Recombinant Immunoproapoptotic Proteins with Furin Site Can Translocate and Kill HER2-Positive Cancer Cells

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Abstract

We previously reported the selective killing of HER2-positive tumor cells by a class of immunoproapoptotic proteins containing single-chain antibody, translocation domain of Pseudomonas exotoxin A (domain II; PEA II), and constitutively active human apoptotic molecules. In this study, a novel class of antitumor immunoproapoptotic proteins was explored to mediate tumor-specific apoptosis both in vitro and in vivo. Three furin cleavage sequences, including a synthetic polyarginine tract, and two furin cleavable sequences from PEA and diphtheria toxin were respectively used to replace PEA II in the previously constructed immunoproapoptotic protein. When produced and secreted by the genetically modified Jurkat cells, the novel targeted proapoptotic proteins selectively bound to HER2, which is overexpressed on tumor cell surface. Followed by receptor-mediated endocytosis and furin cleavage in the endosome, the recombinant proteins could translocate into the cytosol, leading to irreversible cell death. Moreover, delivery of these proteins by either i.m. plasmid injection or i.v. injection of plasmid-vectored proteins selectively bound to HER2, which is often overexpressed on the tumor cell surface. Three furin cleavage sequences, including a synthetic polyarginine tract, and two furin cleavable sequences from PEA and diphtheria toxin were respectively used to replace PEA II in the previously constructed immunoproapoptotic protein. When produced and secreted by the genetically modified Jurkat cells, the novel targeted proapoptotic proteins selectively bound to HER2, which is overexpressed on tumor cell surface. Followed by receptor-mediated endocytosis and furin cleavage in the endosome, the recombinant proteins could translocate into the cytosol, leading to irreversible cell death. Moreover, delivery of these proteins by either i.m. plasmid injection or i.v. injection of plasmid-vectored proteins selectively bound to HER2, which is often overexpressed on the tumor cell surface.

Introduction

Immunotoxins are hybrid proteins composed of plant or bacterial toxins linked with a targeting molecule, such as a monoclonal antibody, an antibody fragment, a growth factor, or a cytokine (1–4). Immunotoxins derive their toxic potency from the toxin and their specificity from the targeting moiety to which they are attached. Typically, the targeting moiety recognizes and delivers the whole molecule to specific receptors expressed on surface of target cells. After binding to the cell surface, conjugates are internalized via a receptor-mediated endocytosis, followed by cleavage-dependent release of active toxin from endosome to cytosol, wherein the toxin functions and eventually kills the cell.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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We have previously reported the generation of a Pseudomonas exotoxin A (PEA)-based immunotoxin, which contains an anti-HER2 single-chain antibody and PEA40 (ε23sFv-PEA40; ref. 5). PEA40 is a cytotoxin composed of PEA domain II (PEA II), which is responsible for the enabling of translocation, and PEA III, which is for catalytic inactivation of protein synthesis. As some other well-demonstrated antitumor immunotoxins (6–8), ε23sFv-PEA40 was shown to have potent and selective cytotoxicity to tumors in culture and nude mouse models. However, most of the immunotoxin clinical trials faced the same obstacle that immunotoxins would arouse strong humoral immune responses (9–14). In most cases, patients with normal immune systems develop neutralizing antibodies against the plant or bacterial-derived toxins (9, 10). Besides, vascular leakage, nonspecific toxicity to vascular endothelial, liver, or renal cells are other side effects of immunotoxin administration (8, 10–14). In short, clinical trial results suggest that it is the foreign toxin fragments that usually cause humoral immune response and therefore limit the clinical utility of immunotoxins.

To minimize nonspecific toxicity and immunogenicity, investigators have set about to explore immunotoxins containing human-derived effector proteins, such as human RNase (15). Similarly, we previously reported the use of human proapoptotic proteins, such as active caspase-3 (16), caspase-6 (17), granzyme B (18), and truncated apoptosis-inducing factor (19). Those human toxic moieties were substituted for the catalytic domain of PEA40, which was conjugated to an anti-HER2 antibody (5). The resulting partially humanized immunotoxins were composed of an anti-HER2 single-chain antibody (20), a PEA translocation domain, and one of the above-mentioned human proapoptotic proteins. Such immunoproapoptotic proteins retained potency to kill HER2-positive tumor cells both in vitro and in vivo (16–19). To further reduce exogenous fragment, we generated truncated variants of PEA II to identify the minimal sequence required for translocation. Our results showed that consistent with previous reports (21), deletion of either the first or last α-helix of PEA II led to translocation activity comparable with the native PEA translocation domain. Strikingly, we observed that when ε23sFv and granzyme B were coupled with a rather small PEA II-derived sequence containing only six amino acid residues around furin cleavage site, this recombinant protein also showed antitumor activity, although the cytotoxicity comparatively decreased compared with the full-length PEA II conjugate. This unexpected finding indicates a crucial role of furin cleavage in the proapoptotic activity of immunotoxins.

Furin is a cellular endoprotease and has been implicated in proteolytically activating large numbers of secreted proteins. Mature furin localizes principally to the trans-Golgi network, the cell surface, and early endosomes (22–24). Furin-mediated cleavage of PEA and PEA-derived immunotoxins is both an obligatory and a
rate-limiting step for their cytotoxic activity (25, 26). In fact, only 5% to 10% of cell-associated PEA can be cleaved by furin within cells (27). In this study, we report the generation of a new class of conjugates, called novel immunoproapoptotic proteins, which contain furin-sensitive sequences. These furin sensitive sequences include sites from PEA (T273RHRQPRGWE282, Fpe), diphtheria toxin (A187GNRVRSVGG196, Fdt), and a synthetic polyarginine tract (RRRRRRRRRRR, R9). We, here, describe the construction and characterization of a novel class of immunoproapoptotic proteins containing these furin cleavage sequences. We present evidence that these novel immunoproapoptotic proteins efficiently and selectively bind to HER2-overexpressing tumor cells, followed by endocytosis, furin-mediated cleavage, and translocation, resulting in induced cell death and reduction of tumor size in nude mice.

Materials and Methods

Plasmids construction. The genes encoding e23sFv, PEA II, and casp3/GrB were generated in our previous study (16, 18). Truncated PEA II fragments were amplified by PCR using a casp3-encoding plasmid as template (16). Furin site sequences were incorporated into casp3, GrB, or enhanced green fluorescent protein (EGFP) by PCR. Generation of the recombinant genes involved a sequential fusion of the genes of a signal peptide. Fragments encoding e23sFv, casp3/GrB, truncated PEA II, or the furin sites were cloned downstream and in frame with DNA encoding a signal sequence (MKHLWFFLLLVAAPRWVLS; Figs. 1 A and 2 A) in the expression vector pCMV. Vector sequences were confirmed by DNA sequencing.

Cell culture and transfection. The human breast tumor cell line SKBR-3, the human gastric cancer cell lines SGC-7901 and AGS, the human hepatocellular carcinoma cell line Hep G2, the human cervical

Figure 1. Selective cytotoxicity of immuno-GrB with truncated PEA II variants. A, schematic diagram of immuno-GrBs containing signal sequence, e23sFv, portions of the PEA translocation domain, and GrB. B, immunofluorescent staining of GrB in HeLa cells 72 h after transient transfection with pCMV or vectors encoding variant constructs described in A. C, time course of cell number in SKBR-3 transfected as described. Columns, mean; bars, SE. D, immunocytochemical staining of GrB in SKBR-3 cells 72 h after transfection with pCMV or variant constructs described in A. Magnification, 400×.
carcinoma cell line HeLa, and the human lymphoma cell line Jurkat were maintained in DMEM or RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in 5% CO2. At 24 h before transfection, cells were seeded in 12-well plates at a density of 1 × 105 cells per well. Transfection was performed with Lipofectamine 2000 (Invitrogen) following the standard procedure. For transient expression, cells were harvested 72 h after transfection, unless stated otherwise. For stable transfection, cells were selected in medium containing 800 µg/mL G418 (Invitrogen) for 2 to 3 weeks.

**Cell viability assay.** For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cells transfected with novel immunopraopotic proteins containing optimal furin-cleavable peptides were cultured in 96-well plates for 24 to 96 h. Cells were then incubated with 20 µL of 1.5 mg/mL MTT per well for 4 h, followed by the addition of 150 µL DMSO. A490 nm values were determined using a Sunrise microplate reader (Tecan). Each assay was performed in triplicate on at least three independent occasions.

**Immunocytochemical and histologic analyses.** Cells on coverslips were transfected with DNA and stained 72 h after transfection. The samples were fixed in 4% paraformaldehyde (Invitrogen) following the standard procedure. For transient expression, cells were harvested 72 h after transfection, unless stated otherwise. For stable transfection, cells were selected in medium containing 800 µg/mL G418 (Invitrogen) for 2 to 3 weeks.

**Figure 2.** Expression and secretion of novel immunopraopotic proteins containing optimal furin-cleavable peptides. A, schematic diagram of novel immunopraopotic proteins comprising signal sequence, e23Fv, furin-cleavable peptides, and proapoptotic proteins or EGFP. B, Western blot analysis of concentrated cell culture medium from Jurkat cells stably transfected with novel immunopraopotic protein genes after 72-h culture. Blots were probed with anti-casp3, anti-GrB, and anti-EGFP antibody, as indicated. C, Western blot analysis of in vitro furin cleavage of concentrated culture supernatants from transfected Jurkat cells. Culture supernatants were concentrated and incubated with recombinant furin at room temperature for 16 h at pH 5.4 or pH 7.2. Cleavage rates were calculated from densitometric estimates.
Results

Immunoproapoptotic proteins containing truncated PEA II can induce HER2-positive tumor cell death. PEA II consists of six a-helices (A-F), and when PEA is internalized into a cell, furin-dependent cleavage occurs between helices A and B (R279-G280). To identify the minimal segment of PEA II required for translocation, we generated three deletion constructs bearing different helices: PEA253-358 (deletion of helix F), PEA275-358 (deletion of helices A and F), and PEA275-280 (deletion of all six helices). The deletion constructs were fused with anti-HER2 single-chain antibody (ε23sFv) and the proapoptotic granzyme B genes, producing a series of truncated immuno-Grb molecules (Fig. 1A). In addition, we generated a full-length PEA II fusion construct as the positive control and a PEA II–null fusion construct as the negative control.

Next, we transfected either HER2-positive human breast tumor SKBR-3 cells or HER2-negative human cervical carcinoma HeLa cells (29, 30) with the PEA II truncated constructs (Fig. 1B). Translocation was assessed indirectly by measurement of Grb-triggered cytotoxicity. The fusion proteins were secreted properly from both SKBR-3 and HeLa cells (data not shown). As expected, transfected HeLa cells proliferated at a rate that was similar to untransfected cells (data not shown), suggesting that expression of the chimeric protein was not toxic. In contrast, transfected SKBR-3 cells exhibited reduced cell number (Fig. 1C) and morphologic changes consistent with increased cell death (Fig. 1D). Interestingly, the smallest construct, containing PEA275-280, led to reduced but detectable cytotoxicity in SKBR-3 cells, indicating the furin site alone is necessary for Grb delivery to the cytosol. As this construct contains the least PEA fragment, it was thus chosen for further optimization of furin cleavage and cytotoxic activity.

Expression and secretion of novel immunoproapoptotic proteins containing optimal furin cleavable sequences. To increase furin-mediated cleavage and release of active effectors to the cytosol, we next attempted to alter the sequence of the amino acids, linking antibody and proapoptotic moieties in three different constructs. In the first construct, we added additional amino acid residues to the PEA275-280 sequence (T279-RHRQPRGWG282, Fpe) to enhance the rate of furin-mediated cleavage (23, 24). In the second construct, we used the furin site from diptheria toxin (A18-GNVRRSVG196, Fdt), which has been reported to facilitate furin-mediated cleavage (27). In the third construct, we used the arginine-rich sequence (RRRRRRRRRRR, R9), a furin cleavage sequence with multiple cleavage sites. These three putative furin-sensitive linkers were cloned between ε23sFv gene, with signal sequence with multiple cleavage sites. These three putative furin-sensitive linkers were cloned between ε23sFv gene, with signal sequence, and apoptosis-inducing protein (casp3 and Grb) genes or the EGFP gene under the control of the CMV promoter (Fig. 2).
Two other plasmids, in which e23sFv and apoptosis-inducing proteins were linked directly or by wild-type PEA II, were also generated as controls (Fig. 2).

CD4⁺ Jurkat cells were then transfected with these expression plasmids and selected by G418 selection for stable expression. Expression of the transfected constructs was confirmed by reverse transcription–PCR (RT-PCR) using primers specific to a 750-bp e23sFv fragment (data not shown). Expression and secretion of fusion proteins was confirmed by Western blot analysis of cell culture supernatants (Fig. 2B). Stably transfected Jurkat cells showed similar proliferation rates compared with parental cells (Supplementary Fig. S1).

**In vitro cleavage of novel immunoproapoptotic proteins by furin.** Secreted fusion proteins from stably-transfected Jurkat cells were collected and subjected to proteolysis with recombinant furin. At pH 7.2, PEA II was uncleavable as previously reported (25, 31, 32). In contrast, cleavage of Fpe (6.9% of total), Fdt (36.4%), and R9 (38.7%) was observed at pH 7.2. At pH 5.4, PEA II, Fpe, Fdt, and R9 were all cleaved more efficiently (39.2%, 49.4%, 68.2%, and 73.7%, respectively). However, the negative control recombinant protein, in which e23sFv directly fused with EGFP, was not cleaved at either pH (Fig. 2C). According to the results, Fdt and R9 were more efficiently cleaved among all of the constructs in both pH conditions. In addition, we observed that although an acidic environment is not obligatory for the cleavage of furin sensitive sites, lower pH, which is similar to the pH environment of endosome, greatly enhances the cleavage rate of these sequences.

**Translocation of novel immunoproapoptotic proteins in SGC-7901 cells.** Jurkat cells stably transfected with e23sFv-Fdt-EGFP or e23sFv-Null-EGFP were cocultured with HER2-positive SGC-7901 cells for 8 h at an E/T ratio of 3:1. Under fluorescence microscope, the majority of SGC-7901 cells were observed taking up the fusion proteins secreted by Jurkat cells (data not shown). To examine the translocation of these fusion proteins in HER2-positive cells, we performed immunofluorescent staining using both anti-EGFP and anti-EEA1 antibodies. EEA1 is a membrand-bound protein component specific to the early endosome (33, 34). The e23sFv-Null-EGFP fusion protein showed a punctate distribution in SGC-7901 cells which colocalized predominantly with the early endosome marker (Fig. 3), suggesting a lack of translocation to the cytosol. In contrast, e23sFv-Fdt-EGFP exhibited both a punctate colocalization with EEA1, as well as a diffuse cytoplasmic fluorescence, indicating translocation of the fusion protein from early endosome to cytosol (Fig. 3). These observations strongly suggest that furin-mediated cleavage leads to cytosolic translocation of immunoproapoptotic proteins in gastric cancer SGC-7901 cells.

**Novel immunoproapoptotic proteins specifically kill HER2-overexpressing tumor cells in vitro.** Recombinant proteins were transiently expressed in HER2-positive SGC-7901 and HER2-negative HeLa cells. Apparent cell death was observed in SGC-7901 cells but not HeLa cells at 48 h after transfection (Fig. 4B), presumably due to the presence of HER2 on the surface of SGC-7901 cells. Indirect immunofluorescent staining showed cells expressing the targeted proteins underwent clear nuclear chromatin condensation 72 h after transfection (data not shown). Next, we investigated degree of apoptosis in transfected SGC-7901 cells by detecting the cleavage of PARP. As expected, PARP was cleaved 72 h after transfection with all constructs containing furin cleavage sites, but not in the null-negative control group (Fig. 4A).

Next, we compared in vitro cytotoxicity of the novel immunoproapoptotic proteins by coculturing transfected Jurkat cells with SGC-7901 cells at a ratio of 3:1. In the case of casp3-conjugated fusion proteins, we observed apparent cell death in the PEA II group 24 h after coculture and a killing rate as high as 80% 4 days after transfection (Fig. 4C, left). Similarly, furin cleavable sequences–linked groups, namely Fpe, Fdt, and R9, showed time-dependent killing activity, but their cytotoxicity was weaker than that of PEA II (Fig. 4C, left). Fpe-containing fusion proteins contributed less to cell killing than Fdt-containing and R9-containing
fusion proteins, possibly due to inefficient furin cleavage. Similar results were obtained with GrB-conjugated fusion proteins, although GrB-mediated killing was not as potent as casp3 (Fig. 4C, right).

To confirm the specificity of the cytotoxicity, e23sFv-Fdt-casp3/GrB-transfected Jurkat cells were cocultured in vitro with four different HER2-positive human tumor cell lines (SKBR-3, SGC-7901, AGS, and Hep G2) and control HeLa cells that express undetectable
levels of HER2 (Fig. 5A). We observed significant killing of SKBR-3, SGC-7901, and AGS cells, moderate killing of Hep G2 cells, and no killing of HeLa cells (Fig. 5B). Consistent with our previous observations, stronger cytotoxicity was observed with casp3 conjugates than with GrB conjugates (Fig. 5B). Taken together, we conclude that immunoproapoptotic proteins are capable of recognizing and destroying HER2-expressing cells, but not cells lacking detectable HER2 expression.

**Secrected e23sFv-Fdt-casp3 effectively suppresses the growth of HER2-overexpressing tumor cells and prolongs survival in nude mice.** The antitumor activity of immunoproapoptotic proteins was evaluated in vivo in a nude mouse xenograft model containing HER2-overexpressing SGC-7901 cells. Because of its potent in vitro antitumor activity, we chose to use e23sFv-Fdt-casp3 in vivo. Nude mice were injected with SGC-7901 cells (see Materials and Methods). One group of mice were given six i.m. doses of Lipofectamine-encapsulated pCMV-e23sFv-Fdt-casp3, pCMV-e23sFv-PEA II-casp3, or empty pCMV every 3 days over the course of the study. e23sFv-Fdt-casp3 expression in muscle cells, as evaluated with an anti-caspase-3 antibody, was found most obvious 24 h after i.m. injection and almost undetectable on day 4 (Fig. 5C). Another group of gastric cancer-bearing mice received three weekly i.v. injections of 2 × 10^7 Jurkat cells expressing e23sFv-Fdt-casp3, e23sFv-PEA II-casp3, or control Jurkat cells. Injected Jurkat cells were found to be localized to tumor tissues, liver, and spleen but hardly detectable in the heart, kidney, and lung (Fig. 5D; Supplementary Fig. S2). Remarkably, Jurkat cells were detected in tumor tissues 1 day after injection and were still evident 1 week later (Fig. 5D).

According to the statistical data, both the Jurkat secreting treated groups and the vector Lipofectamine treated groups led to

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**Figure 5.** Selective cytotoxicity of immunoproapoptotic proteins–expressing Jurkat cells upon HER2-overexpressing tumor cells. A, RT-PCR and Western blot analyses of HER2 level in SKBR-3, SGC-7901, AGS, HeLa, and Hep G2 cells. B, e23sFv-Fdt-casp3–expressing and e23sFv-Fdt-GrB–expressing Jurkat cells were cocultured with SKBR-3, SGC-7901, AGS, HeLa, and Hep G2 cells for indicated times, and the percentages of cell killing were determined by cell counting. Columns, mean; bars, SE. C, e23sFv-Fdt-casp3 gene expression in muscle cells. pCMV-e23sFv-Fdt-casp3 (10 μg) mixed with Lipofectamine was injected in the right posterior limb of athymic mice. At each indicated time, animal was sacrificed and frozen sections were made. e23sFv-Fdt-casp3 expression was detected with an anti–caspase-3 antibody. D, Jurkat nuclei were stained by Hoechst 33258 before i.v. injection. Athymic mice bearing HER2-positive gastric cancer were sacrificed at each indicated time. Specimens and frozen section were made and examined under fluorescence microscope. Bar, 10 μm.
a decrease in tumor volume and prolonged mouse survival time compared with controls (Fig. 6A and B). However, the Jurkat secreting groups were more efficient than the vector Lipofectamine groups in reducing tumor size ($P < 0.05$), suggesting that the recombinant protein, which is continuously produced by the transduced cells inside tumors, accumulated locally to exert its antitumor effects. Interestingly, no obvious differences were observed between e23sFv-Fdt-casp3–injected and e23sFv-PEA II-casp3–injected groups, regardless of the method of delivery (Fig. 6A and B).

We next examined the localization of recombinant proteins in the mice that received genetically modified Jurkat cells. Immunohistochemical staining confirmed that secreted proteins were localized to tumor tissues, but not heart, liver, spleen, lung, kidney (Fig. 6C), or tumor tissues of control mice (data not shown). There was neither histologic abnormality nor apoptosis in liver, lung, kidney, or heart (Supplementary Fig. S3). In contrast, numerous apoptotic cells in tumor tissues were detected by TUNEL analysis (Fig. 6D), suggesting that immunoproapoptotic proteins secreted by the transfected cells in vivo can effectively target and suppress cancer cells overexpressing HER2.

**Discussion**

Obstacles facing immunotherapy are the limited accessibility of antibodies or antibody conjugates to solid tumors and the difficulty in obtaining tumor-specific killer cells by genetically modifying lymphocytes. In our previous work, we established an antibody-directed and cell-mediated cancer therapy strategy, which combines the specificity of antibodies, the potent cytotoxicity of proapoptotic proteins, and the intrinsic ability of lymphocytes to home and penetrate tissue (16–19). The potent in vivo antitumor activity of such a strategy is probably a result of the migration of the lymphocytes to tumors as a targeted toxin carrier and production and accumulation of the targeted toxins inside tumors as a producer. Here, we optimized this strategy by reducing exogenous PEA fragment. We designed three novel, short furin sensitive sites, of ~10 amino acids each, to be used as linkers in combination with an anti-HER2 single-chain antibody and the proapoptotic proteins caspase-3 and granzyme B. We predict that these furin cleavable sites might further attenuate nonspecific cytotoxicity, thus improving its utility as an anticancer treatment.

![Figure 6](https://www.aacrjournals.org/doi/10.1158/0008-5472.CAN-06-2254)
In this study, we have described the construction and functional characteristics of novel immunoprotapoptotic proteins, which included a poly-arginine tract (R9) or a furin site sequence from PEA (amino acids 273-282, Fpe) or diphertheria toxin (amino acids 187-196, Fdt). Our *in vitro* results revealed that these novel immunoprotapoptotic proteins killed HER2-overexpressing tumor cells, but not HER2-negative cells. Moreover, these novel immunoprotapoptotic proteins were also functional *in vivo*, as they led to reduced tumor sizes in a murine xenograft model of HER2-overexpressing gastric cancer cells.

Unlike previously reported immunotoxins, which have been shown to cause immune systems to develop neutralizing antibodies and thus prevent retreatment, our newly generated novel immunoprotapoptotic proteins consist mainly of humanized antibodies and human apoptotic effectors. These novel immunoprotapoptotic proteins possess putatively minor immunogenicity and therefore reduce the possibility of inducing immune response and systematic toxicity, suggesting improved therapeutic utility for long-term treatment of tumors that overexpress HER2.

In this study, three short sequences (Fpe, Fdt, and R9) were examined as potential furin-sensitive sites. Fpe, which lacked a basic residue in the P2 position, exhibited relatively low cleavage efficiency, whereas Fdt and R9 exhibited relatively high cleavage efficiency *in vitro*. Given that poly-argi-nine meets the most stringent furin specificity requirements (24) and that R-X-X-R is the minimal furin cleavage site (22–24), R9 was predicted to be the suitable sequence for furin cleavage, with six possible cleavage sites. Since arginine-rich sequences have been shown to function as protein transduction domain (PTD) (refs. 35–38), we suspected that after furin cleavage, cleaved R9 might aid in translocating the proapoptotic molecule from endosome to cytosol, thus leading to enhanced proapoptotic activity. However, our results showed that the R9-Casp3/Grb conjugates did not show enhanced cell killing compared with Fdt counterparts (Fig. 4B and C). One explanation for this observation is that with six possible cleavage sites, the remaining linker might be inadequate to aid translocation. We also noticed that our R9 conjugates did not show nonspecific cellular uptake as PTD usually does when PTD is fused to either the N or COOH terminus of a protein (35–38). This may be due to the accessibility of R9 to the surface of the cell membrane as a result of steric hindrance of the antibody and proapoptotic moiety.

Both active caspase-3 and granzyme B were used as proapoptotic molecules and both proved to be efficient at cell killing, suggesting the furin-sensitive linker may serve as a universally useful strategy by which to introduce effector proteins. Our results indicate that furin cleavage is a key step in the targeted killing process. However, the mechanism of translocation is not clear thus far. In our work, granzyme B was less cytotoxic than caspase-3. However, novel immunoprotapoptotic proteins containing granzyme B would still be useful in the treatment of tumors deficient in the caspase-dependent apoptotic pathway (39, 40). Given that almost any antigen can be targeted on the cell surface, this approach would be applicable to treatment of cancer, autoimmune diseases, and virus infection.

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