Cleavage of Notch1 by granzyme B disables its transcriptional activity

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INTRODUCTION

The immune system uses two types of cells as its primary defense against cells and viral pathogens, i.e. NK (natural killer) cells and CTLs (cytotoxic T-lymphocytes). Both types of cytotoxic cells harbour cytotoxic granules that are released upon target cell recognition. These granules contain serine proteases, also called granzymes, and the pore-forming protein perforin. Although perforin pores facilitate the entry of granzymes into the target cell, the latter trigger apoptosis by cleaving critical death substrates [1].

Granzyme-mediated cell death is the main pathway for cytotoxic lymphocytes to kill virus-infected and tumour cells. A major player in this process is GrB (granzyme B), which triggers apoptosis in both caspase-dependent and caspase-independent pathways. A caspase-independent substrate of GrB is the highly conserved transmembrane receptor Notch1. The GrB cleavage sites in Notch1 and functional consequences of Notch1 cleavage by GrB were unknown. In the present study, we confirmed that Notch1 is a direct and caspase-independent substrate of GrB. We demonstrate that GrB cleaved the intracellular Notch1 domain at least twice at two distinct aspartic acids, Asp1860 and Asp1961. GrB cleavage of Notch1 can occur in all subcellular compartments, during maturation of the receptor, at the membrane, and in the nucleus. GrB also displayed perforin-independent functions by cleaving the extracellular domain of Notch1. Overall, cleavage of Notch1 by GrB resulted in a loss of transcriptional activity, independent of Notch1 activation. We conclude that GrB disables Notch1 function, probably resulting in anti-cellular proliferation and cell death signals.

Key words: cleavage, granzyme B, Notch signalling, proteolysis, γ-secretase, serine protease.
and in living cells independent of its activation. Importantly, GrB cleavage occurs in all subcellular compartments and results in a loss of Notch1 transcriptional activity.

MATERIALS AND METHODS

Cell lines

HeLa FRT cells were generated by transfecting the FRT-LacZeo plasmid according to the manufacturer’s instructions (Invitrogen). Single integration was determined using Southern blotting (results not shown). pcDNA5-FRT-Notch1 constructs were stably integrated into the FRT site after selection of HeLa cells with 200 μg/ml Hygromycin B. The selected single integrant cells were maintained at 200 μg/ml Hygromycin B.

GrB production and purification

Active recombinant human GrB and inactive control GrB-SA were expressed in Pichia pastoris and purified by cation-exchange chromatography as described previously [12,13]. GrB preparations were dialysed against TBS (Tris-buffered saline; 50 mM Tris/HCl, pH 7.4, and 150 mM NaCl) and stored at −80°C. GrB, but not GrB-SA, was active as determined by the small synthetic chromogenic substrate IETD-pNA (Ile-Glu-Thr-Asp aldehyde; Bachem) (results not shown).

Plasmids and vectors

All mNotch1 plasmids were initially cloned into pCS2 + 6Myc as described previously [14]. Notch1 full-length, LNR, NEXT and NICD constructs were constructed as described previously (shown in Figure 1), HA–N1FL–6MT (where HA is haemagglutinin, N1FL is full-length Notch1 and 6MT is 6Myc tag) was a gift from R. Kopan (Washington University, St. Louis, MO, U.S.A.). Mutations in Notch molecules were introduced by PCR-directed cloning. For in vitro transcription, all mNotch1 variants were cloned in the pSensor backbone (Promega), replacing the luciferase. For stable expression in FRT cell lines, Notch was subcloned into the pcDNA5 vector (Invitrogen). A promoter fragment containing 12× CSL synthetic binding sites in tandem (provided by S. Boyle, Washington University, St. Louis, MO, U.S.A.) was subcloned into pGL4.24 (Promega) and was used for Notch transcription assays. For normalization of transcription assays, a CMV (cytomegalovirus)-driven Gluc (Gaussia luciferase; NEB) tag was used. N-terminally Gaussia luciferase-tagged N1FL was created using PCR-directed cloning from the pGluc-Basic (NEB).

In vitro translation

Notch1 was produced in vitro using the TNT® SP6 Coupled Wheat Germ Extract System (Promega) in combination with FluroTect™ GreenLys in vitro Translation Labeling System (Promega). Gels were analysed on a Typhoon scanner (GE Healthcare).

GrB assay

Cells were pre-treated with 100 μM Z-VAD-FMK in serum-free medium for 1 h. Cells were washed twice with PBS before incubation with the GrB mixture, which consisted of the perforin analogue SLO (streptolysin O; 5 μg/ml), Z-VAD-FMK (100 μM), GrB or GrB-SA (diluted in TBS). The mix was added to the cells for 30 min at 37°C, after which the cells were washed with serum-free medium and lysed by the addition of 17 μl of 4× Laemmli buffer to 50 μl of serum-free medium. In case of γ-secretase inhibition, cells were treated with DBZ (dibenzazepine; 200 nM) for 16 h before GrB incubation. For inhibition of ER (endoplasmic reticulum)–Golgi transport, brefeldin A (1 mg/ml stock in methanol) was used at a concentration of 2 μg/ml for 4 h at 37°C. GrB activity was blocked using up to 300 nM of the inhibitor Ac-IETD-CHO (N-acetyl-Ile-Glu-Thr-Asp aldehyde; Merck). The caspase-3/7 GLO assay (Promega) was used according to the manufacturer’s instructions.

Western blotting

Samples were boiled for 5 min at 95°C and run on an SDS/PAGE gel (8%), blotted on to a PVDF membrane, and incubated with antibodies overnight at 4°C. Antibodies used were anti-Myc (9E10) 1:5000, anti-cleaved caspase 3 (Asp175, Cell Signaling) 1:1000, anti-cleaved caspase 9 (Asp757, Cell Signaling) 1:1000, anti-HA (12CA5) and anti-actin (MP Biomedicals) 1:10000.

Transcription assays

HeLa cells were transfected with a pGL4.24 12× CSL. Firefly luciferase reporter, pBasic-CMV Gaussia luciferase was used as a transfection control. Cell samples were lysed using Passive Lysis Buffer and medium was collected, spun down and diluted in Passive Lysis Buffer before analysis with the Dual luciferase kit (Promega) on a luminometer. Firefly values were corrected for the secreted Gaussia luciferase values, resulting in relative light units (RLU).

Cell preparation for GrB incubation

HeLa FRT Notch1 wild-type cells were grown in a T165 flask until confluence. Cells were washed 3 times with 20 ml of PBS, then incubated with 14 ml of hypotonic PBS (5% PBS in double-distilled H2O) for 15 min at room temperature (25°C). Cells were washed with vesiculation buffer (100 mM NaCl, 50 mM sodium

Figure 1 Notch constructs

(A) Schematic overview of Notch1 constructs used in the present study and (B) location of C- and N-terminal epitope tags. EGF, epidermal growth factor; FL, full length; PEST, Pro-Glu-Ser-Thr motif.
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Figure 2  In vitro cleavage of Notch1 by GrB in a dose-dependent manner
(A) SDS/PAGE analysis showing that in vitro produced NEXT–6Myc proteins with incorporated fluorescently labelled lysine residues are cleaved upon GrB incubation into 55 kDa and 45 kDa fragments as indicated by arrows. *, an alternative translation product. (B) Immunoblotting shows both the 55 kDa and 45 kDa fragments are C-terminally 6Myc tagged. (C) Similar to NEXT, the shorter NICD construct is also a substrate of GrB resulting in two fragments of 55 and 45 kDa. (D) Similar to NEXT, these fragments are also C-terminally tagged with 6Myc. Molecular mass markers are indicated in kDa.

RESULTS

Notch1 is directly cleaved in vitro by GrB
To investigate cleavage of Notch1 by GrB in more detail, we produced a Notch1 protein mimicking the membrane-bound form that lacks most of the extracellular domain (termed NEXT), containing a C-terminal Myc epitope tag (Figure 1). NEXT proteins are constitutively active and a substrate for γ-secretase [15,16]. NEXT–6Myc recombinant protein was produced in vitro and visualized by SDS/PAGE after incorporation using fluorescently labelled lysine residues.

In vitro translated NEXT proteins were incubated with increasing concentrations of a catalytically active GrB and a control mutant GrB-SA, which attenuates cleavage activity. GrB cleavage of Notch1 was completely inhibited using a GrB-specific inhibitor, ruling out possible contaminants present in the P. pastoris culture (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/437/bj4370313add.htm). We observed the appearance of several cleavage products with increasing concentrations of GrB within 60 min of incubation (Figure 2A). Even at the lowest concentration tested (5 nM GrB) a cleavage product with an estimated size of 55 kDa was observed. With higher concentrations of GrB, an additional cleavage product of around 45 kDa was detected. At the highest concentrations, the GrB-SA also generated the 55 kDa cleavage product; however, to a much lesser extent than the wild-type GrB. Under these conditions, no 45 kDa product was observed with the GrB-SA, whereas full conversion from the 55 kDa precursor had occurred with wild-type GrB. Others have previously reported that the single SA mutation attenuated but does not completely abrogate granzyme activity in vitro [17,18].

Apoptosis assay
Cells were grown in 96 well plates. At 8 h after GrB incubation cells were trypsinized and centrifuged at 250 g for 5 min. Cells were resuspended in 50 μl Annexin V-binding buffer (140 mM NaCl, 4 mM KCl, 0.75 mM MgCl₂, 10 mM Hepes and 1.5 mM CaCl₂, pH 7.4) containing Annexin-V-Fluos (Roche) and 1 μg/ml propidium iodide and incubated for 15 min at room temperature. Before FACS analysis, samples were diluted with 150 μl Annexin V-binding buffer. In vitro translated NEXT proteins were incubated with increasing concentrations of a catalytically active GrB and a control mutant GrB-SA, which attenuates cleavage activity. GrB cleavage of Notch1 was completely inhibited using a GrB-specific inhibitor, ruling out possible contaminants present in the P. pastoris culture (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/437/bj4370313add.htm). We observed the appearance of several cleavage products with increasing concentrations of GrB within 60 min of incubation (Figure 2A). Even at the lowest concentration tested (5 nM GrB) a cleavage product with an estimated size of 55 kDa was observed. With higher concentrations of GrB, an additional cleavage product of around 45 kDa was detected. At the highest concentrations, the GrB-SA also generated the 55 kDa cleavage product; however, to a much lesser extent than the wild-type GrB. Under these conditions, no 45 kDa product was observed with the GrB-SA, whereas full conversion from the 55 kDa precursor had occurred with wild-type GrB. Others have previously reported that the single SA mutation attenuated but does not completely abrogate granzyme activity in vitro [17,18]. To further investigate GrB cleavage products, we made use of the C-terminal Myc epitope. Myc immunoblotting revealed that both the 55 and the 45 kDa products could be readily detected, supporting the notion that the 45 kDa product was produced from the 55 kDa precursor (Figure 2B). To see whether sequences within the transmembrane or extracellular domains were required for GrB cleavage, we also investigated whether the non-membrane bound form, called NICD (mimicking the γ-secretase cleaved form of Notch1), was also a substrate for GrB. In vitro produced NICD1 proteins

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were also efficiently cleaved by GrB, producing similar cleavage products of 55 and 45 kDa respectively (Figures 2C and 2D). These results indicate that the intracellular domain of Notch1 is a direct substrate of GrB in vitro.

GrB cleaves Notch1 in vivo independent of caspases

To address whether GrB also cleaves Notch1 under physiological conditions, we produced HeLa cells stably expressing a full-length constitutively active Notch1 receptor carrying a mutation (L1594P) similar to those found in human acute T-cell leukemias [19]. N1L1594P HeLa cells show ligand-independent Notch1 cleavage and activity as shown by immunoblotting and transcriptional reporter assays, which is fully blocked by incubation with the γ-secretase inhibitor DBZ (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/437/bj4370313add.htm).

Next, we incubated GrB with N1L1594P cells, in combination with SLO acting as a pore-forming protein allowing GrB to enter the cell (Figure 3A). Immunoblotting revealed a dose-dependent appearance of a 55 kDa fragment reaching a maximum with 450 nM GrB. This 55 kDa fragment appeared similar to the 55 kDa fragment observed by in vitro cleavage of Notch1 by GrB. Notably at lower concentrations of GrB, little if no cleavage occurred compared with 600 nM catalytic site mutant GrB-SA. Interestingly, whereas in vitro cleavage by GrB led to the consecutive cleavage of the 55 kDa precursor to produce a smaller 45 kDa cleavage product, this was not readily observed in the Notch1-expressing stable cell line exposed to GrB; only upon enrichment could the fragment be detected (see Figure 5B). Importantly, Notch1 cleavage could only be detected in the presence of both GrB and SLO, indicating that cellular uptake of GrB was required for cleavage (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/437/bj4370313add.htm).

To address whether the cleavage of Notch1 in vivo is caused by GrB through activation of a caspase-dependent pathway, we incubated cells with GrB and the cell-permeable pan-caspase inhibitor Z-VAD-FMK, which irreversibly binds to the catalytic site of caspases and inactivates its proteolytic activity including autoproteolysis. Incubation of GrB with HeLa N1L1594P cells caused a dose-dependent increase in the appearance of the 55 kDa cleavage product largely independent of caspase activity (Figure 3B, upper panel). Under these conditions, Z-VAD-FMK efficiently blocked caspase autoproteolysis as shown by immunoblotting for activated caspase-3 (Figure 3B, lower panel) and by a caspase-3/7 profluorescence substrate assay (see Supplementary Figure S4 at http://www.BiochemJ.org/bj/437/bj4370313add.htm). Finally, Z-VAD-FMK blocked GrB-induced apoptosis as measured by annexin V/propidium iodide labelling and flow cytometric analysis (see Supplementary Figure S4). Thus GrB cleavage of Notch1 in vivo does not depend on caspase-dependent cleavage.

To address whether prolonged GrB activity led to a full conversion of the 55 kDa fragment and the formation of additional cleavage products, we incubated HeLa N1L1594P with 300 nM GrB for 30 min, after which cells were washed and incubated again for various amounts of time. We observed robust cleavage and production of the 55 kDa fragment after 30 min of GrB incubation. Continued incubation for up to 120 min reduced the presence of the 55 kDa cleavage product (Figure 3C). Furthermore, this reduction was accompanied by a concomitant reduction of the Notch1 full-length unprocessed and S1-cleaved Notch1 TMIC (transmembrane and intracellular domain) isoforms. By Myc immunoblotting, we could not detect the formation of the 45 kDa product nor of any other additional cleavage products generated from the precursor fragment within 2 h after GrB addition (Figure 3C). Taken together, these results indicate that GrB cleaves Notch1 in vivo independent of caspases.

GrB cleavage is γ-secretase-independent

Notch activity requires proteolytic processing by a metalloprotease and γ-secretase [15]. To address whether GrB-induced
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Figure 4  Cleavage of Notch by GrB is independent of activation

N1L1594P HeLa cells were treated with the γ-secretase inhibitor DBZ (GSI), inhibiting the activating cleavage of Notch1. Upon GrB incubation, 55 kDa fragments could still be observed on the Myc immunoblot. The Val1744 immunoblot stains Notch S3 cleavage products. Molecular mass markers are indicated in kDa.

cleavage is dependent on γ-secretase activity, we pre-incubated HeLa N1L1594P cells with the γ-secretase inhibitor DBZ, after which GrB (with Z-VAD-FMK) was added for 30 min and cell extracts were analysed by immunoblotting. Whereas DBZ efficiently blocks cleavage of Notch1 at Val1744, it did not influence GrB cleavage. Both in the presence and absence of DBZ, the 55 kDa cleavage product was readily observed (Figure 4). These data point to a role for GrB in cleavage of the membrane-bound Notch1 isoforms as well as the S3/γ-secretase cleaved NICD. This is supported by the observation that the full-length uncleaved Notch1 isoform also appears to be a GrB substrate (Figures 3C and 4).

GrB cleaves Notch in all subcellular compartments

The previous experiments support a role for GrB cleavage of Notch1 independent of γ-secretase. Our results indicate that both the truncated constitutively active and full-length unprocessed forms of Notch1 are GrB substrates. To investigate in which subcellular compartments Notch is cleaved by GrB, we determined cleavage patterns in a step-by-step manner. First, to address whether full-length unprocessed Notch1 is a GrB substrate, we treated HeLa N1L1594P cells with brefeldin A, an inhibitor of anterograde ER–Golgi transport. Brefeldin A treatment blocked Notch S1 processing as expected [20,21], leading to an accumulation of the full-length unprocessed form of Notch at the expense of TMIC (Figure 5A). Upon incubation of these cells with GrB, cleavage of the unprocessed full-length precursor into the 55 kDa fragment was observed.

Next, we determined if the cell surface-bound signalling-competent Notch receptor is also a substrate for GrB. To address this, we enriched for plasma membrane-bound Notch1 receptors from cells using an osmotic based vesiculation process [22], whereby vesicles bud off the plasma membrane containing surface molecules. To circumvent the presence of S2 and S3 Notch-processing fragments in these vesicles, we used HeLa cells expressing the wild-type Notch1 receptor and all preparations contained the γ-secretase inhibitor. We incubated isolated vesicles directly with GrB and addressed whether membrane-bound Notch1 is a substrate. A clear enrichment of membrane-bound Notch1 (TMIC) and very little unprocessed Notch1 on the membrane was observed as expected [23]. Notch cleavage products of 55 kDa and 45 kDa were readily detected in vesicles exposed to GrB but not to GrB-SA. Furthermore, a loss of the unprocessed full-length isoform was observed as well (Figure 5B). These results indicate that the membrane S1-cleaved signalling-competent Notch1 receptor is also a GrB substrate.

Both our in vitro and in vivo data suggest that NICD1 may be a direct GrB substrate (Figure 2). To address if NICD1 is a direct substrate in living cells, HeLa cells transfected with NICD1 were treated with GrB and lysates were analysed on immunoblots. In the absence of active GrB, NICD1 was expressed at the expected molecular mass as determined by C- and N-terminal antibodies (Figure 5C). Upon incubation with GrB, the expected 55 kDa processing fragment was observed by Myc immunoblotting. This indicates that the membrane-cleaved activated Notch1 isoform NICD is also a GrB substrate.

So far, our results have shown that all intracellular forms of Notch are subject to GrB cleavage, but recent observations point to a role of extracellular activity of GrB which is perforin-independent [24–26]. Therefore, we investigated whether GrB can induce cleavage in the mature cell surface-exposed NECD (Notch extracellular domain) as well. We expressed a full-length Notch1 protein fused to Gaussia luciferase at the extracellular N-terminus and with a 6Myc epitope tag at the intracellular C-terminus (Gluc-Notch1 wild-type). Release of Gaussia luciferase in the medium can be measured by a sensitive enzymatic assay directly on cell culture medium. HeLa cells were transfected with Gluc–Notch1 wild-type together with the multimerized 12× CSL binding site containing the Notch reporter plasmid driving firefly luciferase expression. The fusion of Gaussia luciferase to the Notch receptor did not induce activation of the receptor (results not shown). These cells were incubated with GrB in the absence of SLO and Gaussia luciferase was measured in the medium 8 h later. A 3-fold significant increase in Gaussia luciferase in the medium was detected when cells were incubated with 300 nM GrB but not with GrB-SA (Figure 5D). Although GrB induced release of Gaussia luciferase into the medium, this did not lead to an increase in Notch transcriptional activity as measured by the 12× CSL, Notch reporter assay.

To determine whether GrB is capable of cleaving Notch in the non-matured intracellularly located NECD, we expressed an N-terminally HA-tagged and C-terminally Myc-tagged Notch molecule (HA–Notch1-6MT) in HeLa cells. After treatment of these cells with GrB in the presence of SLO, we analysed the N-terminus on HA immunoblots. As expected, Myc immunoblots showed the GrB-induced expected 55 kDa fragment, in addition HA blots showed a concomitant reduction in size from the full-length unprocessed Notch1 isoform of approximately 55 kDa (Figure 5E). Interestingly, we did not observe additional cleavage products, suggesting that NECD is not a direct substrate for GrB.

Identification of GrB cleavage sites in Notch

There are five predicted GrB cleavage sites with an aspartic acid residue in the P1 position within the Notch1 intracellular domain (Figure 6A) [4,27]. To identify the main cleavage...
GrB action in different subcellular locations was analysed. (A) Brefeldin A (BFA)-treated cells, blocking ER to Golgi transport, show cleavage of Notch upon GrB incubation. (B) Fractions enriched for plasma membrane-bound Notch1 show cleavage upon GrB treatment. Arrow indicates the 45 kDa fragment. (C) In HeLa cells expressing activated Notch molecules, GrB induces efficient cleavage. (D) HeLa cells expressing N-terminally tagged Gluc–Notch1 were incubated with GrB in the absence of perforin. Gaussia luciferase activity was measured in the medium, and the 12× CSL-driven Notch activity was determined from lysates. A significant (*P = 0.0495) increase was observed upon 300 nM GrB, which did not lead to Notch activation. The histogram is representative of at least two independent experiments in triplicate, and the P-value was calculated using a Kruskall–Wallis non-parametric test. (E) Expression of N-terminally tagged HA–Notch-FL–6Myc indicated the NECD was not a direct substrate of GrB. Arrow indicates HA-tagged NECD. Molecular mass markers are indicated in kDa.

Figure 5  Intracellular and extracellular GrB cleavage of Notch

GrB action in different subcellular locations was analysed. (A) Brefeldin A (BFA)-treated cells, blocking ER to Golgi transport, show cleavage of Notch upon GrB incubation. (B) Fractions enriched for plasma membrane-bound Notch1 show cleavage upon GrB treatment. Arrow indicates the 45 kDa fragment. (C) In HeLa cells expressing activated Notch molecules, GrB induces efficient cleavage. (D) HeLa cells expressing N-terminally tagged Gluc–Notch1 were incubated with GrB in the absence of perforin. Gaussia luciferase activity was measured in the medium, and the 12× CSL-driven Notch activity was determined from lysates. A significant (*P = 0.0495) increase was observed upon 300 nM GrB, which did not lead to Notch activation. The histogram is representative of at least two independent experiments in triplicate, and the P-value was calculated using a Kruskall–Wallis non-parametric test. (E) Expression of N-terminally tagged HA–Notch-FL–6Myc indicated the NECD was not a direct substrate of GrB. Arrow indicates HA-tagged NECD. Molecular mass markers are indicated in kDa.

site of GrB in Notch, we performed mutation analysis on the three conserved aspartic acid residues most proximal to the membrane (VLPD1823, VDAD1860 and VISD1902). Either or both of the mutations D1823Q and D1902E did not result in an abrogation of Notch cleavage since both 55 and 45 kDa fragments could be observed in vitro (Supplementary Figure S5 at http://www.BiochemJ.org/bj/437/bj4370313add.htm). The aspartic acid mutant D1860A, however, revealed a blockade in Notch processing demonstrated by the accumulation of the full-length unprocessed NEXT molecule as well as a loss of the 55 kDa fragment (Figure 6B). The 45 kDa fragment, however, could be detected. This indicates that the 55 kDa fragment results from a cleavage at VDAD1860. Interestingly, a shift in cleavage was observed, resulting in a 65 kDa fragment, reflecting cleavage at the more proximal aspartic acid residue VLPD1823. By combining the D1860A and D1823Q mutations, we
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Figure 6 Cleavage occurs at Asp1860 and Asp1961

(A) The diagram shows the five predicted cleavage sites in the intracellular part of the Notch1 receptor. (B) In vitro mutation analysis reveals that wild-type Notch1 NEXT–6Myc shows the two cleavage fragments, whereas mutation of VDAD1960 shows a loss of the 55 kDa fragment, yet a new 65 kDa fragment appears, which upon additional mutation of VLPD1823 is lost, maintaining the 45 kDa fragment. Only upon an additional third mutation of VSAD1961 is a complete loss seen in Notch cleavage upon GrB treatment. *, an alternative translation product. (C) This could be confirmed in vivo by GrB incubation of HeLa cells expressing Notch1 LNR–6Myc carrying the various mutations as determined by Myc immunoblotting. Actin staining of the same immunoblots serves as a loading control. Molecular mass markers are indicated in kDa.

observed an accumulation of full-length NEXT and a loss of the 65 and 55 kDa fragments respectively. However, the 45 kDa fragment could still be observed, indicating that GrB is capable of cleaving this site directly, independently of cleavage at Asp1860, albeit less efficient. Only after mutating D1961A was production of the 45 kDa fragment blocked and full-length NEXT protein observed (Figure 6B). Myc immunoblotting revealed that, in addition to the 55 and 45 kDa fragments, the larger 65 kDa fragment was also observed (results not shown).

In order to determine whether the cleavage sites identified in vitro correspond to the in vivo situation, we transfected HeLa cells with truncated Notch1 LNR constructs containing the same cleavage site mutations. As observed in vitro, mutation of D1823Q and D1902E did not abrogate Notch cleavage in cells (results not shown); however, the single D1860A mutant shows an accumulation of the full-length Notch precursor and TMIC, and the appearance of the 65 kDa fragment similar to in vitro cleavage of recombinant substrates (Figure 6C). Mutating both D1823Q and D1860A completely blocked cleavage in cells as well. In this cell-based assay we could detect the 45 kDa fragment in cells in the double/triple mutant, indicating that cleavage can take place at this site in vivo and in vitro independent of a proximal cleavage. The additional mutation at D1961A resulted in a complete loss of cleavage as seen in the in vitro results (Figure 6C). These results indicate that the main GrB cleavage sites in Notch1 are the aspartic acid residues Asp1860 and Asp1961.

GrB cleavage disables Notch activity

To address whether GrB cleavage affects Notch activation, we performed gene reporter assays to measure the transcriptional response upon Notch1 activation in the presence or absence of GrB. HeLa N1L1594P cells were transfected with a multimerized 12× CSL-binding site reporter driving firefly luciferase expression pre-incubated with DBZ and Z-VAD-FMK, after which they were exposed to GrB for 30 min, washed and allowed to recover for an additional 4 h, when extracts were analysed for reporter gene luciferase activity. As expected, DBZ reduced reporter gene activity up to 95% in HeLa N1L1594P cells that normally have high constitutive Notch activation (Figure 7). Exposure of cells to 300 nM GrB for 30 min significantly reduced transcriptional output after 4 h, suggesting that GrB cleavage of Notch1 reduces Notch1 signalling activity. Post incubation of GrB-treated cells for longer durations resulted in a non-specific inhibition of reporter activity due to SLO alone (results not shown).

These results demonstrate that GrB cleavage of Notch leads to the inactivation of Notch1 by interfering with the ability of NICD to activate gene transcription.

DISCUSSION

GrB is essential for NK cell- and CTL-induced cell death in pathogen-infected and tumour cells. This cell death is mainly
mediated by the action of granzymes packaged in cytotoxic granules that are released into the target cell upon recognition. Previously, using NK-mediated cytotoxicity, Loeb et al. [4] showed that physiological levels of GrB are able to cleave Notch1 in a caspase-independent manner. It cleaves Notch1 into at least a single fragment at an unidentified site. In the present study, we confirmed that GrB cleaves the Notch1 receptor irrespective of caspase activation. Proteolysis of Notch by GrB results in at least two fragments in vitro, cleaving after Asp1860 and Asp1961. Cell-based assays show that GrB cleaves Notch1 at the same sites only in the presence of the pore-forming SLO, demonstrating that GrB cellular entry is required to cleave Notch. Furthermore, we show that GrB can cleave Notch receptors irrespective of its activity and location; it cleaves the immature Notch precursor, the cell-surface-bound receptor as well as the membrane-cleaved activated NICD. Notch1 cleavage by GrB inactivates the signalling cascade directly by blocking transcriptional activity.

Notch receptors are GrB substrates irrespective of their activation status. Our results indicate that GrB can cleave Notch at multiple levels, during maturation, at the cell surface and after release from the membrane in the nucleus. Previous results indicate nuclear access of GrB to be primarily caspase-dependent [28]. In the present study, making use of caspase inhibitors during the course of GrB treatment, we observe GrB cleavage of NICD, suggesting that GrB may also enter the nucleus and cleave Notch independent of caspase activity. Cleavage of the Notch effector NICD by GrB leads to direct inactivation of transcriptional activity. However, cleavage of the precursor and membrane-bound forms of the receptor results in a lower number of intact Notch molecules at the cell surface able to receive a signal, thereby decreasing signalling capacity indirectly. Thus GrB can inactivate Notch signalling at all levels, either directly or indirectly.

The optimal substrate cleavage site of GrB is P4 I/VXXD-P1 (where X is any amino acid) with critical isoleucine/valine and aspartic acid residues at P4 and P1 respectively. Considering the Notch1 proteins used in the present study, this leaves five possible GrB scissile bonds, at VLPD1823, VDAD1860, VISD1902, VSAD1961 and VLLD2071, all of which are conserved between mouse and human Notch1. We systematically mutated the P1 aspartic acids and found that mutation of both Asp1823 and Asp1902 in Notch still resulted in cleavage by GrB, similar to wild-type controls in vitro and in vivo (results not shown). Only upon mutation of Asp1860 was a cleavage defect observed both in vitro and in vivo. Strikingly, this induced a shift in cleavage in vitro to VLPD1823, resulting in a 65 kDa fragment which disappeared upon additional mutation of Asp1923. This could indicate that cleavage takes place at two residues simultaneously. However, experiments using the single Asp1823 mutant ruled out the possibility of subsequent cleavage starting at Asp1923 (results not shown). The D1823Q/D1860A double mutant still showed the 45 kDa cleavage fragment to be present, demonstrating that GrB is able to cleave this site directly, although less efficiently. Mutation of Asp1961 showed a loss of the smaller 45 kDa fragment, revealing the second cleavage site of GrB. Strikingly, in vivo the 45 kDa fragment is also produced, but detection was more difficult for reasons unknown. Only upon high Notch expression or enrichment of Notch molecules could the 45 kDa fragment be detected in living cells. Detection of the 45 kDa fragment may also be hampered because of processing by other proteases or degradation by the proteasome.

GrB cleavage sites at Asp1860 and Asp1961 both resulted in a loss of the RAM (RBP-Jk-associated molecule) domain, although part of the ankyrin repeats were maintained. The RAM domain is required for NICD binding to CSL, as well as inducing derepression of CSL target genes [29], thus loss results in a block of transcription. The CDC10/ankyrin repeats, responsible for CSL binding [30], can activate Notch signalling in the absence of a RAM domain [31–33]. However, this is several-fold lower than wild-type NICD1. Since part of the ankyrin repeats was removed by the second cleavage at Asp1961, this may further attenuate Notch1 transcriptional activity. Thus both cleavage scenarios lead to a loss of transcriptional activity, thereby effectively blocking transcriptional activation.

Loeb et al. [4] suggested that cleavage of Notch1 would lead to an increase in apoptosis in tumour cells. This coincides with our findings that cleavage of Notch1 results in a block of transcriptional activity. Inhibition of apoptosis by Notch1 appears to occur via several mechanisms. Notch1 has been shown to protect cells from apoptosis acting via INK (c-Jun N-terminal kinase) activation [34], by inhibiting p53 [35–37] or the PKB (protein kinase B/Akt pathway [37,38]. Breast epithelial cells are protected against apoptosis due to the induction of PKB/Akt signalling in response to Notch1 activation [39]. In addition, down-regulation of Notch1 in pancreatic cells leads to increased apoptosis [40]. In tumour cells, NICD1 can prevent the degradation of XIAP (X-linked inhibitor of apoptosis protein) thereby increasing cell survival [41]. Loss of Notch1 signalling in either of these cases would lead to a loss of anti-apoptotic signals, which may eventually lead to cell death. This supports the hypothesis that GrB cleavage may attenuate Notch1-mediated cell survival and thereby promote apoptotic potential in tumour cells. T-cell proliferation and maturation is a highly Notch1-dependent process, resulting in various types of T-cells. In a subset of T-cells, mature Th2 cells, cell death induced by GrB is caspase independent [42]. Consistent with this, genetic loss or inhibition of GrB leads to Th2 proliferation. Since Th2 cells depend on Notch1 activation for their proliferation [43], this suggests that GrB could trigger cell death by down-regulating Notch1 signalling by directly cleaving the receptor. This would ensure control over Th2 lymphocyte numbers and activity after execution of their function in the immune response, thereby tightly controlling cell numbers.

Figure 7 GrB cleavage results in a block of transcription

Transcriptional Notch1 activation assay shows a significant (P = 0.0495) decrease in activity upon GrB treatment. The histogram is representative of at least two independent experiments in triplicate, and the P-value was calculated using a Kruskal–Wallis non-parametric test. GSI, γ-secretase inhibitor DBZ.
to avoid an excessive number of activated T-cells. However, in
CTLs, Notch1 is thought to positively regulate the expression of
GrB and perforin by directly binding to their promoters [44]. One
could see the GrB cleavage of Notch1 as an activating cleavage,
thereby inducing an auto-regulatory loop by up-regulating GrB
and perforin. However, we have clearly shown that cleavage of
Notch1 by GrB leads to inactivation of signalling, making it
very unlikely that within the CTLs there is such a positive auto-
regulatory loop. The function of GrB cleaving Notch1 within
T-cells therefore remains obscure.

The plasma of healthy individuals contains low levels of GrB,
whereas patients suffering from viral infections, inflammation, or
auto-immune diseases show a several-fold increase in GrB levels
[24, 45, 46]. This could be due to leakage out of the immunological
synapse, explaining the higher GrB levels in patients since the
number of immune reactions is increased. However, recently it
was found that GrB is actively secreted into the extracellular
milieu [26]. Moreover, perforin-independent functions of GrB
have been reported [24–26]. In the present study we show that the
extracellular domain of Notch1 receptor is a substrate for cleavage
by GrB in the absence of perforin. We observed a significant 3-
fold increase in GrB-induced Notch1 shedding; however, without
the induction of transcriptional activation. This suggests that
the cleavage must take place at the N-terminal end upstream of the
NRR domain, since removal of this domain leads to activation of
Notch. Ligand binding to Notch is thought to induce a substantial
conformational change of the NRR domain leading to consecutive
S2 and S3 cleavage, producing NICD [16, 47, 48]. The GrB-
induced cleavage of NECD, however, does not lead to a confor-
maticational change that is sufficient for receptor activation.
However, this cleavage could shed off the ligand-binding domain, leaving
the receptor inactive at the cell surface and unable to signal. In
this way, GrB can inactivate the receptor indirectly, resulting in
an increased pro-apoptotic environment. Interestingly, there is a
good correlation between the cleavage fragments observed using N- and C-terminal epitope tags after GrB cleavage. Whereas Myc
immunoblotting detects the appearance of the 55 kDa cleavage
product, the N-terminal HA tag detects a similar reduction in the
size of the full-length precursor. Importantly, no other cleavage
events were observed, suggesting that the Gausis luciferase re-
lease in the medium seen after GrB exposure of cells is an indirect
effect of GrB. Thus the NECD is cleaved by a protease which is
activated by GrB. It will be interesting to identify the proteolytic
activity induced by GrB responsible for this NECD cleavage.

Collectively, our results demonstrate that Notch1 is an efficient
direct substrate for GrB. Cleavage of Notch1 leads to inactivation
of signalling both by the action of intracellular as well as
extracellular GrB. Direct cleavage of active NICD, but also down-
regulating total numbers of Notch1 precursors and receptors at
the membrane, decreases signalling capacity. This down-regulates
anti-apoptotic and pro-proliferative signals, achieving a faster and
more efficient apoptotic response. This example clearly shows
an important caspase-independent function of granzymes. Since
malignant cells identified by CTLs will not only encounter secreted
GrB, but a mixture of granzymes [49], it will be of interest
to determine the caspase-independent spectrum of substrates of
the granzymes altogether to gain more insight into the cytosolic
killing mechanisms involved in cancer.

AUTHOR CONTRIBUTION
Marc Vooijs, Geert van Tetering, Niels Bovenschen and Paul J van Diest conceived and
designed the experiments. Geert van Tetering and Jan Meeldijk performed the experiments.
Geert van Tetering analysed the data. Geert van Tetering and Marc Vooijs wrote the
manuscript.

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SUPPLEMENTARY ONLINE DATA

Cleavage of Notch1 by granzyme B disables its transcriptional activity

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Figure S1 Specification of GrB activity

(A) In vitro translated NEXT fragments are cleaved in the presence of 5 nM GrB. This activity could be fully blocked by 300 nM of the GrB inhibitor Ac-IETD-CHO as assessed by fluorimetric detection of lysine residues (A) as well as Myc immunoblotting (B). Molecular mass markers are indicated in kDa.

Figure S2 HeLa FRT N1 L1594P cell line characteristics

(A) Myc immunoblot of N1 L1594P HeLa cell lysates showing Notch is expressed and cleaved. PC, precursor; S1, S2 and S3 indicate cleavage products of Notch1. Molecular mass markers are indicated in kDa. (B) 12 × CSL transcriptional activation assay showing Notch is active in N1 L1594P HeLa cells, which can be attenuated by the γ-secretase inhibitor DBZ (GSI).

Figure S3 GrB action requires cell entry

Upper two panels, immunoblot showing N1 L1594P HeLa cells incubated with GrB in the absence or presence of SLO. Only upon co-incubation with SLO was GrB able to cleave Notch in the intracellular domain. Lower two panels, Caspase immunoblot demonstrating caspases are only activated upon co-incubation of SLO with GrB. The pan-caspase inhibitor Z-VAD-FMK (zVAD) inhibits the activating autocleavage of caspase 3. Actin staining of the same immunoblots serves as a loading control. Molecular mass markers are indicated in kDa.

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Figure S4  Z-VAD-FMK blocks caspase-dependent cell death

(A) Caspase GLO assay of N1 L1594P HeLa cells shows that upon GrB treatment both caspase 3 and 7 are activated. Both caspase-3 and caspase-7 activity is inhibited in Z-VAD-FMK (zVAD)-treated cells. Staurosporine is used as a positive control for caspase activation.

(B) Annexin V and propidium iodide FACS plots demonstrating that incubation with 600 nM GrB induces cell death. Upon treatment with Z-VAD-FMK, cell death can be prevented. Percentages ±S.D. are given in the Table below.

### Table

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Live cells (%)</th>
<th>Apoptotic cells (%)</th>
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<tbody>
<tr>
<td>No treatment</td>
<td>88.47% ± 4.86%</td>
<td>3.75% ± 2.09%</td>
</tr>
<tr>
<td>GrB 600 nM</td>
<td>23.78% ± 4.95%</td>
<td>46.53% ± 6.88%</td>
</tr>
<tr>
<td>GrB 600 nM z-VAD-fmk</td>
<td>83.68% ± 7.12%</td>
<td>7.47% ± 4.49%</td>
</tr>
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</table>

Figure S5  Possible NEXT-GrB site single mutants

The single site mutations at D1823Q and D1902E do not interfere with GrB processing of Notch1. Double D1823Q/D1902E Notch mutants are still processed normally by GrB. Only upon additionally mutating Asp1860 is cleavage affected.