

Granzyme B Proteolyzes Receptors Important to Proliferation and Survival, Tipping the Balance toward Apoptosis^{*S}

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Granzyme B is critical to the ability of natural killer cells and cytotoxic T lymphocytes to induce efficient cell death of virally infected or tumor cell targets. Although granzyme B can cleave and activate caspases to induce apoptosis, granzyme B can also cause caspase-independent cell death. Thirteen prospective granzyme B substrates were identified from a cDNA expression-cleavage screen, including Hsp70, Notch1, fibroblast growth factor receptor-1 (FGFR1), poly-A-binding protein, cAbl, heterogeneous nuclear ribonucleoprotein H', Br140, and intersec-tin-1. Validation revealed that Notch1 is a substrate of both granzyme B and caspases, whereas FGFR1 is a caspase-inde-pendent substrate of granzyme B. Proteolysis of FGFR1 in pros-tate cancer cells has functionally relevant consequences that indicate its cleavage may be advantageous for granzyme B to kill prostate cancer cells. Therefore, granzyme B not only activates pro-death functions within a target, but also has a previously unidentified role in inactivating pro-growth signals to cause cell death.

Natural killer (NK)⁴ cells and cytotoxic T lymphocytes (CTLs) are the primary defense of the immune system against viral infection and intracellular pathogens. These cytotoxic cells can also kill a variety of cancer cells, giving support to the immunosurveillance hypothesis (1, 2). Indeed, tumor immuno-therapy approaches aim to treat cancers with NKs and CTLs that have potent anti-tumor recognition and killing activities. To that end, it is important to elucidate the mechanisms and

pathways that are important to the ability of NKs and CTLs to kill a particular cancerous cell.

NK cells and CTLs induce the death of their targets by either tumor necrosis factor family death receptor/death ligand inter-action or by granule exocytosis. Granule exocytosis is critical for the efficient killing of virally infected or tumor cell targets (1, 3). Upon target recognition, killer cells release cytotoxic gran-ules, the contents of which cross the immunological synapse and enter the target cell with the assistance of perforin, a puta-tive pore-forming protein. Perforin may enable escape from endocytic vesicles or may allow entry directly through pores in the plasma membrane (1).

Granzymes are serine proteases that are released from the cytotoxic granules to induce target-cell death. There are five human granzymes, termed A, B, H, K, and M. Granzyme B (GrB) can initiate apoptosis by cleaving and activating several members of the caspases, the intracellular pro-apo-ptotic cysteine proteases (4, 5). GrB is required for efficient target cell lysis (6) and DNA fragmentation (7), and treat-ment of target cells with GrB and a delivery agent such as perforin results in these and other common hallmarks of cell death, including nuclear condensation, phosphatidylserine exposure, mitochondrial depolarization, and cytochrome c release (1).

It has also become clear that GrB can induce cell death inde-pendently of caspases. GrB has a similar preference to caspases for cleaving proteins C-terminal to aspartic acid residues (8, 9). GrB has been shown to cleave many caspase substrates such as poly(ADP-ribose) polymerase (PARP) (10), inhibitor of caspase-activated DNase (7), nuclear lamins (11), and Bid (12). Although the morphologies associated with caspase-independ-ent cell death caused by GrB are chiefly characterized by mito-chondrial disruption (13) and lysis (6), the exact cleavage events leading to these hallmarks have not all been completely eluci-dated. This is especially true in light of the fact that GrB can cause apoptosis in the absence of Bid, Bax, and Bak (14). More-over, GrB may cleave other substrates to ensure target cell death but that do not result in a currently recognizable physio-logical trait. Elucidation of all GrB substrates will unveil a more detailed mechanism of cytotoxic cell death.

To identify substrates of GrB, a cDNA expression and cleav-age screen was employed. Several new prospective substrates of granzyme B were identified, and three, Hsp70, FGFR1 and Notch1, were validated. We show here these proteolytic events as well as how the cleavage of FGFR1 by GrB could be important in targeting prostate cancer.

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⁴ The abbreviations used are: NK, natural killer cell; CTL, cytotoxic T lymphocyte; GrB, granzyme B; PARP, poly(ADP-ribose) polymerase; z, benzyl-oxycarbonyl; FMK, fluoromethyl ketone; FGFR1, fibroblast growth factor receptor-1; FGFR1c, FGFR-intracellular; ERK, extracellular signal-regulated kinase; CI, caspase inhibitors zVAD-FMK and zDEVD-FMK; PI, propidium iodide; GI, GrB inhibitor L-038597; BP, lipid protein-delivery reagent BioPORTER; NTM, Notch1-TM; E:T, effector to target ratio; NICD, Notch intra-cellular domain.

EXPERIMENTAL PROCEDURES

Reagents—The caspase inhibitor zDEVD-FMK was purchased from Alexis Biochemicals. The caspase inhibitor zVAD-FMK was purchased from EMD Biosciences and Alexis Biochemicals. When present, both caspase inhibitors are used in equimolar amounts and are designated as CI. The Notch1 antibody Nic927 was a gift from Anthony Capobianco (University of Cincinnati). Antibodies to Lamin B (C-20), Myc (9E10), PARP (H-250), Notch1 (C-20), and Flg (H-76) were purchased from Santa Cruz Biotechnology. Another FGFR1 antibody (Ab-2) was from EMD Biosciences. Purchased from Cell Signaling Technologies were antibodies to PARP, Notch1 (6A5), FGFR1, phospho-FGFR1, phospho-ERK, and ERK. Production of rat granzyme B (GrB) was previously described (8). Human GrB was a generous gift from either Dr. Nancy Thornberry (Merck) or Dr. Sandra Waugh Ruggles (Catalyst Biosciences). The small molecule GrB inhibitor L-038597 (GI) was developed and described in a previous study (15) and was a generous gift from Dr. Nancy Thornberry (Merck).

Plasmid Construction—All inserts were ligated into the Invitrogen mammalian expression vector pcDNA 3.1 Myc/Hisx6. Each overexpressed protein therefore was C-terminally Myc/Hisx6-tagged. The Notch1-ic clone was generated from a Notch1-ic vector that was a generous gift from Anthony Capobianco (University of Cincinnati). The insert coding for Notch1-ic residues 1759–2556 was liberated by BamHI and XbaI digestion and ligated into the expression vector. The plasmids for FGFR1-ic, FGFR1-VSAD, and FGFR1-VSAN were generated from the clone for mouse FGFR1 (IIIc isoform), a generous gift from Dr. David Ornitz (Washington University at St. Louis). The primers used to generate FGFR1-ic were 5'-CAC AAG CTT AAG ATG AAG AGC GGC ACC AAG-3' and 5'-GCC GAA TTC GCG CCG TTT GAG TCC ACT GTT GGC-3'. The latter primer was also used along with the forward primer 5'-CAC AAG CTT ATG TGG AGC TGG AAG TGC CTC CTC TTC TG-3' to generate the full-length insert for cloning both FGFR1-VSAD and FGFR1-VSAN. The FGFR1-VSAN clone was made from FGFR1-VSAD using the QuikChange site-directed mutagenesis kit (Stratagene) and the mutagenesis primers 5'-CAG ACA GGT AAC AGT GTC AGC TAA CTC CAG TGC ATC CAT GAA CTC-3' and its reverse complement. Human FGFR1-gBEC was cloned by reverse transcription-PCR on mRNA prepared from LNCaP cells using the RNeasy Mini Kit and Omniscript reverse transcriptase (both from Qiagen) and the primers 5'-CAC AAG CTT ATG TGG AGC TGG AAG TGC CTC CTC TTC TG-3' and 5'-GCC GAT ATC GTC AGC AGA CAC TGT TAC CTG TCT GCG-3'.

Cell Lines and Culture—All cell lines were available from the American Type Culture Collection (ATCC.) K562 chronic myelogenous leukemia, Jurkat T-cell leukemia, LNCaP, and PC3 prostate cancer cell lines were all propagated in RPMI 1640 media containing 10% fetal bovine serum and penicillin/streptomycin. NK-92 human natural killer cells were cultured in RPMI 1640 containing 10% fetal bovine serum, 10% fetal calf serum, glutamine, non-essential amino acids, sodium pyruvate, β -mercaptoethanol, penicillin and streptomycin, and 100

units/ml interleukin-2. All cells and transfectants were maintained in a humidified 37 °C, 5% CO₂ incubator.

Small Pool cDNA Screening—A cDNA library was constructed from RNA isolated from a 14-day post coitus mouse embryo as previously described (16). Briefly, the cDNA library was cloned into the high copy number plasmid pCS2+ to yield 200,000 independent clones. The library was divided into pools of ~100 unique plasmids. [³⁵S]Methionine-labeled protein pools were prepared directly from the cDNA pools using the coupled transcription-translation rabbit reticulocyte lysate system (Promega) (17). Briefly, 5 μ l of transcription-translation mix was incubated at 30 °C with 0.1 μ g of pooled DNA. After 2 h, half of the labeled protein reaction mix was incubated with 1 nM recombinant rat granzyme B, 50 μ M CI, and 50 μ M Ac-IETD-FMK in a buffer containing 100 mM Hepes, pH 7.4, and 100 mM NaCl. After 1 h, loading dye was added to each reaction mix, and the mixture was heated at 65 °C for 10 min. The samples were loaded side-by-side onto 10–20% Tris-glycine gels and separated by SDS-PAGE. Separated proteins were visualized by autoradiography and scored for cleavage by the disappearance or appearance of bands in the granzyme B-incubated samples that were not found in the control samples.

Proteolysis of Hsp70 in Vitro—Purified Hsp70 protein was purchased from Stressgen. Hsp70 protein at 0.64 μ M was incubated at 37 °C with 50 nM human GrB in GrB activity buffer containing 50 mM Na HEPES, pH 8, 100 mM NaCl, and 0.01% Tween 20. Aliquots of 15 μ l containing 670 ng of Hsp70 were taken at specific intervals and mixed with sample loading buffer. Samples were loaded onto 4–20% Tris-glycine gels and separated by SDS-PAGE. Separated proteins were visualized by Coomassie staining.

In Vitro Transcription-Translation—Plasmids encoding FGFR1-ic and Notch1-ic were subject to T7 Quick Coupled *in vitro* transcription-translation (Promega) in the presence of [³⁵S]methionine. Samples were then diluted into GrB activity buffer and incubated with human GrB at 37 °C for 1–3 h. Aliquots were separated by SDS-PAGE; gels were imaged by autoradiography.

Lysis and Immunoblotting—Cytoplasmic lysates 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 assays described below were lysed in Lysis Buffer including the Complete Protease Inhibitor Mixture tablets from Roche Applied Science (Lysis Buffer plus PI) to stop proteolysis. After incubating on ice for 30 min, lysates were spun at 10,000 rpm for 10 min at 4 °C to remove the insoluble fraction. 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. Proteins were then transferred to nitrocellulose or polyvinylidene difluoride membranes and blocked in TBST containing 5% milk or 5% bovine serum albumin, as per the antibody manufacturer's instructions. 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 detection system. The locations of pre-stained protein markers were traced by overlaying the

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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. Samples were then subjected to SDS-PAGE and immunoblotting.

NK-mediated Cytotoxicity—NK-92 effectors (E) and K562 targets (T) were collected, counted, and mixed at varying E:T ratios as in Ref. 6, with 2×10^5 K562s in 24-well plates. Killing was stopped at intervals from 1 to 6 h by collecting and lysing in Lysis Buffer plus PI.

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 media containing 2% serum. Cells were stimulated with 200 ng/ml CH-11 anti-Fas antibody (Upstate) and incubated from 2 to 6 h. At the indicated time intervals, aliquots were lysed in Lysis Buffer plus PI.

GrB Delivery to LNCaPs—Human GrB was delivered to LNCaP cells with the lipid protein-delivery reagent BioPORTER (BP, Gene Therapy Systems). BioPORTER has been used for intracellular delivery of a variety of proteins (18). This approach has been used to deliver GrB and is an excellent method for analyzing components of cytotoxic granules (19). GrB-BP complexes were established in QuikEase Single-Use tubes according to the manufacturer's instructions and delivered to pre-seeded LNCaPs at 75 nM GrB. Cells were incubated and time points were collected for lysis in Lysis Buffer plus PI. For flow cytometry analysis, serum was added to 30% after 3–4 h of GrB delivery, and samples were incubated for another 2–3 h. The cells were incubated overnight in media containing 10% serum. The following morning cells were harvested, stained with Annexin V-FITC (BioVision), and analyzed on a FACS-Calibur according to the manufacturer's instructions. Dead cells were counted as the Annexin V-positive (FL1) population.

Transient Transfections—Mammalian expression plasmids with FGFR1-VSAD, FGFR1-VSAN, or FGFR1gBEC were transiently transfected into LNCaPs or PC3s with Lipofectamine 2000 (LF) reagent (Invitrogen) according to the manufacturer's instructions. Briefly, LNCaPs were seeded in 500 μl of media without antibiotics so that $\sim 0.5\text{--}2 \times 10^5$ cells were in one well of a 24-well plate the day of the transfection. A DNA-LF complex was formed in OPTI-MEM by combining 0.8 μg of plasmid DNA with 2 μl of LF; the complex was then added to one well of a 24-well plate. PC3s were transfected in 12-well (trypan blue) or 96-well (proliferation) plates and scaled accordingly. After 6 h all cells were changed into fresh media and incubated for 24–48 h, depending on the experiment.

FGF2 Stimulation—LNCaPs were transiently transfected with FGFR1-VSAD as outlined above to monitor FGFR1 cleavage. At 36 h post transfection, cells were incubated with or without GrB-BP complexes in serum-free media as above. After 6 h, transfectants were stimulated for 10 min in the presence or absence 50 ng/ml purified recombinant FGF2 (R&D Systems) and 50 $\mu\text{g}/\text{ml}$ heparin. Samples were then lysed in Lysis Buffer plus PI containing the phosphatase inhibitors 1 mM sodium orthovanadate and 25 mM β -glycerophosphate,

pH 7.3. To monitor overexpressed FGFR1-VSAD or FGFR1-VSAN directly after FGF2 stimulation, the receptors were immunoprecipitated from the lysate with anti-Myc antibodies and protein G-Sepharose. The pulled-down receptors were separated by SDS-PAGE and subjected to immunoblotting for both their Myc tags and for phospho-FGFR1.

Trypan Blue Staining and Proliferation Assay—Viability of PC3s transiently overexpressing FGFR1-VSAD or FGFR1-gBEC for 48 h was determined by trypan blue staining (Invitrogen). Cells that were still adherent were collected by mild trypsinization, washed, resuspended in complete media, and counted on a hemocytometer. The data are presented as the average percent dead and alive cells, and error bars represent one standard deviation above and below the mean of at least three independent transfection experiments. Proliferation of PC3s 48 h post transfection was assessed with the CellTiter 96 Aqueous One Solution cell proliferation assay (Promega) according to the manufacturer's instructions. Briefly, 20 μl of Aqueous One Solution reagent was added to the cells in 100 μl of media. The cells were incubated for 2 h, and then the absorbance at 490 nm was recorded. Readings are presented as the mean A_{490} with error bars representing one standard deviation above and below the mean of six independent transfections. A Student's *t* test with a two-tailed distribution was calculated to determine the statistical significance of FGFR1gBEC from FGFR1-VSAD transfectants.

RESULTS

An *in Vitro* cDNA Expression Cleavage Screen Identified Several Substrates of Granzyme B—To identify new GrB substrates, a cDNA library containing $\sim 200,000$ unique cDNAs was screened. The library was divided into pools each containing ~ 100 unique plasmids. The cDNA pools were *in vitro* transcribed and translated, and the resulting [^{35}S]methionine-labeled protein pools were incubated in the presence or absence of GrB and the caspase inhibitors zVAD-FMK and zDEVD-FMK (CI). Proteins were separated by SDS-PAGE, visualized by autoradiography, and scored for caspase-independent proteolysis.

Cleavage-positive pools were split into smaller sub-pools, which were then re-transformed and subjected to a subsequent round of DNA purification and *in vitro* transcribed and translated and screening. Fig. 1A is an autoradiograph of seven sub-pools from one initial positive pool. In sub-pool #5, a band at ~ 47 kDa decreased in intensity and a smaller band at 40 kDa was enriched when GrB was added. Positive sub-pools like #5 continued to be split into smaller sub-pools and screened until individual cDNAs were isolated. The cDNAs were sequenced and subjected to a BLAST search. The cDNA isolated from positive sub-pool #5 was identified as the fibroblast growth factor receptor-1 (FGFR1), and other prospective GrB substrates identified were: Notch1, cAbl, Hsp70, heterogeneous nuclear ribonucleoprotein H', Br140, intersectin-1, and poly-A-binding protein. Five additional clones matched cDNAs that have undefined protein identities. Lamin B, a known GrB substrate (11), was also isolated, demonstrating the efficacy of the screen.

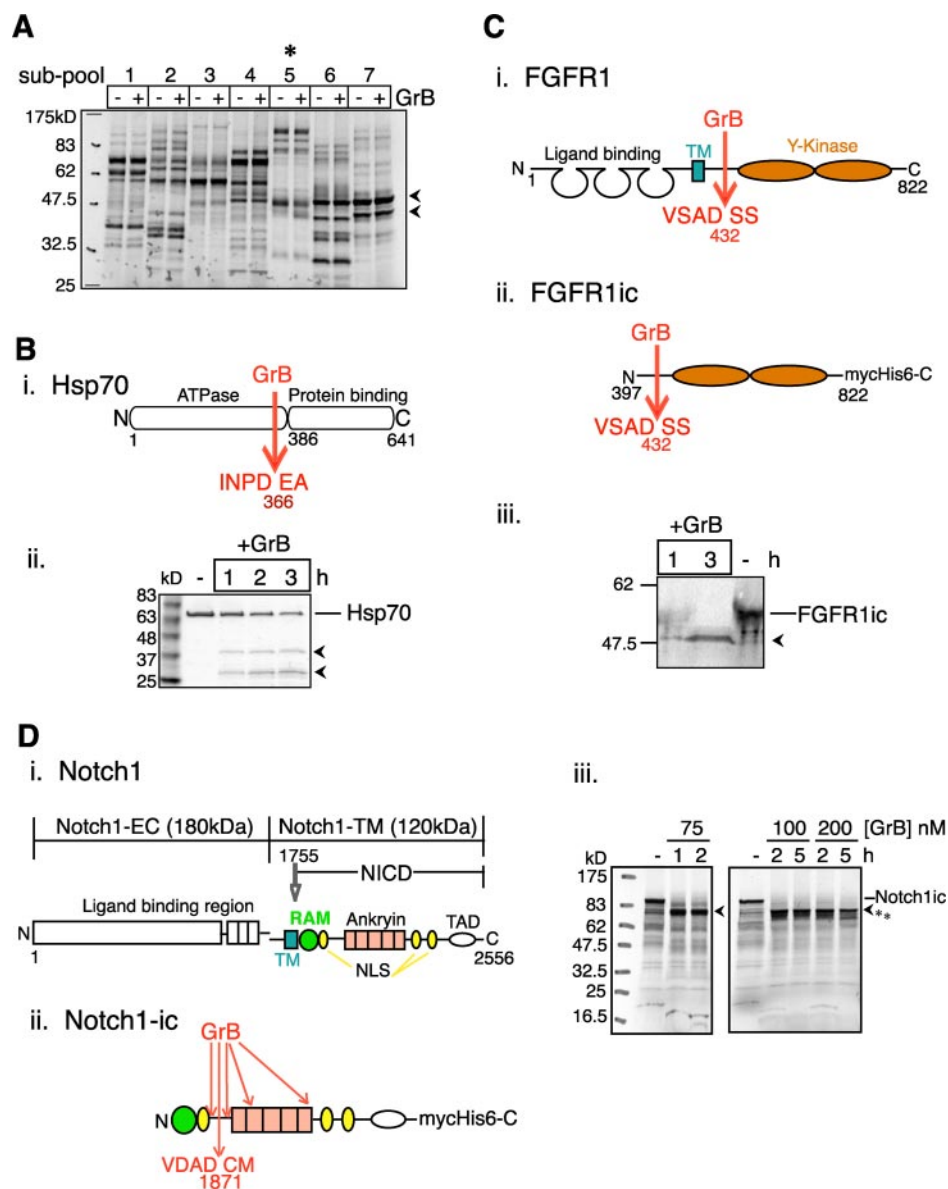


FIGURE 1. Proteolysis of new GrB substrates *in vitro*. A, identification by autoradiography of positive cDNA sub-pool #5. B, panel i, schematic of Hsp70 protein indicating the possible GrB cleavage site at D366, and panel ii, *in vitro* cleavage of purified recombinant Hsp70 by GrB. C, panel i, schematic of FGFR1 with three extracellular Ig-C2-like loops for ligand binding, the transmembrane (TM) region, and the tyrosine kinase domain (Y-Kinase). The prospective GrB cleavage site is at Asp-432. Numbers indicate residue position. Panel ii, FGFR1-intracellular (FGFR1ic). Panel iii, autoradiograph showing proteolysis of *in vitro* transcribed and translated, [³⁵S]Met-labeled, FGFR1ic incubated with or without GrB. D, panel i, domains of the Notch1 receptor. Please see the text for descriptions. Notch1-EC, Notch1 extracellular polypeptide; NLS, nuclear localization sequence; TAD, transactivation domain; RAM, RAM domain. Panel ii, schematic of Notch1-intracellular (Notch1-ic). Autoradiograph, panel iii, depicting proteolysis of *in vitro* transcribed and translated, [³⁵S]Met-labeled, Notch1-ic upon incubation with GrB. Arrowheads indicate major proteolytic products, and asterisks indicate minor products.

Each Identified Substrate Has a Prospective Granzyme B Cleavage Site—GrB has a strict requirement for cleaving C-terminal to Asp residues due to Arg-226 in its P1 pocket (20, 21). Armed with this fact as well as the well characterized extended substrate specificity of the enzyme (8, 9), protein sequences were searched for possible cleavage sites. Table 1 presents the prospective GrB cleavage sites within the identified proteins. According to protease substrate nomenclature, the (N-terminal to C-terminal) P4 thru P2' residues are listed, with the scissile bond located between P1 and P1'. The optimal substrate of GrB is the sequence (I/V)EPD~SG. However, specificity profil-

ing data as well as research into the structural determinants of the specificity of GrB (22) has revealed that the P1 and P4 sites are of critical importance, whereas GrB can accommodate a broader range of residues at the other positions. Depicted in Table 1, the importance of the P4 thru P2' sites is proportional to the size of the letter. Other known substrates of GrB such as caspase 3, PARP, and Bid are cleaved at sites that are a close but not exact match to IEPD SG (Table 1). This understanding created an appropriate sieve for eliminating poly-A-binding protein, which has a less than optimal P4 Leu, from further investigation.

Hsp70, FGFR1, and Notch1 Are Substrates of Granzyme B in Vitro—The ability of GrB to proteolyze Hsp70 was assessed *in vitro* by incubating purified recombinant Hsp70 with 50 nM GrB (Fig. 1B). The cleavage products that appear as early as 1 h (h) are 40 and 30 kDa, as expected given the predicted cleavage site at Asp-366.

Proteolysis by GrB of FGFR1 and Notch1 (Fig. 1, C and D, respectively) was first confirmed *in vitro* by incubating GrB with *in vitro* transcribed and translated peptides corresponding to the intracellular portions of each transmembrane protein (Fig. 1, C (panel ii) and D (panel ii)). The mobility shift of intracellular FGFR1 (FGFR1ic, Fig. 1C), after incubation with GrB, confirms that it is a substrate of GrB *in vitro*, and the size change is consistent with cleavage at Asp-432. Intracellular Notch1 (Notch1-ic, Fig. 1D), which runs at ~85 kDa, appears to be cleaved at one major site yielding a product of ~75 kDa

(arrowhead) but also appears to be proteolyzed further (asterisks) upon longer incubation with increasing concentrations of GrB. The banding pattern of the cleavage products is consistent with proteolysis occurring in the region of Notch1-ic that aligns to the location of the predicted cleavage sites (Fig. 1D and Table 1).

FGFR1 and Notch1 Are Caspase-independent Substrates of Granzyme B—Proteolysis was next confirmed by treating human K562 chronic myelogenous leukemia lysates with GrB. Using cell extracts is a valid approach, because GrB enters the cytoplasm of target cells with the assistance of perforin to induce apoptosis. Aliquots were removed over time, separated

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TABLE 1

Prospective granzyme B cleavage sites within each identified substrate

Substrate Identified	Putative cleavage site(s) P4-P3-P2-P1 ~ P1'-P2'
GrB optimal substrate	I/V _E P _D SG
Hsp70	INPD366 EA
FGFR1	VSAD432 SS
Notch1	VLPD1834 LD
	VDAD1871 CM
	VISD1914 FI
	VSAD1973 AQ
	VLLD2083 HF
cAbl	VASD36 FE
hnRNP H'	VEVD94 WV
	IGPD324 GR
PABP	LEID589 NS
Br140	VEVD102 LH
Intersectin 1	IDQD271 GK
Lamin B	VEVD231 SG*
Pro-caspase 3	IETD175 SG**
PARP	VDPD535 SG***
Bid	IEAD75 SE****

* From Ref. 11.

** From Refs. 4 and 5.

*** From Ref. 10.

**** From Ref. 12.

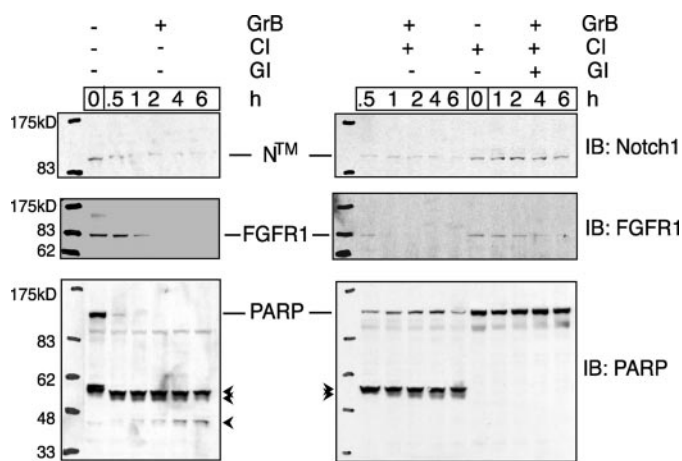


FIGURE 2. Proteolysis of GrB substrates in K562 lysates. K562 cell lysates were incubated with or without 50 nM GrB in the presence or absence of 20 μ M GI and/or 50 μ M CI. Aliquots were removed and lysed at the indicated times. Samples were separated by SDS-PAGE and subjected to immunoblotting (IB) with primary antibodies specific for the indicated GrB substrates.

by SDS-PAGE, and immunoblotted for Notch1, FGFR1, or PARP (Fig. 2). Notch1 exists on the cell surface as a heterodimer (Fig. 1D, panel i); the peptide that contains the transmembrane and intracellular portions of the receptor is ~120 kDa and is known as Notch1-TM (N^{TM}). Within only 1 h, both FGFR1 and N^{TM} have been proteolyzed upon incubation with GrB (Fig. 2, left panels). Including CI did not prevent cleavage, indicating that proteolysis is independent of caspase activation (Fig. 2,

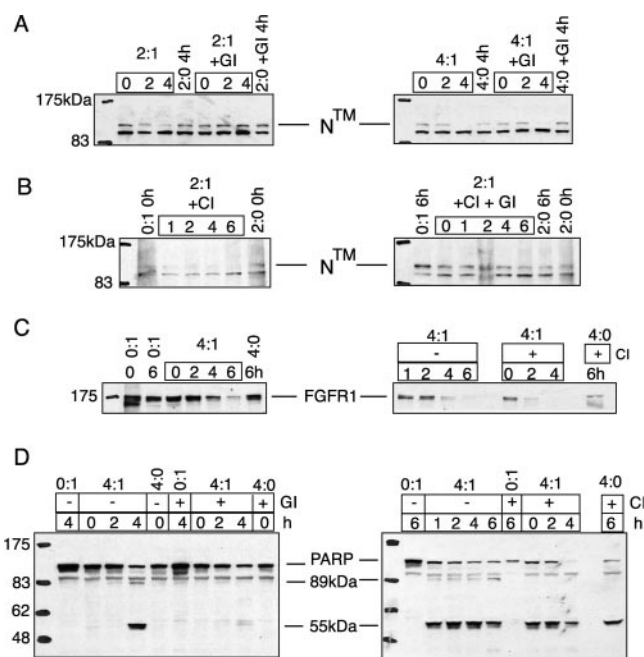


FIGURE 3. Proteolysis of GrB substrates during NK-cell mediated cytotoxicity. NK-92 (E) cells were added to K562 targets (T) at an E:T of either 2:1 or 4:1 and incubated from 1 to 6 h. Immunoblots show N^{TM} during cell killing in the presence or absence of 20 μ M GI (A), and in the presence of CI with or without GI (B). NK-92s incubated alone at the indicated concentrations are in lanes marked 2:0 or 4:0. K562 targets incubated alone are shown in lanes marked 0:1. C, immunoblot showing FGFR1 during cell killing with or without 50 μ M CI. D, immunoblot showing proteolysis of PARP during cell killing with or without CI and with or without GI.

right panels). However, N^{TM} cleavage appears to be less efficient in the presence of CI when comparing the 2-h time points, indicating that N^{TM} may also be a caspase substrate. PARP, a substrate of both GrB and caspases, was also monitored in this system (bottom panels). The addition of a small molecule-specific granzyme B inhibitor (GI, right panels) largely blocked the proteolysis of the full-length proteins.

Notch1 and FGFR1 were also confirmed to be proteolyzed during NK cell-mediated killing of K562 targets (Fig. 3). NK cells kill K562s, which do not express a death receptor, exclusively by granule exocytosis. Potent NK-92 effectors (E) were co-incubated with K562 targets (T), and proteolysis was monitored by immunoblotting.

Notch1 cleavage during NK-mediated cell killing of K562s is presented in Fig. 3 (A and B). N^{TM} is proteolyzed within 2 h and almost completely by 4 h at an E:T of 2:1 (Fig. 3A, left). At 4:1, N^{TM} is totally absent at 4 h (Fig. 3A, right). The proteolysis is not due to NK cells killing themselves, because the 2:0 and 4:0 controls show no destruction of the N^{TM} band. Furthermore, the addition of GI (Fig. 3, A and B) blocks efficient cleavage, and therefore proteolysis of these substrates is due to GrB and cannot be rescued by other granzymes. The addition of CI (Fig. 3B, left) shows that N^{TM} is cleaved during cell killing in a caspase-independent manner. The antibody used to detect N^{TM} in these experiments recognizes a cross-reactive species at 100 kDa.

FGFR1 is also proteolyzed during NK-mediated cytotoxicity to a significant extent by 4 h and completely by 6 h under an E:T of 4:1 (Fig. 3C). FGFR1 is also a caspase-independent substrate during cell killing (Fig. 3C, right panel). In fact, proteolysis of

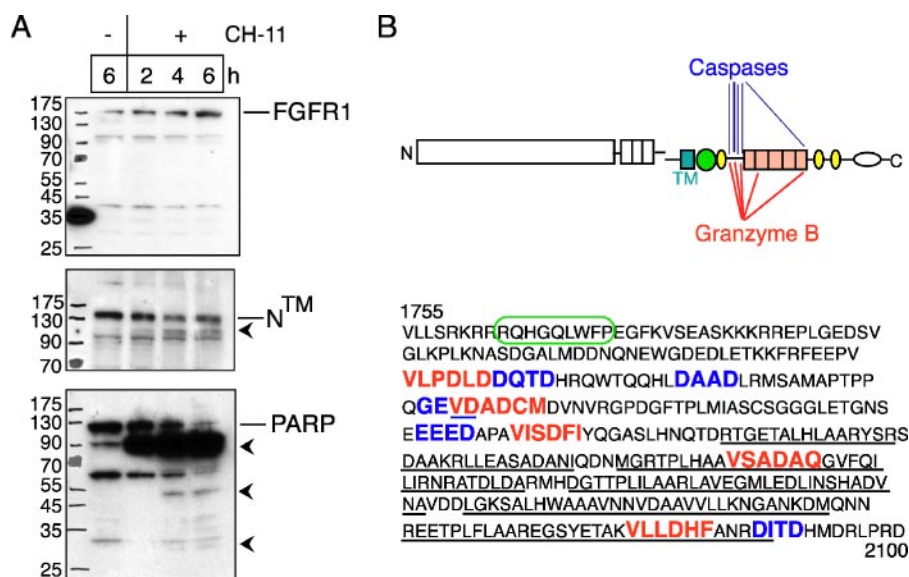


FIGURE 4. **Notch1 is also a caspase substrate, whereas FGFR1 is unique to GrB.** *A*, immunoblots to each prospective substrate after Jurkat cells were treated with CH-11 antibody. *B*, prospective caspase and GrB cleavage sites align to the same regions within Notch1. The schematic, *top*, and primary amino acid sequence from residues 1755–2100, *bottom*, indicate the location of possible caspase (blue) and GrB (red) cleavage sites. The green oval represents the RAM domain, and the underlined regions are the ankyrin repeats. The caspase site GEVD overlaps with the GrB site VDAD-CM.

FGFR1 seems to occur more efficiently by 2 h in the presence of CI. This may indicate that, without apoptotic amplification by caspases, GrB can cleave other substrates more robustly. The cytotoxic cell would therefore still induce the death of a target whose caspase cascade is blocked.

Proteolysis of PARP was also assessed during the cell killing experiments (Fig. 3D). At either 2:1 or 4:1 PARP is cleaved, and the 89-kDa caspase product and 55-kDa GrB products appear (8, 10). The presence of CI does not prevent the GrB product from appearing, but the 89-kDa species is no longer generated. Similarly, the addition of GI blocks proteolysis of PARP altogether, because GrB is blocked from cleaving PARP and from activating caspases.

The immunoblots presented in Figs. 2 and 3 are representative of at least three independent experiments. Supplemental Fig. S1 presents the compiled data from validating FGFR1 and Notch1 proteolysis in both GrB-treated K562 cell lysates and during NK-mediated cell killing of K562s. Proteolysis was quantified using densitometry and is presented as the percent mean intensity of the full-length protein over time. Results were normalized to the zero hour (lysates) or K562 alone (cell killing) negative controls. It can be concluded from Figs. 2, 3, and S1 that FGFR1 and Notch1 are indeed caspase-independent substrates of GrB.

It must be noted that our antibodies fail to reliably recognize cleavage products of either FGFR1 or Notch1. Therefore, it cannot be ruled out that in K562s, GrB targets the receptors for complete degradation. This possibility is explored under “Discussion.”

Notch1 Is Also a Caspase Substrate, whereas FGFR1 Is Unique to Granzyme B—Although proteolysis by GrB during NK-mediated cell killing of both Notch1 and FGFR1 is caspase-independent, it is possible that they are also caspase substrates. Many substrates of GrB can also be cleaved by caspases, and if so, are cleaved by the caspases at distinct aspartic acid residues.

To determine if these receptors are caspase substrates, the caspase cascade must be activated in the absence of GrB. The other method by which an NK or CTL kills its target is by the interaction of a death receptor like Fas with its ligand, FasL. This interaction results in caspase activation. Jurkat T-cell leukemia cells, which express Fas, were stimulated by the anti-Fas antibody CH-11, a surrogate for FasL. Aliquots were removed from 0 to 6 h, lysed, separated by SDS-PAGE, and subjected to immunoblotting. Under these conditions, the full-length PARP at 120 kDa is cleaved very efficiently to its major caspase product at 89 kDa in as little as 2 h (Fig. 4A, *bottom panel*).

In this system, NTM is cleaved by caspases, and a cleavage product appears as early as 2h (Fig. 4A, *middle*). Notch1 has several prospective

caspase cleavage sites that could create this ~100-kDa product (Fig. 4B). In fact, the prospective caspase cleavage sites align to the same regions as the GrB cleavage sites, between the RAM domain and the ankyrin repeats as well as the C-terminal portion of the ankyrin repeats (Fig. 4B).

FGFR1 is not proteolyzed upon Fas stimulation of Jurkat cells and therefore is not a caspase substrate (Fig. 4A, *top*). It is worth noting that the amount of FGFR1 does appear to increase during CH-11 stimulation, but the significance of this is not known.

Granzyme B Can Kill LNCaP Prostate Cancer Cells and in the Process Cleaves FGFR1—The effects of FGFR1 proteolysis by GrB are best analyzed in a cell in which FGFR1 is important. Because a wealth of evidence exists to implicate signaling through FGFR1 in promoting the growth and progression of prostate cancer (23), we chose to investigate if proteolysis of FGFR1 by GrB could have anti-prostate cancer consequences.

GrB was delivered into LNCaP prostate cancer cells with the lipid-mediated protein delivery reagent BioPORTER (BP). As demonstrated by PARP proteolysis as well as Annexin V staining, delivery of GrB results in cell death (Fig. 5, *B* and *C*). FGFR1 is simultaneously proteolyzed upon incubation with GrB·BP (Fig. 5A). Proteolysis of FGFR1 does not occur when LNCaPs are incubated alone or with BP only.

The FGFR1 antibody used in Fig. 5A also recognizes a faint band corresponding to the predicted FGFR1 cleavage product at ~55 kDa. To visualize proteolysis more easily, we constructed a C-terminally Myc/His-tagged clone of full-length FGFR1 (FGFR1-VSAD, Fig. 6, *A* (*panel i*) and *B*). LNCaPs were transiently transfected with FGFR1-VSAD and at 36 h post transfection were treated with GrB·BP. The *top panel* of Fig. 6B shows that FGFR1-VSAD was indeed cleaved by GrB, and a cleavage product at ~55 kDa was generated. This band is consistent with the receptor being proteolyzed at Asp-432.

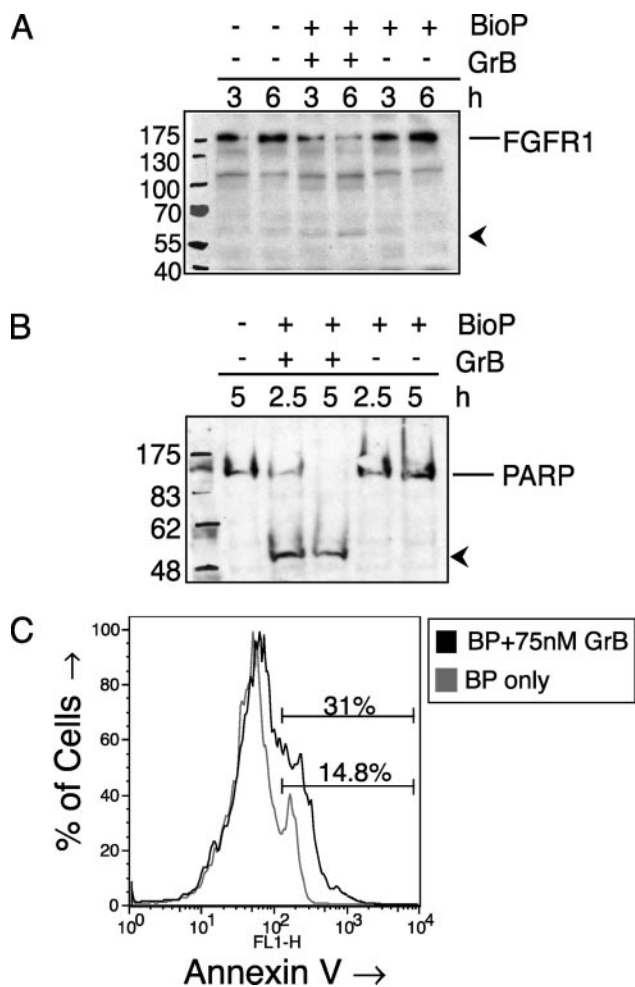


FIGURE 5. GrB can kill LNCaP prostate cancer cells and concurrently cleaves FGFR1. GrB at 75 nM was delivered into LNCaPs with BP. *A* and *B*, aliquots were removed over time, lysed, and analyzed by immunoblotting to FGFR1 (*A*) and PARP (*B*). *C*, LNCaPs treated with BP alone (gray) or 75 nM GrB-BP (black) for 6 h were stained with Annexin V-FITC (FL-1) and subjected to flow cytometry. The percentage of cells gated as Annexin V-positive is indicated beside each trace.

Granzyme B Proteolysis of FGFR1 in LNCaPs Results in the Shearing of the Extracellular Ligand-binding Region from the Kinase Domain—To confirm that the cleavage site in FGFR1 was at the predicted Asp-432, an Asp to Asn mutant FGFR1 was cloned (FGFR1-VSAN, Fig. 6*A*, panel *ii*). LNCaPs were transiently transfected with either FGFR1-VSAD or FGFR1-VSAN and then treated with GrB-BP. Immunoblotting with an anti-Myc primary antibody (Fig. 6*B*) reveals this single mutation prohibits GrB from cleaving FGFR1-VSAN, confirming that proteolysis of FGFR1 occurs at Asp-432. The bottom panels of Fig. 6*B* demonstrate that FGFR1-VSAN is functional and able to become autophosphorylated upon FGF2 stimulation. Therefore the resistance of FGFR1-VSAN to GrB proteolysis cannot be attributed to a structural rearrangement caused by the mutation but instead is the result of destroying the site of proteolysis.

Signaling through FGFR1 in LNCaPs Is Incapacitated upon Granzyme B Delivery—Upon ligand-induced dimerization and activation of FGFR1, the receptor becomes phosphorylated and a signaling cascade proceeds, often through phosphorylation of ERK1/2 (24). Because GrB cleavage of FGFR1 separates the

kinase domain of the receptor from the extracellular FGF binding domain, this proteolysis should disrupt signaling through the receptor. LNCaP cells expressing FGFR1-VSAD were incubated with GrB-BP and subsequently stimulated by the basic fibroblast growth factor (FGF2), the ligand of FGFR1 that induces a signal transduction cascade that results in ERK1/2 stimulation. Immunoblotting analysis (Fig. 6*C*) demonstrates that phosphorylation of ERK1/2 (P-ERK) due to FGF2 stimulation is impaired after GrB delivery.

Overexpression of the N-terminal Cleavage Product of FGFR1 Causes PC3 Prostate Cancer Cells to Cease Proliferating and Die—Studies overexpressing a mutant FGFR1 lacking the kinase domain have been used to demonstrate the vital importance of signaling through FGFR1 in the survival of prostate and other cancers (25). This truncated FGFR1 acts as a dominant negative by shunting the signaling pathway upon dimerization with endogenous full-length receptors. To test whether or not the FGFR1 cleavage product generated by GrB can act similarly, a mutant termed FGFR1-gBEC (FGFR1-granzyme B extracellular, Fig. 6*A*, panel *iii*) was cloned that lacks the intracellular portion of the receptor C-terminal to Asp-432. Expression of FGFR1-gBEC in transiently transfected PC3 prostate cancer cells was confirmed by immunoblotting (Fig. 6*D*). PC3s express both FGF2 and FGFR1, thus promoting proliferation in an autocrine fashion (23). Cleavage of FGFR1 by GrB might be particularly detrimental to prostate cancer that has progressed to this FGF autocrine signaling state. Indeed, overexpression of FGFR1-gBEC in PC3s drastically halted proliferation as compared with PC3s expressing FGFR1-VSAD or cells treated with the transfection reagent alone (Fig. 6*E*). Moreover, overexpression of FGFR1-gBEC resulted in significant death compared with overexpression of FGFR1-VSAD (Fig. 6*F*). Cleavage of FGFR1 by GrB therefore produces a cleavage product that can function as an anti-proliferation and pro-death protein in prostate cancer.

DISCUSSION

Granzyme B (GrB) is critical for the efficient killing of virally infected or tumor cell targets by NK and CTL cells. Because many viruses and cancer cells activate a variety of immune evasion strategies, cytotoxic cells should be able to cause cell death by multiple pathways. Indeed, GrB may cut specific “situationally significant” substrates that are vital to the ability of GrB to ensure cell death in cases where other apoptotic pathways are inhibited. Therefore, discovering new substrates of granzyme B will give a more complete understanding of the mechanism of cytotoxic cell death.

We chose to employ a systematic cDNA expression cleavage screen to identify GrB substrates. Now that this approach has been established and has generated fresh insights into this well known protease, it may prove useful for de-orphaning proteases of little-known function.

This study has identified and confirmed FGFR1 to be a caspase-independent substrate of GrB and one that may be important for GrB to kill prostate cancer cells. FGFR1 is cleaved by GrB *in vitro* and is targeted upon addition of GrB to K562 lysates and during NK-mediated killing. FGFR1 is not cleaved by caspases and is therefore a substrate unique to GrB.

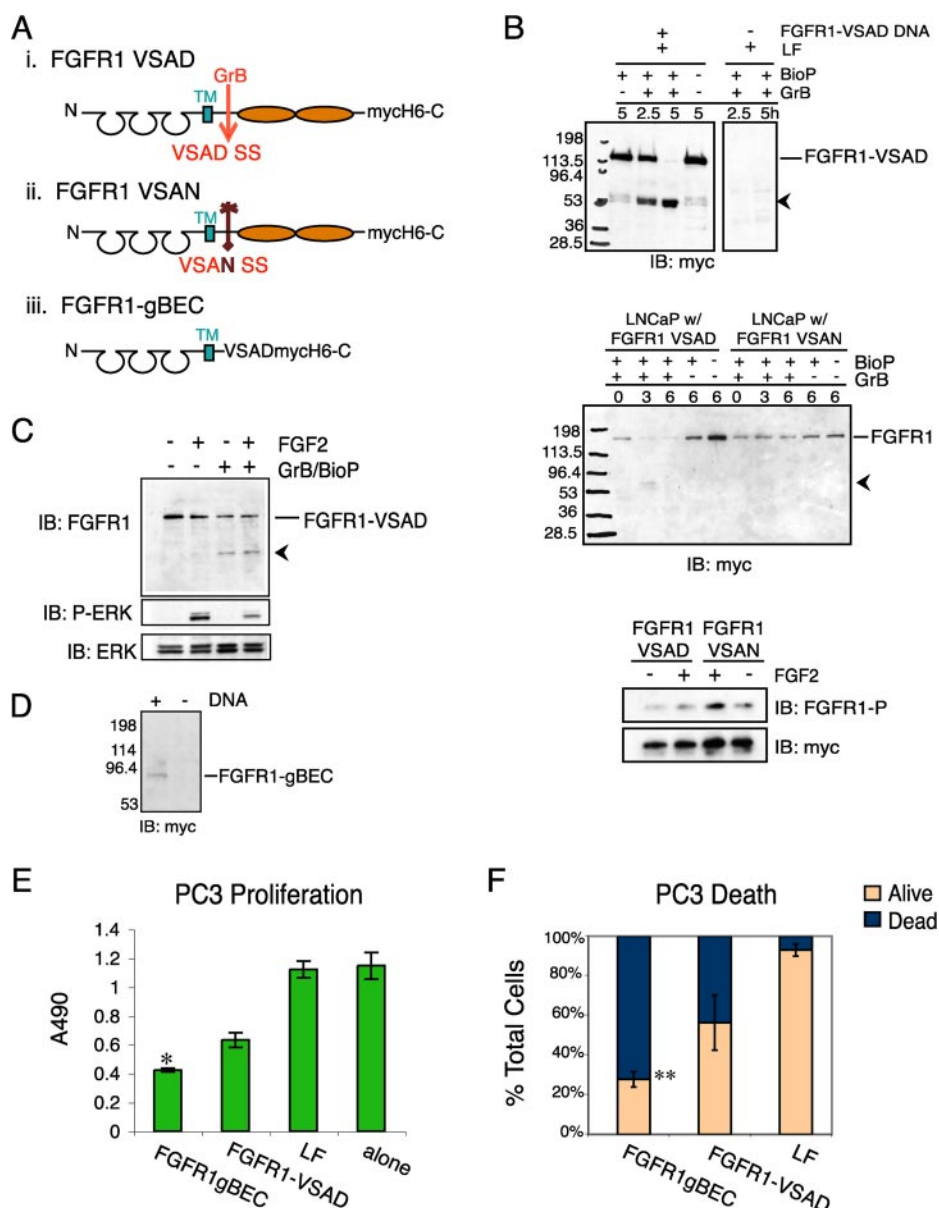


FIGURE 6. GrB proteolysis of FGFR1 has functionally relevant consequences. A, FGFR1 constructs expressed in experiments in B–E. B, proteolysis of FGFR1 by GrB occurs at Asp-432. Anti-Myc immunoblots showing expression and cleavage of FGFR1-VSAD in LNCaPs upon GrB-BP treatment 36 h post transfection. The middle panel also shows that GrB does not cleave FGFR1-VSAN expressed in LNCaPs. In the bottom panels, cells 36 h post transfection were treated with FGF2, and the overexpressed receptors were immunoprecipitated with an anti-Myc antibody and assessed for autophosphorylation. C, GrB prevents efficient ERK stimulation by FGF2. LNCaPs transfected with FGFR1-VSAD were treated with GrB-BP and then stimulated with FGF2. D–F, overexpression of FGFR1-gBEC causes PC3 cells to stop growing (E) and die (F). D, expression of FGFR1-gBEC in PC3s 24 h post transfection. E, absorbance at 490 nm after an MTS proliferation reagent was added to cells expressing the indicated constructs. F, percentage of dead (blue) and live (cream) PC3s expressing FGFR1gBEC or FGFR1-VSAD determined by trypan blue staining. Error bars in both E and F represent one standard deviation above and below the mean of at least three independent experiments. *, $p < 0.0001$; **, $p < 0.004$.

A host of evidence strongly implicates signaling through FGFR1 in promoting the development and progression of several cancers, especially prostate cancer (23). Expression of FGF1, FGF2, and FGFR1 is increased in prostate cancers, and as tumors become progressively less differentiated, expression patterns of the FGFs and FGFR1 indicate a shift from paracrine to autocrine signaling (26, 27). Mouse models have also indicated that expression of FGFR1 can promote tumorigenesis and proliferation (28–30). Increased FGF signaling through FGFR1

in prostate cancer has also been implicated in promoting angiogenesis, increased motility and invasiveness, and androgen independence (23).

Our analysis has revealed that GrB has the intrinsic ability to kill LNCaP prostate cancer cells in culture. We also show that GrB cleaves FGFR1 upon entering prostate cancer cells and that proteolysis results in the shearing of the extracellular and transmembrane regions from the intracellular tyrosine kinase domain.

Proteolysis of FGFR1 by GrB in prostate cancer cells results in the shunting of a pro-proliferation and pro-survival signal in these cells. GrB prevents efficient phosphorylation of ERK1/2 upon FGF2 stimulation, indicating that GrB can shut off proliferation signals while also initiating cell death. Furthermore, the extracellular cleavage product of FGFR1 can act as a dominant negative by causing prostate cancer cells to cease proliferating and die. These results further confirm that disrupting FGFR1 signaling could be an attractive approach in targeting and treating prostate cancer. The functional repercussions that we have elucidated clearly indicate that proteolysis of FGFR1 would help the death process by curtailing survival signals. Our results may suggest that immunotherapy that results in granule exocytosis-induced cell death could be advantageous in targeting prostate cancer.

Notch1 is often referred to as an arbiter of cell fate, because signaling through this transmembrane receptor during development results in differentiation, proliferation, and/or survival of many different tissues (31). This study has identified Notch1 to be a substrate of both GrB and caspases, and has confirmed its proteolysis *in vitro* and in cell-based assays. There are several potential caspase and GrB sites within Notch1, and they align to the same regions on the intracellular side of the receptor (Fig. 4B). To understand the implications of these cleavages, the functional domains within the receptor must be outlined. During Notch1 signaling, ligand binding results in a series of proteolytic events that release the Notch intracellular domain (NICD, Fig. 1D, panel i) (31). NICD translocates to the nucleus where it mediates cell fate decisions. NICD interacts with its

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many transcriptional co-regulators through its ankryin repeats, RAM domain, and the C-terminal transcriptional activation domain (Fig. 1D, panel i) (31). Although the ankryin repeats are required for NICD to interact with its known partners, the necessity of the RAM domain, however, is co-regulator-specific. NICD can inactivate transcription by inhibiting the activator AP-1, and the RAM domain is absolutely critical for binding (32). AP-1 activation sites are found in the promoters of many immunomodulatory and apoptosis-related genes, including *FAS* and *FASL* (33). Cleavage of Notch1 by GrB or caspases could relieve transcriptional repression and allow for increased amplification of pro-apoptotic signals within the target cell.

GrB proteolysis could result in the complete abrogation of Notch1 function in certain situations. Rather than the generation of a modified NICD lacking the RAM domain, GrB proteolysis may lead to subsequent degradation of the receptor, because a cleavage product was not always detectable. NICD can be subjected to ubiquitination and proteasomal degradation (34), and cleavage by GrB and/or caspases could result in the same fate. Furthermore, GrB and caspases may effectively destroy Notch1 themselves, because there are multiple potential GrB and caspase cleavage sites within NICD (Fig. 4B).

Notch1 has been given a role in protecting against activation-induced cell death of activated T-lymphocytes, by binding to and inhibiting the orphan nuclear hormone receptor family member Nur77 (35). Nur77 is thought to promote activation-induced cell death through its transcriptional activation (36, 37). Nur77 may also induce apoptosis by leaving the nucleus, translocating to the mitochondria, and stimulating cytochrome *c* release (38). Accordingly, NICD binding to Nur77 inhibits its relocalization (39). GrB and/or caspase proteolysis of Notch1 within an activated T-lymphocyte could free Nur77 to fulfill its pro-apoptotic functions. GrB and granule exocytosis are thought to have a role in mediating lymphocyte homeostasis (40, 41). Indeed, humans with genetic perforin defects develop the lymphoproliferative disease familial hemophagocytic lymphohistiocytosis in infancy or early childhood (1). Future studies beyond the scope of this report are necessary to ascertain the significance of Notch1 proteolysis by GrB and caspases, but outlined above are certainly provocative scenarios in which Notch1 signal disruption could potentiate the ability of a CTL or NK-cell to kill its target.

Six of the prospective GrB substrates identified in this study can be classified with other known GrB substrates. GrB activates pro-death pathways within the cell by proteolyzing the caspases, inhibitor of caspase-activated DNase, and Bid, all of which can also be caspase targets (1). Similarly, cAbl is a known caspase substrate that has pro-apoptotic activity upon proteolysis (42). GrB also targets substrates involved in cellular homeostasis and integrity, such as cytoskeletal components (43, 44), many nuclear proteins and parts of the transcription and translation machinery (45). Four of the cDNAs identified, heterogeneous nuclear ribonucleoprotein H', poly-A-binding protein, intersectin-1, and Br140, are similar to GrB substrates in this category (45, 46). GrB can proteolyze DNA damage-responsive proteins such as PARP and the DNA-dependent protein kinase. Hsp70, another kind of stress-induced protein, was identified and shown to be a substrate of granzyme B *in vitro*. Hsp70 is an

intriguing target given its anti-apoptotic roles in preventing apoptosome formation (47) and in blocking apoptosis-inducing factor nuclear accumulation (48).

Notch1 and FGFR1 do not fit into previously identified categories of GrB substrates. Both receptors actively transmit signals from the extracellular environment, and their proteolysis could guarantee the death of a target cell by isolating it from outside survival cues. Here we have presented evidence of how GrB proteolysis of FGFR1 has "situationally significant" consequences in prostate cancer cells. Because different tumor cells take on a variety of immune evasion approaches, cytotoxic cells should be equipped to induce cell death by redundant pathways. The identification of Notch1 and FGFR1 as substrates of GrB shows that this protease not only turns on pro-death pathways but also shuts down pro-proliferative signals to further "tip the balance" toward apoptosis.

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