Cell binding, internalization and cytotoxic activity of human granzyme B expressed in the yeast *Pichia pastoris*

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Granzyme B (GrB) is an apoptosis-inducing protease of cytotoxic lymphocytes. We have investigated intracellular and extracellular effects of human GrB using recombinant protein expressed in the yeast *Pichia pastoris*. GrB was rapidly taken up by HeLa cells, and accumulated in vesicular structures in the cytoplasm. There it remained inactive and could not be liberated by the endosomolytic reagent chloroquine, indicating that the vesicular structures are distinct from late endosomes and lysosomes. Direct cytosolic delivery of GrB with a cationic lipid-based transduction reagent, however, resulted in the induction of apoptotic cell death.

After prolonged incubation at or above 125 nM, GrB on its own induced pronounced morphological changes in human tumour cells, leading to partial loss of contact to the culture support. This extracellular effect was dependent on enzymatic activity and could be reversed by removal of the protein, suggesting GrB-dependent cleavage of extracellular matrix components as the underlying mechanism.

Key words: apoptosis, cellular uptake, extracellular matrix, granzyme B, *Pichia pastoris*, procaspase 3.

INTRODUCTION

Cytolytic T-lymphocytes (CTLs) and NK (natural killer) cells are highly specialized effectors of the immune system which are able to recognize and eliminate potentially harmful targets such as virus-infected, non-self or transformed cells by the induction of target-cell apoptosis. Studies with CTLs from knockout mice indicate that this cytotoxicity is mainly exerted by secretion of toxic effector molecules from cytotoxic granules [1]. These granules contain as main constituents the membrane-disrupting protein perforin and a family of serine proteases termed granzymes. So far, granzymes have only been identified in mammals, and are exclusively expressed in activated CTLs, immature T-cells, $\gamma\delta$ T-cells and NK cells. Eight murine granzymes are known (GrA–GrG and GrM), and five different granzymes (GrA, GrB, GrH, GrK and GrM) have been identified in humans [1]. Of these, GrB (granzyme B) was recognized as the most potent inducer of apoptotic cell death when applied together with perforin. Target cells exposed to CTLs from grB^{−/−} mice only show delayed DNA fragmentation [2,3], which has been attributed to the remaining GrA activity [4]. GrB is the only serine protease known to date that shares the unusual substrate specificity of caspases and cleaves its substrates after specific aspartate residues [5,6]. It is commonly accepted that GrB, once inside the cytosol of a target cell, induces apoptosis by mimicking caspase activity and activating caspase 3 and other caspases [7–12]. In addition, GrB was found to cleave central caspase substrates [13], such as the BH3-only protein Bid [14–16] and ICAD (inhibitor of the caspase-activated DNase) [17,18].

So far, it is not understood in detail how GrB and perforin co-operate to induce apoptotic cell death. While GrB directly activates apoptotic pathways via proteolysis of substrate proteins, perforin is apparently required to allow GrB access to these targets within the cytosol [19] or the nucleus [20,21]. In the ab-

sence of perforin activity, GrB is able to enter target cells by a mechanism that is concentration-dependent and saturable [19], but the molecule then localizes in membrane-enclosed vesicles where it remains inactive [19,22]. Induction of apoptosis is only observed after addition of perforin, which therefore is thought to function as an endosomolytic agent to release GrB from endosome-like vesicles [19,23].

Initially, the cation-dependent MPR [M6Ph (mannose-6-phosphate) receptor]/insulin-like growth factor II receptor was described as a target receptor for GrB, mediating uptake of the protease via endocytosis [24]. This receptor, which is ubiquitously expressed and engaged in protein sorting, also serves to transport newly synthesized GrB into the secretory vesicles of CTLs [25,26]. Cells overexpressing MPR were found to be more sensitive towards GrB than parental cells, and binding of GrB to the cell surface could be inhibited by addition of the monosaccharide M6Ph as a competitor [24]. However, subsequent reports demonstrated cell binding and uptake of GrB independent of the presence or function of MPR, indicating that MPR is not, or at least not an exclusive GrB receptor [27–29]. Recently, the dependence of GrB-mediated cytotoxicity on the presence of a functional dynamin pathway was analysed [30]. Dynamin is a critical factor in many endocytic pathways, and hence is most likely required for receptor-mediated uptake of GrB if this were its mechanism of cellular entry. Significant reduction of the cytotoxic activity of GrB was observed against cells lacking functional dynamin, suggesting that GrB indeed enters cells mainly via receptor-mediated endocytosis, and that this is a prerequisite for the induction of apoptosis [30].

With a few exceptions so far, studies investigating GrB activities have been carried out using endogenous enzyme purified from NK cell lines. Lately, recombinant GrB was successfully produced in *Escherichia coli*, but the generation of mature enzyme required refolding *in vitro* and removal of a prodomain by proteolytic

Abbreviations used: Ac-IETD-CHO, *N*-acetyl-Ile-Glu-Thr-Asp-aldehyde; Ac-IETD-pNA, *N*-acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide; CTL, cytolytic T-lymphocyte; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EGFR, epidermal growth factor receptor; GrB, granzyme B; TGF α , transforming growth factor α ; GrB-T, GrB-TGF α ; IL-2, interleukin 2; IPTG, isopropyl β -D-thiogalactoside; mAb, monoclonal antibody; M6Ph, mannose-6-phosphate; MPR, M6Ph receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NK cell, natural killer cell; PBMC, peripheral blood mononuclear cells; RT, reverse transcriptase.

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cleavage [29,31]. This bacterially expressed form of GrB is not glycosylated, affecting its ability to interact with cell surface receptors [29]. While previously the production of enzymatically active murine [32], rat [6] and human [33] GrB has also been reported in eukaryotic expression systems, such recombinant proteins have not yet been employed for mechanistic studies.

In the present paper, we describe the functional characterization of recombinant human GrB produced in the yeast *Pichia pastoris*. Without requiring further manipulation *in vitro*, mature enzymatically active protein could be purified from yeast culture supernatants at high yields. Using the recombinant protease, we investigated uptake of GrB by cultured tumour cells and induction of apoptosis. Upon prolonged incubation of cells with GrB in the absence of a perforin-like activity, we observed a dramatic change in cellular morphology, which could be attributed to an extracellular activity of the enzyme.

EXPERIMENTAL

Cells and culture conditions

The human tumour cell lines HeLa (cervix adenocarcinoma), A431 (epidermoid carcinoma), SKBR3, MDA-MB468 and MDA-MB453 (breast carcinoma) (A.T.C.C.) were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin.

Construction of GrB expression vectors

cDNA of human pre-pro-GrB was derived by reverse transcription of RNA from human PBMC (peripheral blood mononuclear cells), followed by PCR using the oligonucleotides 5'-AAACTCGAGATGCAACCAATCCTGC-3' and 5'-TATGAG-CTCTTAGTAGCGTTTCATG-3' as primers. The PCR product was digested with SacI and XhoI and inserted into the respective restriction sites of pBIKS+ (Stratagene, Heidelberg, Germany), resulting in plasmid pBIKS-GrB. A cDNA fragment encoding mature GrB was amplified from pBIKS-GrB as a template using the oligonucleotides 5' XhoI GrB 5'-ATTCTCGAGAAAA-GAATCATCGGGGGACATGAG-3' and 3' NheI GrB 5'-TTT-GCTAGCCCGTAGCGTTTCATGG-3'. Following digestion with XhoI and NheI, the PCR product was inserted between the XhoI and XbaI sites of pBIKS+. Subsequently, a double-stranded oligonucleotide encoding a Myc epitope (Myc) recognized by mAb (monoclonal antibody) 9E10 [34] and a His₆ tag (His) was ligated between the XbaI and NotI sites. Then, the entire GrB-Myc-His sequence was subcloned as an XhoI, NotI fragment into the respective sites of the yeast expression vector pPIC9 (Invitrogen, Karlsruhe, Germany), resulting in plasmid pPIC9-GrB.

As a control construct, an enzymatically inactive GrB mutant was generated by exchanging the codon of the active site serine residue at position 183 of mature GrB with a sequence coding for alanine. Site-directed mutagenesis was performed by PCR using oligonucleotide primers GrB S183A-sense 5'-TTTAA-GGTGACGCTGGAGGCCCTCTTG-3' and 3' NheI GrB (see above). The resulting PCR product served as 3' primer in a second PCR reaction together with 5' XhoI GrB (see above) and pBIKS-GrB-Myc-His as a template to amplify the entire mutant GrB. The XhoI, NheI digested fragment was subcloned into pPIC9 as described for wild-type GrB to produce the expression plasmid pPIC9-GrB_{S183A}.

Expression and purification of mature GrB

P. pastoris GS115 cells (Invitrogen) were transformed with the pPIC9 expression plasmids via electroporation, and positive

expression clones were selected according to the manufacturer's recommendations. For large-scale protein expression, single colonies were grown in 50 ml of buffered (pH 8) glycerol complex medium for 2 days at 30°C. To induce expression of recombinant proteins, cultures were diluted 5-fold in methanol-containing buffered (pH 8) methanol complex medium to a D_{600} of approx. 1, and cultured for another 4 days at 25°C in the presence of 2% (v/v) methanol. Methanol was again supplemented every 24 h. To harvest culture supernatants, yeast cells were removed by centrifugation at 7500 g. Supernatants containing the recombinant proteins were adjusted to pH 8, passed through a 22 µm filter and applied to an Ni²⁺-saturated chelating Sepharose column (Amersham Biosciences, Freiburg, Germany) equilibrated with PBS (pH 8). Specifically bound proteins were eluted with 250 mM imidazole in PBS (pH 8). Protein fractions containing GrB were identified by SDS/PAGE and immunoblotting with the Myc tag-specific antibody 9E10, or the GrB-specific mAb 2C5 (Santa Cruz Biotechnology, Heidelberg, Germany), pooled, dialysed against PBS (pH 7.4) and stored at -80°C until use.

Deglycosylation of GrB

Purified GrB (1 µg) in 10 µl of PBS was denatured by addition of 1 µl of 10% (w/v) SDS solution and incubation at 95°C for 10 min. One unit of N-glycosidase F (Roche Diagnostics, Mannheim, Germany) and 0.5% Triton X-100 were added in a total reaction volume adjusted to 100 µl with PBS, and the reaction was incubated at 37°C overnight before analysis by SDS/PAGE and immunoblotting.

Analysis of GrB enzymatic activity

To quantify enzymatic activity, varying amounts of purified GrB or GrB_{S183A} were incubated for 3 h at 37°C in 96-well plates with 200 µM synthetic peptide substrate Ac-IETD-pNA (acetyl-Ile-Glu-Thr-Asp-p-nitroanilide; Alexis, Grünberg, Germany) and reaction buffer (10 mM Hepes, pH 7.4, 140 mM NaCl and 2.5 mM CaCl₂) in a total volume of 100 µl per sample. Cleavage of substrate was determined by measuring the absorbance at 405 nm with a microplate reader (corrected for background by subtracting A_{490}). Peptide substrate incubated in reaction buffer without protein served as blank. To determine enzyme kinetics, 100 µl reaction mixtures each containing 30 nM of native human GrB purified from IL-2 (interleukin 2)-activated lymphocytes (Alexis) or recombinant human GrB purified from *P. pastoris* and 8–500 µM Ac-IEPD-pNA substrate (Alexis) were incubated at room temperature (23°C). Initial rates were measured in duplicates for each substrate concentration. Kinetic constants were derived from a Lineweaver-Burk plot.

Cleavage of a GrB target protein was determined using recombinant procaspase 3 as a physiological substrate. cDNA of procaspase 3 was derived by reverse transcription of RNA from human PBMC, followed by PCR using the oligonucleotides 5'-TTAATAGGATCCATATGGAGAACACT-3' and 5'-AACCA-GAGCTCTAGAGAGTGATAAAAATAG-3'. The PCR product was digested with NdeI and XbaI, and inserted downstream of an IPTG (isopropyl β-D-thiogalactoside)-inducible tac promoter in the respective sites of the bacterial expression vector pSW50 [35], in addition containing 3' of procaspase 3 an oligonucleotide sequence coding for an epitope tag recognized by CD24-specific mAb SWA11 [36] and a His₆ tag. For bacterial expression, the resulting plasmid pSW5-caspase-3 was transformed into *E. coli* strain XL1-Blue (Stratagene). A single colony was grown at 37°C to a D_{600} of 0.4–0.6 in LB (Luria-Bertani) medium containing 100 µg/ml ampicillin. Protein expression was induced

with 1 mM IPTG for 1 h at 37°C before cells were harvested by centrifugation. Cell pellet from 1 litre of culture was resuspended in urea-containing buffer (PBS containing 8 M urea), and cells were disrupted by ultrasonification. Recombinant procaspase 3 was purified from clarified lysates via Ni²⁺-affinity chromatography, and refolded by dialysis against PBS containing 20 mM DTT (dithiothreitol) and 10% (v/v) glycerol, followed by decrease of the DTT concentration to 2 mM in a second dialysis step.

Procaspace 3 (100 ng per sample) was incubated for 14 h at 37°C with various amounts of purified GrB or GrB_{S183A} in a total volume of 40 µl in 10 mM Hepes (pH 7.4), 140 mM NaCl and 2.5 mM CaCl₂. Samples were analysed for cleavage of procaspase 3 by SDS/PAGE and immunoblotting. Procaspase 3 and the p12 cleavage product were visualized by probing the blot with mAb SWA11, followed by HRP (horseradish peroxidase)-coupled secondary antibody and chemiluminescent detection using the ECL[®] kit (Amersham Biosciences).

Analysis of binding, uptake and intracellular localization of GrB

HeLa cells were grown on coverslips, and treated with 250 nM (10 µg/ml) recombinant GrB purified from *P. pastoris* in normal growth medium on ice or at 37°C for 1 h. To compete for potential binding of GrB to MPR, in some samples, cells were pre-incubated with 100 mM of the monosaccharide M6Ph (Sigma, Deisenhofen, Germany) for 10 min prior to addition of 250 nM GrB in the presence of 100 mM M6Ph. Binding experiments were also performed with equimolar amounts of commercially available bacterially expressed GrB (Alexis). To detect bound or internalized GrB, cells were fixed with 4% (w/v) paraformaldehyde in PBS for 10 min, permeabilized in 0.1% Triton X-100 in PBS for 5 min, and incubated with 500 ng/ml mAb 2C5 in 3% BSA/PBS, followed by Alexa Fluor 488 donkey anti-mouse IgG (Molecular Probes, Leiden, The Netherlands) diluted 1:1000 in 3% BSA/PBS. Then, samples were analysed with a Nikon Eclipse TE300 fluorescence microscope (Nikon, Düsseldorf, Germany) or a Leica TCS SL laser scanning microscope (Leica Microsystems, Bensheim, Germany).

Intracellular delivery of GrB and detection of apoptosis

The cationic lipid-based transduction reagent BioPorter (Gene Therapy Systems, San Diego, CA, U.S.A.) was used to deliver GrB into the cytosol of HeLa cells. The reagent was applied according to the manufacturer's instructions. Briefly, 1 µl each of BioPorter reagent dissolved in chloroform was dried in a reaction tube, and rehydrated by addition of 10 µl of protein solution in HBS buffer (20 mM Hepes, 140 mM NaCl and 0.75 mM Na₂HPO₄, pH 7.4) to form GrB–BioPorter complexes. HeLa cells were seeded at a density of 5×10^5 cells/well in 96-well plates and were grown overnight. Then, cells were washed with serum-free DMEM, before protein preparations diluted in 90 µl of serum-free DMEM were added. After incubation for 4 h at 37°C, 10% fetal bovine serum was added, and the cells were incubated for another 14 h. Induction of apoptosis was analysed using the Cell Death Detection ELISA plus (Roche Diagnostics) according to the manufacturer's recommendations. The degree of apoptosis was quantified by measuring the absorbance at 405 nm in a microplate reader. To determine the relative number of apoptotic cells, HeLa cells were treated with GrB–BioPorter complexes for 5 h. Then, cells were either stained with Hoechst 33342 dye and apoptotic morphology was analysed by bright field and fluorescence microscopy, or the number of dead cells was determined by Trypan Blue staining followed by microscopy. A minimum of 50 cells per field was evaluated in triplicate.

Cell viability assays

Effects of GrB on cell viability were analysed in MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] metabolization assays. Cells were seeded in 96-well plates at a density of $1.5\text{--}2 \times 10^4$ cells/well and incubated for 14 h at 37°C with increasing concentrations of purified GrB proteins in triplicate in the absence or presence of 100 µM chloroquine. Then, 10 µl of 10 mg/ml MTT (Sigma) in PBS was added to each well, and the cells were incubated for another 3 h. Cells were lysed by the addition of 90 µl of 20% SDS in 50% dimethylformamide (pH 4.7). After solubilization, colour development due to formation of the brown formazan metabolite was quantified by determining the absorbance at 590 nm in a microplate reader. The percentage of viable cells was calculated from these data relative to cells grown in the presence of chloroquine but without the addition of GrB (set to 100%). To determine cell viability after long-term exposure to GrB in the absence of chloroquine, MTT was added after 48 h of treatment and the percentage of viable cells was calculated relative to cells grown in the absence of GrB (set to 100%). Control cells were treated with 1 mM of the protein kinase inhibitor staurosporine.

Analysis of GrB-induced morphological changes

HeLa, A431, SKBR3, MDA-MB453 or MDA-MB468 cells were seeded in 96-well plates at a density of $1\text{--}4 \times 10^4$ cells/well and treated with different concentrations of purified GrB at 37°C. Control cells were incubated with PBS or enzymatically inactive GrB_{S183A}, or were pretreated with 100 µM of GrB-specific aldehyde peptide inhibitor Ac-IETD-CHO (*N*-acetyl-Ile-Glu-Thr-Asp-aldehyde; Alexis) for 30 min before addition of GrB. As a positive control for the induction of apoptosis, cells were treated with 100 µM staurosporine. Morphological changes of cells were analysed by light microscopy at the indicated time points.

RESULTS

Production of recombinant human GrB in *P. pastoris*

In CTLs and NK cells, GrB is initially expressed as an inactive precursor protein. This pre-pro-GrB carries an N-terminal signal peptide that directs packaging of the protein into secretory granules (Figure 1A, upper panel). The enzymatic activity of the protease is strictly controlled by the activation dipeptide Gly-Glu, which is cleaved by dipeptidyl peptidase/cathepsin C during transport into storage vesicles [37].

For expression of recombinant GrB, cDNA of human pre-pro-GrB was derived by RT (reverse transcriptase)–PCR from mRNA of PBMC and subsequently used as a template for amplification of a cDNA fragment encoding the mature form of GrB (amino acid residues 21–247) as described in the Experimental section. This cDNA fragment was then inserted into the yeast expression vector pPIC9, which allows stable integration of the cloned construct into the genome of the methylotrophic yeast *P. pastoris* in one or more copies (Figure 1A, lower panel). The plasmid pPIC9-GrB provides the methanol-inducible alcohol oxidase AOX1 promoter for highlevel expression, and the α -factor signal sequence from *Saccharomyces cerevisiae* for secretion of recombinant protein into the culture supernatant. During secretion, this signal peptide is cleaved off by the *Pichia* protease KEX2 (kexin), producing mature human GrB.

After the induction of expression with methanol, recombinant GrB was purified from culture supernatants under native conditions by single-step Ni²⁺-affinity chromatography to over 90%

Figure 1 Expression of human GrB in the yeast *P. pastoris*

(A) Endogenous mammalian GrB is expressed as a pre-proenzyme containing an N-terminal secretion signal (residues 1–18) for transport of the protein into secretory vesicles, and an inhibitory Gly-Glu dipeptide (residues 19–20) (upper panel). During packaging into cytotoxic granules, signal peptide and activation dipeptide are removed by signal peptidase and cathepsin C/dipeptidyl peptidase, generating mature GrB (residues 21–247). The catalytic serine residue in the active centre of GrB is Ser¹⁸³. For expression in the yeast *P. pastoris*, cDNA encoding mature human GrB (amino acids 21–247) was genetically fused 3' to the α -factor signal peptide (SP) of *S. cerevisiae* in the expression vector pPIC9 (lower panel). During export of the protein, the signal peptide is removed by the *P. pastoris* protease KEX2, producing the N-terminus of mature GrB. Synthetic Myc (M) and His₆ tags (H) are included at the C-terminus of the gene product. (B) Recombinant GrB was purified from yeast culture supernatants by Ni²⁺-affinity chromatography. Eluate fractions were analysed by SDS/PAGE and Coomassie Blue staining (lane 1) and immunoblotting with anti-GrB mAb (lane 2). (C) To demonstrate N-linked glycosylation of yeast-expressed GrB, purified protein was treated with N-glycosidase F before immunoblot analysis.

purity as judged by SDS/PAGE analysis (Figure 1B, lane 1). Yields were 1–2 mg of purified GrB per litre of yeast culture. The identity of the protein was confirmed by immunoblot analysis with a GrB-specific mAb (Figure 1B, lane 2). Like endogenous GrB isolated from NK cell lines [38], the recombinant molecule produced in *P. pastoris* is glycosylated and has an apparent molecular mass of approx. 40 kDa. Treatment of purified protein with N-glycosidase F reduced the molecular mass to approx. 30 kDa (Figure 1C), which corresponds to the calculated molecular mass of non-glycosylated GrB. As a control protein for subsequent experiments, an inactive GrB mutant GrB_{S183A} was constructed by exchanging the catalytic serine residue at position 183 of the mature GrB sequence with an alanine [39]. The mutated protein was expressed in *P. pastoris* and purified from culture supernatants as described above for wild-type GrB (results not shown).

Enzymatic activity of recombinant GrB from *P. pastoris*

To analyse the enzymatic activity of the recombinant proteins, GrB-specific colorimetric peptide substrate Ac-IETD-pNA was incubated with increasing concentrations of purified GrB or GrB_{S183A}. As shown in Figure 2(A), GrB but not GrB_{S183A} cleaved

Figure 2 Recombinant GrB from yeast displays enzymatic activity

(A) GrB-specific peptide substrate Ac-IETD-pNA was incubated with the indicated concentrations of purified GrB or GrB mutant GrB_{S183A} for 3 h at 37 °C. Cleavage of substrate was determined by measuring the absorbance at 405 nm. (B, C) To analyse cleavage of a physiological GrB substrate, bacterially expressed procaspase 3 was incubated with the indicated concentrations of purified GrB for 14 h at 37 °C. GrB pretreated with GrB-specific peptide aldehyde inhibitor Ac-IETD-CHO (B) or enzymatically inactive GrB_{S183A} (C) were included as controls. Samples were analysed by SDS/PAGE and immunoblotting. Procaspase 3 and the p12 cleavage product were detected with mAb SWA11 [36] recognizing an epitope tag included at the C-terminus of recombinant procaspase 3. Bands corresponding to procaspase 3 and p12 are indicated by arrows. In addition, in (B, lanes 2, 3 and 5) and (C, lane 2), intermediate caspase 3 cleavage products are detected by the antibody.

the synthetic substrate in a concentration-dependent manner, resulting in a quantifiable release of *p*-nitroaniline. Kinetic constants for hydrolysis of the peptide substrate Ac-IETD-pNA were in a similar range as those measured for native human GrB from IL-2-activated lymphocytes and previously reported for recombinant rat GrB (Table 1 and [6]). In addition, the enzymatic activity of purified GrB was tested in an *in vitro* cleavage assay using recombinant human procaspase 3 as a physiological substrate [7]. Procaspase 3 was expressed in *E. coli*, solubilized in urea-containing buffer, purified under denaturing conditions by Ni²⁺-affinity chromatography, and refolded as described in the Experimental section. At its C-terminus, the recombinant procaspase 3 carries an epitope tag recognized by anti-CD24

Table 1 Kinetic constants for hydrolysis of Ac-IEPD-pNA by native and recombinant GrB

GrB	k_{cat} (s^{-1})	K_{m} (10^{-5} M)	$k_{\text{cat}}/K_{\text{m}}$ ($10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$)
Native human	4.34 ± 0.53	7.38 ± 1.13	5.87 ± 1.62
Recombinant human	5.23 ± 1.20	6.16 ± 1.83	8.49 ± 4.46
Recombinant rat*	4.16 ± 0.05	5.70 ± 0.40	6.66 ± 0.32

* Kinetic constants for recombinant GrB from rat are taken from [6].

antibody SWA11 [36] to allow immunological detection of full-length p32 as well as the p12 caspase 3 subunit and intermediate p12-containing cleavage products. Upon incubation with purified recombinant GrB, procaspase 3 was cleaved in a concentration-dependent manner, producing the p12 subunit detected by immunoblot analysis with SWA11 antibody (Figures 2B and 2C, lanes 2 and 3). Processing of procaspase 3 could be inhibited by pretreatment of GrB with the GrB-specific tetrapeptide aldehyde inhibitor Ac-IETD-CHO (Figure 2B, lanes 4 and 5). Likewise, prolonged incubation of procaspase 3 with mutated GrB_{S183A} did not result in the generation of p12 subunit (Figure 2C, lanes 4 and 5), demonstrating that cleavage of procaspase 3 depends on GrB activity. Importantly, this processing of procaspase 3 did not result in enzymatic caspase 3 activity, probably due to incorrect folding of the recombinant caspase. Fragmentation of caspase 3 observed in this assay was therefore solely due to GrB activity, and not to autocatalytic cleavage by processed caspase 3. These results show that recombinant human GrB expressed in the yeast *P. pastoris* is enzymatically active and processes typical GrB substrates.

Binding of recombinant GrB to the surface of tumour cells and internalization into intracellular vesicles

Binding and internalization of recombinant GrB was investigated by immunofluorescence analysis with GrB-specific antibody using HeLa cells as a model. Following incubation of the cells with GrB at 4°C, the protein localized to the cell membrane (Figure 3A). At 37°C, rapid uptake of GrB into the cells was found, which could be observed as early as 10 min after addition of the protein, and resulted in a punctate staining within the cytoplasm, suggesting localization of GrB inside vesicular structures (Figure 3B). Similar results were obtained with human A431 epidermoid cancer cells (results not shown). In contrast, neither effective binding to the cell surface nor internalization into the cells could be observed when HeLa cells were incubated with the same amount of commercially available recombinant GrB expressed in bacteria (Figure 3C).

Internalization of recombinant GrB from *P. pastoris* into HeLa cells did not depend on enzymatic activity of the protease, since the inactive mutant GrB_{S183A} could also be detected inside the cells, and showed a staining pattern very similar to that of wild-type GrB (Figure 3E). Cell binding and intracellular uptake of GrB could not be inhibited by high concentrations of M6Ph as a competitor for potential binding to MPR (Figure 3I), demonstrating that also yeast-expressed GrB can be taken up independently from MPR [27–29].

GrB-induced apoptosis

To further study the activity of yeast-expressed GrB, we investigated the ability of the protein to induce apoptotic cell death.

Figure 3 Binding and uptake of recombinant GrB

HeLa cells were incubated with 10 $\mu\text{g}/\text{ml}$ of recombinant GrB purified from *P. pastoris* supernatants at 4°C to detect binding of the protein to the cell surface (A), or at 37°C to allow intracellular uptake (B). Cells were also incubated with the same amount of commercially available GrB expressed in bacteria (C). Control cells were treated with PBS (D). To confirm that the cell binding activity of GrB is independent of its enzymatic activity, the enzymatically inactive GrB mutant GrB_{S183A} was analysed in a similar experiment (E, F). To investigate the dependence of GrB binding on interaction with MPR, HeLa cells were treated with yeast-expressed GrB either in the absence (G, H) or presence (I, J) of 100 mM M6Ph as a competitor. Cells were washed, fixed, consecutively incubated with anti-GrB and anti-mouse Alexa Fluor 488 antibodies, and analysed by confocal laser scanning (A–D) or standard fluorescence (E–J) microscopy. Nuclei were stained with propidium iodide. Merged images are shown in (A–D) and separate images of corresponding fields are shown in (E–J).

Figure 4 Induction of apoptosis upon delivery of recombinant GrB into the cytosol

The cationic lipid-based transduction reagent BioPorter was used for intracellular delivery of GrB. (A) Uptake of GrB was confirmed, 4 h after addition of GrB–BioPorter complexes to HeLa cells, by immunofluorescence analysis with anti-GrB and anti-mouse Alexa Fluor 488 antibodies. Cells treated with BioPorter in the absence of GrB served as a control. (B) After treatment of cells with GrB–BioPorter complexes for 14 h, induction of apoptosis was analysed using a nucleosome ELISA. This assay allows detection of cytoplasmic nucleosomes, indicative of apoptotic DNA fragmentation. Control cells were treated with BioPorter in the absence of GrB, uncomplexed GrB, or complexes containing the enzymatically inactive mutant GrBs183A as indicated. (C) In a similar experiment, HeLa cells were treated with GrB–BioPorter complexes containing GrB expressed in yeast (GrB) or in bacteria (GrB *E. coli*), or with control reagents as indicated. After 5 h, cells were either stained with Hoechst 33342 dye and apoptotic morphology was analysed by bright field and fluorescence microscopy (left panel), or the number of dead cells was determined by Trypan Blue staining followed by microscopy (right panel). A minimum of 50 cells per field was evaluated in triplicate. GrB concentrations given in (B) and (C) were calculated from the amount of free GrB used for complex formation with BioPorter. (D) The ability of the endosomolytic reagent chloroquine to release GrB from intracellular vesicles was analysed in MTT cytotoxicity assays. Human HeLa and MDA-MB468 tumour cells were treated with purified recombinant GrB or a GrB-T fusion protein in the presence of 50 or 100 µM chloroquine as indicated. After 14 h, cell viability was determined by the addition of MTT. Control cells were treated in the absence of GrB.

In a physiological context, this activity requires the membrane-disrupting protein perforin. In cell culture assays, the function of perforin can be replaced by replication-deficient adenovirus [19], or by certain pore-forming bacterial toxins [23]. These are thought to serve as endosomolytic reagents permitting delivery of GrB into the cytosol of target cells.

To promote transport of recombinant GrB across cellular membranes, we made use of the cationic lipid-based transduction reagent BioPorter, which has previously been shown to deliver apoptosis-inducing proteases into cells [40]. In contrast with the punctuate intracellular staining pattern found after uptake of GrB in the absence of a particular delivery reagent (Figure 3B),

4 h after addition of GrB–BioPorter complexes to HeLa cells a homogeneous distribution of the protease throughout the cytosol was observed (Figure 4A, upper left panel), suggesting efficient cytosolic delivery. In contrast with treatment with control complexes, intracellular delivery of GrB induced morphological changes indicative of apoptosis such as membrane blebbing and release of apoptotic bodies (Figure 4A, lower left panel). To confirm induction of apoptosis, after treatment of HeLa cells with GrB–BioPorter complexes and incubation overnight, the cells were analysed using a nucleosome ELISA (Figure 4B). With this assay, nucleosomes are detected in cytoplasmic extracts of apoptotic cells which are indicative of fragmentation of the cellular DNA.

While no apoptosis was observed in cells treated with GrB in the absence of BioPorter, or with BioPorter complexes containing the inactive mutant GrB_{S183A}, active GrB delivered into HeLa cells via BioPorter induced significant DNA fragmentation in a concentration-dependent manner. To detect induction of apoptosis at the single-cell level, in a similar experiment HeLa cells were treated with GrB–BioPorter complexes or control reagents for 5 h before the relative number of apoptotic cells was determined. Under these conditions, apoptotic morphology was induced in more than 50 % of cells with 100 nM GrB, whereas BioPorter-mediated delivery of the same amount of inactive mutant GrB_{S183A} had no significant effect (Figure 4C, left panel). BioPorter-mediated intracellular delivery of GrB expressed in bacteria had a very similar cytotoxic effect on HeLa cells (Figure 4C, right panel), demonstrating that differences between bacterially and yeast-expressed GrB only affect cell binding and uptake into vesicles, but not enzymatic activity or interaction with intracellular substrates.

The basic reagent chloroquine is commonly used as an endosomolytic reagent to enhance cytosolic delivery of therapeutic molecules upon endocytic uptake [41,42]. Chloroquine accumulates in acidic compartments such as late endosomes and lysosomes, where it interferes with the pH equilibrium, finally leading to osmotic rupture of the vesicles. We performed cell viability experiments to test whether chloroquine could also be used to release GrB from intracellular vesicles, thereby allowing access to its cytosolic substrates and induction of apoptosis. HeLa and MDA-MB468 cells were treated with GrB for 14 h in the presence of chloroquine, before viability of cells was analysed in MTT metabolization assays, as described in the Experimental section. The results are shown in Figure 4(D). In contrast with BioPorter as a delivery reagent for GrB, the addition of chloroquine did not support GrB-mediated cell death. In sharp contrast, when GrB was expressed in *P. pastoris* as a fusion protein with the EGFR (epidermal growth factor receptor) ligand TGF α (transforming growth factor α) [43], treatment of EGFR overexpressing MDA-MB468 cells with low concentrations of this GrB–T (GrB–TGF α) fusion protein in the presence of chloroquine induced massive cell killing. In the absence of chloroquine, GrB–T had no effect, confirming that under the chosen experimental conditions chloroquine indeed functioned as an endosome release reagent.

These results suggest that GrB enters cells by a mechanism distinct from classical receptor-mediated endocytosis, and that it does not accumulate in late endosomes or other vesicular structures affected by chloroquine. Redirection of GrB to a pathway involving classical receptor-mediated endocytosis, however, results in routing to an acidic environment and allows chloroquine-mediated release into the cytosol.

Induction of morphological changes in cultured tumour cells

As shown above, for rapid induction of target cell apoptosis by GrB, a delivery reagent functionally equivalent to perforin is required. Nevertheless, cultures of adherent tumour cells showed significantly altered morphology in response to treatment with GrB in the absence of perforin activity (Figure 5). However, in contrast with apoptosis induced by GrB after delivery into the cytosol, this effect required relatively high protein concentrations (at or above 125 nM) and extended incubation times (20–48 h). While low concentrations of GrB (25 nM) had no visible effect (Figure 5B), at higher GrB concentrations HeLa, A431 and SKBR3 cells rounded up and partially lost contact to the culture dish (Figures 5C, 5D, 5J, 5K, 5N, 5O, 5R and 6A). Similar results were also found for MDA-MB453 and MDA-MB468 breast carcinoma cells (results not shown). Yet, cells did not

display typical apoptotic morphology. In particular, no membrane blebbing and no production of apoptotic bodies were observed in cells exposed to GrB, while treatment of the cells with staurosporine did induce these effects (Figure 5E). The rounded cells remained attached to the culture dish and did not float in the culture medium. Addition of GrB-specific peptide aldehyde inhibitor Ac-IETD-CHO to the cells completely prevented morphological changes induced by GrB (Figures 5L and 5P). Likewise, cells treated with the inactive mutant GrB_{S183A} remained unaffected (Figures 5F–5H and 6A), indicating that morphological alterations were caused by GrB enzymatic activity. When the effects of prolonged exposure to high concentrations of GrB on cell viability were analysed, a reduction of approx. 20 % was found after 48 h, which at concentrations up to 250 nM could be completely blocked by a GrB-specific inhibitor (Figure 6B).

Interestingly, after removal of the protease, most of the cells quickly regained their normal morphology. After overnight treatment of HeLa cells with 125 or 300 nM GrB (Figures 7B and 7C), the rounded cells were detached from the culture dish by pipetting, washed and replated in a fresh medium. As early as 30 min after replating, cells were indistinguishable from untreated controls and continued to grow without any visible alterations. Cellular morphology 3 h after replating is shown in Figures 7(E) and 7(F). The data are quantified in Figure 7(G). These results suggest that the observed effects are not due to cleavage of intracellular GrB target proteins, but rather appear to be the result of extracellular GrB activity such as processing of components of the extracellular matrix, leading to detachment of adherent cells from the culture support. Only a small reduction in the number of viable tumour cells was observed, suggesting that this effect depends on indirect mechanisms rather than direct activation of apoptosis pathways.

DISCUSSION

Cytotoxic granules of T and NK cells in addition to GrB contain perforin and a number of other proteases. Isolation of endogenous mammalian GrB therefore requires laborious purification procedures, and carries the risk of contamination with other molecules involved in target cell lysis. Extending earlier work by other groups [6,32,33], we have expressed the processed, mature form of human GrB as a secreted protein in the yeast *P. pastoris*. As a eukaryotic organism, *P. pastoris* shares many of the advantages of other eukaryotic expression systems such as folding, processing and post-translational modification of heterologous proteins. Fusion of the yeast mating type α -factor signal peptide to the protein of interest allows secretion of recombinant protein in native and glycosylated form into the culture supernatant. In contrast with proteins expressed in *S. cerevisiae*, however, hyperglycosylation does not occur and the glycosylation pattern resembles that of human proteins [44,45].

Since endogenous GrB purified from lysates of NK cell lines [38] is heavily glycosylated, glycosylation might be important for overall function of the protein. Recombinant GrB from *P. pastoris* also carried significant glycosylation, resulting in a markedly increased apparent molecular mass of 40 kDa compared with 27 kDa for non-glycosylated GrB. The recombinant protease was enzymatically active and bound to cultured tumour cells, followed by intracellular uptake. Immunofluorescence staining of GrB-treated cells demonstrated localization of GrB in vesicular structures inside the cytoplasm, which is in agreement with the results obtained with GrB purified from NK cells [20,22]. Upon delivery of recombinant GrB into the cytosol of HeLa cells using a cationic lipid-based transduction reagent, apoptotic cell

Figure 5 Exposure to recombinant GrB induces morphological changes in cultured tumour cells

Established human HeLa (**A–L**), A431 (**M–P**) and SKBR3 (**Q–R**) tumour cells were treated for 20 h (**A–H**) or 48 h (**I–R**) with 25 nM (**B**), 125 nM (**C, J, N, R**), 250 nM (**K, O**) or 625 nM (**D**) of GrB purified from *P. pastoris* supernatant. Control cells were incubated with PBS (**A, I, M, Q**) or 1 μ M staurosporine to induce apoptotic morphology (**E**). To investigate dependence of the observed effects on enzymatic activity of GrB, cells were either treated with 25, 125 or 625 nM of the enzymatically inactive protein GrBs183A (**F–H**) or pretreated for 30 min with 100 μ M of GrB-specific peptide aldehyde inhibitor Ac-IETD-CHO before the addition of 125 nM GrB (**L, P**). Representative microscopic fields are shown.

morphology and DNA fragmentation were detected, indicating GrB-mediated induction of apoptosis. Hence, GrB expressed in the yeast *P. pastoris* displays all the activities described so far for the endogenous mammalian enzyme, demonstrating that the recombinant protein is fully functional.

MPR was initially described as a target receptor for GrB, implying that a M6Ph modification of GrB might be responsible for binding to cells [24]. However, subsequent reports demonstrated cell binding and uptake of GrB independent of MPR, indicating that MPR is not, or at least not an exclusive GrB receptor [27–29]. In agreement with the latter studies, we could not block binding and internalization of recombinant GrB into HeLa cells by addition of M6Ph, even at concentrations as high as 100 mM. In another study, the dependence of GrB uptake on dynamin, a component critical for many endocytic pathways, was

investigated [30]. It was found that the predominant pathway for entry of GrB requires dynamin, i.e. involves receptor-mediated endocytosis. Taken together, these findings suggest that GrB enters target cells via receptor-mediated endocytosis, yet binds to structures on the cell surface other than MPR.

Non-glycosylated GrB expressed in *E. coli* was recently reported to bind to monocytes and subpopulations of B cells via heparan sulphate chains present on the cell surface, followed by uptake into lysosomal compartments [29]. However, in this study, the binding of GrB to heparan sulphate appeared irrelevant for GrB-mediated apoptosis, since removal of such binding sites or saturation with enzymatically inactive GrB did not inhibit NK-cell-mediated lysis [29]. This indicates that another, heparan sulphate-independent mode of uptake must exist for GrB, which is apparently the most relevant mechanism for induction of

Figure 6 GrB-induced morphological changes do not result in massive tumour cell death

(A) Effects of high concentrations of GrB on the morphology of HeLa cells after incubation for 20 h were quantified by determining the number of cells with normal or altered morphology in the experiment shown in Figures 5(A)–5(H). Approximately 100 cells per field were evaluated in triplicate. (B) The influence of high concentrations of GrB on cell viability was analysed in MTT cytotoxicity assays. HeLa cells were treated with 125–625 nM of purified recombinant GrB or GrB in the presence of 100 μ M of GrB-specific peptide aldehyde inhibitor Ac-IETD-CHO as indicated. After 48 h, cell viability was determined by the addition of MTT. Control cells were treated in the absence of GrB or with 1 mM staurosporine.

apoptosis by NK cells. In our hands, glycosylated GrB expressed in yeast, but not commercially available non-glycosylated protein from bacteria, readily bound to HeLa cells, followed by rapid uptake. As previously reported for NK and T-cells [29], cell-type-specific differences in the heparan sulphate density might explain why recombinant GrB from *E. coli* did not bind to HeLa cells. However, this does not explain the excellent binding of recombinant GrB from *P. pastoris* to these cells. Consequently, glycosylation or another form of post-translational modification only present in eukaryotic cells might be required to enable binding of GrB to an alternative, as yet unidentified cell surface structure. Once the critical step of cell binding was bypassed by intracellular delivery with the BioPorter transduction reagent, GrB expressed in *E. coli* or *P. pastoris* displayed indistinguishable cytotoxic activity. This demonstrates that the differences between bacterially and yeast-expressed GrB only affect cell binding and uptake into vesicles, but not enzymatic activity or interaction with intracellular substrates.

Following internalization into target cells, GrB localizes in vesicular structures inside the cytoplasm where it remains inactive. This was described by others for GrB purified from mammalian cells [19,20,22], and could be confirmed in the present study for recombinant protein from yeast. Induction of apoptosis, however, is only observed after the addition of a perforin-like activity, which is thought to liberate GrB from these vesicles, allowing access of the protease to the cytosol. In the experiments presented here, GrB expressed in yeast could not be released from

Figure 7 GrB-induced morphological changes are reversible upon removal of the protease

HeLa cells were treated for 20 h with 125 nM (B) or 300 nM (C) recombinant GrB. Then, they were removed from the culture dish by pipetting, washed and reseeded for 3 h at high cell densities in a fresh medium without addition of GrB (E, F). Control cells were incubated with PBS (A, D). Representative microscopic fields are shown. (G) Effects were quantified by determining the number of cells with normal or altered morphology. Approximately 100 cells per field were evaluated in triplicate.

cytoplasmic vesicles by the endosomolytic reagent chloroquine. Chloroquine accumulates in acidic compartments such as late endosomes and lysosomes, where it interferes with the pH equilibrium, finally leading to osmotic rupture of the vesicles [41]. It therefore appears unlikely that during a killer cell attack, GrB is primarily routed to acidic compartments, as would be expected upon classical receptor-mediated endocytosis and has also been suggested for heparan sulphate-bound GrB from *E. coli* [29]. Moreover, the membrane-disrupting potential of perforin is strongly decreased at pH values below 7 [46], making it questionable that its role is to release GrB from acidic compartments such as late endosomes or lysosomes. Target structures for GrB on the cell surface might therefore also control routing of the protease to an intracellular compartment different from lysosomes.

Retargeting of GrB to alternative cell surface receptors, however, can change its mode of uptake and intracellular routing. In a previous report by Liu et al. [47], it was found that perforin significantly enhanced the cytotoxic activity of GrB, but only slightly increased the cytotoxicity of a bacterially expressed GrB–scFvMel antibody fusion protein which internalizes via binding to the melanoma-specific antigen gp270. Experiments from our own laboratory show that retargeting of GrB by fusion to the EGFR ligand TGF α and expression of the chimaeric protein in yeast now resulted in effective killing of EGFR-expressing cells in the presence of chloroquine [43]. This indicates that redirection of GrB to a pathway involving classical receptor-mediated endocytosis can deliver GrB to an acidic environment sensitive to chloroquine.

To further characterize the activity of recombinant GrB, we also analysed its effect on the growth of different human tumour cell lines in the absence of perforin or a functional equivalent. Interestingly, despite its inability to enter the cytosol, we observed a significant antiproliferative effect of the protein after extended incubation times. Upon treatment with GrB, cells underwent dramatic changes in overall morphology which were dependent on enzymatic activity, leading to partial loss of contact to the culture support and cessation of proliferation. However, during the first 24–48 h of incubation, typical signs of apoptotic morphology could not be detected, indicating that this effect is distinct from rapid induction of apoptosis by the concerted action of GrB and perforin. In contrast, our results suggest that GrB is also able to attack cells extracellularly, most likely by cleaving components of the extracellular matrix, which can then result in detachment of the cells from the culture dish. In agreement with this, most of the cells were able to recover and regain their normal morphology upon removal of GrB from the culture medium.

GrB was previously reported to degrade the chondroitin sulphate proteoglycan aggrecan [48,49]. Aggrecan is a major component of the extracellular matrix synthesized by chondrocytes, and cleavage of aggrecan by GrB has been proposed to contribute to cartilage destruction in rheumatoid arthritis [48,49]. When we analysed expression of aggrecan mRNA in tumour cell lines by RT-PCR, we indeed found aggrecan transcripts in HeLa, but not in A431 and SKBR3 cells (results not shown). Nevertheless, upon treatment with GrB, these three tumour cell lines displayed very similar morphological changes. Consequently, while aggrecan could be a possible target for GrB in the extracellular matrix of HeLa cells, as recently suggested, other extracellular matrix proteins might be susceptible to cleavage by the protease [50]. Whether such attack of extracellular matrix by GrB serves a physiological function or results from 'cleavage by chance' due to the presence of cryptic GrB recognition sites in the target proteins remains to be investigated.

Taken together, our results show that recombinant human GrB expressed in the yeast *P. pastoris* closely resembles endogenous GrB from mammalian cells in its behaviour towards target cells. In addition, using the recombinant molecule, we found an extracellular activity of GrB, which might also be relevant for certain physiological or pathophysiological conditions. The relative ease of production of larger quantities of GrB in recombinant form will now allow more detailed mutational analysis of the enzyme, and could help to identify sequence elements and post-translational modifications that might be critical for target cell binding, entry and intracellular routing.

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