Granzyme K Displays Highly Restricted Substrate Specificity That Only Partially Overlaps with Granzyme A*

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Granzymes are serine proteases stored in cytolistic granules of cytotokic lymphocytes that eliminate virus-infected and tumor cells. Little is known about the molecular mechanism and function of granzyme (Gr)K. GrK is similar to GrA in that they are the only granzymes that display tryptase-like activity. Both granzymes induce cell death by single-stranded nicking of the chromosomal DNA by cleaving the same components of the endoplasmic reticulum-associated SET complex. Therefore, GrK may provide a backup and failsafe mechanism for GrA with redundant specificity. In the present study, we addressed the question of whether GrK displays identical substrate specificity as GrA. In peptide- and protease-proteomic screens, GrK and GrA displayed highly restricted substrate specificities that overlapped only partially. Whereas GrK and GrA cleave SET with similar efficiencies likely at the same sites, both granzymes cleaved the pre-mRNA-binding protein heterogeneous ribonuclear protein K with different kinetics at distinct sites. GrK was markedly more efficient in cleaving heterogeneous ribonuclear protein K than GrA. GrK, but not GrA, cleaved the microtubule network protein β-tubulin after two distinct Arg residues. Neither GrK cleavage sites in β-tubulin nor a peptide-based proteomic screen revealed a clear GrK consensus sequence around the P1 residue, suggesting that GrK specificity depends on electrostatic interactions between exosites of the substrate and the enzyme. We hypothesize that GrK not only constitutes a redundant functional backup mechanism that assists GrA-induced cell death but that it also displays a unique function by cleaving its own specific substrates.

Important players in the immune defense against tumor cells and virus-infected cells are cytotoxic T lymphocytes and natural killer cells (1, 2). These immune cells predominantly destroy their target cells by releasing the content of their cytolistic granules, containing the pore-forming protein perforin and a set of serine proteases known as granzymes. In humans, five different granzymes (GrA, GrB, GrH, GrK, and GrM)2 have been identified that all induce cell death by cleaving critical intracellular substrates. Although GrA and GrB have been extensively studied, little is known about the functions and mechanisms of the other granzymes.

The GrA cell death pathway is characterized by single-stranded DNA damage, apoptotic morpholgy, mitochondrial dysfunction, and loss of cell membrane integrity and occurs independent of caspases and the GrB-induced apoptotic routes (1–5). GrA is targeted inside the mitochondrion (6), where it triggers an increase in reactive oxygen species and loss of transmembrane potential (3, 5). After mitochondrial damage, GrA targets a 270–440-kDa endoplasmic reticulum-associated complex (SET complex) that contains three GrA substrates, i.e. nucleosome assembly protein SET (4), DNA-binding protein HMGT-2 (7), and base excision repair enzyme Ape1 (8). Cleavage of SET by GrA allows the SET complex component DNase NM23H1 to make single-stranded nicks in the chromosomal DNA. GrA also facilitates DNA damage by cleavage of Ape1 and the double-stranded DNA repair protein Ku70 (9). GrA is a highly specific serine protease in that only ~10 substrates have been identified and verified as physiological substrates within cells (1, 2).

Far less is known about the molecular mechanism and function of GrK. GrK is similar to GrA in that they are closely linked on the same chromosome and that they are the only granzymes that display tryptase-like activity, i.e. both granzymes cleave after basic residues Arg and Lys (10). This tryptase-like activity contributes to cytotoxic lymphocyte-induced target cell death (11). GrA-deficient mice or cytotoxic lymphocytes thereof have normal expression levels of GrK and display almost normal cytolytic activity against tumor targets (12, 13). Therefore, it is believed that GrK provides a backup and failsafe mechanism for GrA with redundant specificity. Indeed, recent investigations have indicated that GrK induces cell death with similar hallmarks as GrA. GrK triggers rapid caspase-independent cell death with nuclear morphological changes, single-stranded DNA nicks, and reactive oxygen species production from mito-

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2 The abbreviations used are: Gr, granzyme; 2D-DIGE, fluorescent two-dimensional difference gel electrophoresis; hnRNP K, heterogeneous nuclear ribonucleoprotein K; MS, mass spectrometry; SA, serine to alanine mutation; Chaps, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
Proteomic Profiling of Granzyme Substrate Specificity

In the present study, we addressed the question of whether GrK indeed displays identical substrate specificity as GrA. We employed peptide- and protease-proteomic screens to show that GrK and GrA display highly restricted substrate specificities that overlap only partially. Whereas three set complex components are cleaved by both GrA and GrK likely at the same sites (14), we show that both granzymes also cleave the pre-mRNA-binding protein heterogeneous ribonuclear protein K (hnRNP K) with different kinetics at different sites. Furthermore, we demonstrate that GrK, but not GrA, cleaves the microtubule network protein β-tubulin after two distinct Arg residues. These data suggest that GrK not only constitutes a redundant functional backup mechanism that assists GrA-induced cell death, but that it also displays a unique function by cleaving its own specific substrates.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Reagents—Jurkat cells were grown in RPMI 1640 medium, supplemented with 10% fetal calf serum, 0.002 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Cell-free protein extracts were generated from exponentially growing Jurkat cells. The cells (10⁶ cells/ml) were washed two times in a buffer containing 50 mM Tris (pH 7.4) and 150 mM NaCl and lysed in the same buffer by three cycles of freeze-thawing. The samples were centrifuged for 10 min at 14,000 rpm (18,400 g) for 10 min. Cell-free extracts (75 µg) were incubated with GrA (1 µM), GrK (1 µM), GrA-SA (1 µM), or GrK-SA (1 µM). After 1 h at 37 °C, the samples were precipitated using the Plus One two-dimensional clean-up kit as recommended by the manufacturer (GE Healthcare) and solubilized in 8 M urea, 2 M thiourea, 4% Chaps, 300 mM dithiothreitol, 2% biotinylated pH 3–10, and 0.004% bromophenol blue (75 µl). Granzyme- and control-treated samples (50 µl of a concentration of 1 µg/µl) were labeled with 400 pmol of either 1-(5-carboxyphenyl)-1'-propylindocarboxyanine halide N-hydroxysuccinimidyl ester (Cy3) or 1-(5-carboxyphenyl)-1'-methylindolodicarboxyanine halide N-hydroxysuccinimid ester (Cy5). Mixtures (1:1) of both samples were labeled with 3-[(4-carboxymethyl)phenylmethyl]-3'-ethyloxcarboxyanine halide N-hydroxysuccinimid ester (Cy2), which functions as internal control. The labeling reactions were stopped by adding 0.2 mM lysine, diluted with rehydration buffer (8 M urea, 2 M thiourea, 4% Chaps, 150 mM dithiothreitol, 1% biotinylated pH 3–10, and 0.002% bromophenol blue), and combined according to the experimental design. The samples (150 µg) were rehydrated passively into immobilized pH gradient strips (24 cm; pH 3–10, nonlinear) for 15 h at room temperature prior to isoelectric focusing in the IPGphor system (GE Healthcare) for 64 kVh. Immobilized pH gradient (IPG) strips were reduced for 60 min in 2% (w/v) dithiothreitol, 6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, 0.375 M Tris, pH 8.8, and alkylated for 30 min in the same buffer containing 2% (v/v) iodoacetamide instead of dithiothreitol. The strips were overlaid to a 12% SDS-PAGE gel (20 × 24 cm), immobilized to a low fluorescent glass plate and electrophoresed for 18 h at 1 W/gel. The Cy2-, Cy3-, and Cy5-labeled images were acquired on a Typhoon 9400 scanner (GE Healthcare) at the following excitation/emission values 488/520, 532/580, and 633/670 nm, respectively. Each condition was performed at least four times, and a dye swap was included to exclude preferentially labeled proteins from the analysis. The relative quantification of matched gel features was performed by using Decyder DIA and BVA software (GE Healthcare). For inter-gel analyses, the internal standard method was used as described (18). Statistical analysis of gel spot quantitation was performed by Student’s t test. p < 0.05 was regarded as statistically significant.
Proteomic Profiling of Granzyme Substrate Specificity

significant. Two-dimensional gels were post-stained by mass spectrometry (MS)-compatible Flamingo staining (Bio-Rad).

**Tandem Mass Spectrometry**—Selected spots were excised robotically (Etan Dalt Spot Cutter; GE Healthcare), and in-gel proteolytic digestion of stained spots was performed essentially as described (19) using trypsin (Roche Applied Science). The samples were subjected to nanoflow liquid chromatography (Agilent 1100 series) and concentrated on a C18 precolumn (100-μm inner diameter, 2 cm). Peptides were separated on an analytical column (75-μm inner diameter, 20 cm) at a flow rate of 200 nL/min with a 60-min linear acetonitrile gradient from 0 to 80%. The liquid chromatography system was directly coupled to a quadrupole time-of-flight (QTOF) Micro tandem mass spectrometer (Micromass Waters, UK). A survey scan was performed from 400–1200 atomic mass units s⁻¹, and precursor ions were sequenced in tandem MS mode at a threshold of 150 counts. The data were processed and subjected to data base searches using Proteinlynx Global Server version 2.1 (Micromass) or MASCOT software version 2.1 (Matrixscience) against SWISSPROT and the NCBI nonredundant data base, with a 0.25-Da mass tolerance for both precursor ion and fragment ion. The identified peptides were confirmed by manual interpretation of the spectra.

**PepChip Protease Analysis**—PepChip Protease arrays were used to determine the extended substrate preference of GrK and GrA. PepChip Protease arrays contain 1,000 random 15-mer peptides and 24 control peptides, each biotin-labeled at the free C terminus via a peptide bond, allowing proteolytic cleavage between the final residue and biotin. The peptides were coupled to the microarray surface via the N terminus and printed in triplicate on microarrays. The reaction mixtures containing GrA (2 μM), GrA-SA (2 μM), GrK (2 μM), and GrK-SA (2 μM) in 20 mM Tris (pH 7.4) and 150 mM NaCl, or buffer alone were incubated on duplicate PepChip Protease arrays, covered with a 25 × 60-mm coverslip for 6 h at 37 °C in a water-saturated incubator. Following incubation, the microarrays were washed three times for 5 min at room temperature in phosphate-buffered saline and 0.1% Tween 20. Biotinylated peptides were detected with fluorescein-labeled streptavidin (DAKO), diluted 1:25 in phosphate-buffered saline, incubated at room temperature for 1 h, followed by washing three times for 5 min at room temperature in phosphate-buffered saline and 0.1% Tween 20. After rinsing with distilled water, the arrays were dried under streaming nitrogen and scanned. The scans were quantified, and the peptide mean values were calculated. The ratios between control (inactive granzyme mutant) and digested (active granzyme) means and digested (active granzyme) means from at least four significant peptide spot values were calculated. The ratios from means that differ at least two standard deviations were considered statistically significant.

**RESULTS**

**GrK and GrA Cleave SET with Similar Efficiency**—We have purified recombinant mature GrK, GrA, and catalytically inactive variants thereof as controls (GrK-SA and GrA-SA). SDS-PAGE analysis confirmed that purified GrA forms the typical dimer, whereas GrK exists as a monomer (Fig. 1A) (20–22). Both granzymes were active because they showed reactivity toward Arg and Lys synthetic chromogenic substrates (data not shown) (10). In agreement with the literature (9, 14, 16), incubation of tumor cell lysates with GrK or GrA resulted in dose-dependent cleavage of SET, which was illustrated by the progressive disappearance of the SET protein band (44 kDa) and the appearance of a 25-kDa cleavage product (p25) (Fig. 1B). Both cleavage patterns were similar, strongly suggesting that GrK and GrA cleave SET at the same site (9, 14, 16). These data indicate that recombinant mature GrK and GrA are active and cleave SET with similar efficiency.

**GrK and GrA Display Restricted Partially Overlapping Macromolecular Substrate Specificities**—We used fluorescence 2D-DIGE in combination with tandem MS to compare the macromolecular substrate specificities of GrK and GrA (Fig. 2). This protease-proteomic screen scans the native proteome of tumor cells for macromolecular substrates of the granzymes. Human tumor cell lysates were incubated with GrK, GrA, or their corresponding inactive controls. Control- and corresponding granzyme-treated lysates were subsequently labeled with green fluorescent-dye (Cy3) and red fluorescent-dye (Cy5), respectively. Both samples were combined and separated on the same two-dimensional gel. Fig. 2 shows representative examples of GrK (Fig. 2A) and GrA (Fig. 2B). A pool of all samples labeled with Cy2 was included as internal standard, allowing adequate comparison between gels. The gels were sequentially scanned at excitation and emission wavelengths specific for each fluorescent label, and the images were overlaid digitally. Spots present in greater abundance in the control sample appeared green and indicate possible granzyme substrates, whereas spots present in greater abundance in the granzyme-treated sample appear red, reflecting the appearance of
Granzyme A (GrA) and Granzyme K (GrK) cleave similar proteins: 22 spots were identified as potential GrA substrates and 14 spots were identified as potential GrK substrates. These Proteomic Profiling of Granzyme Substrate Specificity

These proteins at the same P1 cleavage sites. Changed spots (Fig. 2) were excised, and we were able to identify 14 protein spots from two-dimensional gels, employing tandem MS. The identity of potential novel GrA and GrK substrates is summarized in Table 1. The substrates that are shared by both granzymes are actin, golgin subfamily A member 2, and hnRNP K. A unique substrate of GrK is β-tubulin, and a unique substrate of GrA is histidine carboxylase. Several spots that consistently changed following granzyme treatment could not be identified, most likely because the protein levels were too low. Taken together, these data indicate that GrK and GrA display restricted macromolecular substrate specificities that overlap only partially.

GrK and GrA Cleave hnRNP K with Different Kinetics at Distinct Sites—One protein that was consistently cleaved by both GrK and GrA is the pre-mRNA-binding protein hnRNP K, a member of the hnRNP family of proteins that directly interact with DNA and RNA through their K homology domains. hnRNP K is indispensable for cell survival and is involved in regulating gene expression at multiple levels, including transcription, RNA splicing, and translation (23). Fig. 3 shows gel images and three-dimensional representations from control and granzyme-treated samples of the spots identified as hnRNP K. GrK completely cleaved hnRNP K, whereas GrA only partially cleaved hnRNP K during the incubation period (Fig. 3A), suggesting differential kinetics. This was confirmed by incubating both granzymes with tumor cell lysate and monitoring hnRNP K cleavage kinetics by immunoblotting. Indeed, GrK was markedly more efficient in cleaving hnRNP K as compared with GrA (Fig. 3B). Different hnRNP K cleavage products were observed following the incubation with GrK and GrA (Fig. 3B), indicating that both granzymes cleave hnRNP K at distinct sites. The ~37-kDa hnRNP K cleavage fragment induced by

potential cleavage products (not affected proteome will appear yellow). Approximately 1500 proteins were resolved in each proteomic screen. For GrK, 14 spots disappeared (~0.9%) (intact substrates), and 22 spots appeared (cleavage products) following the incubation of tumor cell lysate with GrK (Fig. 2C) (log peak volume change >1.5-fold, p < 0.05). For GrA, 30 spots disappeared (~2%) and 55 spots appeared following the incubation of tumor cell lysate with GrA (Fig. 2C) (log peak volume change >1.5-fold, p < 0.05). The relatively low number of decreased spots for GrK (~0.9%) and GrA (~2%) indicate that macromolecular substrate specificities of both granzymes are highly restricted and that GrK has an ~2-fold more restricted substrate specificity as compared with GrA. Six proteins were cleaved and shared by both granzymes, indicating that the macromolecular substrate specificities of both granzymes partially overlap in ~16%. Of the cleavage products that appear during granzyme incubation, 14 spots are shared by both granzymes, strongly suggesting that GrA and GrK cleave

were combined and separated by two-dimensional gel electrophoresis. Protein spots shared between the two samples appear yellow. Protein spots that are reduced in abundance after protease digestion (protease substrates) are green, and new spots (cleavage products) that appear after protease digestion are red. B, Jurkat tumor cell freeze/thaw lysates (100 μg) were incubated with 1 μM GrA (labeled red) or 1 μM GrK-SA (labeled green) for 60 min at 37 °C. The samples were combined and separated by two-dimensional gel electrophoresis. Protein spots shared between the two samples appear yellow. Protein spots that are reduced in abundance after protease digestion (protease substrates) are green, and new spots (cleavage products) that appear after protease digestion are red. B, Jurkat tumor cell freeze/thaw lysates (100 μg) were incubated with 1 μM GrA (labeled red) or 1 μM GrK-SA (labeled green) for 60 min at 37 °C. These experiments were repeated four times. Spot numbers correspond to the numbered spots in Table 1. C, GrK/GrK-SA and GrA/GrA-SA gels were matched, and GrK-SA/GrA log peak volume ratios (x axis) were plotted against GrA-SA/GrA log peak volume ratios (y axis). Changed protein spots are labeled green (intact substrates), red (cleavage fragments), and yellow (not affected proteome). Protein spot intensity was regarded changed when peak volume ratios were >1.5-fold (dotted lines) and p < 0.05.

FIGURE 2. GrK displays restricted macromolecular substrate specificity that only partially overlaps with GrA. A, Jurkat tumor cell freeze/thaw lysates (100 μg) were incubated with 1 μM GrK (labeled red) or 1 μM GrK-SA (labeled green) for 60 min at 37 °C. The samples were combined and separated by two-dimensional gel electrophoresis. Protein spots shared between the two samples appear yellow. Protein spots that are reduced in abundance after protease digestion (protease substrates) are green, and new spots (cleavage products) that appear after protease digestion are red. B, Jurkat tumor cell freeze/thaw lysates (100 μg) were incubated with 1 μM GrA (labeled red) or 1 μM GrK-SA (labeled green) for 60 min at 37 °C. These experiments were repeated four times. Spot numbers correspond to the numbered spots in Table 1. C, GrK/GrK-SA and GrA/GrA-SA gels were matched, and GrK-SA/GrA log peak volume ratios (x axis) were plotted against GrA-SA/GrA log peak volume ratios (y axis). Changed protein spots are labeled green (intact substrates), red (cleavage fragments), and yellow (not affected proteome). Protein spot intensity was regarded changed when peak volume ratios were >1.5-fold (dotted lines) and p < 0.05.

TABLE 1

| Spot number | Protein identity | Shared with spot number | Change | Swiss-Prot accession number |
|-------------|------------------|-------------------------|--------|-----------------------------|
| 1           | Actin            | 10                       | Increase | P63261                       |
| 2           | Actin            | 11                       | Increase | P63261                       |
| 3           | β-Tubulin        | Decrease                 | Q13509  |
| 4           | β-Tubulin        | Increase                 | Q13509  |
| 5           | β-Tubulin        | Increase                 | Q13509  |
| 6           | Golgin subfamily A member 2 | 14 | Increase | Q08379                      |
| 7           | Heterogeneous nuclear ribonucleoprotein K | 12 | Decrease | P61978                       |
| 8           | Heterogeneous nuclear ribonucleoprotein K | Increase | P61978 |
| 9           | Actin            | Decrease                 | P63261  |
| 10          | Actin            | 1                        | Increase | P63261                       |
| 11          | Actin            | 2                        | Increase | P63261                       |
| 12          | Heterogeneous nuclear ribonucleoprotein K | 7 | Decrease | P61978                       |
| 13          | Histidine decarboxylase | Increase | P19113  |
| 14          | Golgin subfamily A member 2 | 6 | Increase | P08379                      |
GrK was also detected by 2D-DIGE, during GrK-mediated proteolysis of hnRNP K (Figs. 2A and 3C). Because the anti-hnRNP K antibody used in Western blotting is directed against residues 100–200 (Fig. 3B), and because MS analysis of the 37-kDa cleavage product (Fig. 2A, spot 8) revealed peptides in the N-terminal region of hnRNP K (Fig. 3D), the detected cleavage product likely represents the N-terminal moiety of hnRNP K. To investigate whether hnRNP K is a direct granzyme substrate, rather than being a substrate of a secondary protease present in tumor cells that is activated by GrK, we expressed and purified recombinant human His-hnRNP K. As shown in Fig. 3E, both granzymes directly cleaved purified His-hnRNP K, and for GrK the 37-kDa cleavage fragment was again evident. Because of extremely low soluble recombinant hnRNP K expression by *E. coli*, we were unable to identify granzyme cleavage sites by N-terminal sequencing. These data indicate that GrK and GrA directly cleave hnRNP K as a novel substrate with different kinetics at distinct sites.

**The Microtubule Network Component β-Tubulin Is a Specific Substrate of GrK**—One protein that was consistently cleaved by GrK, but not by GrA, and followed from our 2D-DIGE proteomic screen is the microtubule network component β-tubulin. This protein controls the integrity of the microtubule network and plays an essential role in cell survival. Previously, we and others have shown that GrM and GrB directly target the counterpart of the tubulin dimer, i.e. α-tubulin (17, 24, 25). Fig. 4 shows images and three-dimensional representations from control and granzyme-treated samples of the spots identified as

**FIGURE 3.** GrK and GrA cleave the pre-mRNA-binding protein hnRNP K with different kinetics at distinct sites. A, representative gel images and three-dimensional representations of hnRNP K during treatment with GrK (spot 7) and GrA (spot 12). B, Jurkat tumor cell freeze/thaw lysates (10 μg) were incubated with GrK (1 μM), GrK-SA (1 μM), GrA (1 μM), GrA-SA (1 μM), or buffer for indicated time points at 37 °C. The samples were immunoblotted, using an antibody directed against human hnRNP K (66 kDa). C, representative gel images and three-dimensional representations of one hnRNP K cleavage product during treatment with GrK (spot 8). D, sequences identified by tandem MS. Light gray (spots 7 and 12) and dark gray (spot 8) shaded text represent the peptides identified in protein spots. E, purified recombinant human His-hnRNP K (100 ng) was incubated with GrK (0–500 nM), GrK-SA (500 nM), GrA (0–500 nM), GrA-SA (500 nM), or buffer for 2 h at 37 °C. The samples were immunoblotted, using an antibody directed against human hnRNP K (66 kDa). The 37-kDa cleavage product is indicated.

**FIGURE 4.** GrK, but not GrA, cleaves the microtubule component β-tubulin. A, representative gel images and three-dimensional representations of β-tubulin during treatment with GrK (spot 3) and GrA. B, representative gel images and three-dimensional representations of the 42-kDa β-tubulin cleavage fragment induced by GrK (Spot 4). C, representative gel images and three-dimensional representations of the 35-kDa β-tubulin cleavage fragment induced by GrK (Spot 5). D, Jurkat tumor cell freeze/thaw lysates (10 μg) were incubated with GrK (1 μM), GrK-SA (1 μM), GrA (1 μM), GrA-SA (1 μM), or buffer for indicated time points at 37 °C. The samples were immunoblotted, using an antibody directed against human β-tubulin.

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**The Microtubule Network Component β-Tubulin Is a Specific Substrate of GrK**—One protein that was consistently cleaved by GrK, but not by GrA, and followed from our 2D-DIGE proteomic screen is the microtubule network component β-tubulin. This protein controls the integrity of the microtubule network and plays an essential role in cell survival. Previously, we and others have shown that GrM and GrB directly target the counterpart of the tubulin dimer, i.e. α-tubulin (17, 24, 25). Fig. 4 shows images and three-dimensional representations from control and granzyme-treated samples of the spots identified as
β-tubulin. GrK, but not GrA, cleaved β-tubulin after 1 h of incubation (Fig. 4A), suggesting that β-tubulin is a GrK-specific substrate. A 42-kDa (Fig. 4B, spot 4) and a 35-kDa (Fig. 4C, spot 5) β-tubulin cleavage fragment appeared in our 2D-DIGE screen following GrK treatment of tumor cell lysate (Fig. 2A). This was confirmed by incubating both granzymes with tumor cell lysate, and β-tubulin cleavage was monitored by immunoblotting. Indeed, β-tubulin was only cleaved by GrK (Fig. 4D). Also a β-tubulin cleavage fragment was detected by Western blot (18 kDa), representing the C-terminal moiety of β-tubulin because the antibody reacts with this part of the protein. These results indicate that GrK, but not GrA, cleaves the microtubule network component β-tubulin in tumor cell lysates.

**GrK Directly Cleaves β-Tubulin after Arg^62 and Arg^282.—**To investigate whether β-tubulin is a specific and direct GrK substrate, rather than being a substrate of a secondary protease present in tumor cells, purified granzymes or their inactive mutants were incubated with purified recombinant human His-β-tubulin. Whereas GrA-treated and control-treated His-β-tubulin remained intact, treatment of purified β-tubulin with increasing concentrations of GrK resulted in the progressive disappearance of the ~52-kDa His-β-tubulin protein and the appearance of four major cleavage products of ~18, 25, 35, and 42 kDa (Fig. 5, A and B). The 42- and 35-kDa β-tubulin cleavage fragments were also detected using 2D-DIGE (Figs. 2 and 4), and the 18-kDa β-tubulin cleavage fragment was detected by immunoblotting (Fig. 4D). This cleavage event already occurred at low nanomolar concentrations of GrK (≤5 nM) and relatively high tubulin concentrations (1 μM), indicating that GrK efficiently cleaves β-tubulin. Under these conditions, neither GrA nor GrK cleaved the microtubule counterpart α-tubulin (Fig. 5, C and D), which is a direct substrate of GrM (17) and GrB (24, 25). N-terminal sequencing of the GrK-mediated β-tubulin cleavage fragments indicated that GrK cleaves β-tubulin at two sites, i.e. Arg^62 and Arg^282. These potential cleavage sites were confirmed by generating three β-tubulin variants in which Arg^62, Arg^282, or a combination thereof was mutated into Ala. Although with different cleavage fragment patterns, both single β-tubulin mutants were still cleaved by GrK with similar apparent kinetics, suggesting that both sites are equally important (Fig. 6A). In contrast, the double mutant completely was resistant for GrK proteolysis, indicating that GrK cleaves β-tubulin at two sites, i.e. Arg^62 in the sequence YVPR^AV and Arg^282 in the sequence QQYR↓AL. Cleavage by GrK after Arg^62 and Arg^282 bisects the two functional domains of β-tubulin (Fig. 6B), and these cleavage sites are conserved in all
human β-tubulin isoforms (Fig. 6C). Apart from P1’ Ala and P4’ Val, the amino acid sequences around P1-Arg and P1-Arg do not show any similarities or a consensus sequence that is shared by both GrK cleavage sites.

**GrK and GrA Prefer P1 Arg and Display Broad Partially Overlapping Extended Substrate Specificities**—It has been well established that both GrK and GrA cleave after an Arg or Lys residue at the P1 position (nomenclature for amino acid positions in substrates is Pn-P2-P1-P1’-P2’-Pn’, with amide bond hydrolysis occurring after P1) (10). Using positional scanning synthetic combinatorial libraries of 4-amino acid peptides, P4-P2 specificities for both GrK and GrA appear broad but distinct, and no clear consensus sequence has been identified (10) (Fig. 6). This technology, however, defines protease substrate sequence preferences that are limited to 4 amino acids and non-prime positions N-terminal to the scissile bond. In the present study, we have developed a novel method to study the extended substrate specificity of proteases that has the potential to study prime sites and nonprime sites that go beyond P4. We have employed a library of 1,000 fully randomized 15-amino acid peptides to compare the extended substrate specificities of human GrK and GrA. The peptide library was incubated with GrK, GrA, or their corresponding inactive variants. Peptides that were cleaved by GrK or GrA are summarized in Table 2. Both GrK and GrA cleaved peptides that are rich in Arg but not Lys residues (Table 2), indicating a preference for Arg over Lys at the P1 position for both granzymes. GrK cleaved 17 peptides (>25% signal reduction and p < 0.05), whereas GrA cleaved 13 peptides (>25% signal reduction and p < 0.05) (Table 2). One peptide was cleaved by GrA probably at the Arg-biotin peptide bond. Why GrA cleaved one peptide that lacks a basic amino acid remains unclear. Interestingly, cleavage of six peptides was shared among both granzymes, indicating that GrK and GrA display overlapping but also unique extended substrate specificities (Table 2). This is consistent with the results obtained from our 2D-DIGE screen (Fig. 2). Following alignment of peptides that harbor a single Arg or Lys residue, however, no clear consensus sequence either N- or C-terminal of the P1 scissile bond could be identified for both granzymes (Table 2). For GrA, there is a tendency for a polar residue at P10 and hydrophobic residues at the P2 and P4 positions. For GrK, there is a tendency for polar residues at P8, P10, and P12, whereas nonpolar hydrophobic residues seem to be preferred at P2 (aromatic), P4, P7 (aliphatic), and P2’ (aliphatic) (Table 2). The lack of a consensus sequence is consistent with known cleavage sites in macromolecular substrates of GrA (7, 20) or GrK (this study) (Fig. 6), suggesting that specificity largely comes from tertiary structure elements. These data indicate that both GrK and GrA prefer P1 Arg and display partially overlapping extended substrate specificities, albeit with no clear amino acid preference at subsites near the cleavage site.

**DISCUSSION**

Among all the granzymes, GrK and GrA are the only two tryptases. This tryptase activity is required for cytotoxic lymphocytes to kill their target cells (11). GrA-deficient cytotoxic cells still contain tryptase activity and normal levels of GrK, and their cytolytic activity is just slightly reduced (12, 13). Therefore, GrK might be a likely candidate to rescue or cooperate with GrA. During the past few years, evidence has been collected that GrK indeed mimics GrA actions. Like GrA, GrK efficiently induces caspase-independent cell death, characterized by mitochondrial and DNA damage. Although the nature of mitochondrial damage remains controversial (2, 14, 15, 26), GrK induces nuclear fragmentation, nuclear condensation, and single-stranded DNA breaks by direct inactivation of three SET complex members that are also cleaved by GrA (2, 14). In this study, we addressed the question of whether GrK indeed displays identical substrate specificity as GrA. We employed peptide arrays and proteome-wide proteomic screens to show that both GrK and GrA display highly restricted substrate specificities that overlap only partially in ∼16% (Fig. 2 and Table 2). Whereas three SET complex components are cleaved by both GrA and GrK likely at the same sites (14) (Fig. 1), we confirmed that both granzymes also cleave the pre-mRNA-binding protein hnRNPK with different kinetics at different sites (Fig. 3). Furthermore, we demonstrate that GrK, but not GrA, cleaves the microtubule network protein β-tubulin after two distinct Arg residues (Figs. 4–6). Apparently, despite their tryptase
activity, both granzymes largely have evolved their own substrate specificity. These data allow us to hypothesize that GrK not only constitutes a redundant functional backup mechanism that assists GrA-induced cell death, but it also displays a unique function by cleaving its own specific substrates.

The proteomic 2D-DIGE method revealed only a limited set of potential GrK (n = 14, ~0.9%) and GrA (n = 30, ~2%) substrates (Fig. 2 and Table 1). The relatively small number of granzyme-induced cleavage events detected in a tumor cell proteome indicates that GrK and GrA display restricted macromolecular substrate specificity. Remarkably, whereas GrB requires a tripeptide synthetic substrate to observe cleavage and is maximal with an idealized tetrapeptide, GrA and GrK readily cleave single amino acid substrates (data not shown) (27, 28). This indicates that the macromolecular substrate specificities of GrA and GrK are not restricted to the active site and fully depend on (i) extended binding sites close to the active site cleft (subsites) that interact with residues around P1 in the substrate and/or (ii) interactions between exosites of the substrate and the enzyme. If subsites around the P1 residue were to play a major role in granzyme specificity, one would expect that GrA and GrK cleave a restricted subset of peptides with considerable primary amino acid sequence homology in our PepChip Protease peptide arrays. Indeed, whereas >50% of the peptides harbor an Arg or Lys residue, both granzymes cleaved only 1–2% of the peptides with significant efficiency (Table 2). Although 1,000 randomly selected 15-mer peptides represent only a small fraction of all possible combinations, we do not find a stringent consensus motif around the P1 cleavage sites in these peptides (Table 2). Likewise, Mahrus and Craik (10) used combinatorial libraries of 4-amino acid peptides to show that P4-P2 subsite specificities of both GrK and GrA appear broad but distinct, and unlike GrB and GrM, no clear P4–P2 consensus sequence could be identified. In agreement with this, GrA and GrK cleave oligo-peptide synthetic substrates only slightly better than single amino acid (Arg or Lys) substrates (27, 28). The lack of a clear consensus sequences around the P1 scissile bond further is consistent with known cleavage sites in macromolecular substrates of GrA (7, 20) or GrK (β-tubulin; Fig. 6). These notions strongly suggest that macromolecular substrate specificity of GrK and GrA largely comes from tertiary structure elements, i.e. exosite–exosite interactions between granzyme and substrate. The GrK molecule is highly positively charged with multiple positively charged patches on its surface (22). These patches could provide additional (electrostatic) docking sites for selection and presentation of substrates to the active site region. This hypothesis is supported by our preliminary observations that the β-tubulin–GrK interaction is completely suppressed with increasing ionic strength, whereas the amidolytic activity of GrK toward a single-amino acid Lys synthetic substrate is only modestly affected by the addition of NaCl.3 A comparable exosite model has also been proposed for the electrostatic interaction between GrA and its substrate SET (21). Therefore, differences in macromolecular substrate specificity between GrK and GrA might, at least in part, be explained by differences in the primary sequence of exosite surface loops (Fig. 7). In this context, it should be noted that GrK is a monomer (22), whereas the active form of GrA exists as a dimeric structure (20, 21) (Fig. 1A). GrA dimer formation is believed to further contribute to GrA specificity in a unique manner by extending the active site cleft (20, 21), a quaternary structural arrangement that is not shared by GrK.

We have identified four novel potential substrates of both GrK and GrA by MS (Table 1). Remarkably, our proteomic screen did not detect one of the known GrK or GrA substrates, including members of the SET complex (1, 2). Although two-dimensional gel electrophoresis is potentially capable of resolving several thousand individual protein spots on a single gel, not all proteins could be visualized because of extremes of molecular weight or charge or because of overlapping gel features in some areas. Furthermore, low abundance proteins are presumably not detected at all. In addition, some of the known granzyme substrates may have been detected on the gels but could not be identified by tandem MS. Nevertheless, our study has demonstrated the feasibility of 2D-DIGE as a potent profiling method to compare macromolecular substrate preferences of different proteases.

The precise physiological role of β-tubulin and hnRNP K cleavage identified in this study is not clear. Both proteins play important roles in cellular physiology that make them potentially relevant for cell survival. β-Tubulin is a component of the microtubule network that is responsible for cell survival, mitosis, motility, maintenance of cell shape, cell signaling, and intracellular trafficking of macromolecules, vesicles, and organelles (29). Down-regulation of α-tubulin by RNA interference or attacking the dynamic behavior of the microtubule network by well established anti-cancer drugs limits mitotic potential and results in the death of tumor cells (24, 29). Normally, α-tubulin assembles with β-tubulin, and this dimer forms polymers to create the microtubule network (29). We and others have previously demonstrated that GrM and GrB cleave α-tubulin, resulting in impaired tubulin polymerization that likely contributes to tumor cell death (17, 24, 25). Strikingly, whereas GrK did not cleave α-tubulin, we showed that GrK efficiently cleaved the β-tubulin counterpart of the tubulin dimer at two distinct sites (Figs. 5 and 6). This raises the possibility that cleavage of β-tubulin by GrK represents a novel cell death pathway of this granzyme that is different from GrA. Apparently,

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3 N. Bovenschen and J. A. Kummer, unpublished results.
multiple granzymes specifically target the different components of microtubule network, and this may be a critical event during cytotoxic lymphocyte-induced death of tumor cells. Knockdown of hnRNP K results in cell death, suggesting that it is an indispensable protein for cell survival (30). hnRNP K mediates translational silencing by binding specific 3′-untranslated region sequences (23, 31); therefore its cleavage could potentially inhibit or rescue the translation of proteins involved in killing of the cell. Indeed, Venables et al. (32) recently demonstrated that down-regulation of hnRNP K promoted a mixture of exon skipping and exon inclusion events, affecting important domains of apoptotic proteins like apoptotic peptidase activating factor 1 and the caspase recruitment domain of NLR family, pyrin domain-containing 1 (32). Whether or not cleavage of hnRNP K by GrA and/or GrK contributes to tumor cell death remains an intriguing question that deserves further study. Next to their roles in cell survival, it has been well established that host cell hnRNP K and microtubules are indispensable for viral entry, replication, and exit (33, 34). Granzymes play a significant role in the elimination of virus-infected cells in vivo (1, 2). This opens the possibility that granzyme-mediated disruption of hnRNP K and/or microtubule function terminates viral production in infected cells during natural killer cell attack.

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