endogenous SERPINB1 and recombinant wtGzmH. All data are representative of at least three separate experiments. n.s., Not significant.
GzmH is not initially covalent in NK cells. The proteolytic activities of all granzymes are inactive in acidic pH granules (1); therefore, GzmH should remain inactive in the granules and be unable to cleave SERPINB1 to form a covalent inhibition complex.

We next determined whether SERPINB1 inhibits the proteolytic activity of GzmH in cells overexpressing SERPINB1. wtSERPINB1 or F343A-SERPINB1 mutant was overexpressed in Jurkat cells, and their stable cell lines were established (Fig. 5F). These transfected Jurkat cells were loaded with wtGzmH with adenovirus. wtSERPINB1 overexpression dramatically suppressed GzmH-induced apoptosis compared with the empty vector control (Fig. 5G). However, F343A-SERPINB1 transfection did not suppress cell death compared with the empty vector control (Fig. 5G, left panel). However, wtSERPINB1 overexpression failed to inhibit GzmB-mediated cytotoxicity (Fig. 5G, right panel). PBMCs were incubated with IL-2 to produce LAK cells. The transfected Jurkat cells were incubated with LAK cells at an E:T ratio of 1. As expected, wtSERPINB1 transfection remarkably suppressed LAK cell–mediated cytolysis compared with the empty vector control (Fig. 5H). F343A-SERPINB1 overexpression almost recovered the cytotoxic capacity of LAK cells. Moreover, CMA, an inhibitor of cytotoxic exocytosis, was mostly able to suppress LAK cell–mediated cytolysis (Fig. 5H). CMK inhibitor markedly reduced LAK cell–mediated cytolysis, which suggests that other Gzm in addition to GzmH participate in the induction of cell–mediated cytolysis.

Overall structures of SERPINB1 and active GzmH

Although wtSERPINB1 failed to crystallize, we were able to determine the crystal structure of the LM-DD-SERPINB1 mutant at 2.9-Å resolution (Table I). The SERPINB1 molecule folds into a typical serpin architecture composed of three large β-sheets and nine α-helices. The two molecules of LM-DD-SERPINB1 in the asymmetric unit adopt a conformation known as the native or stressed state of the serpin (11), in which the RCL is flexibly exposed and only five strands exist in the A sheet (Supplemental Fig. 1E).

The two LM-DD-SERPINB1 molecules could be assembled into two types of asymmetric units. The first one is that the two monomeric molecules stand upside down (Supplemental Fig. 1A). However, in addition to the main chain orientation, the difference density map of chain A could always be traced to the symmetric molecule of chain B at the converged position of Cys944 (Supplemental Fig. 1B). Therefore, we adopted the second asymmetric unit, in which two LM-DD-SERPINB1 molecules are linked by a disulfide bridge (Fig. 6A). Cys944 at the P1′ position in the RCL mediates the disulfide bond formation. RCL normally contains 17 residues and is tethered between β-sheets A and C (28). In our structure, residues 332–341 in chain A and 330–341 in chain B are disordered because they lack clear electron density. Residues 342–349 [from P2 to P6′, nomenclature described by Schechter and Berger (29)] are visible with well-defined electron density, as shown in Supplemental Fig. 1B. Other than the disulfide bond, there is little contact between the two LM-DD-SERPINB1 molecules (Fig. 6A); therefore, this dimer is unlikely to be physiologically relevant. Actually, we did not observe any dimer formation.

Table I. X-ray diffraction data collection and refinement statistics

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Highest-resolution shell statistics are shown in parentheses.

*Rmerge is defined by Rmerge = \[\sum_{h} \left( |F_{o}(h) - \langle |F_{o}(h)\rangle \right) / \sum_{h} |F_{o}(h)|\] where \(\langle F_{o}(h)\rangle\) is the mean of the observations \(F_{o}(h)\) of reflection h.

*Rwork is defined by Rwork = \[\sum_{h} \left( |F_{o}(h) - |F_{c}(h)|/ \sum_{h} |F_{o}(h)|\right)\] for a selected subset (5%) of the reflections that was not included in prior refinement calculations.
FIGURE 6. Overall structures of SERPINB1 and active GzmH, as well as the distorted suicide mechanism. (A) Overall structure of LM-DD-SERPINB1 variant. The three β-sheets and nine helices (hA–hI; cyan) are highlighted. The exposed RCL is shown in magenta, and the missing residues in RCL are denoted as dotted lines. Cys344, which mediates the disulfide bond, is depicted as spheres. (B) Twelve molecules of wtGzmH in (Figure legend continues)
in YTS cell lysates or isolated granules (Supplemental Fig. 1C, 1D).

We previously reported the crystal structures of inactive GzmH mutant (D102N-GzmH) (10). In this study, we solved the crystal structure of enzymatically active GzmH (wtGzmH). Interestingly, the 12 wtGzmH molecules in the asymmetric unit are assembled into two six-petal rings (Fig. 6B). wtGzmH is a monomer in solution, and this six-petal ring is probably not physiologically relevant. All 12 chains adopt the classical serine protease fold with similar rigid structures. RMS distances among equivalent Co atoms of the 12 molecules are no more than 1.0 Å, suggesting that they are identical structures overall (Supplemental Fig. 2A). There are recognizable differences in the conformation of several of the loops on the surface, away from the active site. We superimposed wtGzmH with D102N-GzmH alone and its complex with substrate. As shown in Supplemental Fig. 2B, wtGzmH remains nearly unchanged with respect to its substrate binding, suggesting that the substrate catalysis of GzmH involves little conformational change.

SERPINB1 inhibits the catalytic activity of GzmH by deformation

Although the structures of several serpin–protease complexes were reported (30, 31), only two crystal structures of the inhibitory complexes have been solved (20, 21). Despite extensive efforts, we were unable to cocrystallize SERPINB1 with wtGzmH. Thus, we modeled the binary Michaelis complex and the inhibitory complex by docking the crystal structure of SERPINB1 to wtGzmH (Fig. 6C). In the native state, the RCL of SERPINB1 (especially Phe343) is exposed to the solvent. Upon encountering GzmH, RCL is inserted into the active site groove as a substrate. Phe343 (P1) fits nicely into the hydrophobic cavity of the S1 pocket (Fig. 6D). Additionally, beneath the S1 pocket, Lys192 forms a strong hydrogen bond with the carbonyl group of Ala335 in SERPINB1, which enhances the interaction. The RKR motif of GzmH (10) interacts tightly with the Asp346 and Asp347 residues. Three salt bridges, one between Lys40 (GzmH) to Asp346 (SERPINB1) and another between Arg39 (GzmH), Arg51 (GzmH) to Asp347 (SERPINB1), respectively, were also identified. Interestingly, Lys74 protrudes into the RKR motif and forms a hydrogen bond with Asp347. Except for the electrostatic interactions as mentioned above, the N\text{\textgamma}l atom of Arg39 forms a hydrogen bond with the carbonyl group of Pro348. We deduced that wtSERPINB1 only maintains the main chain interactions with the RKR motif and, thus, lacks those electrostatic interactions. This may explain why LM-DD-SERPINB1 augments the inhibition efficiency against GzmH than wtSERPINB1.

Once the RCL inserts into the binding sites of GzmH, the catalytic triad is activated, and Ser195 of GzmH attacks the peptide bond between Phe343 and Cys344 of SERPINB1. After acylation of Ser195, the covalent complex forms by pulling GzmH by the RCL from one pole to another to generate a new β-sheet S4A (Fig. 6C). To coordinate with the dramatic conformational changes, GzmH is distorted and inactivated. The catalytic triad is disordered to move Ser195 ~5 Å away from its catalytic partner His57 (Fig. 6E), which is consistent with previous reports (20, 21).

Discussion

Gzm-mediated cytotoxicity plays a critical role in immune responses against infections and tumors. However, the balance between activation and inhibition of the proteolytic cascade must be tightly modulated to avoid self damage. In a recent study, we crystallized the enzymatically inactive D102N-GzmH alone and in complex with its synthetic substrate and inhibitor and solved their crystal structures (10). We defined the mechanisms of substrate recognition and enzymatic activation. In this study, we identified SERPINB1 as a potent intracellular inhibitor for human GzmH. Upon cleavage of the RCL at Phe343, SERPINB1 formed an SDS-stable covalent complex with GzmH in YTS and primary NK cells. Overexpression of SERPINB1 suppressed GzmH- or LAK cell–mediated cell death. We tried for years to crystallize human active GzmH and full-length SERPINB1 and failed to obtain the complex structure. Through molecular modeling, SERPINB1 inhibits GzmH by deformation, which is reminiscent of other serpin–protease complexes.

Cytotoxic granules are specialized secretory lysosomes in NK cells. Because of their high density, it was possible to separate secretory lysosomes by self-forming Percoll gradients (24). In the isolated supernatants, three serpin family members SERPINB1/ SERPINB8/SERPINB9 were present in NK cells in addition to the granule components perforin, GzmB, and GzmH. SERPINB9 was reported to be a physiological inhibitor for human GzmB (12), which protects lymphocytes from GzmB-induced injury. A recent study showed that SERPINB4 is an intracellular inhibitor for human GzmM that suppresses GzmM-mediated cell death (13). We showed that SERPINB1 and SERPINB8 resides in YTS cells; however, it is unknown whether they inhibit Gzm.

SERPINB1, also known as monocyte/neutrophil elastase inhibitor, was identified in human monocytes/neutrophils to act as a specific and efficient inhibitor for elastase, cathepsin G, and proteinase-3 (32). SERPINB1 is highly expressed in the cytosol of neutrophils and prevents infection-induced tissue injury (33). Moreover, SERPINB1 can maintain a healthy neutrophil reserve in the bone marrow (34), suggesting that it plays a crucial role in neutrophil development and homeostasis. We demonstrated that SERPINB1 also resides in NK cells, and its overexpression suppresses GzmH- or LAK cell–mediated cytolyis. Notably, SERPINB1 forms a canonical SDS-stable serpin–protease complex with both recombinant and native forms of GzmH. These data suggest that SERPINB1 inhibits GzmH through a conformational-change suicide mechanism.

Based on our structural superimpositions, the overall structures and steric positions of active centers of active GzmH and its mutant D102N-GzmH are similar. However, active GzmH was crystallized
GzmH needs bulky aromatic amino acids to compensate for its hydrophobic cavity at the S1 position. SERPINB1 harbors Phe343 in the RCL, consistent with the catalytic specificity preference of GzmH. Mutation of Phe343 to Ala completely abolishes the inhibitory effect of SERPINB1, as well as the covalent complex formation. We concluded that Phe343 is the acting P1 residue for GzmH cleavage. Actually, Phe343 locates at the P2 position of RCL, effectively shortening the RCL by one residue relative to most serpins. Other chymotrypsin-like proteases, such as cathepsin G, chymase, and elastase, were also reported to interact with SERPINB1 via Phe343 (25), indicating that a similar inhibition mechanism was adopted by SERPINB1. The acting P1’ site (actually the P1 site of RCL) residue Cys344 was identified to inactivate the neutrophil elastase (25). Although Cys344 mediates the disulfide bridge formation in our crystal structure, no dimeric molecules were detected in NK cells. Therefore, we defined the SERPINB1 molecule as a disulfide bond–tethered monomer in the crystal structure. GzmH prefers P3’ and P4’ acidic residues, as we (8) and other investigators (10) reported. We modified the hydrophobic residues Leu346 and Met347 to Asp at the P3’ and P4’ positions and demonstrated that the double mutations of SERPINB1 led to improved inhibitory effect. Structural modeling provides further insights into the SERPINB1 binding and inhibitory mechanism for GzmH.

Serpin family members play pivotal roles in the inhibition of serine proteases in blood coagulation, mental diseases, and innate immune responses. SERPINB1 was first identified as a fast-acting proteinase inhibitor produced in monocytes and neutrophils (35). Because of the lack of a cleavable hydrophobic signal sequence, SERPINB1 resides mainly in cytosol, but it is also detectable in bronchoalveolar lavage fluid during lung inflammatory diseases (36, 37). Studies using SERPINB1-deficient mice established the significant role for SERPINB1 in protecting lung antimicrobial proteins from proteolysis during microbe infection and its regulatory role for SERPINB1 in protecting lung antimicrobial proteins in alveolar lavage fluid during lung inflammatory diseases (36, 37). Immune responses. SERPINB1 was first identified as a fast-acting serine proteases in blood coagulation, mental diseases, and innate immune responses. SERPINB1 led to improved inhibitory effect. Structural modeling using Modeller (15:5.6.1–5.6.30).


