Analysis of Protein Processing by N-terminal Proteomics Reveals Novel Species-specific Substrate Determinants of Granzyme B Orthologs*

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Using a targeted peptide-centric proteomics approach, we performed in vitro protease substrate profiling of the apoptotic serine protease granzyme B resulting in the delineation of more than 800 cleavage sites in 322 human and 282 mouse substrates, encompassing the known substrates Bid, caspase-7, lupus La protein, and fibrillarin. Triple SILAC (stable isotope labeling by amino acids in cell culture) further permitted intra-experimental evaluation of species-specific variations in substrate selection by the mouse or human granzyme B ortholog. For the first time granzyme B substrate specificities were directly mapped on a proteomic scale and revealed unknown cleavage specificities, uncharacterized extended specificity profiles, and macromolecular determinants in substrate selection that were confirmed by molecular modeling. We further tackled a substrate hunt in an in vivo setup of natural killer cell-mediated cell death confirming in vitro characterized granzyme B cleavages next to several other unique and hitherto unreported proteolytic events in target cells. Molecular & Cellular Proteomics 8:258–272, 2009.

Because macromolecular properties present in protease substrates guide cleavage recognition, specificity, and efficiency beyond canonical cleavage sites, the necessity to determine protease substrates directly in a natural proteome and even in a species-specific context strikingly became important to fully elucidate proteolytic actions. Together with recent advances in the development of protease-targeted activity-based probes, systematic high throughput methods with broad applicability for the identification of (individual) in vitro and in vivo protease substrate repertoires have recently emerged (1).

Granzyme B (GrB), a serine protease that recognizes aspartic acid in the substrate P1 position, is contained within the secretory granules of cytotoxic T lymphocytes and natural killer (NK) cells (2) and gains entry into transformed or virally infected target cells by the pore-forming protein perforin (3). Once delivered in targets cells, GrB can promote apoptosis either by activation of the caspase cascade (4) or by directly cleaving substrate proteins (5–10). Next to a few reported extracellular (11) and viral substrates (12) only about 60 possible cellular mammalian GrB substrates have been identified to date mainly by non-systematic approaches. Only for a few of these, physiological relevance was shown and occasionally in a species-specific context (13–15) as it was only recently found that human and mouse granzyme B signal via overlapping as well as distinct apoptotic pathways.

The substrate specificity of mouse, human, and rat GrB was profiled previously by positional scanning combinatorial libraries of short tetrapeptides from P4 to P1 and, using phage display, for mouse and human GrB somewhat extended to P2 (13, 15–17). By further showing that Bid is a very poor substrate for mGrB, in sharp contrast to its very efficient cleavage by hGrB, contradictory results obtained by using recombinant GrB from different species were elucidated (13–15). Next to GrB, GrA was also reported to display altered substrate specificity and functionality fueled by structural differences between its orthologs (15, 18). Furthermore intracellular serine protease inhibitors (serpins) regulate granzymes in species-specific ways (15, 19).

1 The abbreviations used are: Gr, granzyme; Bid, BH3-interacting domain death agonist; COFRADIC, combined fractional diagonal chromatography; NK, natural killer; PBMC, peripheral blood mononuclear cells; SILAC, stable isotope labeling by amino acids in cell culture; z-DEVD-fmk, N-benzyloxycarbonyl-Asp-Glu-Val-Asp fluoromethyl ketone; z-VAD-fmk, N-benzyloxycarbonyl-Val-Ala-Asp(O-methyl)fluoromethyl ketone; mGrB, mouse granzyme B; hGrB, human granzyme B; FACS, fluorescence-activated cell sorting; AcD₃, trideuterocacetyl.
When mapping the evolutionary history of GrB by phylogenetic tree analysis, the rodent homologs are located further away from human (and other primates) than, for example, dog or cow GrB (Fig. 1A). This pattern also extends to other granzymes (e.g., GrA and GrK), pointing to an altered, species-specific rate of granzyme evolution. Eventually multiple gene duplications after separation of the rodent line have resulted in a high number of paralogs that are subject to high specialization pressure. Interestingly when calculating the phylogenetic tree only over residues that are part of the substrate binding pocket, the evolutionary distance between human and rodent species is substantially reduced (Fig. 1B). 

**Fig. 1.** A and B, phylogenetic relationship of various mammalian granzymes. Phylogenetic lineage trees of selected granzymes (A, B, and K) from seven mammalian species were calculated using the full sequence (A) or the substrate binding pocket residues (B) and rooted with the distant homolog human enteropeptidase precursor. C, ClustalX multiple sequence alignment of mammalian granzymes A, B, and K. Multiple sequence alignment of the substrate binding pocket residues of granzymes A, B, and K from human, chimpanzee, macaque, mouse, rat, dog, and cow is shown. Insertions of non-binding pocket residues are replaced by three or more consecutive gap columns. Positions of residues involved in the formation of active sites are highlighted in red (numbering according to human GrB sequence), conserved residues are marked as *, and highly conserved residues are marked as :.
binding pocket (Fig. 1C), the expected order of similarity (primate < rodent < other) is restored for granzymes A and K but not B (Fig. 1B); this is indicative for considerable alteration in specificity determinants disclosed in GrB orthologs. Indeed although human and mouse GrB have a high overall sequence identity (70% in 231 aligned residues), there are a number of mutations of residues in the binding pocket that can be placed in the context of altered substrate specificity given that discrepancies in cleavage specificity and efficiency between mouse and human GrB have been observed on a restricted substrate set (i.e. the mouse and human substrates Bid and caspase-3).

GrB is known to cleave short synthetic peptide substrates poorly compared with natural proteins (17), and we therefore mapped caspase-independent, hGrB- and mGrB-mediated processing events directly on their natural substrates by proteome-wide analysis using the N-terminal peptide COFRADIC sorting procedure for the discovery of novel (neo)-N termini raised by proteolytic events (20, 21). Besides a high number of uncharacterized cleavages that could only have been discovered by this approach, specificity profiles of mGrB and hGrB revealed specific macromolecular properties in substrates that were confirmed by modeling P9–P8’ consensus peptides within the GrB three-dimensional structures. Data obtained from our in vitro GrB substrate analyses were further extrapolated to the more complex in vivo proteolytic processing present in a cellular setup of K-562 target cells subjected to NK cell-mediated cell death.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human Jurkat cells (TIB-152, American Type Culture Collection [ATCC], Manassas, VA), human K-562 (CCL-243, ATCC), and mouse YAC1 cells (TIB-160, ATCC) were grown in RPMI 1640 medium containing 10% fetal bovine serum (26400-044, Invitrogen). For YAC1 cells, L-arginine to proline conversion was not detectable), 57.5 \( \mu \)M \(^{15} \)N\(^4 \)labeled L-arginine (Cambridge Isotope Laboratories, Andover, MA) (22) at a concentration of 230 \( \mu \)M for Jurkat cells (i.e. 20% of the suggested concentration present in RPMI 1640 medium at which L-arginine to proline conversion was not detectable), 57.5 \( \mu \)M for K-562 cells (5% of the suggested concentration), and 575 \( \mu \)M for YAC1 cells (50% of the suggested concentration). Media were supplemented with 10% dialyzed fetal bovine serum (26400-044, Invitrogen), 100 units/ml penicillin (Invitrogen), and 100 \( \mu \)g/ml streptomycin (Invitrogen). For YAC1 cells, \( \beta \)-mercaptoethanol (M7522, Sigma) was added fresh upon subcultivating cells at a final concentration of 55 \( \mu \)M. Cell populations were cultured at 37 °C and 5% CO\(_2\) for at least six population doublings for complete incorporation of the labeled arginine.

**Reagents**—Recombinant mouse GrB was from Sigma (G9278), and human GrB was from Calbiochem (368043). The caspase-3/7-specific inhibitor N-benzoyloxycarbonyl-Asp-Glu-Val-Asp fluoromethyl ketone (z-DEVD-fmk) and the pan-caspase inhibitor N-benzoyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone (z-VAD-fmk) were used at a final concentration of 100 \( \mu \)M. The granzyme A/B serine protease inhibitor 3,4-dichloroisocoumarin was used at a final concentration of 500 \( \mu \)M (287815, Calbiochem), and cycloheximide (C-6255, Sigma) was used at 20 \( \mu \)g/ml.

**NK Cell Isolation**—PBMC from buffy coats were isolated by gradient centrifugation on Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). Freshly isolated peripheral blood-derived non-activated NK cells were isolated from PBMC by depletion of T lymphocytes, B lymphocytes, and macrophages/monocytes using an NK Cell Isolation kit II and auto-MACS magnetic separator (Miltenyi Biotec, Bergisch-Gladbach, Germany) by negative selection. Their purity was determined by FACS analysis (FACSscan, BD Biosciences) using FITC-conjugated anti-CD3 (130-080-401, Miltenyi Biotec) and phycoerythrin-conjugated anti-CD56 (130-090-755, Miltenyi Biotec) as the percentage of CD56\(^+\)/CD3\(^-\) population. NK cells were purified phe- notypically to >95%. Enriched NK cells from peripheral blood were seeded at 100,000 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 10 mM HEPES, and 100 units/ml recombinant human interleukin-2 (D2050, R&D Systems).

**Co-culture of Cells: in Vivo Setup**—Substrate cleavage following granule-mediated apoptosis was induced by incubating purified NK cells with the Fas-negative target cell line K-562 for 4 h with an effector:target ratio of 0.5:1. This occurred in the presence of the caspase-3/7 inhibitor (z-DEV-fmk) and the pan-caspase inhibitor (z-VAD-fmk) both at a concentration of 50 \( \mu \)M and 20 \( \mu \)g/ml cycloheximide. Postlytic artifacts were monitored (and excluded) by co-lysis of co-cultured and control cells in the presence of protease inhibitors (Complete protease inhibitor mixture tablet (11697498001, Roche Diagnostics) and the granzyme A/B-specific inhibitor 3,4-dichloroisocoumarin (final concentration, 500 \( \mu \)M) in lysis buffer (50 mM sodium phosphate buffer, pH 7.5, 100 mM NaCl, 1% CHAPS, and 0.5 mM EDTA). After lysis for 5 min on ice, guanidinium hydrochloride was added to reach a final concentration of 4 M. Isolation of N-terminal peptides by COFRADIC was performed as described previously (20, 23).

**Confirmation of Uncharacterized GrB-mediated Cleavage Specificity via hGrB Treatment of Synthesized Peptides**—The following peptides holding hGrB cleavage sites were synthesized using Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry on an Applied Biosystems 433A Peptide Synthesizer: AQGVISASASNLDDFY, AQGVISA-

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before mixing. Isolation of N-terminal peptides by COFRADIC and sample processing was performed as described previously (20, 24).

Mass Spectrometric Analysis and Peptide Identification by Mascot—ESI LC-MS/MS analysis was performed as described before (25). ESI-Q-TOF MS/MS peptide fragmentation spectra were converted to pkf files using the MassLynx® software (version 4.1, Waters Corp.), and ESI-ion trap MS/MS spectra were converted to mgf files using the Automation Engine software (version 3.2, Bruker). Peptides were identified using a locally installed version of the Mascot database search engine version 2.1 (Matrix Science) and the Swiss-Prot (version 53.2 of UniProtKB/Swiss-Prot protein database, containing 269,293 sequence entries of which 16,602 and 13,316 entries, respectively, originated from human and mouse) and TrEMBL databases (version 35.0 of UniProtKB/TrEMBL protein database, containing 3,874,166 sequence entries comprising 53,146 human and 52,403 mouse entries) were searched with restriction to human or mouse proteins. Truncated peptide databases made by DBToolkit (26) were searched in parallel to pick up protein processing events more efficiently (e.g. Refs. 20 and 21). The following search parameters were used. Peptide mass tolerance was set at 0.2 Da, and peptide fragment mass tolerance was set at 0.1 Da with the ESI-Q-TOF as the selected instrument for peptide fragmentation rules for the Q-TOF Premier data. For ion trap data, peptide mass tolerance was set at 0.5 Da, and peptide fragment mass tolerance was set at 0.5 Da with the ESI ion trap as the selected instrument for peptide fragmentation rules. Endoproteinase Arg-C/P was set as the enzyme with a maximum number of one missed cleavage. Variable modifications were set to methionine oxidation, pyroglutamate formation of N-terminal glutamine, pyrocarbamidomethyl formation of N-terminal alkylated cysteine, deamidation of asparagine, acetylation of the N terminus, and z-DEVD-fmk-mediated inhibition of downstream caspase-3 activity (31) (supplemental Fig. 1). To reduce changes in protein conformation, cells were lysed by freeze-thawing. Recombinant mouse and human GrB was added to lysates in concentrations that resulted in an overall comparable efficiency of cleavage of species-specific caspase-3 (supplemental Fig. 2). In addition, efficient cleavage of mouse and human Bid was mediated by hGrB as opposed to mGrB (supplemental Fig. 2 and supplemental Table 1). These observations are consistent with previous reports using active site-titrated human and mouse GrB and z-DEVD-fmk. Western analysis demonstrated z-VAD-fmk-mediated inhibition of downstream caspase-3 activity (31) (supplemental Fig. 1). To reduce changes in protein conformation, cells were lysed by freeze-thawing. Recombinant mouse and human GrB was added to lysates in concentrations that resulted in an overall comparable efficiency of cleavage of species-specific caspase-3 (supplemental Fig. 2). In addition, efficient cleavage of mouse and human Bid was mediated by hGrB as opposed to mGrB (supplemental Fig. 2 and supplemental Table 1). These observations are consistent with previous reports using active site-titrated human and mouse GrB and z-DEVD-fmk.

**Phylogenetics of Mammalian Granzymes—** Homologs of granzymes A, B, and K were collected from the National Center for Biotechnology Information (NCBI) non-redundant database (27) using BLAST (Basic Local Alignment Search Tool) (28) and aligned with ClustalX (29). Phylogenetic trees were created using PhyloWin (30) with the bootstrapped (1,000 steps) neighbor-joining method and observed divergence as distance measurement (Table I).

**RESULTS**

**Cataloging Substrates of Protease Orthologs—** Potential physiological GrB targets were probed in cell-free lysates preconcentrated with the pan-caspase inhibitors z-VAD-fmk and z-DEVD-fmk. Western analysis demonstrated z-VAD-fmk-mediated inhibition of downstream caspase-3 activity (31) (supplemental Fig. 1). To reduce changes in protein conformation, cells were lysed by freeze-thawing. Recombinant mouse and human GrB was added to lysates in concentrations that resulted in an overall comparable efficiency of cleavage of species-specific caspase-3 (supplemental Fig. 2). In addition, efficient cleavage of mouse and human Bid was mediated by hGrB as opposed to mGrB (supplemental Fig. 2 and supplemental Table 1). These observations are consistent with previous reports using active site-titrated human and mouse GrB and z-DEVD-fmk.
FIG. 2. A, experimental setup for the identification of mGrB and hGrB substrates and the relative quantification of the degree of processing. SILAC (using $^{12}\text{C}_6\text{-},^{13}\text{C}_6\text{-},$ and $^{13}\text{C}_6^{15}\text{N}_4\text{-labeled l-arginine}$) was used to analyze two different z-VAD/z-DEVD-fmk-pretreated cell lysates (human K-562 or mouse YAC1 cells). The in vitro approach to study processing by human and/or mouse GrB is schematically outlined. $^{12}\text{C}_6\text{-}$ and $^{13}\text{C}_6\text{-labeled lysates were treated with mGrB and hGrB, respectively, and }^{13}\text{C}_6^{15}\text{N}_4\text{-labeled lysates served as a control. After incubation and inactivation of GrB by adding guanidinium hydrochloride, equal amounts of proteome preparations were mixed, and N-terminal peptides were isolated by the N-terminal COFRADIC procedure using an enriched sample of }\alpha\text{-amino-blocked peptides obtained after a strong cation exchange (SCX) prefractonation step (24). B, identification of hGrB substrates in NK cell-mediated cell death. B shows the in vivo setup to monitor NK cell-mediated proteolysis events in K-562 target cells. Three different cell populations and two different cell types were analyzed in a single proteomics experiment: untreated, L-$^{13}\text{C}_6^{15}\text{N}_4\text{-arginine-labeled K-562 cells (+ cycloheximide, z-VAD-fmk, and z-DEVD-fmk) and co-cultured, L-$^{13}\text{C}_6\text{-arginine-labeled K-562 cells (+ cycloheximide, z-VAD-fmk, and z-DEVD-fmk) with unlabeled NK cells. NK and K-562 cells$}
both were active on the same mixture of potential substrates. As such, single $^{12}$C$_{6}$- and $^{13}$C$_{6}$-labeled peptides indicate, respectively, unique mGrB and hGrB substrates, whereas couples spaced by 6 mass units indicate neo-N termini raised by both orthologs. The ratio of ion signal intensities of such couples further is indicative for the difference in substrate cleavage efficiency between human and mouse GrB.

Neo-N Termini Reveal Unique and Extended GrB Substrate Properties—Following COFRADIC-based N-terminal peptide sorting and LC-MS/MS analysis, 14,726 peptides, of which 11,263 were in vivo $\alpha$-N-acetylated and/or in vitro $\alpha$-N-trideuterioacetylated (76.5%), were identified. 6,404 of these peptides were in vivo $\alpha$-N-acetylated, and 4,859 were in vitro $\alpha$-N-trideuterioacetylated. Of all N-terminal peptides, 7,787 (69%) started at position 1 or 2, whereas 3,476 peptides (31%) had a start position beyond position 2 and could thus be indicative for proteolytic processing (among others, including the constitutive removal of mitochondrial transit sequences). Overall these N-terminal peptides led to the identification of 2,059 human and 1,093 mouse proteins. More than 800 cleavage sites located in 322 human and 282 mouse proteins were identified, thereby constituting by far the most comprehensive list of potential GrB substrates hitherto reported. Our list includes 17 previously characterized GrB substrates (supplemental Tables 1 and 2) next to 32 known caspase substrates processed at non-canonical group III caspase cleavage sites (these caspasases, including caspase-6, -8, and -9, display a GrB-like cleavage preference), thus validating our approach. A snapshot of physiologically interesting GrB substrates reported to function in regulation and induction of apoptosis is shown in Fig. 3.

The general amino acid conservation in the complete set of hGrB substrates cleaved at Asp (i.e. 585 cleavages in K-562, Jurkat, and YAC1 lysates) aligned with the cleavage site as anchor point is shown in Fig. 4A. Unexpectedly hGrB preferences are found from P4 to P6’ and additional, although less strong conservation from P6’ to P9’ (see below). The significance of amino acid variability restriction measured by Shannon entropy was further estimated by comparison with a background/random set. As such, statistically significant sequence variability differences from positions P4 to P9’ were observed with $p < 0.001$ (supplemental Fig. 3). This is a clear extension of the observation that residues N-terminal to the scissile bond determine hGrB-mediated cleavage (32, 33). We further observed that mouse GrB displayed comparable extended substrate specificity although with different amino acid preferences.

To complement substrate sequence conservation based on amino acid identity we next analyzed aligned hGrB substrates sequence motifs for physical property constraints in which the pattern of conservation was emphasized because the statistical signal of individual amino acid type preferences is merged when they share similar physical properties. Therefore, we created non-redundant sets of all hGrB substrates (allowing maximal redundancy of 30% sequence identity over 40 positions) identified as neo-N-terminal peptides. Then we calculated the correlation of the frequencies of all amino acids at all motif positions with a library of roughly 700 physical properties as described previously (34). The properties with the highest correlation over single and multiple positions can be mapped to a basic set of amino acid properties corresponding to positive and negative charge (35), hydrophobicity (36), and small or aromatic amino acids (37). Fig. 4B shows these five signature properties as normalized deviation from the overall database average (Swiss-Prot version 50). An additional filter was applied that removes noise within the expected error of the experiment, estimated as standard deviation observed over all positions between the two independent experiments (K-562 and YAC1) for each physical property. We identified preferences for negative charges at P3 as well as from P2’ to P8’. Close to the cleavage site there are slight dispreferences for positive charges (around P1). We also detected a tendency for hydrophobic residues at P4, which is followed by less hydrophobic amino acids at P3. Furthermore positions P3, P2, and P1’, surrounding the cleavage were co-cultured for 4 h at an effector to target ratio of 1:2. The inset shows a K-562 cell being targeted by approximately five NK cells (note the big difference in cellular volume). To rule out postlytic processing, co-lysis of co-cultured and control cells in the presence of 3,4-dichloroisocoumarin (3.4 DCI) was performed after which N termini were isolated as described for A. C–H, illustration of the different categories of isolated N-terminal peptides found in K-562 freeze-thaw lysates treated with mouse recombinant GrB ($^{12}$C$_{6}$), human recombinant GrB ($^{13}$C$_{6}$), or untreated ($^{13}$C$_{6}$N$_{15}$). C, unaltered protein-N termini; these peptides were identified as Ac-$^{2}$AAAAEGVLATR$^{12}$ (Ac denotes an $\alpha$-acetylated amino group) from the biogenesis of lysosome-related organelles complex-1 subunit 2, and the ion intensities of their three different forms are about equal, indicating that this peptide was not affected by GrB. D, unique neo-N terminus (VTTD $\downarrow$ AcD$_{6}$-148DETFEKNNFER$^{147}$ where AcD3 denotes a triodeuterioacetylated $\alpha$-amino group) generated only by mouse recombinant GrB in the pericentriolar material 1 protein. E, unique neo-N terminus (VMD $\downarrow$ AcD$_{6}$-168KSKGVPSVKTSGSKER$^{169}$) formed as in D but now generated exclusively through the action of human recombinant GrB in the scaffold attachment factor B protein. F, neo-N termini of the polyuridyrimidine tract-binding protein (generated at AAVD $\downarrow$ AcD$_{6}$-173AGMAGGSPVLR$^{185}$) that, according to the ion intensities, appears to be 2 times more efficiently cleaved by mouse versus human recombinant GrB. G, neo-N termini of the BCL2/adenovirus E1B 19-kDa protein-interacting protein 2 (IEAD $\downarrow$ AcD$_{6}$-25ILAITG-PEDOPGSLEVNGKVR$^{25}$) generated with similar efficiency by mouse and human recombinant GrB. H, this peptide was identified as Ac-$^{2}$AIDNKEESLQDLDVEEVEEEETGTKLKAR$^{25}$ of the nucleosome assembly protein 1-like and is indicative for how our analysis may monitor indirect cleavage events, in this case cleavage mediated by human recombinant GrB (disappearance of the $^{13}$C$_{6}$ isotopic envelope). In a separate peptide fraction, the neo-N terminus generated after cleavage at LDQQ 15 was also identified following human recombinant GrB treatment (supplemental Table 1). The amino acid residues in bold indicates the P1 position (or P1’ variant) in the cleavage site within the protein substrate.
age site, tend to be occupied by small residues, which are indicative for size restrictions in the peptide binding pocket. Overall the primary substrate specificities reported in the literature, albeit more restricted by the nature of the types of analyses used, correlate well with our general observed cleavage preference of hGrB (and mGrB; data not shown) (13, 15–17).

Alternative Cleavage Specificities of GrB—Nearly 25% of all GrB cleavages occurred C-terminal to residues other than Asp. Such alternative cleavages were generated by both orthologs. Highly similar amino acid patterns, such as the key determining residues at P4, surrounding Asp and non-Asp cleavage events were found, suggesting that primary GrB cleavage can indeed occur with alternative specificities (supplemental Fig. 4).

To validate GrB alternative cleavage preferences, we synthesized 16-mer peptides containing such sites together with their Asp variants as in vitro substrates for hGrB. In this way, the monitored cleavage yield in Glu → Asp and Ser → Asp converted peptides indicates that cleavage occurred after a glutamate or serine residue although with lower kinetics than Asp-converted peptides (Fig. 5A). hGrB-mediated cleavage at Asn36 (neo-N terminus: LALLEEIEAENR) in the mouse and at Asp37 (neo-N terminus: LALMEEMAEAEHR) in the human DNA polymerase catalytic subunit further indicates that substrate cleavage arises even when a species-specific suboptimal P1 residue replacement occurred (supplemental Table 1) and highlights the importance of non-P1 residues in determining cleavage susceptibility.

Differential Substrate Proteolysis Reveals Distinct Human and Mouse GrB Specificities—Given that discrepancies between mouse and human GrB specificity have been observed at the peptide and protein level (13–15), we next performed an in-depth comparison of the differences (taking into account ion intensity ratios of peptides generated by human and/or mouse GrB cleavage in a given proteome; see above) between mGrB and hGrB substrates.

When neo-N-terminal peptides are ranked by their mean mGrB:hGrB cleavage ratio, it became apparent that Lys-starting peptides were by far more tolerated by mGrB compared with their Glu-starting counterparts (Table II). To further distinguish differentially accommodated amino acids from P5 to P5′ in their respective subsites of human and mouse GrB, a modified two-sample WebLogo (38) was created using the human and mouse Asp-specific data sets (Fig. 4C). Statistically significant residues (p < 0.05) are plotted with the size of the amino acid proportional to the difference observed in cleavage efficiency between human and mouse GrB on their respective substrates. In addition, Fig. 4D plots the substrate specificity differences between the human and mouse GrB substrate.

Fig. 3. 25 GrB substrates with known function in apoptotic processes. Proteins are marked in a gray background according to the function(s) they fulfill in apoptotic regulation/induction and whether they display a proapoptotic or antiapoptotic role.

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The significant enhancement of cleavage efficiency observed when Gly (Fig. 4C) and in general small amino acids (Fig. 4D) are present at P2 for mGrB substrates confirms a previously assigned preference (15) and thus validates our approach for mapping sequence-related specificities. Glu at P4 was found to increase substrate binding for hGrB (39). As P4 is predicted to be in close proximity to Arg of mature hGrB, a salt bridge formation can be established in contrast to Ile occupying this position in mGrB. This explains why in general P4 Glu-containing sites were more efficiently cleaved by hGrB compared with mGrB where lysine and hydrophobic residues seem to enhance mGrB-mediated cleavage. Furthermore although charged residues at P1 are proposed to destabilize the catalytic triad, mGrB seemingly cleaves substrates holding a P1 Lys on average 14-fold (deduced from proteomics data) more efficiently compared with hGrB. To further illustrate this, a peptide, LEADKGK-

Fig. 4 A, sequence logo of the aspartic acid cleavage sites identified by proteome-wide screening for hGrB substrates. Cleavage site motifs for proteases are given as Pn…P2-P1P1’-P2’…Pn’ with cleavage occurring between P1 and P1’ N- and C-terminal, respectively, to the scissile bond. Residues carboxy-terminal to the scissile peptide bond are indicated as prime side (P’) residues and amino-terminal residues as non-prime side (P) residues. 585 experimentally identified hGrB Asp-specific cleavages from proteome-wide substrate analysis performed on Jurkat, K-562, and YAC1 cell lysates were used to create a WebLogo (49). P10 to P10 positions are indicated. The amino acid heights are indicative for their degree of conservation at the indicated position. B, physical property characteristics of hGrB substrates (in K-562 and YAC1 cells) as significant deviations from general database averages. Physical property characteristics for P2 to P20 positions are indicated. The weak arginine conservation seen from P11 to P15 is probably due to the N-terminal COFRADIC sorting procedure used that selects for Arg-C-specific peptides with average length of 10–15 amino acid residues. C, weighted differential sequence logo of hGrB and mGrB aspartic acid-specific motifs. A modified two-sample WebLogo was created (38) using the obtained human and mouse Asp-specific data sets (462 differential data points). Statistically significant residues with a p value threshold of 0.05 around the conserved P1 Asp are plotted with the size of the amino acid proportional to the difference observed in cleavage efficiency between human and mouse GrB on their respective substrates. P5 to P5 residues are indicated. On the y axis the height of the symbol is proportional to the mean difference in intensity ratios of mouse versus human GrB formed neo-N termini. D, physical property characteristics of mGrB versus hGrB motifs as significant deviations from general database averages. Significant preferences for key physical properties at specific motif positions are shown. Positive values indicate a preference for human GrB, and negative values indicate a preference for mouse GrB.

data set as physical property preferences and shows that such differences are expected in the P3 to P5’ region.

The significant enhancement of cleavage efficiency observed when Gly (Fig. 4C) and in general small amino acids (Fig. 4D) are present at P2 for mGrB substrates confirms a previously assigned preference (15) and thus validates our approach for mapping sequence-related specificities. Glu at P4’ was found to increase substrate binding for hGrB (39). As P4’ is predicted to be in close proximity to Arg of mature hGrB, a salt bridge formation can be established in contrast to Ile occupying this position in mGrB. This explains why in general P4 Glu-containing sites were more efficiently cleaved by hGrB compared with mGrB where lysine and hydrophobic residues seem to enhance mGrB-mediated cleavage. Furthermore although charged residues at P1 are proposed to destabilize the catalytic triad, mGrB seemingly cleaves substrates holding a P1 Lys on average 14-fold (deduced from proteomics data) more efficiently compared with hGrB. To further illustrate this, a peptide, LEADKGK-
LEYD, based on the optimal P and P' sequence requirements in mGrB cleavage sites and including a P1 glycerine (shown here to discriminate between mouse and human GrB cleavage specificity), was synthesized. In the setups analyzed, this peptide was almost exclusively cleaved by mGrB (Fig. 5, C and D). However, because a P1' lysine selection by mGrB on peptide substrates has not previously been reported, this amino acid might not be the most optimal residue for mGrB cleavage to occur. On the other hand, the presence of cooperative residues that are generally overlooked by positional scanning approaches and can be easily overseen in phage display studies can result in an overall increased cleavage efficiency. Negatively charged and small residues at P1 glycerine are better tolerated by hGrB; this can be explained by the presence of Lys-Arg at positions 40 and 41 in hGrB (Fig. 1C).

**Structural Analysis of the GrB Substrate Motif—** We further analyzed our findings in the context of the complex between human GrB and its substrate peptide. The available structure (Protein Data Bank code 1IAU) is from hGrB crystallized with the bound tetrapeptide IEPX (32) where X is a non-reactive analog of aspartate. It should be noted that it is difficult to argue about structural constraints of neighboring positions given the original Protein Data Bank entry. Consequently we modeled the much longer 17-mer peptide EEEEEVEADSEEEEEEE, which corresponds to the consensus sequence of the hGrB substrates identified in this work, into the binding pocket of the crystal structure aligned to the coordinates of the IEPX peptide (Fig. 6A). The model was energy-minimized in a short simulated annealing molecular dynamics simulation with the AMBER99 force field (40) using standard parameters as implemented in Yasara 7 (41), which allows both the backbone and side chains to be optimized. Interestingly the extended regions of the peptide are very well accommodated in an extension of the binding groove during the simulation. Furthermore two positively charged loops of hGrB embrace the highly negatively charged peptide.

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**Fig. 5. Validation and determination of alternative hGrB cleavage specificity.** Reverse-phase HPLC UV absorbance chromatogram profiles of the substrate peptides AQQVISASASNLDFFY (gray arrow, A) and of the Ser → Asp variant AQQVISADASNLDFFY (gray arrow, B) are shown for the indicated time points of hGrB incubation. The precursor peptide, derived from the pericentriol material 1 protein, is cleaved at a previously uncharacterized cleavage site with an unknown cleavage specificity of human GrB (serine residue). The product peak ASNLDFFY (confirmed by MS/MS analysis) is indicated with a black arrow in both panels. The other product peaks AQQVISAS and AQQVISAD eluted at earlier time points. C and D, validation and determination of species-specific GrB cleavage. Reverse-phase HPLC UV absorbance chromatogram profiles of the substrate peptide LEADKGKLEYD (striped arrow) for the indicated time points of hGrB (C) and mGrB (D) incubation are shown. The precursor peptide was synthesized based on the (sub)optimal P and P' sequence requirements for mGrB cleavage sites and includes a P1' lysine and P2' glycerine (shown to discriminate between mouse and human GrB cleavage specificity). LEADKGKLEYD is a far better substrate for mGrB as compared with hGrB. The product peaks LEAD (left peak) and KGKLEYD (right peak) were confirmed by MS analysis and are indicated with a black arrow. mAU, milliabsorbance units. The amino acid written in bold indicates the P1 position (or P1 variant) in the cleavage site within the peptide substrate.
TABLE II

List of trideuteroacetylated (AcD₃), internal peptides starting with either a lysine or glutamate and raised by cleavage C-terminal to aspartic acid in mouse and/or human GrB-treated mouse YAC1 or human K-562 lysates for which the mGrB:hGrB ratios (or single ¹²C/single ¹³C, respectively, indicating unique mouse or human GrB substrate) were determined. Peptides are classified according to their mean mGrB:hGrB ratios. UniProt database primary accession number, start/end, site (P4–P1), sequence, and highest Mascot ion score of the identified spectra are given. Glutamate-starting peptides holding a glycine at the P₂/H₁₁₀₃₂ position are underlined. SUMO, small ubiquitin-like modifier; snRNP, small nuclear ribonucleoprotein; ARF, ADP ribosylation factor.

| Mean mGrB: hGrB | Accession no. | Start | End | P4–P1 | Modified sequence | Highest score | Description |
|-----------------|---------------|-------|-----|-------|-----------------|--------------|-------------|
|                 | Single ¹³C    |       |     |       |                 |              |             |
| 0.038           | Q27635        | 180   | 189 | FNAD  | AcD₃-EFEDMVAEKR  | 47           | 60 S ribosomal protein L10 |
| 0.048           | Q8BT18        | 837   | 844 | MQAD  | AcD₃-ECDDEEEIVDMSNR | 34 | Protein phosphatase 1G |
| 0.065           | P17987        | 360   | 370 | ICDD  | AcD₃-ELILIKNTKAR | 49 | Nucleolin |
| 0.077           | Q19338        | 408   | 420 | VTOD  | AcD₃-ELKEVFEDAMEIR | 49 | Ubiquitin C-terminal hydrolase 10 |
| 0.080           | Q3UHX2        | 62    | 74  | LDSD  | AcD₃-ESEDEDDDYQQKR | 76 | Nucleolin |
|                 | Single ¹²C    |       |     |       |                 |              |             |
| 0.086           | Q6A068        | 146   | 156 | IDMD  | AcD₃-EDELEMLSEAR | 51 | Cell division cycle 5-related protein |
| 0.090           | P46277        | 129   | 152 | VTGD  | AcD₃-EYNEVISIDOGPAGTFCDYLDAGLAR | 56 | L3 small nucleolar RNA-associated protein 18 homolog |
| 0.097           | Q6ZWW3        | 56    | 69  | MVSD  | AcD₃-EYEILSLSEALEAR | 70 | Ubiquitin C-terminal hydrolase 10 |
| 0.099           | P09405        | 410   | 422 | ITED  | AcD₃-EELKEVFEDAMEIR | 70 | Nucleolin |
| 0.100           | Q75533        | 484   | 495 | VDUD  | AcD₃-ELKEVFEDAMEIR | 70 | Nucleolin |
| 0.109           | Q9ERU9        | 2132  | 2142 | VGTG  | AcD₃-ELKEVFEDAMEIR | 70 | Nucleolin |
| 0.114           | P27659        | 215   | 234 | FGQD  | AcD₃-EMIDIVGVTGKGYKGVTSR | 96 | 60 S ribosomal protein L3 |
| 0.135           | Q7Z456        | 532   | 550 | LSSD  | AcD₃-KETIEIIDAKDLEKLKR | 65 | Kinesin family member 21A |
| 0.157           | P70268        | 204   | 217 | AAPP  | AcD₃-EAQGDPELGAVELR | 80 | Serine/threonine protein kinase N1 |
| 0.162           | Q9R0C0        | 36    | 47  | TSPD  | AcD₃-EGLIEFFPVDVR | 49 | Ubiquitin C-terminal hydrolase 10 |
| 0.165           | Q99549        | 146   | 156 | IDMD  | AcD₃-EDELEMLSEAR | 86 | Cell division cycle 5-like protein |
| 0.187           | Q9JLJ8        | 90    | 101 | WEYD  | AcD₃-EDELEMLSEAR | 86 | Cell division cycle 5-like protein |
| 0.198           | Q88HN1        | 84    | 93  | LEGD  | AcD₃-EDELEMLSEAR | 62 | γ-Taxilin |
| 0.201           | Q9EST5        | 241   | 261 | VDED  | AcD₃-EDELEMLSEAR | 52 | Acidic leucine-rich nuclear phosphoprotein 32 family member B |
| 0.220           | Q9EST5        | 244   | 261 | DEED  | AcD₃-EDELEMLSEAR | 52 | Acidic leucine-rich nuclear phosphoprotein 32 family member B |
| 0.303           | Q6KCD5        | 1086  | 1098 | ISSD  | AcD₃-EDELEMLSEAR | 81 | Nipped-B-like protein |
| 0.696           | Q7TOI3        | 28    | 36  | LAYD  | AcD₃-EDELEMLSEAR | 77 | Acidic leucine-rich nuclear phosphoprotein 32 family member E |
| 1.031           | P97822        | 230   | 243 | DYYD  | AcD₃-EDELEMLSEAR | 77 | Acidic leucine-rich nuclear phosphoprotein 32 family member E |
| 1.207           | Q8K2D3        | 215   | 227 | ALFD  | AcD₃-KAAVFEEIDTYER | 72 | Enhancer of mRNA-deacetylase 3 |

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The crystal structure coordinates of human and rat GrB were then used to develop a homology model of the mouse GrB structure (Fig. 6B). Although the protease adopts the same overall structure, the model predicts a number of differences in the extended substrate binding cleft that could be indicative for the altered substrate interactions observed. Apparent from Fig. 6B, the surfaces of the binding pockets exhibit different physical properties. For example, the residue to the right of positions P7 to P5 is a positively charged lysine (Lys202) in mouse compared with a neutral asparagine (Asn218) in human. In addition, the closure of the two loops in the human model is not confirmed in mouse GrB, which lacks these attracting positive charges. The linear continuation of the binding groove at P2 is gated by an arginine in hGrB (Arg41), whereas the isoleucine (Ile28) in mGrB leaves a structural gap that could form a potential alternative exit for (a subset of) substrate peptides in mGrB. Overall the altered substrate interaction observed in the crystal and modeled GrB structures correlates well with the different substrate specificities observed between mGrB and hGrB.

In Vivo GrB Cleavage in NK Cytotoxic Granule-mediated K-562 Cell Death—To further validate our in vitro results, we studied proteolysis in the Fas-negative K-562 cell line challenged with natural killer-mediated cell death. Cleavage of poly(ADP-ribose) polymerase and caspase-3 in K-562 cells after incubation with lymphokine-activated killer cells was verified by Western blotting (supplemental Fig. 1A).

The proteomics experiment was performed with purified, interleukin-2-activated human NK cells, which display an increased level of granzyme B expression (supplemental Fig. 1B). NK cells were incubated with L-[13C6]arginine-labeled K-562 cells for 4 h with an effector:target ratio of 0.5:1 in the presence of z-DEVD-fmk, z-VAD-fmk, and cycloheximide (Fig. 2B). This approach allows direct allocation of substrate processing in target cells. To monitor postlytic proteolytic processing that may occur when NK cells are lysed (42), co-lysis of co-cultured K-562 and K-562 control cells (13C6,15N4-labeled L-arginine) in the presence of the granzyme A/B-specific inhibitor 3,4-dichloroisocoumarin was performed. According to our knowledge this is the first quantitative proteome-based approach to study proteolytic cascades and their resulting cleavage actions in mixed cell populations.

Proteome analysis resulted in the confirmation of 14 GrB cleavage events in vivo (Table III). This apparently undersized
number could reflect the most efficient GrB substrates but represents a small fraction of the *in vivo* GrB proteolytic events here identified in target cells (43) because in total 38 unique *in vivo* cleavages were identified here, further pointing to the expected activation of several other proteases next to GrB. Indeed because we already have evidence for potential *in vivo* µ-calpain- (43), GrA-, and Omi (21)-mediated process-
ing, this observation reflects the activation of complex proteolytic networks (besides activity of orphan granzymes) inside dying K-562 cells. As an illustration, Bid cleavage during K-562 killing was documented by identification of tBid in the target cells (Table III and supplemental Fig. 5). Interestingly the verification of an *in vitro* identified glutamate-specific cleavage event in the nuclease-sensitive element-binding protein 1 at VQGE ↓ V217M further exemplifies the *in vivo* existence of the alternative cleavage preferences *in vitro* displayed by hGrB.

**DISCUSSION**

Here we identified over 800 GrB processing events in GrB-challenged proteome preparations. The biological relevance of these GrB-specific cleavages needs to be determined, and several of these substrate cleavages could well be “bystander effects” in analogy with the reported caspase substrate repertoire (44). Conservation of mouse and/or human GrB cleavage in 100 orthologous mouse and human proteins, however, further highlights the existence of potentially more relevant GrB substrates than assumed; here at least 100 corresponding cleavage sites from both orthologous substrates were identified (supplemental Table 3). In sharp contrast, pioneering proteomics studies resulted in the identification of only one or no cleavage site in 30 and 15 observed mGrB-specific protein changes, respectively (4, 6). As few of the discrepancies between the substrate specificities displayed by orthologous granzyme B members have been comprehensively documented (13, 15–17), the triple SILAC labeling method used here not only allowed us to attribute cleavages to a specific GrB ortholog but also enabled quantification of differences in subsite selections. Such a systematic approach to map differences in orthologous or paralogous proteases has not been described previously.

Our catalog points to alternative and unexpected GrB cleavage specificities: although the substrate P1 position mainly harbors the expected aspartate, the binding pocket of GrB tolerates other amino acids like related proteases such as caspase-3 (32). Interestingly for the majority of non-Asp cleavages, P4 is generally occupied by Val, Ile, or Leu, which is one of the major determinants in the substrate specificity displayed by GrB (supplemental Fig. 4) and thus points to direct GrB processing. Furthermore although we cannot completely rule out activation of other proteases in the lysate by GrB, several non-Asp GrB cleavages were reported: cleavage at Glu is a known cleavage site specificity of granzyme B (39) and in our list accounts for 45% of all non-Asp-specific cleavages. Previous studies also reported the ability of granzyme B to cleave ester substrates after Asn, Met, and Ser (45, 46) that was postulated, however, to be rather “unspecific” (17) (compared with the specific amide hydrolysis by granzyme B). Noteworthy is the fact that in our analysis Asn-, Met-, and Ser-specific cleavages account for the majority of non-Asp/Glu-specific cleavages. Finally N-terminal sequencing of the

![Figure 6](https://via.placeholder.com/150)

**Fig. 6.** The extended specificity determinants differ between human and mouse GrB. Three-dimensional surface representations of the hGrB P9–P8 consensus peptide (EEEEVEDSEEEEEEE) modeled in the binding pocket of the hGrB structure (A) and the modeled mGrB structure (panel B) are shown. GrB coloring is according to amino acid residue properties (blue represents positive charge, red represents negative charge, green represents large polar residues, cyan represents small polar residues, and gray represents hydrophobic residues).
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### TABLE III

| Description                                                                 | Accession no. | Site          | Identified peptide | Start | End   | K-562 | YAC1 |
|-----------------------------------------------------------------------------|---------------|---------------|-------------------|-------|-------|-------|------|
| A-kinase anchor protein 8                                                   | O43823        | VAAD          | AcD58-VAEVIATAVR  | 577   | 587   | 1.60  | ND   |
| BH3-interacting domain death agonist (BID)                                 | P55957        | IEDD          | AcD80-SESEQDIIR   | 76    | 84    | 0.06  | ND   |
| Eukaryotic translation initiation factor 2 subunit 2                        | P20042        | PTED          | AcD14-KDELAEEDTR  | 52    | 62    | 14.5  | ND   |
| Eukaryotic translation initiation factor 3 subunit 1                        | O75822        | WDAD          | AcD33-AFSVDPVR    | 18    | 26    | 0.09  | 0.29 |
| Importin α-4 subunit                                                       | O00629        | ICED          | AcD10-SIDGGDYR    | 60    | 67    | 0.46  | 0.62 |
| Microtubule-associated protein 4                                            | P27816        | SLAD          | AcD71-ALTEPSDIEGEIKR | 9    | 23    | 0.15  | ND   |
| Nucleosome-sensitive element-binding protein 1                              | P67809        | VOGE          | AcD68-VMEGADNQGAGEQGR | 217  | 231   | ND    | 0.07 |
| Nucleolin<sup>a</sup>                                                       | P19833        | VTQD          | AcD44-ELKEFEDAAERI | 408  | 420   | 0.08  | 0.10 |
| Polyglutamine-binding protein 1                                             | O60828        | IAEDD         | AcD71-YDPPDVEYATR | 33    | 44    | ND    | Unique hGrB |
| Natural killer cell-specific antigen KLIP1                                   | Q9GZM5        | VDAD          | AcD71-AADAAAAAEEEGFLGKFKGQ | 66  | 91    | ND    | ND   |
| Serine/threonine protein kinase RIO2                                        | Q9BVS4        | IKED          | AcD53-SLSESEADAR  | 380   | 389   | ND    | ND   |
| Seryl-ribose synthetase, cytoplasmic                                       | P49591        | ISND          | AcD33-EDVDRNVER   | 149   | 157   | ND    | ND   |
| Vimentin                      <sup>b</sup>                                      | P08670        | IDVAD         | AcD39-VSKPDLTAALR | 260   | 270   | 0.26  | 0.22 |
| Zinc finger CCHC domain-containing protein 8                                | Q6NZY4        | VDAD          | AcD52-ALTELLEEQQR | 514   | 525   | 0.08  | 0.06 |

<sup>a</sup> Indicates known GrB cleavage site.

<sup>b</sup> Indicates known caspase substrate.

<sup>c</sup> Amino acid counterpart in mouse homologous site identified in the in vitro screen.

6-kDa fragment generated by treatment of SPI6 with mGrB or hGrB also showed that cleavage occurred at a cysteine (Cys339), which was a previously unrecognized cleavage specificity of GrB (15).

However, in vivo validation of such alternative sites in cellular models is complicated by the presence of different proteolytic activities in cellular systems besides those of GrB. The fact that such novel cleavage preferences were hardly found in other studies can be explained by the presence of residues far beyond the cleaved site that steer the efficiency of cleavage. Previous studies have indeed largely neglected amino acid preferences beyond the P4 to P2<sup>+</sup> positions. By directly focusing on the protein level, we have now found extended "acidic patches" or "stretches" in preferred hGrB substrates C-terminal to the cleavage site as opposed to the complete absence of such motifs in substrates of the basic protease granzyme A.<sup>2</sup> In general, a high correlation with the previously observed acidic P4<sup>+</sup> to P2<sup>+</sup> residues is increased manifold and can complement our protease substrate profiling studies.

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By using an equivalent targeted proteomics approach, in vivo proteolytic cleavages created in a mixed cellular context were backtracked to their exact cellular origin. Noteworthy here was the fact that eight of the in vivo identified substrates displayed an acidic P4<sup>+</sup> residue, which was hypothesized to discriminate early and late GrB targets as high and lower affinity substrates, respectively (39). This observation might indicate that processing of early apoptotic mediators was preferentially sampled here and that secondary cleavage events were missed, possibly partially explaining the high discrepancies in numbers of in vivo versus in vitro substrates identified. Furthermore because the apoptotic process is induced non-synchronously in the target cells (in contrast to simultaneous cleavage on protein substrates when GrB is added to a lysate), proteolytic events raised by GrB may give rise to protein fragments that are either further processed yielding novel N termini that were not detected in vitro or generate unstable protein fragments that were subsequently removed by the proteasome. Finally and in contrast with in vitro screening, in vivo cleavage susceptibility of a substrate will also depend on substrate localization and conformation in a particular cellular context and/or phenotype (47, 48). Additionally a surprising observation was the identification of the in vitro identified GrB-generated N terminus in the eukaryotic
translational initiation factor 2 subunit 2 starting with a lysine at P1′ shown to be much less accommodated by hGrB compared with mGrB, indicating that even suboptimal cleavage events can occur as an early event in vivo.

In conclusion, we demonstrated here that the combined use of the N-terminal COFRADIC approach and triple SILAC labeling to identify protein cleavage sites on the natural protein level allowed us to analyze and compare ortholog-specific substrate cleavages in an in vitro approach that was easily traceable and linkable to proteolytic events taking place in a dual cell-based in vivo setup. We therefore believe that intraexperimental evaluation of multiple parameters will allow a clear dissemination of (ortholog-specific) proteolytic pathways.

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