Targeted induction of apoptosis by chimeric granzyme B fusion proteins carrying antibody and growth factor domains for cell recognition

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Abstract

The serine protease granzyme B (GrB) of cytotoxic lymphocytes efficiently induces apoptosis by direct activation of caspases and cleavage of central caspase substrates. We employed human GrB as an effector function in chimeric fusion proteins that also contain the EGFR ligand TGF α or an ErbB2-specific single-chain antibody fragment (scFv) for selective targeting to tumor cells. GrB-TGF α (GrB-T) and GrB-scFv(FRP5) (GrB-5) molecules expressed in the yeast *Pichia pastoris* were bifunctional, cleaving synthetic and natural GrB substrates, and binding specifically to cells expressing EGFR or ErbB2 target receptors. Upon cell binding the chimeric molecules were internalized into intracellular vesicles, but could be released into the cytosol by the endosomolytic reagent chloroquine. Treatment with picomolar to nanomolar concentrations of GrB-5 and GrB-T resulted in selective and rapid tumor cell killing, accompanied by clear signs of apoptosis such as chromatin condensation, membrane blebbing, formation of apoptotic bodies and activation of endogenous initiator and effector caspases.

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Abbreviations: CTL, cytotoxic T-lymphocyte; EGFR, epidermal growth factor receptor; GrB, granzyme B; mAb, monoclonal antibody; NK, natural killer; scFv, single-chain antibody fragment

Introduction

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are highly specialized effectors of the immune system, which are able to recognize and eliminate virus-infected or transformed cells. Upon contact with their targets, they secrete the contents of cytotoxic granules, which efficiently induce apoptotic cell death within minutes to a few hours.^{1,2} Together with the pore-forming protein perforin, the serine-protease granzyme B (GrB) was recognized as the most

potent effector molecule present in these cytotoxic granules.³ Thereby perforin is required to allow GrB access to substrate proteins inside target cells by mediating its release from intracellular vesicles, where the protease accumulates after uptake.⁴ GrB is the only serine protease known to date that shares the substrate specificity of caspases, and cleaves its substrates after specific aspartate residues.⁵ Once inside the cytosol of a target cell, GrB induces apoptosis by mimicking caspase activity and activating caspase-3 and other caspases.^{6–10} In addition, GrB was found to cleave central caspase substrates,¹¹ such as the BH3-only protein Bid^{12,13} and the inhibitor of the caspase-activated DNase (ICAD).¹⁴

Here, we have employed recombinant human GrB to selectively eliminate tumor cells that express the epidermal growth factor receptor (EGFR) or the closely related ErbB2 (HER2) receptor tyrosine kinase. EGFR and ErbB2 over-expression have been found in many tumors of epithelial origin, and have been shown to contribute to cellular transformation.^{15,16} These growth factor receptors are accessible from the extracellular space, making them attractive targets for monoclonal antibodies (mAbs),¹⁷ and antibody-derived reagents such as immunotoxins.¹⁸ For targeting to EGFR and ErbB2, we fused the proapoptotic protease GrB to the ErbB2-specific single-chain antibody fragment (scFv) scFv(FRP5),¹⁹ or to transforming growth factor α (TGF α)²⁰ as a natural EGFR ligand. In previous attempts to utilize GrB as an apoptosis-inducing effector, recombinant derivatives were expressed in *Escherichia coli* as inactive precursors, requiring *in vitro* cleavage after purification from bacterial lysates to yield enzymatically active GrB molecules.^{21–23} This necessity for *in vitro* activation might explain the more moderate cytotoxic activities described for these molecules. We have followed a different strategy expressing the chimeric GrB molecules as secreted proteins in the yeast *Pichia pastoris* without the need for refolding or *in vitro* activation. GrB-scFv(FRP5) (GrB-5) and GrB-TGF α (GrB-T) molecules purified from yeast culture supernatants retained enzymatic GrB activity, and in the presence of an endosomolytic reagent displayed high and selective cytotoxicity towards tumor cells expressing the respective target receptors.

Results

Chimeric GrB fusion proteins are expressed in *P. pastoris* at high yields

cDNA encoding mature human GrB (amino-acid residues 21–247) was fused via a (Gly₄Ser)₄-His₆ linker to sequences encoding the ErbB2-specific scFv antibody fragment scFv(FRP5), or the natural EGFR ligand TGF α in the yeast expression vector pPIC9 (Figure 1a). In the resulting expression plasmids pPIC9-GrB, pPIC9-GrB-5 and pPIC9-GrB-T, expression of mature GrB and GrB-scFv(FRP5) (GrB-5) and

GrB-TGF α (GrB-T) fusion proteins carrying C-terminal myc and His₆ tags is controlled by the methanol-inducible alcohol oxidase AOX1 promoter, and the gene products are secreted into the culture supernatant via the α -factor signal sequence from *Saccharomyces cerevisiae*. During secretion, this signal peptide is cleaved off, producing the N-terminus of mature human GrB.

Recombinant GrB derivatives were purified from culture supernatants by single-step Ni²⁺-affinity chromatography to 80–90% purity as judged by SDS-PAGE analysis (Figure 1b, lanes 1–3). Yields were 1–2 mg of purified proteins per liter of culture. Identity of the proteins was confirmed by immunoblot analysis with myc-tag-specific mAb 9E10 (Figure 1b, lanes 4–6). The proteins migrated with an apparent molecular mass significantly higher than calculated from the amino-acid sequences (38 versus 27 kDa for GrB; 40 versus 36 kDa for GrB-T; 70 versus 58 kDa for GrB-5). This was due to glycosylation as demonstrated by treatment of the recombinant proteins with N-glycosidase F (data not shown). As controls, inactive GrB_{S183A}, GrB_{S183A-5} and GrB_{S183A-T} mutants, which contain an alanine instead of the active site serine 183,²⁴ were constructed and produced in *P. pastoris* as described above (data not shown).

Recombinant GrB derivatives cleave synthetic and physiological GrB substrates

Colorimetric assays were performed using the GrB-specific peptide substrate Ac-IETD-pNA. GrB-5 and GrB-T fusion proteins like unmodified GrB cleaved this substrate with

Figure 1 Expression of granzyme B fusion proteins in the yeast *P. pastoris*. cDNA encoding mature human GrB (amino acids 21–247) was genetically fused to the α -factor signal peptide (α) of *S. cerevisiae* in the expression vector pPIC9. As cell recognition domains, sequences encoding the ErbB2-specific antibody fragment scFv(FRP5) or the EGFR ligand TGF α were fused to the 3' end of GrB via a (Gly₄Ser)₄ linker (L) and a polyhistidine tag (H) (a). A myc-epitope (M) and a second polyhistidine tag (H) are included at the C-termini of the gene products. During export of the GrB-scFv(FRP5) (GrB-5) and GrB-TGF α (GrB-T) fusion proteins, the signal peptide is removed by the *P. pastoris* protease KEX2, producing the N-terminus of mature GrB. Chimeric GrB-T (b, lanes 2, 5) and GrB-5 (b, lanes 3, 6) proteins were purified from yeast culture supernatants by Ni²⁺-affinity chromatography. Eluate fractions were analyzed by SDS-PAGE and Coomassie staining (b, lanes 1–3), and immunoblotting with myc-tag-specific mAb 9E10 (b, lanes 4–6). Unmodified recombinant GrB produced in *P. pastoris* following a similar procedure (b, lanes 1, 4) is shown for comparison

comparable activity in a concentration-dependent manner (Figure 2a). High concentrations of the inactive mutants GrB_{S183A-5} and GrB_{S183A-T} were unable to process the substrate. In addition, as a physiological GrB substrate recombinant human procaspase-3 was incubated with GrB derivatives and cleavage reactions were analyzed for production of p12 caspase-3 subunit by SDS-PAGE and immunoblotting (Figure 2b). Thereby incubation with GrB, GrB-5 and GrB-T, but not inactive GrB_{S183A-5} and GrB_{S183A-T} proteins resulted in complete processing and disappearance of procaspase-3, and detection of increased amounts of the p12 subunit.

Chimeric GrB-5 and GrB-T proteins bind specifically to tumor cells

Binding of GrB-5 and GrB-T to ErbB2 and EGFR on the surface of tumor cells was investigated by FACS analysis with human MDA-MB453 cells which express high levels of ErbB2 but no detectable EGFR, and MDA-MB468 cells which highly overexpress EGFR but have no detectable ErbB2.²⁵ After incubation with purified fusion proteins followed by myc-tag-specific mAb 9E10 and phycoerythrin-conjugated secondary antibody, strong binding of GrB-5 to MDA-MB453 cells, and of GrB-T to MDA-MB468 cells was detected, indicating functionality of the fusion proteins' cell recognition domains (Figure 2c). Only very weak binding of the fusion proteins to the respective control cells was observed, which possibly reflects interaction of the GrB domain with structures on the cell surface^{26,27} (and unpublished data). Specific binding to cells expressing ErbB2 or EGFR was also found for the mutant proteins GrB_{S183A-5} and GrB_{S183A-T}, but not for unmodified GrB (data not shown).

Next cell killing activity of GrB-5 and GrB-T towards tumor cells expressing the respective target receptors was analyzed. To test GrB-T, MDA-MB468 and MDA-MB453 cells were used. Cytotoxic activity of GrB-5 was evaluated using murine Renca-lacZ/ErbB2 cells expressing human ErbB2 and ErbB2-negative Renca-lacZ cells as a control.²⁸ The cells were incubated with increasing concentrations of the fusion proteins for 14 h, before cell viability was determined in MTT assays. At concentrations up to 1 μ g/ml, the chimeric GrB proteins had no effect on the survival of tumor cells irrespective of the presence of their cognate target receptors on the cell surface (Figure 2d). This indicates that specific cell recognition and enzymatic activity alone are insufficient to facilitate GrB-induced cell death.

GrB fusion proteins are internalized into intracellular vesicles

We investigated internalization of the fusion proteins in human A431 epidermoid cancer cells that express both, EGFR and ErbB2 (Figure 3a). After incubation for 2.5 h, in immunofluorescence analysis with a GrB-specific antibody the proteins were no longer detected at the cell surface, indicating efficient uptake. Instead punctuate staining within the cytoplasm was found for GrB-5 and GrB-T, suggesting localization inside vesicular structures such as endosomes. Diffuse intracellular

staining indicative of cytosolic localization was not observed. To investigate whether GrB fusion proteins could induce cell death if delivered directly to the cytosol, we performed microinjection experiments with GrB-5 using Renca-lacZ

cells, which are ErbB2 negative and therefore unable to internalize the protein via receptor-mediated endocytosis. After 4 h, the majority of cells that were injected with GrB-5 displayed apoptotic morphology with membrane blebbing, formation of apoptotic bodies and nuclear DNA condensation (Figure 3b). After 20 h, 68% of cells injected with GrB-5 had disintegrated into small cell fragments and could no longer be identified on the culture slides (Figure 3c). In contrast, injection of cells with the mutant protein GrB_{S183A}-5 similar to injection with PBS had no effect (4% of apoptotic cells after 20 h; Figure 3c), confirming that induction of apoptosis was dependent on the specific activity of GrB.

GrB-5 and GrB-T selectively kill tumor cells

The basic reagent chloroquine accumulates in acidic compartments such as late endosomes and lysosomes leading to osmotic rupture of the vesicles.²⁹ We tested whether chloroquine could release internalized GrB fusion proteins from intracellular vesicles, thereby allowing access to cytosolic GrB substrates and induction of apoptosis. As described above, EGFR expressing MDA-MB468 and ErbB2-positive Renca-lacZ/ErbB2 cells were treated with increasing concentrations of GrB-T or GrB-5 in the presence of 50 μ M chloroquine. MDA-MB453 and Renca-lacZ cells served as controls. After 14 h, survival of cells was determined in MTT assays (Figure 4a). In contrast to treatment in the absence of the endosomolytic reagent, incubation of the cells with GrB-T and GrB-5 in the presence of chloroquine resulted in potent and concentration-dependent cytotoxicity towards tumor cells expressing the respective target receptors (IC₅₀ of 10 ng/ml, corresponding to 0.25 nM for GrB-T on MDA-MB468 cells; IC₅₀ of 20 ng/ml, corresponding to 0.29 nM for GrB-5 on Renca-lacZ/ErbB2 cells). MDA-MB453 control cells displayed a slight reduction in the number of viable cells at the highest GrB-T concentration of 1 μ g/ml, which might be due to the expression of small amounts of EGFR below the detection limit of EGFR

Figure 2 Chimeric GrB-T and GrB-5 fusion proteins are enzymatically active and bind to the surface of EGFR and ErbB2 overexpressing tumor cells. GrB-specific peptide substrate Ac-IETD-pNA was incubated with the indicated concentrations of purified GrB-T or GrB-5 fusion proteins for 1 h (a). Unmodified GrB and fusion proteins containing the enzymatically inactive GrB mutant GrB_{S183A} were included as controls. Cleavage of substrate was determined by measuring the absorbance at 405 nm. As a physiological GrB substrate, bacterially expressed procaspase-3 was incubated with the indicated concentrations of purified GrB, or GrB-5 or GrB-T fusion proteins for 14 h (b). Enzymatically inactive GrB_{S183A}-5 and GrB_{S183A}-T proteins were included as controls. Samples were analyzed by SDS-PAGE and immunoblotting. Procaspase-3 and the p12 cleavage product were detected with mAb SWA11 recognizing an epitope tag included at the C-terminus of recombinant procaspase-3. Specific binding of GrB-5 and GrB-T proteins to the surface of tumor cells was investigated by FACS analysis using human breast carcinoma cells which express high ErbB2 (MDA-MB453), or high EGFR levels (MDA-MB468) (c). Bound proteins were detected with myc-tag-specific mAb 9E10 followed by phycoerythrin-conjugated secondary antibody. Cytotoxic activity of GrB-T and GrB-5 fusion proteins (d). EGFR overexpressing MDA-MB468 and MDA-MB453 control cells (left panel), or murine Renca-lacZ/ErbB2 renal carcinoma cells stably transfected with human c-erbB2 cDNA and ErbB2-negative Renca-lacZ control cells (right panel) were incubated for 14 h with the indicated concentrations of GrB-T (left panel) or GrB-5 (right panel). Then the relative number of viable cells in comparison to PBS treated controls was determined using an MTT metabolism assay

Figure 3 GrB-5 and GrB-T proteins are taken up into intracellular vesicles. Human A431 epidermoid cancer cells expressing both target receptors EGFR and ErbB2, were grown on coverslips, treated with GrB-5 or GrB-T for 1 h, washed and incubated for another 1.5 h at 37°C to allow intracellular uptake (a). Cells were fixed, consecutively incubated with anti-GrB and anti-mouse Alexa Fluor 488 antibodies, and analyzed by confocal laser scanning microscopy. Nucleic acids were stained with propidium iodide. Merged images are shown. Cytosolic delivery of GrB-5 protein induces apoptotic cellular morphology. Purified GrB-5 was microinjected into the cytosol of Renca-lacZ cells together with GST-eGFP fusion protein for detection (b). Control cells were injected with GST-eGFP and enzymatically inactive GrB_{S183A}-5 or PBS. After 4 h, injected cells were identified by fluorescence microscopy (middle panels), and cellular morphology was analyzed by phase contrast microscopy (upper panels). Nuclear morphology was analyzed by staining of cellular DNA with Hoechst 33342 dye (lower panels). Identical fields are shown. Apoptotic cells are identified by arrows. For quantification, the number of intact eGFP-containing cells was determined 20 h after microinjection (c). Total numbers of injected cells from three independent experiments are indicated

immunoblots.²⁵ ErbB2-negative Renca-lacZ cells remained unaffected at the GrB-5 concentrations applied.

To confirm that even under conditions of effective endosome release cell killing activity of the GrB fusion proteins was strictly dependent on specific recognition of the ErbB2 and EGFR target receptors, Renca-lacZ/ErbB2 cells were incubated with GrB-5 and chloroquine in the presence of an excess of parental ErbB2-specific mAb FRP5,³⁰ or a recombinant ErbB2 protein fragment encompassing the FRP5-binding epitope.³¹ Addition of the competitors markedly reduced GrB-5-induced cell killing (Figure 4b). Likewise, competition of binding of GrB-T to EGFR by the antagonistic EGFR-specific mAb C225¹⁷ resulted in a drastic reduction of its cytotoxicity towards MDA-MB468 cells. In addition to specific cell recognition, enzymatic GrB activity was required to induce cell killing (Figure 4c). The mutant proteins GrB_{S183A}-5 and GrB_{S183A}-T were unable to kill Renca-lacZ/ErbB2 or MDA-MB468 cells in the presence of chloroquine. Also enzymatically active GrB lacking a cell targeting domain could not induce cell death.

GrB fusion proteins induce apoptotic morphology

Cytotoxic activity of GrB-T and GrB-5 was also tested on A431 tumor cells that expresses both target receptors. In the

presence of chloroquine, treatment of the cells for 14 h with GrB-T or GrB-5, but not unmodified GrB resulted in concentration-dependent cell killing quantified in MTT assays (Figure 5a). When cellular morphology was analyzed, A431 cells treated with GrB-T or GrB-5 displayed typical signs of apoptosis indicated by membrane blebbing and formation of apoptotic bodies (Figure 5b). In addition, chromatin condensation and fragmentation of the nuclei comparable to the effects induced by staurosporine could be detected after incubation of the cells with enzymatically active fusion proteins, but not upon treatment with GrB_{S183A}-T or GrB_{S183A}-5 (Figure 5c). Similar results were found with GrB-T for MDA-MB468 but not for MDA-MB453 cells, and with GrB-5 for Renca-lacZ/ErbB2 but not for Renca-lacZ cells. Signs of apoptosis could be observed as early as 2 h after addition of fusion proteins, which corresponds well with the kinetics described for the GrB/perforin system (data not shown).

GrB-5 and GrB-T activate endogenous caspases

We analyzed activation of endogenous caspase-3 in cytoplasmic extracts of A431 cells treated with GrB-5 or GrB-T, using the colorimetric caspase-3 peptide substrate Ac-DEVD-pNA. In addition, we investigated activation of

Figure 4 Addition of an endosomolytic reagent facilitates specific cell killing activity of GrB fusion proteins (a). EGFR overexpressing MDA-MB468 and MDA-MB453 control cells (left panel), or ErbB2 expressing Renca-lacZ/ErbB2 and ErbB2-negative Renca-lacZ cells (right panel) were incubated for 14 h with the indicated concentrations of GrB-T (left panel) or GrB-5 (right panel) in the presence of the endosomolytic reagent chloroquine. The relative number of viable cells in comparison to controls only treated with chloroquine was determined using an MTT metabolism assay. Cell killing is dependent on specific binding to target receptors and enzymatic GrB activity (b, c). Renca-lacZ/ErbB2 cells were incubated with 75 ng/ml of GrB-5 and 50 μM chloroquine in the absence of competitor, or in the presence of parental ErbB2-specific mAb FRP5 or recombinant ErbB2 protein (b, open bars), or with 100 ng/ml of GrB-5, enzymatically inactive GrB_{S183A-5}, or an equimolar concentration of unmodified GrB in the presence of 50 μM chloroquine (c, open bars). In the case of GrB-T, MDA-MB468 cells were treated with 100 ng/ml of the fusion protein and 50 μM chloroquine in the absence or presence of the antagonistic EGFR-specific mAb C225 (b, filled bars), or with 100 ng/ml of GrB-T, enzymatically inactive GrB_{S183A-T}, or an equimolar concentration of unmodified GrB in the presence of 50 μM chloroquine (c, filled bars). After 14 h, the relative number of viable cells was determined in MTT assays

caspases-8 and -9 acting upstream of caspase-3, with the fluorimetric peptide substrates Ac-IETD-AFC (caspase-8) and Ac-LEHD-AFC (caspase-9). All three caspases were activated following treatment of A431 cells with GrB-5 or GrB-T in the presence of chloroquine (Figure 6a). In the absence of the endosomolytic reagent, only background activities were found. Caspase activation was dependent on both, specific cell recognition and release of GrB fusion proteins from the endosomal compartment, since independent from the presence or absence of chloroquine, GrB lacking a cell targeting domain did not induce activation of the caspases. As expected, addition of the broad-range caspase inhibitor zVAD-fmk to the cells completely blocked caspase activities.

Chimeric GrB proteins cleave endogenous procaspase-3

Processing of endogenous caspase-3 in A431 cells treated with GrB-5 or GrB-T was analyzed by immunoblotting with a

mAb recognizing an epitope at the N-terminus of caspase-3. In the absence of zVAD-fmk, both p20 and p17 caspase-3 fragments were detected indicating cleavage and activation of caspase-3 (Figure 6b). In the presence of zVAD-fmk, caspase-3 p20 subunit was still produced, but p17 was absent. This is in agreement with a mechanism relying on GrB activity for initial cleavage of caspase-3 to generate p20, followed by autocatalytic processing to yield p17.⁷ Only this latter step could be blocked by the caspase inhibitor zVAD-fmk. Confirming the results obtained in the enzymatic assays, specific cell recognition and endosome release were required to allow GrB access to caspase-3. Treatment of the cells with unmodified GrB, or retargeted GrB in the absence of chloroquine did not result in caspase-3 cleavage.

To investigate the effect of caspase inhibition on cell killing by retargeted GrB, A431 cells were treated with GrB-5 and chloroquine in the presence or absence of zVAD-fmk for 14 h, before cell viability was determined in an MTT assay (Figure 6c). Importantly, while inhibition of caspases reduced

Figure 5 Cell killing by GrB-T and GrB-5 proteins is associated with signs of target cell apoptosis. A431 epidermoid cancer cells expressing EGFR and ErbB2 were incubated for 14 h with the indicated concentrations of GrB-5 or GrB-T in the presence of 100 μ M chloroquine. GrB lacking a targeting domain was included as a control. Then the relative number of viable cells in comparison to controls only treated with chloroquine was determined using an MTT metabolization assay (**a**). Morphology of A431 cells after incubation for 14 h with 0.5 μ g/ml of GrB-T, 1 μ g/ml of GrB-5 or 0.5 μ g/ml of GrB in the presence of 100 μ M chloroquine (**b**). Cells treated with PBS and chloroquine are shown as a control. Nuclear morphology of A431 cells was investigated after treating the cells for 14 h with 0.5 μ g/ml of GrB-T or enzymatically inactive GrBs183A-T, or 1 μ g/ml of GrB-5 or enzymatically inactive GrBs183A-5 in the presence of 100 μ M chloroquine (**c**). Control cells were incubated with PBS and chloroquine, or apoptosis-inducing staurosporine. Nuclei were stained with Hoechst 33342 dye before analysis of cells under a fluorescence microscope

cell killing by GrB-5, significant cytotoxic activity was still observed indicating a contribution of caspase-independent mechanisms to GrB-induced cell death.

Discussion

Recombinant antibody or growth factor toxins combine specific recognition of tumor cells with selective delivery of a potent cytotoxic effector molecule.^{18,32} However, due to the bacterial or plant origin of their effector domain, patients treated with such molecules can develop neutralizing antitoxin antibodies, which precludes prolonged or repeated treatment cycles that might be necessary to achieve long-lasting

therapeutic effects. Consequently, attempts are being made to reduce immunogenicity and employ cytotoxic effector functions of human origin for the construction of immunotoxin-like molecules. Here, we have derived novel chimeric proteins which carry human GrB as an apoptosis-inducing effector domain, and performed a detailed analysis of their *in vitro* activities. Specific targeting of GrB to tumor cells expressing high levels of the cellular proto-oncogenes EGFR or ErbB2 was achieved by fusion to the EGFR ligand TGF α or an ErbB2-specific scFv antibody fragment, and resulted in selective and efficient cell killing.

Different strategies have been proposed to exploit the process of cellular self-destruction for the ordered elimination of tumor cells. One approach is based on the fusion of tumor-specific cell-targeting domains to cell-death inducing cytokines such as tumor necrosis factor, CD95L or TRAIL.³³ These cytokines act via death receptors on the tumor cell surface, and are therefore dependent on an intact extrinsic apoptosis pathway in the target cell. Owing to its ability to bypass common apoptosis resistance mechanisms in tumor cells,³⁴ the serine protease GrB is emerging as a promising alternative effector function for immunotoxin-like molecules. GrB acts more downstream in the apoptosis cascade, and similar to recombinant toxins, functions via an enzymatic activity. It shares the substrate specificity of caspases and cleaves both, cellular caspases and various caspase target proteins.^{6–11} Like an effector caspase, GrB activates the apoptosis machinery directly and at different levels. Therefore, cell death can be induced even if one pathway is blocked.^{1,2} GrB has previously been employed in a fusion protein containing VEGF to target endothelial cells,²¹ and in chimeric proteins or immunoconjugates carrying antibody fragments for recognition of cancer cells via binding to a melanoma antigen or Lewis Y.^{22,23} These recombinant GrB proteins were expressed in *E. coli* as inactive precursors, requiring *in vitro* cleavage after purification from bacterial lysates, and in one case also refolding to yield enzymatically active molecules. In another approach, the problem to produce a functional tumor-specific GrB fusion protein was solved by coincubating tumor cells with engineered T cells releasing such a molecule.³⁵

We have followed a different strategy expressing the ErbB2-specific GrB-5 and EGFR-specific GrB-T molecules as secreted proteins in the yeast *P. pastoris*, thereby bypassing the need for refolding or *in vitro* activation, and resulting in large amounts of active recombinant proteins after a single purification step. Attachment of the C-terminal antibody and growth factor domains did not affect enzymatic activity of GrB. The chimeric GrB-5 and GrB-T molecules were able to cleave a synthetic GrB substrate and recombinant procaspase-3 as a natural target protein in a manner indistinguishable from unmodified GrB. Both fusion proteins were bifunctional, in addition to enzymatic activity also displaying specific binding to cells expressing high levels of the ErbB2 or EGFR target receptors. When GrB-5 was directly injected into the cytosol of tumor cells, we observed induction of apoptosis, which was strictly dependent on enzymatic GrB activity. GrB-5 and GrB-T added from the outside, however, were unable to kill ErbB2 or EGFR expressing tumor cells unless an endosomalolytic activity was present.

Figure 6 Treatment with GrB-5 and GrB-T results in activation of endogenous caspases. A431 cells expressing EGFR and ErbB2 target receptors were treated for 14 h with 1 μ g/ml of GrB-5, 0.5 μ g/ml of GrB-T or 0.5 μ g/ml of GrB in the presence or absence of 100 μ M chloroquine and 100 μ M of the caspase inhibitor zVAD-fmk as indicated. Activation of endogenous caspases-3, -8 and -9 in response to treatment was investigated by addition of the colorimetric caspase-3 peptide substrate Ac-DEVD-pNA, or the fluorimetric peptide substrates Ac-IETD-AFC (caspase-8) or Ac-LEHD-AFC (caspase-9) to whole cell extracts (a). Cleavage of substrates was quantified by measuring the absorbance at 405 nm or fluorimetric analysis. To detect cleavage of caspase-3, cell extracts were analyzed by SDS-PAGE and immunoblotting with a caspase-3 specific antibody recognizing an epitope within the p17 subunit (b). To investigate the effects of caspase inhibition on GrB-5 induced cell killing, A431 cells were treated with 100 ng/ml of GrB-5 and 100 μ M chloroquine in the presence or absence of 100 μ M zVAD-fmk as indicated (c). After 14 h, the relative number of viable cells was determined using an MTT assay

Wild-type GrB is able to internalize into target cells, but localizes in vesicular structures inside the cytoplasm where it remains inactive.^{4,36} Induction of apoptosis is only observed after the addition of perforin, which is thought to liberate GrB from these vesicles, allowing access of the protease to the cytosol. Also, GrB-5 and GrB-T were found to accumulate in intracellular vesicles without affecting cell survival. However, when the endosomolytic reagent chloroquine was present, GrB-5 and GrB-T were able to kill tumor cells at low concentrations. Importantly, this cell killing activity still required interaction with ErbB2 or EGFR target receptors and enzymatic activity of the GrB domain. Chloroquine has successfully been employed as an endosome release agent in animal models,³⁷ and is being used as a drug for the treatment of malaria and other diseases in humans.³⁸ Therefore, a requirement for cotreatment with chloroquine might not severely limit therapeutic applicability of GrB fusion proteins. 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.²⁹ Therefore, retargeting of GrB to ErbB2 or EGFR must have resulted in routing to an acidic environment sensitive to chloroquine, as expected upon uptake of the fusion

proteins via classical receptor-mediated endocytosis, but not typical for wild-type GrB. The membrane-disrupting potential of perforin is strongly decreased at pH values below 7,³⁹ making it unlikely that its role is to release GrB from late endosomes or lysosomes. Furthermore, while unmodified GrB expressed in yeast was also found in intracellular vesicles when added to cells at high concentrations (unpublished data), these vesicles did not belong to a chloroquine-sensitive, acidic compartment. The endosomolytic reagent was unable to release unmodified GrB into the cytosol indicated by a lack of cytotoxic activity.

Selective cytotoxic activity was also described for bacterially expressed GrB fusion proteins targeting cell surface antigens other than ErbB2 or EGFR.^{21–23} Thereby no endosomolytic activity was provided, suggesting different intracellular routing of chimeric GrB molecules depending on the type of target receptor. However, also in these cases addition of an endosomolytic reagent might have been beneficial, indicated by the slower kinetics of apoptosis induction and cell killing, and the requirement for 10–300 times higher protein concentrations to achieve effects similar to GrB-5 and GrB-T.^{21–23} The requirement for effective endosome release was also shown for a chimeric GrB protein

which similar to GrB-5 targets ErbB2, but in an approach for adoptive cell therapy was expressed in gene modified T cells. There the translocation domain of a bacterial toxin was included in the molecule to achieve cytoplasmic delivery of an active GrB fragment in target cells.³⁵ In the presence of chloroquine, GrB-5 and GrB-T were able to specifically kill target cells with IC₅₀ values in the picomolar to nanomolar range, whereas nontarget cells were not affected at considerably higher concentrations. These cytotoxic activities of the chimeric GrB derivatives are comparable to those of *Pseudomonas* exotoxin A fusion proteins which employ the same cell targeting domains.^{20,28} Importantly, while such immunotoxins require incubation times of 40 h for maximum cell killing, high cytotoxic activity of the GrB fusion proteins was found after 14 h of incubation, and apoptotic morphology of target cells was observed as early as 2 h after addition of chimeric GrB molecules (data not shown), following kinetics similar to the GrB/perforin system.

A more detailed analysis of the events following treatment with GrB-5 and GrB-T was performed in A431 cells which carry both target receptors. Upon treatment of the cells with chimeric GrB proteins and chloroquine we observed hallmarks of apoptosis such as activation of initiator and effector caspases, chromatin condensation and formation of apoptotic bodies. Both, p20 and p17 caspase-3 fragments were generated, demonstrating cleavage and activation of endogenous caspase-3. In the presence of the broad-range caspase inhibitor zVAD-fmk only p20 subunit was produced, which is in agreement with a mechanism that relies on GrB activity for initial cleavage of caspase-3 to generate p20, followed by autocatalytic processing to yield p17.⁷ Importantly, even in the presence of the caspase inhibitor A431 cells were killed by GrB-5, albeit to a lesser extent. This ability of GrB fusion proteins to also initiate caspase-independent cell death pathways, possibly through cleavage of Bid or ICAD,^{13,14} could be relevant for elimination of tumor cells with a block in caspase-dependent apoptosis.

Our data show that bifunctional, immunotoxin-like proteins employing human GrB as an effector domain can be readily expressed at high yields in the yeast *P. pastoris*. Upon specific binding to ErbB2 or EGFR on the surface of tumor cells, the chimeric molecules are internalized and can be released into the cytosol by an endosomolytic reagent, resulting in rapid and efficient cell killing. These reagents might be useful to study GrB-induced apoptosis without the requirement for perforin, but also hold promise for further development as targeted therapeutics of human origin.

Materials and Methods

Construction of GrB fusion proteins

A cDNA fragment encoding mature human GrB was amplified by PCR with the oligonucleotides 5'-GrB 5'-ATTCTCGAGAAAAGATCATCGGGG ACATGAG-3' and 3'-GrB 5'-TTTGCTAGCCCGTAGCGTTTCATGG-3' from full-length GrB cDNA. The PCR product was fused to a sequence encoding a myc and a His₆ tag, and subcloned into the yeast vector pPIC9 (Invitrogen, Karlsruhe, Germany), resulting in plasmid pPIC9-GrB. Likewise, fusion genes encoding the GrB domain followed by a (Gly₄Ser)₄ linker, an internal His₆ tag, the ErbB2-specific single-chain antibody

fragment scFv(FRP5),¹⁹ or the EGFR ligand TGF α ,²⁰ and C-terminal myc and His₆ tags were assembled in pPIC9, resulting in plasmids pPIC9-GrB-5 and pPIC9-GrB-T. Enzymatically inactive GrB mutants were derived from constructs encoding active enzyme by site directed mutagenesis via PCR using the oligonucleotide primers S183A-s 5'-CCTTTAAGGGG ACGCTGGAGGCCCTCTTG-3' and S183A-as 5'-CAAGAGGGCCTCCA GCGTCCCCCTTAAAGG-3'.

Expression and purification of recombinant proteins

P. pastoris GS115 cells (Invitrogen) were transformed with pPIC9 plasmids via electroporation, and positive clones were selected according to the manufacturer's instructions. Single colonies were grown in 100 ml buffered (pH 8) glycerol complex medium for 2 days at 30°C. For induction of expression, cultures were diluted 10-fold in methanol-containing buffered (pH 8) methanol complex medium to an OD₆₀₀ of one, and cultured for another 4 days at 25°C. Yeast cells were removed by centrifugation, supernatants were adjusted to pH 8, and applied to a Ni-NTA superflow column (Qiagen, Hilden, Germany). Specifically bound proteins were eluted with 250 mM imidazole, 1 M NaCl in PBS, pH 8. Protein fractions containing GrB proteins were identified by SDS-PAGE and immunoblotting with myc-tag-specific antibody 9E10, or GrB-specific mAb 2C5 (Santa Cruz Biotechnology, Heidelberg, Germany), pooled, concentrated, dialyzed against PBS and stored until use at -80°C.

GrB activity assays

Chimeric GrB molecules were incubated for 1 h at 37°C in 96-well plates with 200 μ M synthetic peptide substrate Ac-IETD-pNA (Alexis, Grünberg, Germany) and reaction buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) in a total volume of 100 μ l per sample. Cleavage of substrate was quantified by measuring absorbance at 405 nm with a microplate reader.

Analysis of binding and uptake of chimeric GrB proteins

MDA-MB468 and MDA-MB453 human breast carcinoma cells, A431 epidermoid cancer cells (ATCC), and murine Renca-lacZ and Renca-lacZ/ ErbB2 cells were cultured as described.^{20,28} For FACS analysis, 3×10^5 trypsinized cells were incubated with 1.5 μ g of GrB-T, GrB_{S183A}-T, GrB-5 or GrB_{S183A}-5 for 1 h at 4°C. Bound fusion proteins were detected upon subsequent incubation of the cells with 1.5 μ g of myc-tag-specific antibody 9E10 followed by 1 : 50 diluted phycoerythrin-conjugated goat anti-mouse IgG (Jackson Immuno Research, West Grove, PA, USA) in PBS at 4°C using a FACScan flow cytometer (Beckton Dickinson, Heidelberg, Germany). To investigate uptake of GrB-5 and GrB-T, A431 cells were grown on coverslips and treated for 1 h at 37°C with 10 μ g/ml of the proteins in normal growth medium. Cells were washed, and incubated for another 1.5 h in fresh medium. Then cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized in 0.1% Triton X-100 in PBS for 5 min, and incubated with 500 ng/ml GrB-specific mAb 2C5 in PBS, 3% BSA, followed by Alexa Fluor 488 donkey anti-mouse IgG (Molecular Probes, Leiden, The Netherlands) in PBS, 3% BSA. Then samples were analyzed with a Leica TCS SL laser scanning microscope (Leica Mikrosysteme, Bensheim, Germany). Microinjection of GrB fusion proteins into the cytosol of Renca-lacZ cells was carried out following established procedures.⁴⁰

Cell viability assays

Cells were seeded in 96-well plates at a density of 1.5 to 2×10^4 cells/well and incubated for 14 h at 37°C with purified GrB proteins in triplicate in the absence or presence of 50 or 100 μM chloroquine. In some experiments, 100 μM of the broad-range caspase inhibitor zVAD-fmk (Alexis) were included. The relative number of viable cells in comparison to cells grown without GrB proteins was determined in MTT assays as described.²⁰ To compete binding to ErbB2 and EGFR, cells were preincubated for 30 min with ErbB2-specific mAb FRP5³⁰ or recombinant ErbB2 protein,³¹ or the antagonistic anti-EGFR antibody C225,¹⁷ before addition of chimeric GrB molecules and determination of cell viability.

Hoechst staining

A431 cells were seeded in 96-well plates and incubated for 14 h at 37°C with 1 $\mu\text{g}/\text{ml}$ of GrB-5, GrB-T, or enzymatically inactive fusion proteins. As a positive control for apoptotic morphology of nuclei, cells were treated with 1 μM of staurosporine. Adherent cells recovered by trypsinization and detached dead cells from sample supernatants were pelleted by centrifugation, resuspended in 50 μl Hoechst staining solution (5 $\mu\text{g}/\text{ml}$ Hoechst 33342, 1 $\mu\text{g}/\text{ml}$ propidium iodide in PBS), incubated for 10 min at room temperature in the dark, and analyzed using a Nikon Eclipse TE300 fluorescence microscope.

Activation of endogenous caspases

A431 cells were incubated for 14 h at 37°C with GrB fusion proteins in the absence or presence of 100 μM chloroquine. Control samples were in addition treated with 100 μM of zVAD-fmk. Cells were lysed in 200 μl NETN buffer (20 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA, 0.5 % NP-40, 10% glycerol, 1 mM DTT) for 15 min on ice, and cell debris was removed by centrifugation. Amounts of cell extracts corresponding to 50 μg of total proteins were analyzed by SDS-PAGE and immunoblotting with caspase-3 specific mAb E-8 (Santa Cruz Biotechnology) followed by HRP-conjugated secondary antibody and chemiluminescent detection with the ECL kit (Amersham Biosciences, Freiburg, Germany). Enzymatic activity of caspase-3 was determined by incubating 50 μg of total cellular proteins for 14 h at 37°C with 266 μM of the colorimetric caspase-3 substrate Ac-DEVD-pNA (Alexis) followed by measuring the absorbance at 405 nm. Enzymatic activity of caspases-8 and -9 was determined by incubating 20 μg of total cellular proteins for 2 h at 37°C with 37 μM of the fluorimetric caspase-8 substrate Ac-IETD-AFC, or caspase-9 substrate Ac-LEHD-AFC (Alexis), in a total volume of 150 μl in reaction buffer (100 mM Tris pH 8, 10% sucrose, 10 mM DTT, 150 mM NaCl, 0.1% CHAPS). Fluorescence was measured with a Bio-Rad Fluoromark™ microplate reader (Bio-Rad, Munich, Germany).

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