The extended substrate specificity of granzyme B (GrB) was used to identify substrates among the chaperone superfamily. This approach identified Hsp90 and Bag1-L as novel GrB substrates, and an additional GrB cleavage site was identified in the Hsc70/Hsp70-Interacting Protein, Hip. Hsp90, Bag1L, and Hip were validated as GrB substrates in vitro, and mutational analysis confirmed the additional cleavage site in Hip. Because the role of Hip in apoptosis is unknown, its proteolysis by GrB was used as a basis to test whether it has anti-apoptotic activity. Previous work on Hip was limited to in vitro characterization; therefore, it was important to demonstrate Hip cleavage in a physiological context and to show its relevance to natural killer (NK) cell-mediated death. Hip is cleaved at both GrB cleavage sites during NK-mediated cell death in a caspase-independent manner, and its cleavage is due solely to GrB and not other granule components. Furthermore, Hip is not cleaved upon stimulation of the Fas receptor in the Jurkat T-cell line, suggesting that Hip is a substrate unique to GrB. RNA interference-mediated reduction of Hip within the K562 cell line rendered the cells more susceptible to NK cell-mediated lysis, indicating that proteolysis by GrB of Hip contributes to death induction. The small effect of RNA interference-mediated Hip deficiency on cytotoxicity is in agreement with the inherent redundancy of NK cell-mediated cell death. The identification of additional members of the chaperone superfamily as GrB substrates and the validation of Hip as an anti-apoptotic protein contribute to understanding the interplay between stress response and apoptosis.

Cytotoxic lymphocytes, including cytotoxic T lymphocytes and natural killer (NK) cells induce the death of virally infected or tumor cell targets through activation of tumor necrosis factor family death receptors or through granule exocytosis (1). During granule exocytosis, the granzymes, a family of serine proteases, are released into the cytoplasm of the target cells with the assistance of perforin, where they induce target cell death. The human granzyme family includes five members that are termed A, B, H, K, and M. Granzymes A and B are the most well characterized and have been implicated as important contributors to target cell death (2–5).

Previous substrate identification efforts have shown that granzyme B (GrB) induces target cell death by cleaving substrates that activate pro-apoptotic activities (i.e. caspase 3 (6)), dismantle the cytoskeleton (i.e. α-tubulin (7–9)), and inactivate proteins important for cellular homeostasis (i.e. DNA-dependent protein kinase catalytic subunit (10)). Recent substrate identification efforts have expanded the third class of GrB substrates to include receptors that transmit pro-proliferative signals from the extracellular environment (11) and members of the heat shock/stress response family (8, 11–13). These efforts and others (reviewed in Refs. 14 and 15) emphasize the critical role that substrate identification plays in understanding GrB-induced death.

We undertook a candidate-based approach to identify new GrB substrates. The extended substrate specificity of GrB has been comprehensively identified by the use of positional scanning substrate combinatorial libraries (16, 17). These data sets provide a four-amino acid “zip code” to identify potential cleavage sites within a protein. Algorithms such as PoPS (18, 19) and GraBCas (20) leverage the comprehensive nature of the positional scanning substrate combinatorial library-derived zip code to go beyond sequence gazing. However, searching the entire proteome for substrates with only the zip code yields too many hits to practically screen. Additional information is required to filter the hits to a reasonable number. Proteomic screens (8, 11) have proven to be useful filters by identifying proteins that are likely cut by GrB. Here, we restricted our search for GrB substrates to the heat shock/stress response family because of the well documented interplay between stress response and cell death (21–23) and the identification of several heat shock proteins as GrB substrates (8, 11, 13).

Heat shock proteins (Hsps) are a family of ubiquitous and highly conserved chaperone proteins that serve a maintenance function in the cell, helping to fold newly synthesized peptides. They are also expressed in response to environmental, physical, and chemical stress, including heat shock, oxidative free radi-

**References**

1. Hostetter, D. R., Loeb, C. R. K., Chu, F., and Craik, C. S. (2007) The extended substrate specificity of granzyme B (GrB) was used to identify substrates among the chaperone superfamily. The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and supplemental Figs. S1–S4.

2. These authors contributed equally to this work.

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6. The abbreviations used are: NK, natural killer; Hip, Hsc70/Hsp70-interacting protein; GrB, granzyme B; Hsp, heat shock protein; Hop, Hsp70/Hsp90-organizing protein; Bag1, Bcl-2-associated athano gene-1; CI, caspase inhibitors; G1, granzyme B inhibitor; z, benzoyloxycarbonyl; FMK, fluoromethyl ketone; PARP, poly(ADP-ribose) polymerase; HPLC, high-performance liquid chromatography; siRNA, small interfering RNA; PI, propidium iodide.
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cals, chemotherapeutic agents, nutrient withdrawal, and irradiation (21–23). Hsps are able to prevent protein aggregation and stimulate the refolding and renaturation of proteins that have misfolded under stress. These functions limit the extent of stress-induced damage and facilitate cellular recovery. Conversely, apoptosis removes damaged or unwanted cells. Both of these opposing mechanisms are so well conserved that it is likely the two evolved together. It is not surprising, then, that components of these two pathways are increasingly found to oppose each other at the molecular level. Indeed, many Hsps have been shown to directly interfere with components of the apoptotic machinery, and this inhibition is sometimes, but not always, dependent on chaperone activity (21, 23, 24). Therefore, Hsps respond to stress by preventing the misfolding and aggregation of proteins while directly antagonizing the apoptotic machinery to tip the balance toward cell survival.

Given the antagonistic interactions between the stress response/heat shock pathway and apoptosis, GrB may also target Hsps to ensure the death of the target cell. This hypothesis is supported by the identification of Hsp70, Hsp70/Hsp90-organizing protein (Hop), and Hsc70/Hsp70-interacting protein (Hip) as GrB substrates (8, 11, 13). To identify additional stress response substrates of GrB, members of the chaperone family of proteins were chosen as candidates for a sequence-based search for GrB cleavage sites. Three putative GrB substrates were identified. Hsp90, the Bcl-2-associated athano gene-1 (Bag1), and Hip were found to be substrates of GrB in vitro, and an additional cleavage site was identified and validated in Hip. Prior to this work, Hsp90 and Bag1 were known chaperone family members with documented anti-apoptotic roles, but Hip was not known to play an anti-apoptotic role and was therefore selected for further investigation. Proteolysis of Hip at both cleavage sites was caspase-independent and GrB-dependent in cell based assays. GrB cleavage is predicted to result in a loss of function by compromising the oligomerization domain of Hip and its Hsc70/Hsp70-interacting domain. To determine whether Hip loss of function made a contribution to natural killer cell-mediated cytotoxicity, RNA interference was used to reduce cellular levels of Hip within the K562 chronic myelogenous leukemia cell line. Hip deficiency rendered the cells slightly more susceptible to natural killer cell-mediated lysis, indicating that the proteolysis of Hip by GrB contributes to death induction. The modest impact of Hip deficiency is consistent with previous work showing that no single substrate or mechanism is required for GrB-mediated death and demonstrates the highly redundant nature of natural killer cell-mediated cytotoxicity.

EXPERIMENTAL PROCEDURES

Reagents—The caspase inhibitor z-DEVD-FMK was purchased from Alexis Biochemicals (catalog number ALX-260-072-M001) and Calbiochem (catalog number 264155). The caspase inhibitor z-VAD-FMK was purchased from EMD Biosciences (catalog number 627610) and Bachem (catalog number N-1560). When present, both caspase inhibitors were used in equimolar amounts (50 μM each) and are designated as CI. Antibodies to PARP were from Santa Cruz Biotechnology (catalog number sc-7150) or from Cell Signaling Technologies (catalog number MA3–413). The anti-Bag1 antibody was from Stressgen (catalog number AAM-400). The anti-Hsp90 antibody (recognizing both α and β isoforms) was from Cell Signaling (catalog number 4874). Both the α-specific (catalog number SPS-771) and β-specific (catalog number SPA-843) anti-hsp90 antibodies were from Stressgen. The antibody to c-Myc was from Santa Cruz Biotechnology (catalog number sc-40). Human GrB was a generous gift from either Dr. Nancy Thornberry (Merck) or Dr. Sandra Waugh Ruggles (Catalyst Biosciences). The small molecule GrB inhibitor I-038597 (GI) was developed as described in Ref. 25 and was a generous gift from Dr. Nancy Thornberry (Merck). Purified recombinant Hip (catalog number SPP-767), Hsp90 (catalog number SPP-770), and Hsp27 (catalog number SPP-715) proteins were from Stressgen.

Cell Lines and Culture—All of the cell lines are available from the American Type Culture Collection. K562 chronic myelogenous leukemia and Jurkat T-cell leukemia cell lines were all propagated in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate. NK-92 human natural killer cells were cultured in RPMI 1640 containing 10% fetal bovine serum, 10% bovine calf serum, 2 mM glutaxam-1 (Invitrogen; catalog number 35050-81), non-essential amino acids (1-AA, 1-Asn, 1-Asp, 1-Glu, 1-Pro, and 1-Ser), 110 μg/ml sodium pyruvate, 0.1 mM β-mercaptoethanol, 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 100 units/ml interleukin-2. All of the cells and transfectants were maintained in a humidified 37 °C, 5% CO₂ incubator.

Plasmid Construction—All of the inserts were ligated into the Invitrogen mammalian expression vector pcDNA 3.1 Myc/His₆ that contains a neomycin resistance gene. Each overexpressed protein, therefore, was C-terminally Myc and His₆-tagged. Human Hip was cloned by reverse transcription-PCR from mRNA prepared from K562 cells by using the RNeasy Mini Kit and Omniscript reverse transcriptase (both from Qiagen). Pfx DNA polymerase, and the primers 5'-GCC GAA TTC CGC TTG ACC TCC TTC-3' (reverse) and 5'-CAC AAG CTT ATG GAC CCC CGC AAA GTG-3' (forward). The reverse transcription-PCR product was cloned into an Invitrogen TOPO TA cloning kit as an intermediate step for blue/white screening and then sequenced. Positive clones were then excised by HindIII and EcoRI digestion and ligated into the pcDNA 3.1 Myc/His₆ vector for mammalian expression. The uncleavable mutant Hip-DDNN was cloned using a Stratagene QuikChange mutagenesis kit first by synthesizing and confirming the D92N single mutant and then subjecting this clone to a second round to make the D180N mutation. The mutagenesis primers used were the following: 5'-GAA GGT GTG ATG CAA CCC CCG CAC TGG-3' (D92N), the complement of primer D92N, 5'-GCC GAT ATT GAA ATG AAC CAC GTG CCT CAA GA-3' (D182N), the complement of primer D92N, 5'-GCC GAT ATT GAA ATG AAC CAC GTG CCT CAA GA-3' (D182N), and the complement of primer D180N.

Proteolysis of Chaperones in Vitro—Purified Hip, Hsp90, or Hsp27 protein at 45 μg/ml was incubated at 37 °C with 50 nM human GrB in GrB activity buffer containing 50 mM Na-HEPES, pH 8.0, 100 mM NaCl, and 0.01% Tween 20. Ali-
quot of 15 μl containing 670 ng of either Hip or Hsp90 were taken at specific intervals and were mixed with sample loading buffer. The samples were loaded onto 4–20% Tris-glycine gels and separated by SDS-PAGE. Separated proteins were visualized by Coomassie Blue staining.

**On-line Capillary Liquid Chromatography-Mass Spectrometry Analysis**—A 1-μl aliquot of the digestion mixture was injected manually into an Eldex capillary liquid chromatography system and separated by a silica-based monolithic reverse phase capillary column (Onyx column from Phenomenex, Torrance, CA) at a flow rate of ~1 μl/min. The HPLC eluent was connected directly to the micro-ion electrospray source of a QSTAR Pulsar QqTOF mass spectrometer (Applied Biosystem/MDS Sciex, Foster City, CA). Typical performance characteristics were >8000 resolution with 30 ppm mass measurement accuracy in mass spectrometry mode.

**Lysis and Immunoblotting**—Cytoplasmic lysates to be treated with GrB were generated by resuspending cells at 1 × 10^7 cells/ml in 50 mM Tris, pH 8.0, 150 mM NaCl and 1% Nonidet P-40, (Lysis Buffer). Aliquots from cell killing or Fas stimulation experiments described below were lysed in lysis buffer including the Complete Protease Inhibitor mixture tablet from Roche Applied Science (product number 11 697 498 001) (Lysis Buffer + PI) to stop proteolysis. After incubating on ice for 30 min, the lysates were spun at 16,000 × g for 10 min at 4 °C to remove the insoluble fraction. The protein concentrations were determined with the BCA protein assay reagent (Pierce), and equal amounts of total protein from each sample were separated by SDS-PAGE. The proteins were then transferred to nitrocellulose or polyvinylidene difluoride membranes and blocked in Tris-buffered saline Triton X-100 containing 5% milk or 5% bovine serum albumin, as per the antibody manufacturer’s instructions. The membranes were then incubated with substrate-specific antibodies, washed, and incubated with horseradish peroxidase-conjugated secondary antibodies. Immunoblots were developed on film with the ECL (Amersham Biosciences Bioscience) or ECL Plus detection systems (GE Healthcare). The locations of prestained protein markers were traced by overlaying the film onto the membrane. Immunoblots presented are representative of at least three independent experiments.

**Confirming Proteolysis in Cell Lysates**—K562 cytoplasmic extracts were generated in the absence or presence of 50 μM CI in lysis buffer. The lysate was incubated with 50 nM human GrB in the absence or presence of 20 μM GI, and aliquots were removed from 1 to 6 h. The samples were then subjected to SDS-PAGE and immunoblotting.

**NK Cell-mediated Cytotoxicity**—NK-92 effectors (E) and K562 targets (T) were collected, counted, and mixed at varying E:T ratios as in Refs. 3 and 11, with 2 × 10^5 K562 cells in 24-well plates. For analysis of proteolysis by immunoblotting, killing was stopped at intervals from 1 to 6 h by collecting and lysing the cells in Lysis Buffer + PI. For analysis by flow cytometry, the cells were mixed as described in Ref. 3. Briefly, 20,000 K562 cells were mixed with 20,000–80,000 NK-92 effectors in a total volume of 50 μl of medium in 5 ml, 12 × 75 mm polystyrene round bottom test tubes (BD Biosciences, catalog number 352054). Prior to mixing, the cells were preincubated for at least 1 h in 50 μM CI; CI concentration was kept constant throughout the experiment. The cells were incubated for 1–6 h at 37 °C, and phosphate-buffered saline was added to 475 μl to stop the experiment. The cells were then washed with 20 μl of an anti-CD56 allophycocyanin-conjugated antibody (Becton Dickinson; catalog number 555518) and 5 μl of propidium iodide (BioVision, catalog number K101) and incubated in the dark at room temperature for 30 min. The samples were then transferred to ice, kept in the dark, and analyzed on a FACS Calibur (Becton Dickinson) according to the manufacturer’s instructions. Dead target cells were counted as the population of PI+, CD56 cells, excluding cellular debris. Analysis was conducted with FlowJo v6.4.1 software.

**Activation of Caspase-mediated Apoptosis in Jurkat T-cell Leukemia Cells**—Jurkat cells were plated in a 96-well plate at 4 × 10^5 cells/well in 200 μl of RPMI 1640 medium containing 2% fetal bovine serum. The cells were stimulated with 200 ng/ml anti-Fas antibody CH-11 (Upstate Biotechnology, Inc.; product number 05-201) and incubated from 2 to 6 h. At the indicated time intervals, the aliquots were lysed in Lysis Buffer + PI.

**Stable Transfectant Generation**—All of the transfections were executed using an Amaza electroporator system and the Nucleofactor Solution V, program T-16, according to the manufacturer’s instructions. For generation of stable cell lines expressing Myc-tagged cleavable Hip and uncleavable Hip-DDNN proteins, 1 million K562 cells were transfected with 2 μg of plasmid DNA. At 48 h post-transfection, the cells were plated in ClonaCell-TCS (Stem Cell Technologies; product number 03814) methylcellulose-based medium containing 1.2–1.7 mg/ml G418 according to the manufacturer’s instructions. Colonies were allowed to form for up to 2 weeks and were then selected and expanded in culture medium containing 1.5 mg/ml G418. Clones expressing the constructs were maintained, expanded, weaned off of G418, and periodically checked for expression of the desired Hip protein.

**siRNA Knock-down of Hip**—All of the transfections were generated by using the Amaza electroporation instrument and solution kits as described above. Of the four siRNA duplexes available from Ambion, siRNA 13835 resulted in optimal Hip knock-down. Hip-deficient cells were compared with either K562 cells transfected with nuclease-free water or the nontargeting siRNA negative control number 4 (Ambion; catalog number 4641). The ON-TARGETplus SMARTpool from Dharmacon (catalog number L-017380-00-0005) was also used to silence Hip expression. Hip-deficient cells were compared with K562 cells transfected with ON-TARGETplus siCONTROL Nontargeting pool (Dharmacon; catalog number D-001810-10-05). On the day of the transfection, 1 million K562 cells were transfected with ~3 μg of Ambion siRNA or Dharmacon siRNA according to the instructions from Amaza. Transfected cells were maintained in 12-well plates at a maximum concentration of 500,000 cells/ml for up to 6 days post-transfection. The transfectants were then either used for lysis and immunoblotting or for cell killing experiments, both described above.

**Statistical Analysis**—All of the analyses were performed using the freely available R language (26). To analyze the asso-
ciation of dead cell ratio in K562 cells with siRNA transfection, a multivariate linear model was fit with dead cell ratio as response and siRNA transfection indicator as the independent variable. To control for length of incubation and K562/natural cell ratio, time and K562/natural cell ratio covariates were added. Time was considered as a continuous variable because it had a strong linear association with the response, whereas K562/natural cell ratio was considered as categorical because there was no obvious trend. To take into account the blocking effect of the experiments being performed in pairs for each time point and K562/natural cell ratio, a random blocking effect for each pair was also included. The model was fit using the nlme package in R (27).

RESULTS

Several Members of the Chaperone Family Are Proteolyzed by Granzyme B in Vitro—Recently, three chaperone family members have been shown to be GrB substrates: Hsp70 (11); the Hsp70-organizing Protein, Hop (8, 12); and the Hsc/Hsp70-interacting Protein, Hip (13). Other chaperone proteins were therefore investigated for their ability to be proteolyzed by GrB. As a first pass approach to determining whether a particular chaperone family member could be a prospective GrB substrate, the amino acid sequence was scanned for putative GrB cleavage sites. GrB has an absolute requirement to cleave C-terminally to aspartic acid residues because of Arg226 in its S1 pocket (28, 29). Moreover, because the extended substrate specificity of GrB has been defined (16, 17), a substrate amino acid sequence can be scanned to predict cleavage sites. The GrB optimal substrate has the sequence (I/V)EPD (supplemental Table S1). Specificity profiling data indicated that the preference of GrB for Ile or Val at P4 is of critical importance, but that its preference at P3 and P2 is much more broad. The likelihood of a site being cleaved can also be evaluated on the basis of the preference of all proteases to cleave specifically at extended β strands (30).

The chaperones selected for cleavage site analysis included those with known and unknown anti-apoptotic roles. Cleavage sites were identified by both sequence gazing and by GraBCas (20). The analysis identified several known GrB substrates including Hsp70, Hop, and Hip (supplemental Table S1). The analysis also identified an additional prospective cleavage site in Hip and Hsp90, Bag1, and Hsp27 as putative GrB substrates (supplemental Table S1 and Fig. 1). Many of the prospective GrB cleavage sites were not represented in the Protein Data Bank; however, both VRAD170-HG in Hsp90 and VRTD175-TG in Hsp90 are in extended β strands (data not shown), suggesting that Hsp90 is a likely GrB substrate.

To first test whether these proteins could be GrB substrates, purified recombinant Hsp90 (both α and β isoforms), Hip, and Hsp27 were incubated with human GrB from 1 to 6 h (Fig. 1). Aliquots were removed at the indicated times, separated by SDS-PAGE, and visualized by Coomassie Blue staining. As shown in Fig. 1, both Hsp90 and Hip are proteolyzed by GrB in vitro, whereas Hsp27 is resistant to proteolysis throughout the time course.

To assess the validity of the cleavage site analysis (supplemental Table S1), the sizes of the proteolytic product bands

FIGURE 1. Proteolysis of purified Hip (A), Hsp90 (B), and Hsp27 (C) protein in vitro. Purified proteins were incubated with granzyme B as described under “Experimental Procedures,” separated by SDS-PAGE, and analyzed by Coomassie Blue staining. The schematics detailing the prospective cleavage sites for each protein are drawn below each gel. An arrow indicates that cleavage at the putative site is supported by analysis of cleavage products on the Coomassie-stained gels. The color-coded arrowheads adjacent to the gel indicate the predictions of which proteolytic product was generated from which prospective cleavage site.

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were compared with the location of the predicted GrB cleavage sites within each substrate (Fig. 1). Hip, which is ~48 kDa, has two potential cleavage sites, at sequences IEPD$_{92}$-TD and INPD$_{180}$-SA (Fig. 1A). Proteolysis at either or both sites would yield products of 12, 13, 24, 25, and/or 36 kDa. The banding pattern by 1 h showed one band at 24 kDa and two at ~12–13 kDa, indicating that GrB cleaves Hip at both prospective cleavage sites. The bands at ~35 and 13 kDa in the initial t = 0 time point may, in fact, be the cleavage products from an extremely rapid proteolysis at the sequence IEPD$_{92}$-TD immediately upon GrB addition. These data are consistent with the sequence INPD$_{180}$-SA as a novel GrB cleavage site within Hip. To confirm the analysis of proteolysis on denaturing gels, recombinant Hip-Myc digested with GrB was characterized using mass spectrometry in conjunction with reverse phase HPLC. Fragments consistent with GrB cleavage at IEPD$_{92}$-TD and INPD$_{180}$-SA were observed (supplemental Fig. S1).

Both isoforms of Hsp90, α and β (schematic shown in Fig. 1B), have several predicted GrB cleavage sites. An alignment of the two isoforms shows that each cleavage site is present in both isoforms of Hsp90 (supplemental Table S1). Consistent with the many predicted cleavage sites, purified Hsp90 is proteolyzed at multiple sites by GrB in vitro. The largest proteolytic products, at ~80 and 72 kDa, are consistent with cleavage at sequences IDED$_{693}$-EV (Fig. 1B, red arrowhead) and INPD$_{631}$-PI (Fig. 1B, orange arrowhead) in Hsp90β, and at sequences IDED$_{701}$-DP and INPD$_{639}$-HS in Hsp90α. The other smaller proteolytic products can be generated by cleavage of Hsp90 at the other sites (Fig. 1B, green, blue, purple, and gray arrowheads).

Substrate validation was next tested by adding purified GrB to various cell lysates. Aliquots of lysate were removed at time points up to 6 h, and the samples were separated by SDS-PAGE and analyzed by immunoblotting to the prospective chaperone substrates. The addition of GrB to HEK cell lysates confirmed the proteolysis of Hsp90 (Fig. 2B) in K562 cell lysates generates an ~75-kDa band within 1 h of incubation with 50 nM GrB, and an additional 70-kDa product appears within 1 h of incubation with 100 nM GrB. To clarify which cleavage sites within Hsp90α and Hsp90β are proteolyzed, varying concentrations of GrB were added to lysate for 2 h and then analyzed by immunoblotting with isoform-specific antibodies (Fig. 2B). The accumulation of an ~19-kDa Hsp90α product is evident at 500 nM GrB and is consistent with cleavage at VRTD$_{175}$-TG. Cleavage at this site is predicted to generate a 19.4-kDa product, and the α-specific antibody recognizes an epitope contained within residues 2–12 of Hsp90α. The accumulation of ~80- and 73-kDa Hsp90β products is evident at 500 nM GrB and is consistent with cleavage at IDED$_{693}$-EV and INPD$_{631}$-HP, respectively. Cleavage at VRAD$_{170}$-HG within Hsp90β is an unlikely explanation for the observed products because a 65-kDa product would be expected. Therefore, GrB differentially proteolyzes both Hsp90α and Hsp90β.

It is also noteworthy that the addition of GrB to K562 lysates cleaves the long form of the cochaperone Bag1, Bag1-L (Fig. 2C). Because a cleavage product does not appear, it is possible that Bag1L is further degraded upon GrB proteolysis. The short
and middle forms of Bag1 do not appear to be proteolyzed. GrB proteolysis of Bag1-L is independent of caspase activity because cleavage is observed in the presence of the caspase inhibitors z-VAD-FMK and z-DEVD-FMK (CI). Therefore, Hip, Hsp90, and Bag1 are all proteolyzed in lysates after incubation with GrB.

**Hip Is a Caspase-independent Substrate of Granzyme B in Cell-based Assays** — The anti-apoptotic roles of Hsp90 (21–23) and Bag1L (31, 32) are well established, but the role of Hip is less clear. Therefore, we hypothesized that cleavage by GrB indicates that Hip has an anti-apoptotic function, and we investigated the role of Hip in NK-induced cell death.

Although the *in vitro* proteolysis of Hip shown in Fig. 1 indicates that GrB is able to cleave Hip directly, it is still possible that in cell-based assays Hip proteolysis may be caused by GrB caspase activation. Therefore, CI were added to the K562 cell lysates before GrB treatment. As Fig. 3A demonstrates, the presence of CI does not prevent Hip proteolysis upon GrB incubation. Proteolysis of PARP was used to monitor both GrB and caspase activity in lysates (Fig. 3B). PARP is cleaved at distinct, nonoverlapping caspase and granzyme B cleavage sites, producing proteolytic products that can be easily resolved by SDS-PAGE. In the presence of CI, full-length PARP was stabilized, but PARP was still cleaved into a 55-kDa band by GrB. In addition, the inclusion of a small molecule specific inhibitor of GrB (GI) blocks proteolysis of Hip (Fig. 3A) and PARP (Fig. 3B). Therefore, proteolysis of Hip by GrB is caspase-independent.

Hip proteolysis was next assessed in NK cell-mediated cell killing of K562 targets (Fig. 4) to demonstrate that cleavage occurs in a biologically relevant context. K562 cells do not express the Fas death receptor and are killed by NK-92 effector cells exclusively by the granule exocytosis pathway. NK-92 effectors (E) were mixed with K562 target cells (T) at an E:T ratio of either 4:1 or 2:1 in the presence (Fig. 4B) or absence (Fig. 4A) of caspase inhibitors and incubated for the indicated times. Proteolysis of PARP was assessed to monitor caspase inhibition (data not shown). Regardless of whether or not CI was present, Hip was proteolyzed during NK-mediated cell killing, and the 24-kDa product was generated, indicating proteolysis of Hip at both of the predicted aspartic acids. Therefore, Hip is a caspase-independent substrate.

To determine whether Hip proteolysis during cell killing was due to GrB and not other granule components, NK-92 cells were preincubated with GI and then mixed with K562 targets, keeping GI present throughout. As shown in Fig. 4B, proteolysis of Hip was completely blocked in the presence of GI.

**Hip Is a Substrate Unique to GrB and Is Not Cleaved by Caspases** — Although GrB proteolysis of Hip is caspase-independent, it is formally possible that caspases may also be able to cleave Hip. The addition of recombinant caspase to purified human Hip did not result in proteolysis (13). To confirm this result in a cell-based assay, Jurkat cells, which express the death receptor Fas, were stimulated with the anti-Fas stimulating antibody CH-11. CH-11 acts as a surrogate for FasL and induces apoptosis via the death receptor and death ligand extrinsic apoptotic pathway, which begins with activation of caspase 8 and results in activation of the caspase cascade. Jurkat cells were stimulated for the indicated times with CH-11 and then lysed in lysis buffer containing protease inhibitors and separated by SDS-PAGE for analysis by immunoblotting. As indicated in Fig. 5, PARP was processed to the 89-kDa caspase-generated proteolytic product after Fas stimulation of Jurkat cells. However, Hip was not proteolyzed. It appeared that Hip increased in abundance during the incubation with anti-Fas, which was a phenomenon noticed when fibroblast growth factor receptor 1 was assessed during Fas-mediated killing of Jurkat cells (11), but the significance of this is not known. Taken
together, these data show that Hip is not a substrate of caspases and is unique to GrB.

**Hip Is Proteolyzed at Both Sequences IEPD₉₂ and INPD₁₈₀**

To confirm the sites of GrB proteolysis within Hip, a double mutant (termed Hip-DDNN; Fig. 6, top panel) was cloned in which both suspected aspartic acids were changed to asparagines. Site-directed mutagenesis has been used previously to confirm the sites of proteolysis within several GrB substrates, including Bid (33), ICAD (34), H₂₉₂₅₁-tubulin (7, 9), and fibroblast growth factor receptor 1 (11). This mutant Hip is C-terminally Myc- and His-tagged; therefore, it migrates at 52 kDa. To more directly compare this mutant Hip with the wild-type protein, a C-terminally Myc- and His-tagged, wild-type cleavable Hip (Hip-Myc; Fig. 6, bottom panel) was constructed. Lysates were generated from K562 cells overexpressing either Hip-DDNN or Hip-Myc. These lysates were treated with GrB, and aliquots were removed after 2–6 h, mixed with sample buffer, and separated by SDS-PAGE for analysis by immunoblotting. The blots were probed with an anti-Myc antibody to monitor the overexpressed Hip. Hip-Myc was proteolyzed (bottom panel), but Hip-DDNN was not (top panel), indicating that the cleavage sites had been successfully removed in Hip-DDNN.

**FIGURE 4.** Hip is a caspase-independent substrate of granzyme B during NK cell-mediated cell killing of K562 target cells. A, proteolysis of Hip at NK/K562 ratios of 2:1 (left) and 4:1 (right). B, Hip is cleaved during cell killing in the presence of caspase inhibitors (CI, left). Proteolysis of Hip during cell killing is completely blocked in the presence of a small molecule granzyme B inhibitor (GI, right). C, granzyme B delivery into K562 cells with the BioPORTER protein delivery reagent results in Hip proteolysis.

**FIGURE 5.** Hip is not cleaved during Fas-stimulated caspase activation and is a substrate unique to granzyme B. Jurkat cells were stimulated by an anti-Fas antibody (Ab) that induces caspase activation and apoptosis. The immunoblots were to Hip (left) and PARP (right).

**FIGURE 6.** Hip is proteolyzed by granzyme B at sequences IEPD₉₂ and INPD₁₈₀. Two Myc-His₆ C-terminally tagged constructs were cloned, one coding for wild-type Hip (Hip-Myc) and the other coding for a full-length Hip in which the two predicted P₁ Asp residues were mutated to Asn (Hip-DDNN). Lysates were generated from K562 cells stably expressing either Hip-Myc or Hip-DDNN and then incubated with granzyme B as described under “Experimental Procedures.” Immunoblots (IB) to the Myc tag enabled monitoring of the cells with overexpressed Hip. Hip-Myc was proteolyzed (bottom panel), but Hip-DDNN was not (top panel), indicating that the cleavage sites had been successfully removed in Hip-DDNN.

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FIGURE 7. K562 cells that do not express Hip are sensitized to NK-92 cell-mediated cytotoxicity. A, siRNA to Hip was transfected into K562 cells with the Amaxa Nucleofection electroporator. Knock-down of Hip protein expression was monitored by immunoblotting. Equal amounts of total protein were loaded in each lane. The cells that did not receive the siRNA were instead transfected with water. Equivalent results were also obtained with a nontargeting siRNA control. Essentially 100% knock-down was achieved by 4 days post-transfection and was maintained through day 6. B, Hip-deficient cells that were knocked down for endogenous Hip expression were used as target cells in NK-92 cell-mediated cytotoxicity assays in the presence of caspase inhibitors. See "Experimental Procedures" for details. Cell death was assessed by flow cytometry. Cytotoxicity was counted as the percentage of target cells staining positive for propidium iodide. NK-92 cells were distinguished from K562 cells in the analysis using an anti-CD56, APC-conjugated antibody. These data are presented as the average percentages of PI+ targets from two-three independent experiments. ± S.D. C, cytotoxicity of Hip-deficient cells was compared with K562 cells transfected with a nontargeting siRNA control. D, cytotoxicity of Hip-deficient cells transfected with a pool of siRNAs designed to minimize off target effects was compared with K562 cells transfected with a pool of nontargeting siRNAs.

pro-survival or anti-apoptotic function to Hip during GrB-induced apoptosis or apoptosis induced by other factors. The fact that GrB targets Hip is a first clue that Hip may have such a biological role.

To test the hypothesis that Hip has an anti-apoptotic function, siRNA specific to Hip mRNA was used to silence Hip expression in K562 cells (Hip-deficient cells). As Fig. 7A demonstrates, the transient transfection strategy employed resulted in almost complete silencing of Hip protein levels by 72 h post-transfection. The knock-down of Hip protein was maintained through 6 days (144 h) post-transfection.

siRNA-mediated Hip-deficient or wild-type target K562 cells were mixed with NK-92 effectors at E:T ratios from 1:1 to 4:1 and incubated at 37 °C for up to 6 h. PI staining was chosen as a parameter for monitoring NK-induced cytotoxicity, because GrB has been shown to be the major effector of NK cell-mediated cell lysis (3). Prior to mixing, all cell populations were preincubated with caspase inhibitors, which were also kept at constant concentrations throughout the experiment. The cells were then assessed by flow cytometry using PI as a marker for cell death. An anti-CD56 antibody conjugated to allophtococyanin was employed to exclude the NK cells from analysis.

Hip-deficient cells were more sensitive to NK cell-induced lysis, because they consistently demonstrated more PI+ cells at each time point and at each E:T ratio (4:1, 2:1, 1:1) examined. An analysis of variance showed that all three fixed effects (siRNA treatment, time, and E:T ratio) were highly significant in the linear model, which is described under “Experimental Procedures,” with treatment and time being most significant (p < 1 × 10⁻¹⁰) and E:T ratio having a p value of 0.001 (supplemental Fig. S2). The dead cell ratio increased by 0.045 when siRNA transfected cells were compared with control, keeping other variables constant, whereas the increase was 0.087 when the time of incubation was increased by an hour while keeping other variables constant. The optimal conditions for observing the increased sensitivity of Hip-deficient cells are shown in Fig. 7B. The p values from performing a t test at each time point are below 0.05, demonstrating a significant difference (data not shown).

The increased sensitivity to lysis is observed when Hip-deficient cells are compared with cells transfected with a nontargeting siRNA (Fig. 7C). Furthermore, the use of a pool of siRNAs designed to minimize off target effects silenced Hip expression (data not shown) and reconstituted the increased sensitivity to lysis (Fig. 7D). The greater sensitivity of Hip-deficient cells to NK-induced lysis demonstrates that Hip can have pro-survival functions and indicates that GrB proteolysis of Hip is important to the efficiency of death induction.

The effect of Hip expression on the sensitivity of other GrB substrates to proteolysis was also examined. Lysates were generated from K562 cells stably overexpressing cleavable Myc-tagged Hip, untreated K562 cells, and K562 cells transfected with the Hip siRNA. These lysates were treated with GrB, and aliquots were removed after 2–6 h, mixed with sample buffer, and separated by SDS-PAGE for analysis by immunoblotting against PARP and caspase 3 (supplemental Fig. S3). The 89-kDa proteolytic product of PARP is produced by caspase cleavage and contains GrB cleavage sites. The sensitivity of the 89-kDa proteolytic product to GrB proteolysis increases as Hip expression decreases. In contrast to PARP, the proteolysis of caspase 3 to its active form is not influenced by Hip expression. These data suggest that Hip expression might set the threshold of GrB required to proteolyze certain substrates.

Hip-deficient cells were also challenged with etoposide, a topoisomerase II inhibitor that has been widely used to couple DNA damage to apoptosis through the mitochondrial pathway (35, 36). Hip could act upstream or downstream of the mitochondrial pathway. If Hip has an anti-apoptotic role that blocks the events downstream of mitochondrial damage induced by GrB, then Hip-deficient cells should be more susceptible to apoptosis inducers such as etoposide. In fact, Hsp70 expression has been shown to protect K562 cells from etoposide-induced apoptosis, requiring high drug concentrations to induce apoptosis in a 24-h period (37). However, Hip-deficient K562 cells are no more sensitive to etoposide than their wild-type counterparts (supplemental Fig. S4). Therefore, Hip does not seem
to be involved in the protective effect exerted by Hsp70 with regards to etoposide. This suggests that the anti-apoptotic role of Hip is actually upstream of or separate from mitochondrial damage and may actually be affecting aspects of GrB-induced apoptosis.

**DISCUSSION**

This study has identified and confirmed Hsp90, Bag1-L, and Hip as substrates of GrB in vitro. Previous work had identified Hip as a substrate (13); however, its role in apoptosis was unknown, and therefore Hip was selected for further investigation. We identified and validated an additional GrB cleavage site at the sequence INPD_{180}-SA. GrB-mediated cleavage at sequences IEPD_{92}-TD and INPD_{180}-SA is observed in vitro with both recombinant protein and in K562 lysates. Hip proteolysis is caspase-independent and GrB-dependent in K562 lysates and during NK cell-mediated killing, confirming that cleavage at both sites occurs in a biologically relevant context. It is unclear why cleavage was not observed at residue 180 by Caruso and Reiners (13). Possible explanations include differences between commercial anti-Hip antibodies and differences between the activities of GrB preparations.

The significance of proteolysis at both sequences IEPD_{92}-TD and INPD_{180}-SA must be discussed in the context of the functional domains of Hip. Hip contains a homo-oligomerization domain at its N terminus, three tetratricopeptide repeats in its central region that are responsible for Hsc/Hsp70 binding, and Hsc70-like and Sti1-like homology regions at its C terminus (38). Hip forms an elongated dimer through its N-terminal homo-oligomerization domain that is capable of binding two Hsc70 molecules (39, 40). Removal of the N-terminal 37 amino acids from Hip abolishes homo-oligomerization and weakens its Hsc70 binding activity (41). Therefore, cleavage at the sequence IEPD_{92}-TD would separate the oligomerization domain from an intact Hsp70 binding region and would likely weaken but not abolish the Hsc70 binding activity of Hip. Cleavage at sequence INPD_{180}-SA would cleave Hip at the end of its second tetratricopeptide repeat within the Hsp70 binding region. The tetratricopeptide repeat is characterized by tandem repeats of a 34-amino acid consensus motif found in chaperones and many other proteins (42). Deletion of the tetratricopeptide repeat region of Hip abolishes Hsc70 binding in a yeast two-hybrid assay (41, 43), and a structural model of Hip and Hsc70 suggest that cleavage at sequence INPD_{180}-SA would disrupt Hsc70 binding (40). Therefore, the structure-function analysis of the functional domains of Hip strongly suggests that proteolysis of Hip by GrB would result in a loss of function.

RNA interference-mediated reduction of Hip levels in K562 cells modestly increased sensitivity to NK-mediated cytotoxicity, demonstrating that Hip is a biologically significant GrB substrate. The modest increase is consistent with the highly redundant nature of NK-induced cytotoxicity. Because many viruses and cancer cells activate a variety of immune evasion and anti-apoptotic strategies, cytotoxic cells should be able to cause cell death by multiple pathways. In fact, cleavage of pro-survival proteins might represent one strategy to counteract these anti-apoptotic attempts and ensure target cell death. Consistent with this hypothesis, no single substrate or mechanism has been identified to date that is required for GrB-mediated death. Inhibition of the caspases with z-FAD-FMK and z-DEVD-FMK only has a minor effect on cell lysis (44). Other RNA interference studies on granzyme substrates show that silencing multiple substrates cannot completely protect or sensitize cells to death (45–47). Indeed, silencing of the Hsp70 cochaperone Hop had no effect on the sensitivity of cells to either GrB-mediated or NK-mediated cytotoxicity (12). The simultaneous silencing of multiple pro-survival proteins might be required to significantly impact NK-mediated cytotoxicity.

There are three observations that shed insight into the nature of the protective/pro-survival role of Hip during cell death. The sensitization of Hip-deficient K562 cells was detected in the presence of caspase inhibitors, indicating that Hip exerts a protective effect against a caspase-independent pathway. In addition, Hip deficiency did not affect etoposide-induced apoptosis, suggesting that the anti-apoptotic role of Hip is actually upstream of or separate from mitochondrial damage and may actually be affecting aspects of GrB-induced apoptosis. The observation that PARP proteolytic sensitivity correlates with Hip expression in lysates suggests that Hip may act as a proteolytic sink. Hip has two GrB cleavage sites and may serve as a competitive inhibitor, preventing the protease from cleaving more important substrates, thereby acting as a sensor to ensure that a threshold level of GrB activity is surpassed. RasGAP has recently been shown to be a sensor for caspase proteolysis, first being proteolyzed by caspases to reveal an anti-apoptotic protein that is then further proteolyzed by increased caspase concentration to remove this anti-apoptotic effect (48). Future experiments that continue to elucidate the nature of the pro-survival function of Hip and the identification of additional GrB substrates will contribute to our understanding of the mechanism of GrB-induced cell death and cell-mediated cytotoxicity.

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