Tools and Technologies

Receptor-Directed Chimeric Toxins Created by Sortase-Mediated Protein Fusion

Andrew J. McCluskey and R. John Collier

Abstract

Chimeric protein toxins that act selectively on cells expressing a designated receptor may serve as investigative probes and/or antitumor agents. Here, we report use of the enzyme sortase A (SrtA) to create four chimeric toxins designed to selectively kill cells bearing the tumor marker HER2. We first expressed and purified: (i) a receptor recognition-deficient form of diphtheria toxin that lacks its receptor-binding domain and (ii) a mutated, receptor-binding–deficient form of anthrax-protective antigen. Both proteins carried at the C terminus the sortase recognition sequence LPETGG and a H6 affinity tag. Each toxin protein was mixed with SrtA plus either of two HER2-recognition proteins—a single-chain antibody fragment or an Affibody—both carrying an N-terminal G5 tag. With wild-type SrtA, the fusion reaction between the toxin and receptor-recognition proteins approached completion only after several hours, whereas with an evolved form of the enzyme, SrtA/C3, the reaction was virtually complete within 5 minutes. The four fusion toxins were purified and shown to kill HER2-positive cells in culture with high specificity. Sortase-mediated ligation of binary combinations of diverse natively folded proteins offers a facile way to produce large sets of chimeric proteins for research and medicine. Mol Cancer Ther; 12(10); 1–9. ©2013 AACR.

Introduction

Many proteinaceous toxins, such as diphtheria and anthrax toxins, act on mammalian cells through a complex sequence of events that begins with binding to a cell-surface receptor.

For diphtheria toxin (DT) to act on mammalian cells, it must be proteolytically cleaved into two, disulfide-linked polypeptide chains, A and B, before or after binding to its receptor, heparin-binding (HB)-EGF (1). The DT-HB-EGF complex is endocyted and delivered to the endosomal compartment, where the B chain inserts into the membrane under the influence of acidic pH (2). This insertion mediates translocation of the catalytic A chain (DTA) across the membrane to the cytosolic face, where reduction of the disulfide linking it to the B chain allows it to be released into the cytosol (3, 4). There, DTA ADP-ribosylates eukaryotic elongation factor-2, blocking protein synthesis and inducing apoptotic cell death (5–7).

Like diphtheria toxin, anthrax toxin acts by modifying intracellular targets and enters cells via the endosomal compartment. However, anthrax toxin is structurally different from diphtheria toxin and is composed of three large (80–90 kDa) nontoxic, monomeric proteins, which interact noncovalently in binary or ternary combinations to cause toxic responses (8–10). Two of the proteins are enzymatic effectors, lethal factor (LF) and edema factor (EF), which modify cytosolic targets (11–13). The third protein, protective antigen (PA), is responsible for binding multiple receptors (14–16), pore formation, and transport of lethal factor and edema factor from the extracellular milieu to the cytosol (17).

In these two toxins and others, the receptor, or receptors, are widely distributed among tissues, allowing the toxin to act on a variety of cell types within a mammalian host. If the receptor-binding function can be disrupted by mutation or chemical modification, there is the possibility of changing receptor specificity by linking the toxin to a heterologous receptor-binding protein (RBP). This fundamental strategy has served as the basis of efforts over many years to develop antineoplastic toxins that selectively kill tumor cells displaying high levels of a specific receptor on their surface (18, 19).

Although chemical methods have also been used to join polypeptides, recombinant DNA has become the method of choice in recent years to generate homogeneous populations of chimeric toxin fusions (19). For recombinant chimeric proteins to be produced in active form, however, neither of the fused entities must interfere with folding of the other. Also, because the physical chemical properties of no two chimeric proteins are identical, purification of each requires special tailoring. Finally, production of some chimeric toxins may be restricted by biosafety concerns.
The discovery of sortases (20) and ways to adapt them to fuse polypeptides presents an alternative path to create chimeric proteins that avoids these disadvantages. Sortases are sequence-specific transpeptidases that covalently anchor surface proteins to the cell wall of Gram-positive bacteria (20, 21). Sortase A (SrtA) from Staphylococcus aureus catalyzes the cleavage of a short-peptide recognition motif (LPXTG) with the concurrent formation of a covalent linkage between the protein carrying this sequence and an oligoglycine-containing substrate (22–24).

Our group and others have shown that targeting the actions of protein toxins to cancer cells, using chemical and recombinant technologies, is an effective approach to treat cancer (18, 19, 25). In the current study, we have used SrtA-mediated fusion of appropriately tagged, natively folded substrate proteins to create four discrete chimeric toxin proteins, all directed to HER2, a cell-surface marker overexpressed on cells in a variety of human cancers (breast, ovarian, and gastric). We first synthesized two HER2-specific RBPs in Escherichia coli and purified them. Both RBPs—a small antibody mimic, or Affibody, known as ZHER2 (26), and a single-chain antibody fragment (scFv), termed 4D5 (27)—carried a penta-Gly sequence at their N terminus. We then used SrtA to fuse each RBP to either a receptor recognition–deficient form of diphteria toxin (mDT) carrying a SrtA-recognition sequence at its C terminus, or alternatively, to mPA, a mutated receptor-binding–deficient form of protective antigen containing a 24-amino acid peptide linker between the VL and VH domain (31) by PCR and cloned into the pETSUMO vector (Invitrogen).

Materials and Methods

Materials

Oligonucleotides were synthesized by Integrated DNA Technologies. A plasmid encoding the gene sequence for anti-HER2 4D5 scFv (4D5) was a generous gift from Gregory Poon (Washington State University, Pullman, WA). The wild-type (WT) SrtA and SrtA* expression plasmids were supplied by Brad Pentelute (MIT, Cambridge, MA). All chemicals were from Sigma-Aldrich, unless otherwise stated. The A431 cell line was from American Type Culture Collection (cat. no. CCL-1555) and the JIMT-1 cell line was from AddexBio (cat. no. C0006005). BT-474 and MDA-MB-468 cell lines were provided by Jean Zhao (Dana-Farber Cancer Institute, Boston, MA) and MDA-MB-231 line by Gregory Poon. The authors did not authenticate the cell lines, but fluorescence-activated cell sorting (FACS) validated HER2 receptor levels. Cells were frozen upon receipt and only low passage number cells were used.

Cell line maintenance

A431 and JIMT-1 cell lines were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 500 U/mL penicillin G and streptomycin sulfate (Invitrogen). All other cell lines were grown in RPMI medium (Invitrogen) supplemented with 10% FCS, 500 U/mL penicillin G and streptomycin sulfate.

Molecular cloning

mDT (residues 1–387 of diphtheria toxin) was cloned into the petSUMO vector (Invitrogen) with a C-terminal glycine-serine repeat (G[S]3) linker, SrtA recognition motif (LPETGG), and hexa-histidine tag, following the standard procedures. mPA, harboring a double mutation (N682A/D683A), was created as described previously (28, 29) and cloned into the pet22b vector (Novagen) with the same C-terminal [GS]3–linker, SrtA recognition peptide, and His6 tag. LPETGG was chosen as the SrtA recognition motif because SrtA more rapidly turns over substrates with a G in the P2 position (30).

Aminoglycine pentapeptides (G5) were recombinantly fused to RBPs: a HER2-specific Affibody, Z4HER2:342 (abbreviated ZHER2), and an anti-HER2 scFv (termed 4D5) containing a 24-amino acid peptide linker between the Vl and Vh domain (31) by PCR and cloned into the petSUMO vector (Invitrogen).

Protein expression and purification

All proteins were expressed and purified from the BL21 (DE3) strain of E. coli (New England Biolabs), under the induction of 1 mmol/L isopropyl β-D-1-thiogalactopyranoside (IPTG), for 2 hours at 30°C. WT SrtA and SrtA* (harboring mutations P94S/D160N/K196T) both lacking the membrane-spanning domain (residues 1–58) were expressed and purified as described by Ling and colleagues (32). mPA-LPETGG-His6 was purified from the periplasm as previously described (28, 33).

mDT-LPETGG-His6, G5-ZHER2, and G5-4D5 were expressed from the petSUMO vector (Invitrogen) as His6-SUMO fusions. Cell pellets were lysed by sonication in lysis buffer (20 mmol/L Tris–HCl pH 8.0, 150 mmol/L NaCl, 10 mmol/L imidazole, 10 mg lysozyme, 2 mg DNAse I, supplemented with a Roche complete protease inhibitor). His-tagged proteins were bound to Ni2+–NTA resin, washed with wash buffer (20 mmol/L Tris–HCl pH 8.0, 150 mmol/L NaCl, and 20 mmol/L imidazole), and eluted with wash buffer supplemented with 250 mmol/L imidazole. The resulting purified proteins were exchanged into imidazole-free buffer (20 mmol/L Tris–HCl, pH 8.0 and 150 mmol/L NaCl) and cleaved by SUMO protease for 1 hour at room temperature to generate mDT-LPETGG-His6 and RBPs displaying free N-terminal oligoglycine peptides. G5-ZHER2 and G5-4D5 were freed from the His6-SUMO tag by Ni2+ affinity chromatography. mDT-LPETGG-His6 was separated from the His6-SUMO tag by size exclusion chromatography on a HiLoad 16/60 Superdex 75 prep grade column attached to an automated Äkta purifier (GE Healthcare Biosciences).
SrtA reactions

mDT-LPETGG-His6 or mPA-LPETGG-His6 (50 μmol/L) was incubated with an excess of either G5-ZHER2 or G5-4D5 (200 μmol/L). Reactions were catalyzed by 5 μmol/L WT SrtA or SrtA* in sortase reaction buffer (50 mmol/L Tris–HCl, 10 mmol/L CaCl2, 150 mmol/L NaCl pH 7.5) at room temperature.

Purification of mTx–RBP conjugates

mTx–RBP fusions were purified from 0.5 mL reactions by sequential Ni2+-NTA and size exclusion chromatography steps (Fig. 1A). Ni2+-NTA resin (250 μL) was added to the ligation reactions to bind the His6-tagged unreacted mTx substrate and SrtA* enzyme. The flow-through fraction was collected, and the resin was washed with an additional 1 mL of wash buffer. The flow-through and wash fractions were pooled and mTx–RBP fusions were separated from unreacted RBP using a HiLoad 16/60 Superdex 200 prep-grade size exclusion chromatography column.

Cytotoxicity and competition assays

Cells were plated in appropriate medium at densities of 3 to 3.5 × 10^4 cells per well in 96-well plates and incubated overnight at 37°C. The following day, cells were exposed to medium supplemented with the toxin conjugate or toxin mixture. For mDT-variants, cells were exposed to eight 10-fold serial dilutions (starting with a final concentration of 100 nmol/L) for 24 hours. For mPA-variants, cells were exposed to 20 nmol/L mPA-ZHER2 or mPA-4D5 plus a 10-fold serial dilution of LFN-DTA (starting with a final concentration of 100 nmol/L) for 4 hours. After the incubation period, toxin-containing medium was removed and replaced with leucine-deficient medium supplemented with 1 μCi of [3H]-leucine/mL (PerkinElmer) and incubated for an additional hour. Plates were washed twice with cold PBS (200 μL) before the addition of 200 μL of scintillation fluid. The amount of [3H]-leucine incorporated was determined by scintillation counting using a Wallac MicroBeta TriLux 1450 LSC (PerkinElmer). Percentage protein synthesis was normalized against untreated cells and plotted versus the concentration of LFN-DTA or diphtheria toxin–variant in GraphPad Prism.

Competition assays were conducted as described earlier in which increasing concentrations of free G5-ZHER2 or G5-4D5 were added to medium containing 20 nmol/L mPA-ZHER2/mPA-4D5 plus LFN-DTA (1 nmol/L) and exposed to BT-474 cells for 4 hours. Percentage protein synthesis was normalized against untreated cells and plotted using GraphPad Prism.

Coculture cytotoxicity assay

Cancer cell lines were seeded (3.5 × 10^4 cells/well) in partitioned sections of a chambered tissue culture slide.
(Thermo Scientific). After an overnight incubation, the medium was removed, and the partitioning element was discarded. The slides were washed with PBS and incubated for 24 hours with RPMI medium containing (i) 20 nmol/L of mPA-ZHER2 with 10 nmol/L LFN-DTA, (ii) 20 nmol/L mPA-4D5 plus 10 nmol/L LFN-DTA, (iii) 100 nmol/L mDT-ZHER2, or (iv) 100 nmol/L mDT-4D5. Following toxin exposure, cells were processed as previously described (25).

Results and Discussion

HER2 is overexpressed in several cancers (34–37) and is the target of U.S. Food and Drug Administration (FDA)–approved protein therapeutics (e.g., trastuzumab and T-DM1), as well as receptor-rediected protein toxins in preclinical stages (25, 38–40). Some classes of toxins, such as diphtheria toxin and anthrax toxin, have evolved an active mechanism of crossing the endosomal membrane and delivering bioactive proteins to the cytosol (17). This endosomal escape mechanism can be exploited to deliver a cytocidal enzymatic "payload," such as the catalytic domain of diphtheria toxin (DTA; refs. 5, 6) used in the current work, or other bioactive polypeptides that modulate intracellular processes.

Ploegh and others have shown the use of SrtA in vitro to incorporate polypeptides (41–43), biochemical handles (e.g., biotin; ref. 44), fluorescent probes (44, 45), peptide nucleic acids (30), sugars (46), lipids (47), unnatural amino acids (43), and chemical groups (32) into a number of structurally distinct proteins. Although SrtA can be expressed in E. coli and purified as a soluble enzyme (23, 48), its use for in vitro protein engineering has been limited by long reaction times (typically 16–24 hours) and the need for large quantities of enzyme (more than 30 μmol/L) to circumvent suboptimal kinetics. The reaction rates were consistent with results reported by Ling and colleagues to ligate peptides with SrtA (25, 49).

Recently, Chen and colleagues evolved SrtA by yeast display to generate mutants with improved kinetics (49). Here, we describe the use of WT SrtA and an evolved SrtA variant (SrtA') to assemble receptor-directed chimeric protein toxins in vitro (Fig. 1). The approach requires two building blocks: (i) a mutated, receptor recognition-deficient toxin protein (mTx) containing a canonical C-terminal SrtA recognition motif (here, LPETF), and (ii) a heterologous RBP carrying an N-terminal oligoglycine peptide (Fig. 1). SrtA catalyzes cleavage of the toxin moiety between Thr and Gly of the recognition peptide and formation of a covalent bond between the carboxyl group of Thr and the amino group of the oligoglycine peptide of the RBP (Fig. 1; refs. 23, 24).

Two HER2-directed single-chain toxins were created by fusing mDT with a HER2-specific Affibody (ZHER2; ref. 26) or humanized a single-chain antibody fragment (4D5; ref. 27); the products were designated mDT-ZHER2 and mDT-4D5, respectively (Fig. 1). The catalytic DTA chain contained within these single-chain toxins served as a cytocidal payload that causes inhibition of protein synthesis and apoptotic cell death upon its delivery to the cytosolic compartment of sensitive cells (5, 6).

We also created two HER2-directed binary toxins. First, we fused ZHER2 or 4D5 to the C-terminus of mPA (28, 29), yielding mPA-ZHER2 and mPA-4D5 (Fig. 1). Protective antigen, the receptor-binding pore-forming component of anthrax toxin, noncovalently binds the enzymatic components of the toxin and delivers them to the cytosol (9, 10). However, the effector moieties of anthrax toxin are not cytocidal toward most cell types, therefore we combined mPA-ZHER2 or mPA-4D5 with LFN-DTA, an effector protein containing the high affinity N-terminal protective antigen–binding domain of the anthrax lethal factor (LFN) with DTA. LFN-DTA binds to mPA-ZHER2 and mPA-4D5 and upon its delivery to the cytosol, the DTA moiety blocks protein synthesis, as with the single-chain toxins. Our previous study (25) using the same panel of cell lines showed that DTA delivery by recombinantly fused mPA-ZHER2 resulted in rapid protein synthesis inhibition and subsequent cell death via apoptosis. In the current study, we used protein-synthesis inhibition as a sensitive readout for delivery of DTA to the cytosol to monitor the functions of the SrtA fusions. DTA thus served as the enzymatic effector moiety of all of the targeted toxins in our study.

The fusion reaction creating mDT-ZHER2, mDT-4D5, mPA-ZHER2, or mPA-4D5 was virtually complete within 5 minutes when 5 μmol/L SrtA' was used as catalyst, whereas the same concentration of WT SrtA required more than 4 hours to achieve the same level of fusion (compare Fig. 2A and B). Reaction rates showed no significant dependence on specific substrate proteins, indicating that the nature of the folded polypeptide entities to which the LPETF and G5 tags were attached mattered little in the SrtA-catalyzed reactions. SrtA' reaction rates were consistent with results reported by Ling and colleagues to ligate peptides with chemical groups to proteins for use in semi-synthesis strategies (32).

SrtA'–ligated fusions were purified by sequential Ni²⁺-NTA and size exclusion chromatography steps to give virtually pure products (yield 20%–65%; Fig. 2C; Table 1). Cancer cell lines expressing various levels of HER2, including a trastuzumab-resistant line isolated from a HER2-positive patient clinically resistant to trastuzumab (50), were incubated with either mPA-ZHER2 or mPA-4D5 plus LFN-DTA for 4 hours or mDT-ZHER2 or mDT-4D5 for 24 hours. Following toxin exposure, protein synthesis was measured over a 1-hour period. All four fusions were able to direct toxin action to HER2-positive cells, and the degree of cell killing was dependent on the level of cell-surface HER2 (Fig. 3; refs. 25, 51). Cells expressing the highest levels were the most sensitive (BT-474; Fig. 3; Table 1), and HER2-negative cells, MDA-MB-468, were unaffected (Fig. 3). The specificity of SrtA'–generated toxin fusions for cells bearing the
cognate receptor was confirmed, and the absence of bystander effects on cells lacking HER2 shown, in experiments conducted with mixed cell populations of HER2-positive and -negative cells (Fig. 4). Some non-specific toxicity was observed for mDT-4D5 toward HER2-negative MDA-MB-468 cells in mixed cell populations. Unlike mPA and LFN-DTA, mDT retains some residual non-specific activity toward even toxin-resistant cells (52), an

Table 1. SrtA+ ligation reaction yields and in vitro activities of HER2-targeted toxin fusions on various cell lines

<table>
<thead>
<tr>
<th>Toxin</th>
<th>% Yieldb</th>
<th>BT-474</th>
<th>JIMT-1</th>
<th>A431</th>
<th>MDA-MB-231</th>
<th>MDA-MB-468</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPA-ZHER2 + LFND1TAc</td>
<td>35+/−6</td>
<td>9.1 × 10⁻¹³</td>
<td>2.3 × 10⁻¹²</td>
<td>2.3 × 10⁻¹¹</td>
<td>6 × 10⁻¹⁰</td>
<td>&gt;1 × 10⁻⁷</td>
</tr>
<tr>
<td>mPA-4D5 + LFND1TAc</td>
<td>20+/−3</td>
<td>1.5 × 10⁻¹²</td>
<td>1.6 × 10⁻¹¹</td>
<td>8.6 × 10⁻¹¹</td>
<td>2.2 × 10⁻⁹</td>
<td>&gt;1 × 10⁻⁷</td>
</tr>
<tr>
<td>mDT-ZHER2d</td>
<td>67+/−4</td>
<td>1.7 × 10⁻¹¹</td>
<td>1.2 × 10⁻⁹</td>
<td>5.4 × 10⁻⁹</td>
<td>&gt;1 × 10⁻⁷</td>
<td>&gt;1 × 10⁻⁷</td>
</tr>
<tr>
<td>mDT-4D5d</td>
<td>38+/−9</td>
<td>1.3 × 10⁻¹¹</td>
<td>5.9 × 10⁻¹⁰</td>
<td>3.0 × 10⁻⁹</td>
<td>8.3 × 10⁻⁸</td>
<td>&gt;1 × 10⁻⁷</td>
</tr>
</tbody>
</table>

EC⁵₀ values were calculated in GraphPad Prism from dose–response curves (presented in Fig. 3).

bMolar ratio of the mTx–RBP product versus mTx after purification. Average of three independent reactions.

cMeasured by [³H]-leucine incorporation after 4-hour toxin exposure.
Effect that is reduced when the protein is attached to an RBP. The decrease in protein synthesis may indicate that 4D5, when fused to mDT, does not have the same steric effects as ZHER2 in shielding off-target toxicity of mDT in mixed populations.

Free ZHER2 Affibody competitively inhibited mPA-ZHER2-dependent cell killing, but not mPA-4D5-dependent killing (Fig. 5); and free 4D5 protected cells against mPA-4D5 plus LF₉-DTA, but not against mPA-ZHER2-dependent killing under the same conditions (Fig. 5). These findings are consistent with structural data showing ZHER2 and a Fab fragment of trastuzumab (from which 4D5 is derived) recognize nonoverlapping HER2 epitopes (53).

The combinations of mPA-ZHER2 or mPA-4D5 with LF₉-DTA were 10— to 100-fold more potent than the corresponding diphtheria toxin fusions (Table 1), as the mPA chimeric toxins acted in a shorter incubation period (4 hours vs. 24 hours) and were able to kill MDA-MB-231, a cell line expressing low levels of HER2 (Fig. 3). The difference in EC₅₀ for the toxin conjugates could be a result of more efficient effector delivery by mPA, combined with its ability to deliver multiple enzymatic effectors (54).

The results we report here have shown the use of SrtA-based protein fusion and its potential use for fusing a broad array of appropriately tagged proteins. Consistent with this versatility, Guimaraes and colleagues were able to link DTA to cholera holotoxin to identify host factors required for intoxication (55). The fact that fusion reactions in our study reached completion within a few minutes when SrtA was used makes this evolved form of SrtA particularly attractive.

SrtA-based protein fusion is appealing from many perspectives. (i) It can circumvent potential problems in expression and/or folding of recombinantly fused polypeptides into their respective active configurations. Thus, although we were unable to express and purify mPA-4D5 as a single-polypeptide fusion, the individual mPA and 4D5 proteins expressed, folded, and underwent rapid SrtA-mediated fusion to yield a biologically active product. (ii) SrtA-based fusion avoids the need to tailor a purification protocol for each individual chimeric protein, as is required when such proteins are produced recombinantly. (iii) Preparation of subsets of pure, appropriately tagged fusion partners opens the possibility of easily preparing large numbers of fusions (the algebraic product of the numbers of entities in the subsets) for testing. With the protocol described here, in which both the sortase enzyme and the toxin substrate carried a His₆ tag, we were able to remove the enzyme, unreacted toxin protein,
and the GGH6 peptide product with a Ni\(^{2+}\)-NTA column. Subsequent removal of unreacted RBP on a size exclusion column yielded the desired chimeric proteins in substantially pure form. (iv) With chimeric toxins in which the individual fusion moieties are nontoxic and only the fusion product displays toxic properties, sortase-based fusion of the purified tagged substrates avoids biosafety issues that may arise in expressing the fused polypeptide \textit{in vivo}.

\section*{Disclosure of Potential Conflicts of Interest}
No potential conflicts of interest were reported.

\section*{Authors' Contributions}
Conception and design: A.J. McCluskey
Development of methodology: A.J. McCluskey
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.J. McCluskey
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.J. McCluskey
Writing, review, and/or revision of the manuscript: A.J. McCluskey, R.J. Collier
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.J. McCluskey
Study supervision: R.J. Collier

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