Hop Cleavage and Function in Granzyme B-induced Apoptosis*

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Granzyme B (GzmB) is a cytotoxic protease found in the granules of natural killer cells and cytotoxic T lymphocytes. GzmB cleaves multiple intracellular protein substrates, leading to caspase activation, DNA fragmentation, cytoskeletal instability, and rapid induction of target cell apoptosis. However, no known individual substrate is required for GzmB to induce apoptosis. GzmB is therefore thought to initiate multiple cell death pathways simultaneously to ensure the death of target cells. We previously identified Hop (Hsp70/Hsp90-organizing protein) as a GzmB substrate in a proteomic survey (Bredemeyer, A. J., Lewis, R. M., Malone, J. P., Davis, A. E., Gross, J., Townsend, R. R., and Ley, T. J. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 11785–11790). Hop is a co-chaperone for Hsp70 and Hsp90, which have been implicated in the negative regulation of apoptosis. We therefore hypothesized that Hop may have an anti-apoptotic function that is abolished upon cleavage, lowering the threshold for GzmB-induced apoptosis. Here, we show that Hop was cleaved directly by GzmB in vitro and in cells undergoing GzmB-induced apoptosis. Expression of the two cleavage fragments of Hop did not induce cell death. Although cleavage of Hop by GzmB destroyed Hop function in vitro, both cells overexpressing GzmB-resistant Hop and cells with a 90–95% reduction in Hop levels exhibited unaltered susceptibility to GzmB-induced death. We conclude that Hop per se does not set the threshold for susceptibility to GzmB-induced apoptosis. Although it is possible that Hop may be cleaved by GzmB as an “innocent bystander” during the induction of apoptosis, it may also act to facilitate apoptosis in concert with other GzmB substrates.

The granzymes are a family of cell death-inducing serine proteases expressed in the granules of cytotoxic lymphocytes. Granzymes A–C, K, and M have each been described to induce death in vitro (2–6). Granzyme B (GzmB) is the most well studied of these enzymes, with >30 substrates described (4, 7), including the key substrates caspase-3 (8); ICAD (inhibitor of caspase-activated DNase) (9, 10); and Bid (11–14), a pro-apoptotic member of the Bcl-2 family. GzmB-mediated death is thought to occur via coordinated activation of mitochondrial pathways and the caspase cascade (15, 16). However, the GzmB death pathway does not appear to depend absolutely on any single substrate or mechanism. Caspase inhibition (10, 17, 18), caspase-3 deficiency (10), ICAD deficiency (10), or Bid deficiency (19–21) can slow or decrease sensitivity to GzmB, but not block it entirely except at low doses (20). It therefore appears that GzmB targets multiple redundant pathways to ensure controlled apoptotic death of the target cell even if the cell expresses endogenous or viral inhibitors of one or more of these pathways.

We (1) and others (22, 23) have recently described several new GzmB substrates identified in proteomic screens of GzmB protease activity. From these studies, two broad classes of GzmB substrates have emerged: first, proteins whose cleavage induces activation of pro-apoptotic mechanisms, including caspases, Bid, and ICAD; and second, structural and structure-related proteins, including lamin B (24), α-tubulin (1, 25, 26), β-actin (1), and ROCK (Rho-associated coiled coil-containing protein kinase II) (27). The importance of directly targeting this second class of substrates is unclear, but presumably, such events contribute to the nuclear collapse, cytoskeletal breakdown, and membrane blebbing characteristic of classical apoptosis. From recent proteomic screens, a third class of substrate has emerged. We identified the Hsp70/Hsp90-organizing protein Hop as a substrate of GzmB (1), and Hsp70 itself was recently shown to be cleaved by GzmB (23). Other heat shock proteins have been found in our screens of granzyme A (GzmA) and GzmB activity, further suggesting that this molecular chaperone machinery can be targeted by granzymes. Indeed, heat shock proteins have been widely implicated in the regulation of apoptosis (reviewed in Ref. 28). Heat shock/stress response proteins may therefore represent a new class of granzyme substrate.

Hop is a 543-amino acid ~60-kDa co-chaperone that mediates the association of the heat shock proteins Hsp70 and Hsp90 (29, 30). Hop expression appears to be largely cytoplasmic (31) and is expressed at detectable levels in all mouse-tissue small interfering RNA; EGFP, enhanced green fluorescent protein; EFYFP, enhanced yellow fluorescent protein; CFSE, 5(6)-carboxyfluorescein diacetate S-succinimidyl ester; NK, natural killer; TPR, tetratricopeptide repeat; ES, embryonic stem.

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The abbreviations used are: GzmB, granzyme B; GzmA, granzyme A; GzmM, recombinant murine granzyme B; Z, benzoyloxy carbonyl; fmk, fluoromethyl ketone; WT, wild-type; rHop, recombinant human Hop; 7-AAD, 7-aminoactinomycin D; GFP, green fluorescent protein; GFPα, high GFP; siRNA, small interfering RNA; EGFP, enhanced green fluorescent protein; EFYFP, enhanced yellow fluorescent protein; CFSE, 5(6)-carboxyfluorescein diacetate S-succinimidyl ester; NK, natural killer; TPR, tetratricopeptide repeat; ES, embryonic stem.

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sues examined (Gene Expression Database, Genomics Institute of the Novartis Research Foundation, expression.gnf.org). The function of Hop has been best characterized in in vitro systems examining the assembly of the progesterone receptor (33, 34) and glucocorticoid receptor (35, 36) into hormone-binding-competent heterocomplexes. In this model, Hop serves as an adaptor protein, tethering Hsp70 and Hsp90 and bringing Hsp90 into proximity with the hormone receptor (30). This scaffolding function of Hop is required in vitro for the efficient assembly of mature hormone receptor capable of binding hormone (34, 36). Hop also enhances the chaperoning of certain protein kinases (37), is required for the in vitro reconstitution of hepadnavirus reverse transcriptase activity (38), and has had several additional functions described, including acting as a ligand for cellular prion protein to promote survival in neurons (39, 40). The only Hop loss-of-function model described to date was performed in yeast (41). Deficiency in the yeast ortholog of Hop (Sti1) results in reduced glucocorticoid receptor activity (39, 40). The only Hop loss-of-function model described to date was performed in yeast (41). Deficiency in the yeast ortholog of Hop (Sti1) results in reduced glucocorticoid receptor activity (41) and, in a model of protein misfolding, a severe defect in the degradation of misfolded protein (42).

Because Hop is implicated in the binding and organization of Hsp proteins and because multiple Hsp-mediated anti-apoptotic mechanisms have been described (28), we hypothesized that Hop orchestrates one or more anti-apoptotic mechanisms and that cleavage of Hop by GzmB destroys this anti-apoptotic function, lowering the threshold for death induction. Here, we further characterize the cleavage of Hop by GzmB and investigate the role of Hop and its cleavage in cell death. We demonstrate that the cleavage products of Hop do not exhibit gain-of-function pro-apoptotic properties. We found that, although cleavage of Hop destroyed its in vitro binding and assembly functions, neither cells expressing ~5–10% of normal Hop levels nor cells overexpressing GzmB-resistant Hop exhibited altered levels of GzmB-mediated death. Therefore, Hop does not set the threshold for death induced by GzmB, although its cleavage may contribute to the death pathway in coordination with other substrates.

EXPERIMENTAL PROCEDURES

Cell Lines, Equipment, and Software—YAC-1 cells (mouse lymphoma) were maintained in K5 medium (RPMI 1640 medium, 5% heat-inactivated fetal bovine serum, 10 mM HEPES, 100 μM each nonessential amino acid, 1 mM sodium pyruvate, 2 mM l-glutamine, 1× penicillin/streptomycin, and 0.57 μM β-mercaptoethanol) in a 5% CO2 incubator at 37 °C. All flow cytometry was performed on a FACScan (BD Biosciences), and data were analyzed using CXP software (Beckman Coulter) or FlowJo (Tree Star, Inc., Ashland, OR). Cell sorting was performed on a MoFlo high-speed cell sorter (Dako). All graphs were created using GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, California), and error bars denote S.D.

In Vitro Cleavage Assays—Recombinant murine GzmB (rGzmB) was produced as described (43). Recombinant human GzmB was a kind gift of Margarita Garcia-Calvo (Merck). YAC-1 freeze/thaw lysates were prepared as described (1); diluted 1:5 in 25 mM Tris-HCl, 30 mM NaCl, and 1 mM dithiothreitol (pH 8.0); and treated with the indicated concentrations of rGzmB for 1 h or the times indicated at 37 °C. In some experiments, as indicated, YAC-1 cells were pretreated with the caspase inhibitors benzoyloxycarbonyl (Z)-DEVD-fluoromethyl ketone (fmk; Sigma) and Z-VAD-fmk (Sigma) at 100 μM each or with vehicle (Me₃SO; Sigma) for 15–30 min prior to freeze/thaw lysis and then diluted 1:5 as described above. In some experiments, transfected populations were isolated by high-speed cell sorting, and more dilute freeze/thaw lysates were prepared in the presence of caspase inhibitors. In vitro transcribed/translated substrates were made from murine wild-type (WT) or D186E Hop cDNA using the TNT quick-coupled system (Promega Corp.) in the presence of [35S]methionine according to the manufacturer’s recommendations. The translated product was treated with 0 or 1 μM rGzmB at 37 °C for the times indicated. Samples were run on an SDS-polyacrylamide gel, and radiolabeled products were detected by autoradiography. Recombinant human Hop (rHop) was produced as described (44) or purchased from Stressgen Bioreagents. 1 μg of rHop was treated with the indicated doses of rGzmB for 0 or 60 min, run on SDS-polyacrylamide gels, and silver-stained using the ProteoSilver kit (Sigma) according to the manufacturer’s instructions.

Western Blot Analysis—Cell lysates were separated on SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes (GE Healthcare), and blotted with the following antibodies: mouse anti-C-terminal Hop (F5) produced as described (29) at 1:1000 dilution, mouse anti-N-terminal Hop (clone 28, catalog no. 610834, BD Biosciences) at 1:500 dilution, mouse anti-α-tubulin (catalog no. sc-5286, Santa Cruz Biotechnology, Inc.) at 1:1000 dilution, horseradish peroxidase-conjugated rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (code ab9385, Abcam) at 1:500 dilution; rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (code ab9485, Abcam) at 1:1000 dilution, goat anti-Bid (catalog no. AF860, R&D Systems) at 1:1000 dilution, rabbit anti-caspase-3 (catalog no. AAS-103, Stressgen Bioreagents) at 1:1000 dilution; or mouse anti-caspase-3 (clone 3G2, catalog no. 9668, Cell Signaling Technology, Inc.) at 1:1000 dilution, mouse anti-Hsp70 (clone BB70) (29) at 1:1000 dilution, and mouse anti-Hsp90 (clone H90-10) (45) at 1:5000 dilution. Secondary antibodies were used at 1:10,000 dilution. Blots were developed using ECL or ECL Plus Western blotting detection reagent (GE Healthcare).

Site-directed Mutagenesis—The codon encoding Asp186 in the murine Hop cDNA was mutated to glutamic acid using the QuickChange mutagenesis kit (Stratagene) with primer 5'CCTCCTTTGGGTTGAACTGGGCAGCATGGAT-3' and its reverse complement (synthesized by Sigma).

Cell Death Reconstitution Assay—In some experiments, YAC-1 cells were pretreated with Z-DEVD-fmk and Z-VAD-fmk (100 μM each) or Me₃SO for 30 min as indicated. Cells were then treated with rGzmB and purified perforin for 60 min at 37 °C as described (19). Cells were analyzed by Western blotting for substrate cleavage or by 7-aminoactinomycin D (7-AAD; Calbiochem) staining, followed by flow cytometric analysis to assess cell death. For death assays with cells transiently cotransfected with green fluorescent protein (GFP) and Hop forms, the viability index for GFP⁻ and high GFP (GFP⁺) populations in each was calculated as described under “Results.” For small interfering RNA (siRNA)-transfected cells, the assay was performed at 52–54 h
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post-transfection. Efficacy of siRNA-mediated knockdown was verified by Western blotting for all experiments.

Transient Plasmid DNA Transfections—Enhanced GFP (EGFP)-C1 and enhanced yellow fluorescent protein (EYFP)-C1 were purchased from Clontech, and pcDNA3.1 was purchased from Invitrogen. For transient cotransfection of GFP and Hop forms, 2 μg of EGFP-C1 and 8 μg of pcDNA3.1 (empty vector) or vector containing WT or D186E Hop cDNA or 4 μg each of vector containing Hop-(1–186) cDNA (N-terminal fragment) and vector containing Hop-(Met-187–543) cDNA (C-terminal fragment) were transfection into 2 × 10^6 YAC-1 cells using a Nucleofector (Amaxa) with cell line solution L and program C-009 following the manufacturer’s instructions. Cells were plated in 1.5 ml of K10 medium (same as K5 medium but with 10% fetal bovine serum) and used in cell death assays or sorted based on GFP expression at 16–20 h post-transfection and used for in vitro cleavage assays and/or Western blotting.

Clonogenic Assays—EGFP-C1 alone or expression vector containing GFP-WT Hop cDNA, GFP-D186E Hop cDNA, GFP-Hop-(1–186) cDNA, Hop-(1–186)-GFP cDNA, GFP-Hop-(187–543) cDNA, Hop-(Met-187–543)-GFP cDNA, or tBid-GFP cDNA (a kind gift of Dr. Honglin Li, Northwestern University) was transiently transfected into YAC-1 cells. In some experiments, 50 μg of supercoiled plasmid DNA was transfected using a BTX electroporator at 300 V, 150 microfarads, and 360 ohms, whereas in other experiments, 8 μg of supercoiled plasmid DNA was transfected using the Nucleofector as described above. For cotransfection experiments, 2 μg of EGFP-C1 was cotransfected with 8 μg of empty vector (pcDNA3.1) or vector containing WT Hop cDNA, D186E Hop cDNA, Hop-(1–186) cDNA, or Hop-(Met-187–543) cDNA or 4 μg each of Hop-(1–186) cDNA and Hop-(Met-187–543) cDNA using the Nucleofector as described above. For all assays, cells were plated in medium following transfection and incubated at 37 °C and 5% CO₂ for 4 h. GFP⁺/H₁⁺ cells (~1% of all cells) were then sorted into 96-well plates and incubated at 37 °C and 5% CO₂. Wells positive for growth were counted 12 days later. Clonogenic potential was normalized to the GFP alone condition (for GFP fusion transfections) or GFP plus empty vector condition (for cotransfections).

In Vitro Protein Binding Assays—Radiolabeled WT Hop, D186E Hop, Hop-(1–186) (N-terminal fragment), or Hop-(Met-187–543) (C-terminal fragment) was transcribed/translated in vitro as described above. Hop70 and Hsp90 immunoprecipitation was performed as described (30). Briefly, 10 μg of antibody (clone BB70 for Hop70 immunoprecipitation (29) and clone H90-10 for Hsp90 immunoprecipitation (45)) was preadsorbed to 10 μl of protein G-Sepharose resin (Sigma). Molar equivalents of radiolabeled Hop forms were added separately to 100 μl of rabbit reticulocyte lysate (1:1 lysate; Green Hectares, Oregon, WI), combined with the immunoresin, and incubated for 30 min at 30 °C. Resin-bound complexes were washed three times with 1 ml of wash buffer (20 mM Tris-HCl (pH 7.4), 50 mM KCl, and 0.5% Tween 20), resuspended in 2× SDS sample buffer, boiled, and separated by SDS-PAGE. Gels were Coomassie Blue-stained to visualize bound proteins, and bound Hop forms were detected by autoradiography of the dried gel.

Progestosterone receptor complexes were assembled in vitro as described (30). Briefly, recombinant chicken progesterone receptor was immunopurified with antibody PR22 (46) pre-adsorbed to protein A-Sepharose, and 10 μl of this resin (containing 1 μg of progesterone receptor) was combined with 200 μl of reticulocyte lysate containing an ATP-regenerating system (47), one of four radiolabeled Hop forms (as described above), and 20 μg/ml geldanamycin (Hsp90 inhibitor) to enrich for intermediate progesterone receptor complexes containing Hop. Reactions were incubated for 30 min at 30 °C and then washed and analyzed as described for Hsp70/Hsp90 immunoprecipitation.

siRNA Transfections—for RNA interference experiments, Dharmacon siGENOME ON-TARGET duplexes D-048388-02 (Hop siRNA-1, 5’-CCAAGGAAUGCCAGCCUGCUUCUU-3’ (sense) and 5’-GUAGGGUCAGUUCUUCCGUU-3’ (antisense)) and D-048388-04 (Hop siRNA-2, 5’-GAGUAGUCGUUACAGUGCUU-3’ (sense) and 5’-AGCAUCUGAAGCAUCUU-3’ (antisense)), a pool of four duplexes targeting hemoglobin β₂-subunit (β-minor chain), HBB-B2 (L-062946-00, Dharmacon, proprietary sequences), and non-targeting control siRNA-1 (Dharmacon) were used. 100 pmol of siRNA duplex was transfected into 2 × 10⁶ YAC-1 cells using the Nucleofector as described for plasmid transfections and then plated in 2 ml of K10 medium. Cells were expanded 24–30 h later and used in experiments or harvested for Western blotting between 48 and 60 h post-transfection.

Etoposide Treatment and γ-Irradiation—For treatment with etoposide (Sigma), siRNA transfectants were plated at 48 h post-transfection at ~5 × 10⁵ cells/ml in 96-well plates in duplicate at the indicated drug concentrations. After 36 h, cell death was assessed by 7-AAD staining and flow cytometry. For γ-irradiation, duplicate transfectants were plated at 48 h post-siRNA transfection, each in duplicate, at ~3 × 10⁵ cells/ml in 12-well plates and then subjected to the indicated dose of radiation. 7-AAD staining and flow cytometric analysis were performed 48 h later. Data were fit using mixed models, with replicates and separate experiments treated as random effects and doses of radiation treated as fixed effects. Least squares means differences were compared using Tukey’s multiple comparison adjustment. Efficacy of siRNA-mediated knockdown was verified by Western blotting at the time of drug treatment or irradiation for all experiments.

Natural Killer Cell-mediated Cytotoxicity Assay—48 h after siRNA transfection, YAC-1 target cells were washed twice with phosphate-buffered saline and labeled with 125 μM 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE; Molecular Probes) at ~5 × 10⁵ cells/ml for 15 min at 37 °C. The labeling reaction was stopped by the addition of an equal volume of K10 medium, and cells were washed once with K10 medium. For preparation of natural killer (NK) effector cells, the following strain-matched 129/SvJ mice were used: WT, Gzma⁻/⁻ (48), Gzmb⁻/⁻/ΔPGK-neo (where PGK is phosphoglycerate kinase) (49), Gzmb⁻/⁻/+PGK-neo (50), and Gzma⁻/⁻ × Gzmb⁻/⁻/+PGK-neo (48). Gzma⁻/⁻ × Gzmb⁻/⁻/ΔPGK-neo mice were generated by crossing Gzma⁻/⁻ mice with Gzmb⁻/⁻/ΔPGK-neo mice to derive F₁ obligate heterozygous offspring for intercrosses. Doubly deficient mice from F₁ intercrosses were identified by PCR geno-
typing. Splenocytes were isolated, and red blood cells were lysed as described (49). Bulk splenocytes were cultured in 24-well plates at 1.5 × 10^7 cells/ml in K10 medium plus 100 ng/ml recombinant murine interleukin-15 (R&D Systems). After 48 h, bulk splenocytes were harvested with vigorous pipetting using cold phosphate-buffered saline, and viable cells were counted using trypan blue. For cytotoxicity assays, cells were plated in 96-well V-bottom plates at effector/target cells ratios of 5:1, 10:1, and 20:1 with a fixed number of CFSE-labeled target cells (1 × 10^5), mixed by gentle pipetting, centrifuged at 1000 rpm for 3 min, and incubated at 37 °C for 4 h. Cells were then harvested, and 2–3 min before flow cytometric analysis, 7-AAD was added for a final concentration of 150 nM. Analysis was performed by gating on CFSE−/H11001 cells and evaluating percent 7-AAD+/H11001 target cells as described (51). Efficacy of siRNA-mediated knockdown was verified by Western blotting.

RESULTS

GzmB Directly Cleaves Hop at Asp^{186} in Vitro and in Cells Undergoing GzmB-induced Death—We previously identified Hop as a GzmB substrate in a proteomic screen (1). To further examine Hop cleavage in vitro, we performed Western blotting of YAC-1 freeze/thaw lysates treated with increasing doses of rGzmB for 0 or 60 min and analyzed by Western blotting. Similar experiments were performed twice with the same results. C-term., C-terminal; α-GAPDH, anti-glyceraldehyde-3-phosphate dehydrogenase antibody. 8. YAC-1 cells were pretreated with the caspase inhibitors Z-VAD-fmk (zVAD) and Z-DEVD-fmk (zDEVD) at a final concentration of 20 μM each or vehicle (Me2SO (DMSO)) prior to freeze/thaw-induced lysis. Lysates were treated and analyzed as described for A. C, 1 μg of rHop was treated with rGzmB for 0 or 60 min at the doses indicated and then analyzed by SDS-PAGE and silver staining. Similar experiments were performed two additional times with the same results. N-term, N-terminal. D, in vitro transcribed/translated WT or D186E Hop was treated with 0 or 1000 nm rGzmB for 0 or 60 min. Samples were separated by SDS-PAGE and visualized by autoradiography. The autoradiograph shown is representative of two similar experiments.

FIGURE 1. GzmB cleaves Hop at Asp^{186}. A, YAC-1 freeze/thaw lysates were treated with the indicated doses of rGzmB for 0 or 60 min and analyzed by Western blotting. Similar experiments were performed twice with the same results. C-term., C-terminal; α-GAPDH, anti-glyceraldehyde-3-phosphate dehydrogenase antibody. B, YAC-1 cells were pretreated with the caspase inhibitors Z-VAD-fmk (zVAD) and Z-DEVD-fmk (zDEVD) at a final concentration of 20 μM each or vehicle (Me2SO (DMSO)) prior to freeze/thaw-induced lysis. Lysates were treated and analyzed as described for A. C, 1 μg of rHop was treated with rGzmB for 0 or 60 min at the doses indicated and then analyzed by SDS-PAGE and silver staining. Similar experiments were performed two additional times with the same results. N-term, N-terminal. D, in vitro transcribed/translated WT or D186E Hop was treated with 0 or 1000 nm rGzmB for 0 or 60 min. Samples were separated by SDS-PAGE and visualized by autoradiography. The autoradiograph shown is representative of two similar experiments.
expected; Me$_2$SO pretreatment allowed complete activation to the p17 form. Therefore, Hop processing by GzmB is caspase-independent.

To demonstrate direct cleavage of Hop by GzmB, we treated rHop with rGzmB. Human Hop is $>97\%$ identical to murine Hop at the amino acid level, including the region immediately surrounding the GzmB cleavage site (data not shown). rGzmB cleaved rHop into two fragments, p40 and p20 (Fig. 1C), in agreement with our previous findings (1). Recombinant human GzmB cleaved Hop (data not shown). Therefore, Hop is a direct substrate of GzmB. In vitro transcribed/translated Hop was also cleaved by rGzmB (Fig. 1D, lane 4). Mass spectrometry data suggested previously that GzmB cleaves Hop after Asp$^{186}$ (1). Mutation of this residue to glutamic acid rendered the transcribed/translated product (D186E Hop) highly resistant to cleavage by rGzmB (lane 8).

We next assessed the cleavage of Hop in cells undergoing GzmB-induced death. Treatment of YAC-1 cells with rGzmB and purified perforin induced cell death as measured by 7-AAD incorporation (data not shown) (10). Cell death was observed after either Me$_2$SO or caspase inhibitor pretreatment. As shown in Fig. 2, Hop was cleaved in a time-dependent fashion in YAC-1 cells undergoing death induced by the addition of rGzmB and perforin. Hop cleavage proceeded at a similar rate in cells pretreated with Me$_2$SO (upper panel, lanes 3–5) or caspase inhibitor (lanes 8–10). The addition of rGzmB alone was insufficient to cause death (data not shown) or to lead to cleavage of Hop (lanes 2 and 7). We judged caspase inhibition to be successful since caspase-3 processing was arrested at the p20 form (lower panel, lanes 8–10). The observed cleavage of Hop was unlikely to be an artifact of cleavage by rGzmB during preparation of lysates for Western blotting for the following reasons. First, cells treated with rGzmB alone did not exhibit Hop cleavage, indicating that Hop cleavage was dependent upon delivery of rGzmB to the cells by perforin. If Hop was cleaved by residual GzmB after the cell lysates were made, no perforin dependence of cleavage would have been demonstrable. Second, an increased incubation time with perforin and rGzmB prior to lysate preparation yielded increased cleavage of Hop (upper panels, lanes 3–5 and 8–10). Lysates were prepared identically at each time point. Third, cell pellets harvested after treatment with perforin and rGzmB were solubilized in SDS- and β-mercaptoethanol-containing loading buffer and boiled immediately, which quickly inactivates GzmB. Taken together, these data demonstrate that Hop is cleaved directly by rGzmB in cells undergoing GzmB-induced death, in agreement with our proteomic data (1).

GzmB-generated Fragments of Hop Do Not Induce Cell Death—To examine the role of Hop cleavage in GzmB-mediated death, we first investigated the possibility that the GzmB-generated Hop fragments take on a novel pro-apoptotic function not associated with full-length Hop. We transiently cotransfected expression plasmids encoding amino acids 1–186 (N-terminal fragment, p20) and amino acids 187–543 (C-terminal fragment, p40) of Hop into YAC-1 cells. To mark the transfected population, the cells were also cotransfected with EGFP-C1. (To verify the ability of GFP to accurately mark the transfected population, we cotransfected YAC-1 cells with EGFP-C1 and EFYP-C1. Virtually 100% of cells expressing high levels of GFP also expressed high levels of YFP and vice versa (data not shown).) Cells cotransfected with both N- and C-terminal Hop fragments plus GFP were analyzed by flow cytometry and, and GFP$^+$ and GFP$^-$ populations were isolated by high-speed cell sorting (Fig. 3A). Western blotting of these populations demonstrated that GFP expression was indeed concordant with the simultaneous expression of the N- and C-terminal Hop fragments and that the expression levels of these fragments approached those of endogenous full-length Hop (Fig. 3B). Therefore, GFP cotransfection accurately marked the population expressing the N- and C-terminal Hop fragments.

To test the hypothesis that Hop fragments are toxic (i.e. their expression leads to cell death), we used a clonogenic assay (Fig. 3, C and D) as described (52). EGFP-C1 was transiently cotransfected with a vector containing the cDNA of full-length Hop or a Hop fragment, and at 4 h post-transfection, GFP$^+$ cells were sorted into 96-well plates. 12 days after sorting, wells were scored for growth. The relative clonogenic potential of cells transiently expressing high levels of Hop or its cleavage products is shown in Fig. 3C. As a positive control, we used a GFP fusion to tBid, the pro-apoptotic truncated form of the Bcl-2 family member Bid. Sorted GFP$^+$ cells transiently expressing tBid-GFP had a greatly reduced clonogenic potential compared with control cells transfected with EGFP-C1 plus empty vector. Transient expression of WT or D186E Hop had no effect on clonogenic potential, as expected. Expression of the N- or C-terminal fragment of Hop or both fragments did not reduce the clonogenic potential, suggesting that the fragments are not toxic. To ensure concordant expression of GFP and the Hop fragments, we also performed the clonogenic assay using GFP fusions to Hop fragments (Fig. 3D). Expression of these fragments with GFP fused to either end did not cause toxicity individ-
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It remained a possibility, however, that despite an inability to induce cell death on their own, Hop cleavage products could contribute to the demise of the cell in the context of GzmB-mediated apoptosis. To investigate this possibility, we tested whether expression of Hop cleavage products could augment GzmB-induced killing. Vectors containing cDNAs of the N- and C-terminal Hop fragments were transiently coexpressed with EGFP-C1 (as shown in Fig. 3A), and the heterogeneous transfected population was treated with GzmB and perforin. Death was evaluated by 7-AAD staining and flow cytometry. The viability index for the GFP~ and GFP~ populations for each sample is shown in Fig. 3E. The viability index was defined as follows. The viability of the GFP~ or GFP~ population was calculated by dividing the number of cells in the sample that were GFP~/7-AAD~ by the number of total cells in the sample. The viability index was arbitrarily set to 100 for samples treated with perforin alone. In control cells transfected with EGFP-C1 and empty vector, both GFP~ and GFP~ populations exhibited a dose-dependent decrease in viability upon GzmB treatment (left panel). The viability index for GFP~ cells was consistently lower than that for GFP~ cells for unclear reasons. Regardless, the difference between the GFP~ and GFP~ populations exhibited a dose-dependent decrease in viability upon GzmB treatment (left panel). The viability index for GFP~ cells was consistently lower than that for GFP~ cells for unclear reasons. Regardless, the difference between the GFP~ and GFP~ populations exhibited a dose-dependent decrease in viability upon GzmB treatment (left panel). The viability index for GFP~ cells was consistently lower than that for GFP~ cells for unclear reasons.

Cleavage of Hop at Asp^186 Destroys Known Binding and Assembly Functions of Hop—The structure of Hop is dominated by three tetratricopeptide repeat (TPR) domains, which can bind the solvent-exposed C-terminal peptide tails of Hsp70 and Hsp90 (31, 33, 53). Specifically, the N-terminal-most TPR domain,

FIGURE 3. Hop cleavage fragments do not induce cell death. A, a schematic of the gating strategy is illustrated. Shown are representative dot plots, gated on 7-AAD~ cells, of untransfected YAC-1 cells (upper panel) and YAC-1 cells cotransfected with three vectors: EGFP-C1 and vectors containing N-terminal (N) and C-terminal (C) Hop fragment cDNAs (lower panel). The GFP~ and GFP~ gates shown were used for high-speed cell sorting for plots in B and for analyses in E. A more stringent GFP~ gate (not shown) was used for experiments in C and D. B, populations sorted from cells cotransfected with EGFP-C1 and both N-terminal (N-term.) and C-terminal (C-term.) fragments as described for A were analyzed by Western blotting using two Hop-specific antibodies, one recognizing each fragment. C, YAC-1 cells were cotransfected with EGFP-C1 and empty expression vector (pcDNA3.1) or vector containing cDNAs of the Hop forms indicated, and 4 h later, the highest GFP expressors (~1% of all cells) were sorted into 96-well plates. Clonogenic potential was normalized to the GFP plus empty vector control. D, EGFP-C1 alone or vectors containing GFP-fused Hop forms were transfected and sorted as described for C. Each condition in C and D was performed at least twice, and error bars represent S.D. E, YAC-1 cells were cotransfected with EGFP-C1 and vectors containing N- and C-terminal Hop fragment cDNAs. 16–20 h later, 1 × 10^5 cells were treated with perforin alone or with the indicated doses of rGzmB for 1 h at 37 °C. Cells were analyzed by 7-AAD staining and flow cytometry, and the viability index was calculated as described under “Results.” The data shown are representative of three experiments.

B

C

D

E

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Hop Cleavage by Granzyme B

TPR1, has been shown to bind Hsp70. Additional domains of Hop and Hsp70 also contribute to this interaction (30, 54–56). Binding to Hsp90 is mediated by the central TPR domain of Hop, TPR2a. Hop can therefore serve as an adaptor for Hsp70 and Hsp90, bringing Hsp70-bound client proteins and both Hsp proteins into a single complex (30). Indeed, Hop is required for the efficient assembly of the progesterone and glucocorticoid receptors into mature, hormone binding-competent complexes in vitro (34, 36).

Asp186, the site at which GzmB cleaves Hop, falls within the amino acid region that separates TPR1 from TPR2a. It therefore amino acid region that separates TPR1 from TPR2a. It therefore participates in the Hsp-tethering function of full-length Hop. We hypothesized that cleavage of Hop at Asp186 by GzmB destroys an as yet undescribed anti-apoptotic function of Hop, thereby lowering the threshold for death induction.

To investigate this possibility, we first tested the performance of the Hop cleavage products in two assays of Hop function (30). Immunoprecipitation of Hsp70 or Hsp90 from rabbit reticulocyte lysate pulls down endogenous Hop by virtue of the Hop-TPR domain interaction. As shown in Fig. 4A (upper panel, lanes 1 – 8), immunoprecipitation of Hsp70 pulled down both endogenous Hop and Hsp90, whereas immunoprecipitation of Hsp90 pulled down endogenous Hop and Hsp70 as visualized by Coomassie Blue staining. When radiolabeled in vitro transcribed/translated WT or D186E Hop was added to the reticulocyte lysate prior to immunoprecipitation, both forms of exogenous Hop co-immunoprecipitated with Hop70, and with equal efficiency (middle panel, lanes 1 and 2). The same result was observed for Hsp90 immunoprecipitation (lanes 5 and 6). However, when an equimolar amount of radiolabeled N-terminal Hop cleavage fragment was added prior to immunoprecipitation, this fragment failed to associate with either Hsp70 or Hsp90 (lanes 3 and 7, respectively). The C-terminal cleavage fragment failed to associate with Hsp70, as expected (lane 4), but did immunoprecipitate with Hsp90 (lane 8). The in vitro transcribed/translated products were analyzed directly by SDS-PAGE to demonstrate proper radiolabeling (lower panel). Taken together, these results indicate that neither the N- nor C-terminal Hop cleavage fragment is capable of binding both Hsp70 and Hsp90, suggesting that GzmB-cleaved Hop would be unable to serve as a scaffold for Hsp proteins and that Hop cleavage may cause the dissociation of certain Hop-Hsp-client protein complexes.

Next, we assessed the ability of Hop cleavage products to participate in the in vitro assembly of an Hsp client, the progesterone receptor (30). Recombinant chicken progesterone receptor was added to reticulocyte lysate along with radiolabeled forms of Hop, and immunoprecipitation of the progesterone receptor was performed (Fig. 4B). Endogenous Hsp70, Hsp90, and Hop were all abundantly represented in the complex pulled down with the progesterone receptor (Coomassie Blue stain; upper panel). Although radiolabeled full-length WT Hop and D186E Hop also associated with these complexes, as expected, the C-terminal Hop fragment associated only very poorly, and the N-terminal fragment failed to associate at all (middle panel). These data demonstrate that Hop cleavage products do not participate in progesterone receptor assembly and suggest that GzmB-cleaved Hop would be unable to support the assembly of the progesterone receptor or other hormone receptors. Taken together, these immunoprecipitation analyses indicate that cleavage of Hop by GzmB destroys known functions of Hop in Hsp binding and hormone receptor assembly. Additionally, these results establish that the D186E mutation does not alter these functions of Hop.

Overexpression of GzmB-resistant Hop Does Not Protect Cells from GzmB-induced Death—Because Hop cleavage by GzmB destroys known functions of Hop, we wished to determine whether Hop harbors an anti-apoptotic function that might also be abolished upon cleavage. To address this hypothesis, we transiently overexpressed a vector containing WT or D186E Hop cDNA in YAC-1 cells. Again, EGFP-C1 was cotransfected, and GFP− and GFP+ populations were sorted using the strategy shown in Fig. 3A. Western blot analysis revealed that WT or D186E Hop was overexpressed by ~2–2.5-fold as estimated by densitometry (Fig. 5A). To demonstrate that D186E Hop expressed in YAC-1 cells was indeed cleavage-resistant, freeze/thaw lysates from sorted cells were treated with rGzmB. The Hop present in GFP− lysates from WT or D186E Hop transfectants was cleaved to completion (Fig. 5B, lanes 2 and 6, respectively). In GFP+ lysates from WT Hop transfectants, Hop was overexpressed (lane 3), but was still cleaved completely by GzmB (lane 4). The p40 fragment was of markedly greater...
abundance in this lysate than its GFP counterpart (lane 2). In contrast, Hop in GFPhi lysates from D186E Hop transfectants was not cleaved to completion. Approximately 25% of full-length Hop remained as estimated by densitometry (compare lane 6). α-Tubulin was cleaved to completion in all lysates, serving as a control for transfection efficiency. The data shown are representative of two experiments. Note that the left panel (empty vector) is identical to the left panel in Fig. 3E because the two experiments were run simultaneously and shared a control.

We then treated GFP plus WT or D186E Hop cotransfectants with rGzmB and performin as described for Fig. 3E. Again, GFPhi cells exhibited lower viability compared with GFP cells in the empty vector-transfected population (Fig. 5C, left panel). Comparison of GFP versus GFPhi populations in WT or D186E Hop transfectants revealed a very similar pattern (middle and right panels, respectively), indicating that overexpression of WT or cleavage-resistant Hop did not confer protection against GzmB-mediated death.

Effect of Hop Knockdowns on the Induction of Cell Death—As a more general means of addressing whether Hop can perform an anti-apoptotic function, we wished to develop a loss-of-function model for Hop in mouse cells. We transfected murine 129/SvJ embryonic stem (ES) cells with an isogenic targeting vector containing arms of 2.7 and 4.8 kb specific for the murine Stip1 (Hop) locus. 637 drug-resistant ES clones were screened by Southern blotting, but no correctly targeted clones were identified (data not shown). Therefore, we turned to an RNA interference approach to reduce cellular levels of Hop. YAC-1 cells were transiently transfected with control siRNA duplexes or with siRNA duplexes specifically targeting levels of Hop. At 48–72 h post-transfection, both Hop siRNAs reduced Hop protein levels to ~90–95% as estimated by densitometry, whereas neither a non-targeting siRNA nor an siRNA targeting the hemoglobin β-minor chain (which is not expressed in YAC-1 cells) altered Hop levels (Fig. 6A). Hop knockdown with Hop siRNA-1 was slightly more efficient, on average, than knockdown with siRNA-2. Hop knockdown did not affect the expression levels of its binding partners Hsp70 and Hsp90 (Fig. 6A).

We next assessed the response of Hop siRNA-transfected cells to the apoptosis-inducing agents etoposide and γ-radiation. At 48 h post-siRNA transfection, cells were treated with etoposide, and cell death was assessed by 7-AAD staining 36 h later (Fig. 6B). No significant difference in susceptibility was found between control cells and cells with reduced levels of Hop. γ-Irradiation was performed at 48 h post-siRNA transfection, and cells were analyzed by 7-AAD staining 48 h later (Fig. 6C). Cells with reduced Hop levels exhibited a small increase in sensitivity to this type of ionizing radiation. Transfection of Hop siRNA-1 resulted in a statistically significant increase in cell death upon irradiation compared across all doses with either of the controls (versus non-targeting control siRNA or β-minor siRNA, p < 0.0001). Hop siRNA-2 transfection yielded a significant difference relative to transfection with non-targeting control siRNA (p = 0.0043), but not relative to transfection with β-minor siRNA (p = 0.055).

We then treated siRNA-transfected YAC-1 cells with rGzmB and perforin at 52–58 h post-transfection. Experiments were performed with cells given no pretreatment (data not shown) or with cells pretreated with Me2SO (Fig. 6D) or caspase inhibitors at 100 μM (Fig. 6E). No difference in susceptibility to GzmB-mediated death was observed between cells transfected with control or Hop-specific siRNAs. Therefore, cellular levels of Hop do not set the threshold for death induced by GzmB, and Hop cleavage by GzmB does not represent a rate-limiting step in the GzmB death pathway.

Hop Knockdown Does Not Alter the Susceptibility of YAC-1 Cells to NK Cell-induced Death—To evaluate the susceptibility of cells with low levels of Hop in a more physiologic death assay, we transfected YAC-1 cells with siRNAs as described above and used them as targets in an NK cell-mediated cytotoxicity assay. Target cells were labeled with CFSE and co-incubated for 4 h with recombinant murine interleukin-15-activated primary murine NK cells. Death of CFSE-labeled target cells was assessed by 7-AAD staining and flow cytometry as described (51). No difference was detected between target cells transfected with control or Hop-specific siRNAs (Fig. 7A).

Interleukin-15-activated murine NK cells expressed both GzmA and GzmB at high levels.5 To examine NK cell cytotox-

5 T. A. Fehninger and T. J. Ley, unpublished data.
icity induced by individual granzymes, we performed the cytotoxicity assay using NK cells derived from different granzyme-deficient mouse strains (48–50). GzmA–/– NK cells, which approximate "GzmB-only" effector cells, did not kill Hop knockdown target cells more efficiently than control target cells (Fig. 7B). To investigate the possibility that GzmB cleavage of Hop lowers the threshold for death induced by other granzymes, GzmB-deficient NK cells were also tested. In addition to being GzmB-deficient, cytotoxic cells from GzmB–/–/PGK-neo mice express reduced levels of granzymes C and F (57), which are found in the GzmB gene cluster; GzmB–/–/ΔPGK-neo cytotoxic cells have restored levels of granzymes C and F (49). Target cells with reduced levels of Hop were no more susceptible than control target cells to GzmB–/–/ΔPGK-neo (Fig. 7C) and GzmB–/–/PGK-neo (Fig. 7D) NK effectors. GzmA–/– × GzmB–/–/ΔPGK-neo and GzmA–/– × GzmB–/–/PGK-neo doubly deficient NK effectors were also used in this assay, and likewise, no differences in susceptibility were observed between control and Hop knockdown target cells (data not shown). Taken together, these data indicate that Hop expression levels do not set the threshold for death induced by granzymes delivered by NK cells.

**DISCUSSION**

In this study, we have examined the consequences of Hop cleavage by GzmB during the induction of cell death. Hop was cleaved directly by GzmB at Asp186 in vitro, generating an N-terminal p20 fragment and a C-terminal p40 fragment, thereby separating the TPR1 and TPR2a domains of Hop. Hop was also cleaved (in a caspase-independent fashion) to its p40 fragment in YAC-1 cells undergoing GzmB-induced death. The Hop fragments generated upon cleavage were not pro-apoptotic because transient coexpression of the two fragments did not directly induce death or...
potentiate GzmB-induced death in YAC-1 cells. Hop cleavage products could neither bind Hsp70 and Hsp90 nor participate in progesterone receptor assembly in vitro, suggesting that cleavage of Hop by GzmB would abolish its ability to act as a scaffold for Hsp70 and Hsp90. However, overexpression of GzmB-resistant Hop did not protect YAC-1 cells from death induced by GzmB. siRNA-mediated knockdown of Hop by 90–95% slightly increased the susceptibility of cells to death caused by GzmB. In agreement with the knockdown data, no such protection was observed. Taken together, these results suggest that the abundance of full-length Hop in a cell does not dictate its susceptibility to GzmB-mediated death.

Although Hop does not appear to set the threshold for the induction of death by GzmB, several possibilities remain for the role of Hop cleavage in the GzmB death pathway. As discussed below, the contribution of Hop cleavage to GzmB-induced death may be too small to measure in our assays because of the redundancy of the GzmB death pathway. However, there is evidence that Hop promotes cell survival, including the survival of neurons in mice (39, 40) and the elimination of misfolded proteins in yeast (42). Alternatively, Hop may be a non-apoptotic substrate whose cleavage contributes to other important features of a controlled death process (7). For instance, Hop is a required component for the in vitro reconstitution of the active reverse transcriptase of hepadnavirus (38), so Hop cleavage by GzmB might abort virus replication in an infected cell attacked by a cytotoxic lymphocyte.

The identification of Hop and Hsp proteins (23) as granzyme substrates signals the emergence of a new class of granzyme substrate. The first GzmB substrates identified (the caspases) anchor the class of pro-apoptotic substrates, which also includes Bid, ICAD, and the GzmA substrates Ape1 and SET. These substrates activate death induction pathways in most cells. Proteins that are part of cytoskeletal, nuclear, and plasma membrane structures make up a second class of substrates, which includes α-tubulin, β-actin, ROCK II, lamin B, and the GzmA substrates lamin A and lamin B (24) and histones H1, H2A, and H3 (61). Direct targeting of the GzmB substrates in this group, all of which are also cleaved by caspases or have functional homologs that are cleaved by caspases (27, 62), may bypass the need for caspase activation to cause the cellular structural changes associated with apoptosis. The finding of a third class of granzyme substrates, the stress response/chaperone proteins, suggests that granzymes may also act to disassemble the normal stress response to a death stimulus. Indeed, heat shock–related proteins might be rational targets for granzymes: Hsp70 and/or Hsp90 has been reported to prevent Bid cleavage (63), to inhibit translocation of Bax to the mitochondria (64), and to negatively regulate apoptosome assembly/activation (65–67). Although it has yet to be demonstrated, Hop itself may participate in one or more of these mechanisms. Hsp proteins may also serve as non-apoptotic substrates; for instance, destruction of these chaperone molecules might inhibit assem-

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A. J. Bredemeyer, M. A. Ford, J. A. Ratner, R. R. Townsend, and T. J. Ley, unpublished data.
bly of viral particles in infected cells (68–71). Interestingly, GzmA has been shown to associate strongly with Hsp27, but not cleave it; the role of this stress response protein in GzmA-induced death remains unclear (72).

GzmB not only targets distinct classes of protein substrates, but also clearly targets multiple independent pathways that can induce apoptosis. Perhaps foremost, activation of the caspase cascade leads to the cleavage of hundreds of substrates (22, 62), many of which perform redundant functions. GzmB cleaves and activates Bid, leading to mitochondrial membrane changes that cause cytochrome c release (12, 32); it depolarizes mitochondria in the absence of Bid (19) and can directly cleave ICAD to activate CAD (caspase-activated DNase) (9, 10). The existence of multiple pathways is further supported by evidence from cells deficient in single GzmB substrates. Genetically defined caspase-3/– mouse embryonic fibroblasts are equally susceptible to perforin/GzmB treatment as WT mouse embryonic fibroblasts in a clonogenic replating assay (10), indicating that GzmB can induce death independently of caspase-3, as had been suggested by studies employing caspase inhibitors (17, 18). Likewise, GzmB is able to kill ICAD/– mouse embryonic fibroblasts, albeit less efficiently (10), revealing that activation of CAD by the cleavage of ICAD is unnecessary for death induction. We reported no difference between the susceptibility of WT and Bid/– mouse embryonic fibroblasts to perforin/murine GzmB treatment (19), whereas Waterhouse et al. (20) recently found Bid/– mouse cells to be relatively resistant to low doses (75 nM) of human GzmB. High doses (1 μM) of either human or murine GzmB can kill Bid/– cells, demonstrating that GzmB can kill cells in the absence of Bid. These findings provide solid evidence that deficiency in single substrates is generally insufficient to prevent GzmB from killing a target cell. It is perhaps not surprising, then, that the reduction of Hop abundance or the expression of GzmB-resistant Hop does not affect susceptibility to death.

These findings highlight the need for new approaches when defining the contributions of individual GzmB substrates. It has been recently suggested that all substrate validation should be performed in the setting of Bcl-2 overexpression and caspase inhibition (7). This approach would remove Bid- and caspase-mediated mechanisms from the alternative pathways at play, increasing the likelihood of detecting a death phenotype. This tactic assumes, of course, that the new substrate under study does not itself act in the same pathway as Bid or caspases. Careful validation of putative granzyme substrates still needs to be performed because it is relatively straightforward to identify gain-of-function pro-apoptotic properties of cleavage products. Such gain-of-function mechanisms, exemplified by the pro-apoptotic activity of tBid, may be easier to detect than the effects of loss-of-function mechanisms. As an alternative means to identify important substrates, a forward genetic approach could be very powerful. In our hands, selection of GzmB-resistant cells has thus far resulted in pan-granzyme or perforin resistance.7 However, mutagenesis or selection methods could be used to identify genetic or epigenetic changes that confer protection against granzyme-induced death pathways. This might well provide new insights into mechanisms by which virus-infected cells and tumor cells escape cytotoxic lymphocyte attack.

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