Valosin-Containing Protein Cleavage by Granzyme K Accelerates an Endoplasmic Reticulum Stress Leading to Caspase-Independent Cytotoxicity of Target Tumor Cells

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Valosin-Containing Protein Cleavage by Granzyme K Accelerates an Endoplasmic Reticulum Stress Leading to Caspase-Independent Cytotoxicity of Target Tumor Cells

Yuming Guo,1 Jun Chen,1 Lei Shi, and Zusen Fan

Granzyme K (GzmK) highly expressed in NK and NKT cells. We recently demonstrated that GzmK induces rapid caspase-independent cell death with ssDNA nicks. Little is known about its molecular mechanisms to mediate caspase-independent cell death. In this study, we found the valosin-containing protein (VCP) is a physiological substrate of GzmK. GzmK cleaves VCP at residue Arg713 in the D2 domain and abrogates its ATPase activity. GzmK can also target other endoplasmic reticulum-associated degradation complex components Ufd1 and Npl4. Disruption of the endoplasmic reticulum-associated degradation pathway after GzmK treatment initiates ubiquitinated protein accumulation leading to xbp1 splicing. These indicate that ubiquitinated protein accumulation triggers endoplasmic reticulum stress in target cells. In support of this, target tumor cells with silenced VCP expression are more sensitive, whereas cells overexpressing VCP are more resistant to GzmK-mediated cytotoxicity. The Journal of Immunology, 2010, 185: 000–000.

Granizes (Gzms)/perforin-mediated cytolysis is the major pathway for killer lymphocytes to eradicate pathogens and tumor cells. GzmK and GzmA are closely linked together on the same chromosome both in mice and humans (1, 2). They are the only two tryptases among all of the Gzms (3). GzmA-deficient CTLs still contain 20% tryptase activity, and their cytolytic activity is just slightly reduced (4). GzmK might therefore be a potent Gzm to rescue the GzmA activity. GzmK expresses at high levels in CD56high NK cells, memory CD8+ T cells, and CD56+ T cells (5). A recent study showed the levels of circulating GzmK are notably elevated in virus-infected patients (6), suggesting that GzmK may play an important role in NK/CTL-mediated virus clearance. We recently demonstrated that human GzmK induces rapid caspase-independent cell death with rapid externalization of phosphatidylserine, nuclear morphological changes, and ssDNA nicks (7). After loading into target cells, GzmK targets the SET complex, resulting in the degradation of selected components, freeing the NM23H1 DNase and leading to ssDNA nicks, which is reminiscent of the dead features of GzmA. We found that GzmK can target the GzmA-associated SET complex and cleave the substrates of GzmA in the SET complex, including SET, Ape1, and HMGB2 (7, 8). GzmK can also induce rapid generation of reactive oxygen species (ROS) and collapse of the mitochondrial inner membrane potential (9). Cytochrome c (cyt c) is released from the mitochondrial outer membrane to the cytosol through a Bid-dependent way. However, GzmA induces a rapid increase in ROS and mitochondrial transmembrane potential loss through a Bid-independent way (10). GzmK cleaves Ape1 and wrecks its redox function. Ape1 cleavage accelerates intracellular ROS accumulation and cell death (8). We recently demonstrated that the tumor suppressor p53 is a specific substrate for GzmK (11). GzmK cleaves p53 at Lys24 and Lys305 to generate three cleavage fragments that harbor strong proapoptotic activities (12). GzmK (11). GzmK cleaves p53 at Lys24 and Lys305 to generate three cleavage fragments that harbor strong proapoptotic activities (12). GzmK cleaves p53 at Lys24 and Lys305 to generate three cleavage fragments that harbor strong proapoptotic activities (12).
cellular biological functions. VCP can form a complex with the Ufd1-Npl4 heterodimer and participate in the ERAD pathway to degrade misfolded proteins that are usually ubiquitinilated for recognition and degradation (18–20). In this study, we identified VCP what to our knowledge is a new substrate of GzmK by affinity chromatography and communoprecipitation assay. GzmK cleaves VCP at residue Asp273 in the D2 domain and abrogates its ATPase activity. GzmK can target other ERAD complex components, Ufd1 and Npl4, to destroy the ERAD pathway for misfolded protein degradation leading to ubiquitinilated protein accumulation, which induces an ER stress leading to cell death.

Materials and Methods

Cell lines, Abs, and reagents

Cells were grown in RPMI 1640 (Jurkat) and DMEM (HeLa) supplemented with 10% FCS, 50 μM 2-ME, 100 U/ml penicillin, and 100 mg/ml streptomycin. Commercial Abs were rabbit antiserum against rat M23H1 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse mAbs against VCP for immunoblotting (BD Pharmingen, San Diego, CA), ubiquitin (Cell Signaling Technology, Beverly, MA), β-actin (BD Pharmingen), goat antisera against Ufd1 (Santa Cruz Biotechnology), HRP-conjugated sheep anti-mouse IgG, HRP-conjugated sheep anti-rabbit IgG, and HRP-conjugated rabbit anti-goat IgG (Santa Cruz Biotechnology). Anti-VCP Ab for immunoprecipitation was a gift of Y. Ye (National Institutes of Health, Bethesda, MD); anti-Npl4 Ab was a gift of O. Steimann (Max-Planck-Institute of Biochemistry, Martinsried, Germany). Mouse mAb against human GzmK was generated through hybridoma screening in our laboratory. This Ab is specific for GzmK and has no cross-reactivity with other Gzms. Rabbit anti-SMA was generated, as described (7). Reagents include Affi-gel 10 resin (Bio-Rad, Hercules, CA), ATP (AMRESCO, Solon, OH), MG132 (Sigma-Aldrich, St. Louis, MO), Lipofectamine 2000 (Invitrogen, Carlsbad, CA), adenovirus (Ad; Ben Yuan Zheng Yang, Beijing, China), annexin-V FITC kit (BD Pharmingen), and streptolysin O (SLO; Sigma-Aldrich). Perfornin was a gift of J. Trapani (Research Division, Peter MacCallum Cancer Centre, Melbourne, Australia).

Plasmids and recombinant protein expression

The plasmids of GzmK and inactive S-AgGzmK were constructed and expressed, as described (7). Active GzmA was expressed in E. coli and purified as GzmK. Active GzmB, GzmM, and GzmH were expressed in E. coli and purified from inclusion bodies, as described (21). The construct of rVCP was a gift of T. Rapoport (Harvard Medical School, Boston, MA). rVCP was expressed in JM109 cells and purified sequentially over Novagen (Madison, WI) nickel column. The short hairpin RNA (shRNA) sequence targeting VCP is as follows: 5′-GATCCCCTTGTTGTATGATGACATTGTTCAAGAGACATACTGCATCATACCCTACGGG-3′. The VCP shRNA was subcloned in the pSUPER vector. The pcDNA3.1-VCP plasmid was a gift of D. Strasser, Walter and Eliza Hall Institute of Medical Research, Parkville, Australia) was transfected for Bcl-2 overexpression.

Affinity chromatography with S-AgGzmK

Purified S-AgGzmK (1 mg) was coupled to 0.5 ml Affi-Gel 10 (Bio-Rad) to generate an affinity column. Cytoplasmic extracts prepared from 5 × 10⁸ HeLa cells treated with Nonidet P-40 lysis buffer (0.5% Nonidet P-40/25 mM KC1/5 mM MgCl2/1 mM PMSF/10 mM Tris-HCl [pH 7.6]) were applied to the column and eluted with gradient NaCl concentrations. The naked resin was used as a negative column control. Fractions were analyzed by SDS-PAGE and followed by silver staining. Protein bands at 97 and 37 kDa were subjected to tryptic digestion and identified by mass spectrometry.

Communoprecipitation and immunoblotting

Abs were preincubated with protein A/G Sepharose (Pharmacia, Peapack, NJ) for 1 h at 4°C. We washed Ab-coated beads twice in PBS buffer before adding them to recombinant proteins (50 μg/ml or cytosolic lysates (5 × 10⁶ cell equivalents in 20 μl Nonidet P-40 lysis buffer) that had been preincubated for 2 h at 4°C with 50 μg/ml S-AgGzmK. After overnight shaking at 4°C, the beads were washed extensively in 1% Nonidet P-40 lysis buffer containing 150 mM NaCl and resolved by SDS-PAGE. The proteins were transferred to nitrocellulose membranes before probing with the indicated Abs.

Preparation of lymphokine-activated killer cells and lymphokine-activated killer cell-mediated cytosis assay

PBMCs from healthy donors (Beijing Red Cross Blood Center) were separated by Ficoll-Hypaque gradient centrifugation. Lymphokine-activated killer (LAK) cells were obtained by incubation with human rIL-2 (1000 U/ml) in 15% FBS/RPMI 1640. For inhibition experiment, HeLa cells were treated with 200 μM GzmK inhibitor Ac-YRFRK-chloromethylketone (CMK) prior to incubation with the IL-2–activated LAK cells. HeLa cells were incubated with LAK cells as the indicated E:T ratios at 37°C for 4 h. The treated HeLa cells were harvested through removing the suspended LAK cells and followed by probing for VCP and β-actin.

Loading GzmK with Ad/perforin/SLO

HeLa or Jurkat cells were washed three times in PBS and resuspended in the loading buffer. Cells (2 × 10⁸) in 50 μl loading buffer were incubated at 37°C for the indicated times with different concentrations of GzmK, S-AgGzmK, and an optimal dose of Ad, perforin, or SLO, as described before (7, 21).

Cleavage assay

Cell lysates prepared from HeLa cells were treated with 0.5% Nonidet P-40 lysis buffer. Cell lysates (2 × 10⁶ cells) were incubated with 1 μM rVCP was incubated with the indicated doses of GzmK or S-AgGzmK or indicated doses of GzmA, B, M, or H for the indicated times in 20 μl cleavage buffer (50 mM Tris-HCl [pH 7.5], 1 mM CaCl₂, 1 mM MgCl₂). For in vivo cleavage assay, cells loaded with GzmK/Ad were lysed in 0.5% Nonidet P-40 lysis buffer. The reaction samples were run in 5% SDS loading buffer and probed by immunoblotting.

Enzymatic activity assays

Enzymatic activities were detected by cleavage of synthetic substrates. The substrates were as follows: Ac-YRFK-pNA (GzmK), Suc-V ANR-pNA (GzmB), Z-ETD-APC (GzmM), Suc-FLF-pNA (GzmH), and Suc-AAPL-pNA (Gzm). Their activities were measured, as described (21).

ATPase activity assay

ATP hydrolysis was measured directly by the conversion of ATP to ADP. Release of inorganic phosphate (Pi) was estimated, as described (23). Briefly, rVCP was incubated with 1 mM ATP at 37°C for 30 min in 100 μl buffer containing 10 mM HEPES-KOH (pH 7.4), 5 mM MgCl₂, and 120 mM KCl. Inhibition by GzmK was performed with 1.5 μM rVCP, 1 mM ATP, and various amounts of GzmK. Reactions were stopped by addition of 200 μl 1% SDS. Samples (0.1 ml) were added to 0.7 ml ascorbate-molybdate reagent, which contains 1% (w/v) ascorbic acid, 1% (w/v) molybdate, and 6 parts of (w/v) ammonium molybdate in 1 N H₂SO₄. Tubes were incubated at 45°C for 20 min, and absorbance was determined at 820 nm.

Intracellular ubiquitinated protein quantification assay

For the analysis of intracellular ubiquitinilated protein, HeLa cells (2 × 10⁸) were treated with GzmK, GzmA, or GzmB plus Ad for the indicated times at 37°C in loading buffer. The reaction samples were run in 5% SDS loading buffer, resolved on SDS-PAGE, and detected by immunoblotting using a ubiquitin Ab. Ubiquitinilated proteins were analyzed by fluorescence microscopy. Briefly, Jurkat cells were treated with the indicated concentrations of GzmK plus Ad for the indicated times, and then plated on polylys-coated slides. After being fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized with 0.05% Triton X-100 for another 5 min, cells were incubated at room temperature for 1 h with 5 μg/ml anti-ubiquitin mAb (Abcam, Cambridge, MA) and 50 μg/ml donkey serum, and then washed twice with PBS. The cells were stained with Alexa488-conjugated donkey anti-mouse IgG. The slides were mounted and observed using a fluorescence microscope (Olympus FV500, Melville, NY).

xbp1 mRNA splicing assay

Total RNA of the treated cells was harvested using TRIzol reagent (Invitrogen). cDNA was synthesized by random hexamers and Superscript III (Invitrogen). The xbp1 mRNA was amplified by using the primers 5′-CTG-GAAAGCAATGCTTTAGA-3′ and 5′-CTGCTGCTCTTCTGGTACG-3′. PCR fragments of the spliced (XBP1s) and the unspliced xbp1 (XBP1u) were visualized through 2% agarose gels with ethidium bromide staining.
Apoptosis assay
Target cells treated with S-AGzmK or GzmK, GzmA, or GzmB plus Ad were stained with annexin V-FTTC and propidium iodide (PI) for flow cytometry using a FACScalibur (BD Biosciences, San Jose, CA). The data were analyzed with CellQuest software.

The cyt c release detection
GzmK-treated cells were fractionated with 0.025% digitonin for 5 min on ice. Mitochondrial (pellet) and cytosolic (supernatant) fractions were obtained for cyt c detection by immunoblotting.

Results
VCP associates with GzmK
Previously, we identified several substrates for GzmK, including SET, Ape1, and HMGB2, which also are in the GzmA-associated SET complex (7, 8). To further investigate other substrates, an inactive GzmK mutant (S-AGzmK) was immobilized with Affi-gel10 resin and passed through HeLa cell lysates for affinity chromatography assay. The S-AGzmK column was eluted with NaCl concentration gradients. After elution with 500 mM NaCl, the eluted fractions were visualized by SDS-PAGE gels and followed by silver staining. The two major bands (97 and 37 kDa) were eluted, as shown in Fig. 1A. The naked resin did not bind to any proteins when washed with >200 mM NaCl. These two bands were cut out and trypsin digested for mass spectrometry analysis. From the peptide fingerprints, 19 experimental masses matched the theoretical masses for VCP with 32.5% protein coverage and 6 theoretical masses for SET with 22.02% protein coverage. We provide complete information for the detected peptides for VCP and SET (Supplemental Table). SET was a previously identified substrate for GzmK, thus confirming the reliability of our experimental system.

To further examine the direct interaction of VCP with GzmK, rVCP was incubated with S-AGzmK for communoprecipitation assay. The anti-GzmK Ab can precipitate rVCP, and the anti-VCP Ab can also precipitate S-AGzmK (Fig. 1C). However, Ig employed as control did not pull down either rVCP or S-AGzmK. S-AGzmK column was eluted with NaCl concentration gradients. After elution with 500 mM NaCl, the eluted fractions were visualized by SDS-PAGE gels and followed by silver staining. The two major bands (97 and 37 kDa) were eluted, as shown in Fig. 1A. The naked resin did not bind to any proteins when washed with >200 mM NaCl. These two bands were cut out and trypsin digested for mass spectrometry analysis. From the peptide fingerprints, 19 experimental masses matched the theoretical masses for VCP with 32.5% protein coverage and 6 theoretical masses for SET with 22.02% protein coverage. We provide complete information for the detected peptides for VCP and SET (Supplemental Table). SET was a previously identified substrate for GzmK, thus confirming the reliability of our experimental system.

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VCP is a substrate of GzmK and cleaved at Arg713
To test whether VCP is cleaved by GzmK, 1 μM rVCP was incubated with different concentrations of GzmK at 37°C for 2 h and probed by anti-VCP Ab. VCP began to be degraded at a low concentration of 0.1 μM GzmK (Fig. 2A). GzmK processed rVCP in a dose-dependent manner, and rVCP was mostly degraded with 1 μM GzmK. By 10 min, rVCP started to be degraded after 0.5 μM GzmK treatment and GzmK degraded rVCP in a time-dependent manner (Fig. 2A). Even with a high concentration of 2 μM S-AGzmK, rVCP was uncleaved. Thus, VCP cleavage requires the enzymatic activity of GzmK. The smaller cleaved products were undetectable in the above experiments, even with Coomassie staining (data not shown). The 17-kDa fragment might be further degraded by GzmK. The cleaved product (∼80 kDa) was the N-terminal truncated fragment determined by N-terminal sequencing. We then determined the cleavage site of VCP using site-directed mutagenesis. Because GzmK cleaves its substrate after Arg or Lys, we generated 22 VCP mutations of Arg or Lys to Ala based on the size of the cleavage product. All mutant VCP proteins were expressed and treated with GzmK and visualized by SDS-PAGE, followed by Coomassie staining. We found that only the R713A mutant was resistant to GzmK cleavage (Fig. 2B), whereas the R700A mutant and others were still cleaved by GzmK (Fig. 2B and data not shown). Cleavage after arginine is consistent with the trypsin activity of GzmK.

To assess whether GzmK degrades native VCP, HeLa cell lysates (2 × 10⁶ cell equivalents) were incubated with different doses of GzmK for 2 h or incubated for different times with 1 μM GzmK. VCP was degraded by GzmK in a dose- and time-dependent manner (Fig. 2C). The same blot was stripped and reprobed for SET and β-actin. The known GzmK substrate SET was cleaved by GzmK with similar kinetics (Fig. 2C, right panel). β-actin was unchanged as a good negative control. To further verify that VCP processing is physiologically relevant, HeLa cells were treated with the indicated concentrations of GzmK in the presence of Ad at 37°C for 6 h. VCP could be cleaved in a dose-dependent manner (Fig. 2D). The truncated 80-kDa products were undetectable in GzmK-loaded cells, which may be due to its low concentration, instability, or degradation by other factors. β-actin was unchanged as a good loading control. Similar results were obtained in Jurkat cells (Fig. 2D, right panel). Furthermore, we found that VCP could be cleaved in a time-dependent manner after loading GzmK (Fig. 2E). The same blot was stripped and reprobed for SET and β-actin. SET was also processed by GzmK with similar kinetics. Our previous study compared several delivery agents (perforin, Ad, SLO, or protein delivery reagent Pro-lect) for the GzmK-loading assay (7). We found that utilizing Ad to load GzmK into target cells results in similar dead features and cleavage of its substrates as perforin-delivered GzmK. To confirm whether VCP can be degraded by GzmK with the physiological delivery protein perforin, we loaded GzmK into target HeLa cells with perforin or SLO at 37°C for 4 h to detect VCP cleavage. Our results indicate that VCP was also cleaved (Fig. 2F). Additionally, the pan caspase inhibitor z-VAD (100 μM) was preincubated with HeLa cells prior to GzmK loading with Ad. We found that z-VAD did not affect VCP cleavage by GzmK (Fig. 2G), although the same concentration of z-VAD could completely inhibit GzmB-induced caspase 3 processing of p21 to p19 (Fig. 2H). However, z-VAD did not affect procaspase 3 processing to p21 by GzmB. β-actin was unchanged as a good loading control. To further determine whether Bid-mediated cyt c release participates in VCP cleavage, Bcl-2 was overexpressed in HeLa cells to examine VCP degradation after GzmK loading (Fig. 2I, left panel). Compared with the vector-transfected cells, Bcl-2 overexpression could not affect VCP cleavage (Fig. 2I, right panel). These results indicate that VCP is directly cleaved by GzmK in its recombinant and native forms in cell lysates or GzmK-loaded target cells.

VCP of target tumor cells is degraded in LAK cell-mediated cytolysis
LAK cells can use perforin/Gzm and Fas/Fas ligand pathways to kill target cells. We pretreated HeLa cells with the inhibitor of cytotoxic granule pathway concanamycin A prior to coincubation with LAK cells, concanamycin A could disrupt LAK cell-mediated cytolysis (data not shown). This finding and our previous report indicate that LAK cell-mediated killing is mainly induced by the perforin/Gzm pathway (11). To further investigate whether VCP is cleaved in cytolysis induced by killer lymphocytes, HeLa cells were incubated with LAK cells at 37°C for 4 h at different E:T ratios. After incubation with HeLa cells for 4 h, LAK cells were still suspended and removed from the plate wells. Treated HeLa cells were harvested by washing with PBS buffer three times and followed by immunoblotting. VCP in HeLa cells was apparently degraded at the E:T ratios 10:1 and 20:1, just slightly cleaved at the E:T ratio 5:1 (Fig. 3A); ~60%–70% HeLa cells at the E:T ratio 20:1 were dead through trypan blue staining. GzmK was expressed in the LAK cells, and anti-GzmK mAb did not react with noncytolytic
FIGURE 1. VCP associates with GzmK. A, HeLa cell lysates were applied to immobilized S-AGzmK affinity column through eluting by gradient NaCl. Naked resin was used as a negative control. B, VCP coelutes with the substrate SET. The two major bands (97 and 37 kDa) were identified as VCP and SET by mass spectrometry. Nineteen trypsin-digested peptides matched theoretical masses for the VCP, and six matched theoretical masses for the SET. C, rVCP binds directly to S-AGzmK. rVCP and S-AGzmK were coincubated in PBS at 4˚C and immunoprecipitated with Abs of GzmK (left), VCP (right), or control Abs. The blots were probed by anti-VCP (left) or anti-GzmK (right) Abs through immunoblotting. D, S-AGzmK can coprecipitate native VCP in the HeLa cell lysates. HeLa cell lysates (2 × 10^5 equivalents) were incubated with S-AGzmK for immunoprecipitation, as in C. FT, flow through; IB, immunoblotting; IP, immunoprecipitation.
FIGURE 2. GzmK cleaves VCP after Arg713 in its recombinant and native forms. A, rVCP is cleaved by GzmK in a dose- and time-dependent fashion. A total of 1 μM rVCP was incubated at 37°C for 2 h with different concentrations of GzmK or 0.5 μM GzmK for the indicated times. Reactions were stopped by 5× SDS loading buffer and probed by anti-VCP Ab. B, GzmK cleaves VCP at Arg713. Purified wild-type, R700A, or R713A recombinant proteins were treated with GzmK and visualized by SDS-PAGE, followed by Coomassie staining. C, GzmK cleaves native VCP in cell lysates. HeLa cell lysates (2 × 10⁶ cell equivalents) were treated with different doses of GzmK at 37°C for 2 h or 1 μM GzmK for the indicated times. The known substrate SET was probed as a positive control. β-actin was unchanged as a loading control. D, GzmK cleaves VCP in cells permeabilized by Ad. HeLa or Jurkat cells were treated with the indicated concentrations of GzmK plus Ad for 6 h. The whole-cell lysates were analyzed by immunoblotting. The same blot was stripped and reprobed by anti-β-actin Ab. β-actin was unchanged as a loading control. E, GzmK cleaves VCP in cells permeabilized by Ad with a time-dependent manner. HeLa cells were treated with 1 μM GzmK plus Ad for the indicated times and probed for the VCP and SET by Western blot analysis. β-actin was unchanged as a loading control. F, VCP cleavage occurs in GzmK-loaded HeLa cells with the perforin or the SLO. HeLa cells were incubated with optimal doses of perforin or SLO plus 1 μM GzmK for 6 h; the whole-cell lysates were analyzed by immunoblotting. G, The caspase pan inhibitor z-VAD cannot abolish GzmK-induced VCP cleavage. HeLa cells were pretreated with or without 100 μM z-VAD before GzmK loading with Ad. H, The z-VAD blocks caspase 3 activation. HeLa cells were pretreated with or without 100 μM z-VAD before GzmK loading with Ad. The samples were subjected to SDS-PAGE and probed for the VCP and caspase 3, as above. I, GzmK cleaves VCP in Bcl-2–overexpressed HeLa cells. Bcl-2 was overexpressed in HeLa cells (left panel). Bcl-2–overexpressed cells or vector-transfected cells were treated with 1 μM GzmK plus Ad for 6 h and probed with anti-VCP and anti-β-actin Abs, as above (right panel).
GRANZYME K TARGETS VCP TO INDUCE CELL DEATH

GzmK targets the ERAD complex of target tumor cells

VCP localizes in the cytosol and ER and delivers the ubiquitinated proteins to the cytosolic 26S proteasome for degradation (25). VCP tightly interacts with polyubiquitinated proteins in the ERAD pathway. This high-affinity binding results from the synergistic binding of VCP and its cofactor Ufd1-Npl4 complex to the polyubiquitin chains (26, 27). VCP, Ufd1, and Npl4 can form a complex to participate in substrate recognition and degradation (20). We therefore wanted to test whether GzmK also degrades the other two ERAD complex components, Ufd1 and Npl4. HeLa cell lysates (2 × 10⁵ cell equivalents) were incubated with different doses of GzmK for 2 h or incubated for different times with 1 μM GzmK. Ufd1 and Npl4 were degraded by GzmK in a time- and dose-dependent manner (Fig. 4A, 4B). To further verify that Ufd1 and Npl4 processing is physiologically relevant, HeLa cells were treated with 1 μM GzmK plus Ad at 37°C for 6 h. These two proteins were cleaved in GzmK-loaded cells (Fig. 4C). The same blot was stripped and reprobed for β-actin. β-actin was unchanged as a good loading control. Taken together, GzmK is able to target the ERAD complex.

GzmK abolishes the ATPase activity of VCP and accelerates ubiquitinated protein accumulation leading to xbp1 splicing

VCP belongs to the AAA family with type II AAA-ATPase activity and regulates many cellular functions. The VCP molecule consists of an N-terminal domain and two ATPase domains named D1 and D2, and these two ATPase domains have different roles in ATPase activity. The D2 domain accounts for the major ATPase activity (15, 28). Residue Arg713 in the GzmK cleavage site is located in a disordered loop (705–731) of the D2 domain. This disordered loop will undergo a dramatic structural change to be more ordered during ATP hydrolysis, a process that is of significant importance for the ATPase activity of the D2 domain (29). We next wanted to determine whether GzmK cleavage interferes with the ability of VCP to hydrolyze ATP. To directly study the ATPase activity, we used a colorimetric assay to measure the Pi release from ATP hydrolysis. Incubation of rVCP with ATP resulted in increasing free phosphates in a dose-dependent manner (Fig. 5A). Preincubation of rVCP (1.5 μM) with GzmK abolished its ATPase activity in a dose-dependent fashion (Fig. 5B). A total of 1.5 μM GzmK completely blocked ATPase activity of rVCP, whereas inactive S-AGzmK exhibited no such effect.

Ubiquitinated protein accumulation in the ER will increase ER stress and trigger cell death (30, 31). VCP provides its ATPase activity and forms a complex with the Ufd1-Npl4 heterodimer to take part in the ERAD pathway for misfolded protein degradation (25, 32). We demonstrated that VCP cleavage by GzmK destroyed its ATPase activity, so we next wanted to assess whether GzmK induces ubiquitinated protein accumulation in target cells. Jurkat cells were treated with buffer, proteasome inhibitor MG132, and 0.5 or 1 μM GzmK plus Ad, respectively, and visualized by immunofluorescence assay with anti-ubiquitin Ab. GzmK caused a dramatic accumulation of intracellular ubiquitinated protein (Fig. 5C). MG132 was used as a positive control. Similar results were obtained through immunoblotting assay (Fig. 5D). The anti-ubiquitin Ab was more sensitive for immunofluorescence staining than Western blot analysis. Additionally, the molecular weights of ubiquitinated proteins were so large, and those proteins were mainly localized on the top of the gel, that it was hard to detect the difference from the gel. Therefore, we quantified these band signals in Fig. 5D by densitometry comparison and marked them. GzmB or GzmA could not significantly induce accumulation of ubiquitinated proteins. These data are representative of at least three separate experiments. Ubiquitinated protein accumulation required GzmK delivery by Ad, as cells treated with either GzmK or Ad alone had no effect. Ad-mediated delivery of the inactive S-AGzmK also had no observable effect (data not shown).

Accumulation of unfolded or misfolded proteins in the ER can initiate the unfolded protein response (UPR) to cause a transcriptional induction pathway for clearing the cytotoxic proteins (33, 34). These three factors include protein kinase R-like ER kinase, activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1), which are initially induced during the UPR. The transcription factor XBP1 is a target of ATF6, and its mRNA is spliced by IRE1 in response to an ER stress (35). We examined xbp1 mRNA splicing in GzmK-treated HeLa cells. HeLa cells were treated with 1 μM GzmK plus Ad or 20 μM MG132 for 4 h at 37°C. xbp1 mRNA was spliced after GzmK treatment (Fig. 5E). MG132 was used to initiate an ER stress and make xbp1 mRNA splicing to generate a spliced form (XBP1s). xbp1 mRNA could not be processed to maintain a total unspliced form (XBP1u) in the mock-treated cells. GzmB or GzmA could not significantly cause xbp1 splicing. β-actin mRNA was amplified as a control. Together, these results indicate that GzmK can cause ubiquitinated protein accumulation and trigger ER stress in target cells.

VCP silencing facilitates GzmK-mediated cell death

To determine whether VCP cleavage affects GzmK-mediated cell death, we silenced VCP expression in HeLa cells (Fig. 6A). We designed three shRNA sequences against VCP mRNA sequence and constructed pSUPER-VCP-shRNA plasmids. Three days after transfection, VCP expression was reduced by 70% by one shRNA as shown in Fig. 6A. β-actin was unchanged in either empty vector-
transfected or pSUPER-VCP-shRNA–transfected cells. To assess whether VCP knockdown accelerates GzmK-induced cell death, GzmK was introduced into VCP-silenced HeLa cells. The HeLa cells were incubated for VCP by immunoblotting. The same blot was stripped and probed by anti–β-actin Ab. β-actin was unchanged as a good loading control. The native GzmK is present in LAK cells. The activated LAK cells (5 × 10^3) by 1000 U/ml IL-2 were probed with anti-GzmK Ab. rGzmK was used as a positive control. The 293A cells were used as a negative control. C, VCP is only cleaved by GzmK, not other Gzms. HeLa cell lysates (2 × 10^5 equivalents) were incubated with 1 μM GzmB, GzmH, GzmM, GzmA, or GzmK at 37°C for 2 h. NM23H1 was probed as a negative control. D, All Gzms have enzymatic activity. The enzymatic activities were detected by cleavage of their synthetic substrates. The relative activity was analyzed by comparing the active Gzms to their solution buffers. Ac-YRFK-pNA (GzmK), Suc-VANR-pNA (GzmA), Z-IETD-AFC (GzmB), Suc-FLF-pNA (GzmH), and Suc-AAPL-pNA (GzmM). E, GzmA or GzmB does not cleave the native VCP. HeLa cell lysates (2 × 10^5 equivalents) were incubated with 1 μM GzmA (left panel) or different doses of GzmB (right panel) at 37°C for 2 h. VCP, caspase 3, SET, β-actin, or NM23H1 was probed by immunoblotting. SET cleavage was used as a positive control, and NM23H1 was used as a negative control for GzmA (left panel). Caspase 3 activation was used as a positive control, and β-actin was used as a negative control for GzmB (right panel). F, The GzmK inhibitor Ac-YRFK-CMK abolishes VCP cleavage in the GzmK-loaded or attacked HeLa cells. HeLa cells were incubated with different ratios of GzmK inhibitor to GzmK for 1 h before treatment with GzmK (1 μM) plus Ad for 6 h (left panel). VCP and SET were detected by immunoblotting. β-actin was unchanged as a loading control. IL-2–activated LAK cells (the indicated E:T ratios) were incubated with HeLa cells at 37°C for 4 h (right panel). For GzmK blocking, HeLa cells were pretreated with 200 μM GzmK inhibitor prior to incubation with IL-2–activated LAK cells. HeLa cells were isolated and probed for VCP by immunoblotting. The same blot was stripped and probed by anti–β-actin Ab. I:K ratio, GzmK inhibitor to GzmK molar ratio.

FIGURE 3. VCP is degraded in LAK cell-mediated cytolysis and only hydrolyzed by GzmK. A, The native VCP is cleaved in LAK cell-mediated cytotoxicity. HeLa cells were incubated with effector LAK cells at E:T ratios of 5:1, 10:1, and 20:1 at 37°C for 4 h. The LAK cells were removed from the HeLa cells. The HeLa cells were detected for VCP by immunoblotting. The same blot was stripped and probed by anti–β-actin Ab. β-actin was unchanged as a good loading control. B, The native GzmK is present in LAK cells. The activated LAK cells (5 × 10^5) by 1000 U/ml IL-2 were probed with anti-GzmK Ab. rGzmK was used as a positive control. The 293A cells were used as a negative control. C, VCP is only cleaved by GzmK, not other Gzms. HeLa cell lysates (2 × 10^5 equivalents) were incubated with 1 μM GzmB, GzmH, GzmM, GzmA, or GzmK at 37°C for 2 h. NM23H1 was probed as a negative control. D, All Gzms have enzymatic activity. The enzymatic activities were detected by cleavage of their synthetic substrates. The relative activity was analyzed by comparing the active Gzms to their solution buffers. Ac-YRFK-pNA (GzmK), Suc-VANR-pNA (GzmA), Z-IETD-AFC (GzmB), Suc-FLF-pNA (GzmH), and Suc-AAPL-pNA (GzmM). E, GzmA or GzmB does not cleave the native VCP. HeLa cell lysates (2 × 10^5 equivalents) were incubated with 1 μM GzmA (left panel) or different doses of GzmB (right panel) at 37°C for 2 h. VCP, caspase 3, SET, β-actin, or NM23H1 was probed by immunoblotting. Caspase 3 activation was used as a positive control, and β-actin was used as a negative control for GzmB (right panel). F, The GzmK inhibitor Ac-YRFK-CMK abolishes VCP cleavage in the GzmK-loaded or attacked HeLa cells. HeLa cells were incubated with different ratios of GzmK inhibitor to GzmK for 1 h before treatment with GzmK (1 μM) plus Ad for 6 h (left panel). VCP and SET were detected by immunoblotting. β-actin was unchanged as a loading control. IL-2–activated LAK cells (the indicated E:T ratios) were incubated with HeLa cells at 37°C for 4 h (right panel). For GzmK blocking, HeLa cells were pretreated with 200 μM GzmK inhibitor prior to incubation with IL-2–activated LAK cells. HeLa cells were isolated and probed for VCP by immunoblotting. The same blot was stripped and probed by anti–β-actin Ab. I:K ratio, GzmK inhibitor to GzmK molar ratio.
results represent at least three independent experiments. Because VCP is a physiological substrate for GzmK, we next wanted to determine whether VCP silencing affects other Gzm-induced cell death. We found that VCP-silenced HeLa cells were not markedly susceptible to GzmA- or GzmB-induced cell death (Fig. 6E, 6F). Taken together, VCP silencing is susceptible to GzmK-induced cell death.

VCP overexpression protects cells from GzmK-induced cell death

Because VCP is an important substrate for GzmK, we next wanted to assess whether overexpression of VCP in target cells protects against GzmK-induced cytotoxicity. We overexpressed VCP in target HeLa cells, as shown in Fig. 7A. VCP-overexpressed HeLa cells were treated with 1 μM GzmK plus Ad for 6 h and followed by flow cytometry analysis. GzmK caused 41.9% dead cells in VCP-overexpressed HeLa cells (Fig. 7B). In contrast, the empty vector-transfected cells resulted in 61.6% dead cells. VCP overexpression was not significantly resistant to GzmA- or GzmB-mediated death (Fig. 7C). Taken together, the results suggest that VCP overexpression is only resistant to GzmK-induced cell death, not GzmA or GzmB.

Discussion

GzmK and GzmA are the only two tryptases among all the five Gzms in humans that are required for NK/CTL-mediated cytotoxicity. Recently, we showed that GzmK induces rapid caspase-independent cell death with ssDNA nicks (36). After loading into target cells, GzmK targets the ER-associated SET complex, resulting in the degradation of some important components such as SET, Ape1, and HMGB2. SET cleavage activates the NM23H1 DNase and causes ssDNA nicks. We previously demonstrated that Ape1 can antagonize ROS generation as an antiredox protein (8). Ape1 cleavage by GzmK abolishes its redox function and triggers ROS accumulation. GzmK can target mitochondria by processing Bid to truncated Bid, leading to release of cyt c and other proapoptotic factors (9, 10). GzmK also cleaves the tumor suppressor p53 at Lys24 and Lys305 to generate three cleavage fragments, which harbor strong proapoptotic activities to sensitize target tumor cells to GzmK-mediated cytotoxicity (11). To elucidate the mechanisms of GzmK-induced cell death, we further identified other physiological substrates for GzmK by using affinity chromatography. We found that VCP is a physiological substrate for GzmK. GzmK enters the cytoplasm of the target cell and targets the components of the ERAD complex (VCP, Npl4, and Ufd1) to disrupt the ERAD pathway, leading to ER stress (Fig. 7D). GzmK also degrades SET to release the DNase NM23H1, enabling it to enter the nucleus for DNA nicking. The ER stress and DNA nicking induce the target cell to undergo caspase-independent cell death.

VCP forms a complex with the Ufd1-Npl4 heterodimer that is essential for the ERAD pathway, a process by which constituent and transient ER proteins are removed from the ER and degraded in the cytosolic 26S proteasome (37). VCP is required to dislocate proteins from the ER to the cytosol in the ERAD pathway. It contains four functional domains, as follows: the N-terminal, D1, D2, and C-terminal domains. The N-terminal domain (aa 1–187) can bind the polyubiquitinated substrates and cofactors (27). The D1 domain (208–459) mainly stabilizes its conformation. The D2 domain (481–761) significantly inhibits the ATPase activity at physiological temperatures, whereas mutation in D1 does not. In contrast, mutation in D2, but not D1, impairs the heat-stimulated ATPase activity (28). DeLabarre and colleagues (38) demonstrated that some major residues in D2 mediate the VCP activity for the ERAD. The C-terminal domain (761–806) is responsible for membrane fusion and nuclear translocation. Our results showed that GzmK can cleave VCP after Arg713, which is localized in the D2 domain that mediates main ATPase activity. VCP cleavage by GzmK at Arg713 certainly destroys the structural feature near the disordered loop (705–731) and disturbs the conformational changes in this region.

FIGURE 4. GzmK targets other ERAD complex components Ufd1 and Npl4. A, GzmK cleaves native Ufd1 and Npl4 in cell lysates with a time-dependent manner. HeLa cell lysates (2 × 10^5 cell equivalents) were treated with 1 μM GzmK at 37°C for the indicated times; the reaction was stopped by loading buffer and analyzed using anti-Ufd1 and anti-Npl4 Abs, respectively. The same blot was stripped and reprobed by anti–β-actin Ab. β-actin was unchanged as a negative control. B, GzmK cleaves native Ufd1 and Npl4 in cell lysates in a dose-dependent manner. HeLa cell lysates (2 × 10^5 cell equivalents) were treated with the indicated concentrations of GzmK at 37°C for 2 h; the cleavage of Ufd1 and Npl4 was tested, as in A. C, GzmK cleaves Ufd1 and Npl4 in cells permeabilized by Ad. HeLa cells were treated with 1 μM GzmK plus Ad for 6 h. The whole-cell lysates were analyzed by immunoblotting. The same blot was stripped and reprobed by anti–β-actin Ab. β-actin was unchanged as a negative control.

8 GRANZYME K TARGETS VCP TO INDUCE CELL DEATH

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supposed to occur during the ATP hydrolysis. Indeed, VCP degradation by GzmK abolishes its ATPase activity.

VCP acts as a molecular chaperon and cooperates with the ubiquitin-proteasome system in mediating misfolded protein degradation. Many newly synthesized proteins are not properly folded and will be ubiquitinated and removed from the ER to the 26S proteasome for degradation. VCP hydrolyzes ATP and provides energy in this process. Abolition of its ATPase function by GzmK dramatically accelerates ubiquitinated protein accumulation. VCP recognizes ubiquitinated substrates and cofactors in the ER. Silenced VCP induces ubiquitinated protein accumulation, leading to xbp1 splicing (data not shown), which is in agreement with pre-
previous reports (32, 39). Accumulation of unfolded or misfolded proteins in the ER can initiate the UPR to cause a transcriptional induction pathway for clearing the cytotoxic proteins (33, 34). In this study, we found that GzmK can rapidly enter into the cytosol after being introduced into target cells (data not shown). GzmK can target VCP and the ERAD complex to abolish its biological functions. Moreover, GzmK can cleave the other two ERAD complex components, Ufd1 and Npl4. Cleavage of Ufd1 and Npl4 will further disrupt the ERAD pathway for ubiquitinated protein degradation and accelerate an ER stress. The transcription factor XBP1 is a target of ATF6, and its mRNA is spliced by IRE1 in response to an ER stress (35).

**FIGURE 6.** VCP silencing accelerates GzmK-mediated cell death. A, VCP was silenced in HeLa cells by transfection of pSUPER empty vector or pSUPER-VCP-shRNA plasmids for 3 d. Equal amounts of whole-cell lysates were subjected to Western blot analysis for anti-VCP Ab. The numbers represent the ratio of VCP to β-actin signal by densitometry compared with the pSUPER-treated cells. The pSUPER-treated cells were normalized to 1. B, VCP silencing accelerates GzmK-induced cell death. VCP-silenced HeLa cells and control cells were treated with 0.5 or 1.0 μM GzmK plus Ad at 37˚C for 4 h and stained with annexin V-FITC and PI, followed by flow cytometry. C, Total dead cells were calculated by annexin V and PI single-positive as well as annexin V/PI double-positive cells shown as means ± SD. **p** < 0.01. D, VCP silencing accelerates GzmK-induced cyt c release. VCP-silenced and control HeLa cells were treated with 1 μM GzmK plus Ad at 37˚C for 6 h. Supernatant and pellet fractions were separated for cyt c detection by immunoblotting. The supernatant β-actin or pellet COXIV was probed as a control. These data are representative of at least three separate experiments. E and F, VCP silencing does not affect GzmA- or GzmB-mediated cell death. VCP-silenced HeLa cells were treated with 1 μM GzmA plus Ad (E) or the indicated concentrations of GzmB plus Ad at 37˚C for 4 h (F) and followed by flow cytometry. Total dead cells were calculated by annexin V and PI single-positive as well as annexin V/PI double-positive cells shown as means ± SD (right panels). Supern, supernatant.
FIGURE 7. VCP overexpression protects target cells from GzmK-induced cell death. A, Overexpression of VCP in HeLa cells. HeLa cells were transfected with pcDNA3.1-VCP or pcDNA3.1 vector for 30 h and harvested for probing with anti-VCP Ab. β-actin was probed as a control. The numbers represent the ratio of VCP to β-actin signal by densitometry compared with the pcDNA3.1 vector-treated cells; the pcDNA3.1 vector-treated cells were normalized to 1. B, VCP overexpression protects HeLa cells from GzmK-induced cell death. VCP-overexpressed or pcDNA3.1 vector-transfected HeLa cells were treated with 1.0 μM GzmK plus Ad at 37˚C for 6 h and stained with annexin V-FITC and PI, followed by flow cytometry (left panel). Total dead cells were calculated by annexin V and PI single-positive as well as annexin V/PI double-positive cells shown as means ± SD. **p < 0.01, right panel. C, VCP overexpression does not influence GzmA- or GzmB-induced cell death. VCP-overexpressed or pcDNA3.1 vector-transfected HeLa cells were treated with 1.0 μM GzmA or 0.3 μM GzmB plus Ad for 4 h, respectively, and stained with annexin V-FITC and PI, followed by flow cytometry (left panel). Total dead cells were calculated by annexin V and PI single-positive as well as annexin V/PI double-positive cells shown as means ± SD (right panel). D, A schematic model for GzmK targeting the ERAD complex. NK/CTL granules containing perforin and Gzms are released into the immunosynapse formed with the target cell. GzmK enters the cytoplasm of target cell and targets the components of ERAD complex (VCP, Npl4, and Ufd1), leading to an ER stress. GzmK also degrades SET to release the DNase NM23H1, enabling it to enter the nucleus for DNA nicking. The ER stress and DNA nicking force the target cell to death.
Previously, we demonstrated that GzmK induced a rapid generation of ROS and collapse of mitochondrial inner membrane potential (8, 9). GzmK trafficking in the target cells needs further investigation via three-dimensional methodology. GzmK cleaves the antioxidant protein Apel to facilitate ROS accumulation in target cells (8). Accumulating evidence suggests that protein folding and production of ROS are closely linked events (40). The ER is an organelle in which proper folding and disulfide formation of proteins are dependent on the redox status within the lumen of the ER. ROS generation will destroy the redox status in the ER and promote unfolded and misfolded protein accumulation and increase an ER stress. In contrast, an ER stress may elicit Ca^{2+} leakage into the cytosol and increase ROS generation in mitochondria. This cross-talk will initiate caspase-independent cell death.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References

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## Supplementary table
**Complete information of the detected peptides for VCP and SET by mass spectrometry**

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