Probing the surface of eukaryotic cells using combinatorial toxin libraries

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The success of proteomics hinges in part on the development of approaches able to map receptors on the surface of cells. One strategy to probe a cell surface for the presence of internalized markers is to make use of Shiga-like toxin 1 (SLT-1), a ribosome-inactivating protein that kills eukaryotic cells [1, 2]. SLT-1 binds to the glycolipid globotriaosylceramide [3, 4], which acts as a shuttle, allowing the toxin to be imported and routed near ribosomes. We investigated the use of SLT-1 as a structural template to create combinatorial libraries of toxin variants with altered receptor specificity. Since all SLT-1 variants retain their toxic function, this property served as a search engine enabling us to identify mutants from these libraries able to kill target cells expressing internalizable receptors. Random mutations were introduced in two discontinuous loop regions of the SLT-1 receptor binding subunit. Minimal searches from screening 600 bacterial colonies randomly picked from an SLT-1 library identified toxin mutants able to kill cell lines resistant to the wild-type toxin. One such mutant toxin was shown to bind to a new receptor on these cell lines by flow cytometry. Toxin libraries provide a strategy to delineate the spectrum of receptors on eukaryotic cells.

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Results and discussion

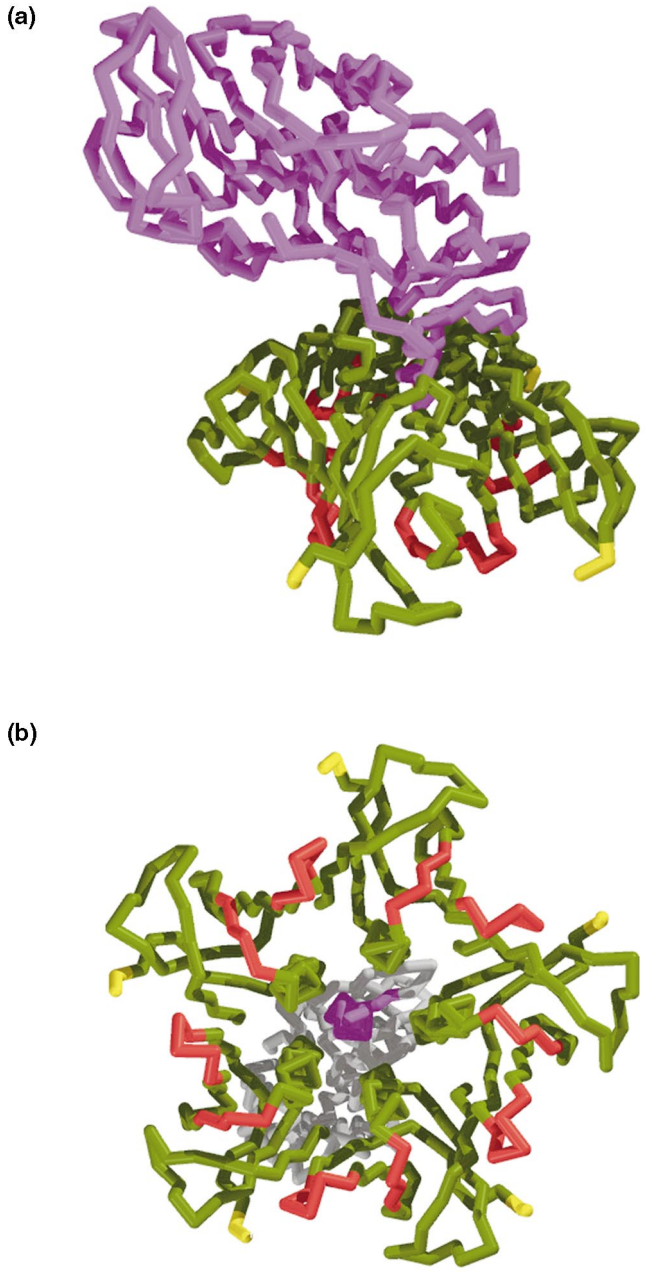
Construction of SLT-1 libraries

Shiga-like toxin 1 (SLT-1) is a ribosome-inactivating protein composed of two functional domains: a catalytic A subunit involved in the blockage of protein synthesis and a pentamer of identical B subunits (Figure 1). The A chain is responsible for the inactivation of eukaryotic ribosomes, acting as a specific N-glycosidase that cleaves a single adenine residue from 28S rRNA [1, 2]. The B subunit pentamer recognizes a glycolipid receptor, globotriaosylceramide [3, 4], also known as CD77, Gb_3, or Pk antigen. Combinatorial SLT-1 libraries were created by randomly mutating two discontinuous regions of the B subunit. The selection of these regions was based on the following knowledge. The B subunit is a small protein composed of only 69 amino acid residues that pentamerizes spontaneously in solution. Structural as well as modeling studies have indicated that three loop regions of the B subunit [5, 6] delimited by residues 15–19, 32–35, and 54–64 are associated with the receptor binding function of the toxin (Figure 1; [7–11]). Two dominant classes of binding sites for CD77 exist on the B subunit pentamer and are referred to as sites I and II [7]. Residues located in regions 15–19 and 30–33, in particular Asp16, Asp17, Phe30, Asn32, and Arg33 contact the carbohydrate receptor or line the binding clefts defining sites I and II. The calculated interaction energy derived from modeling studies suggested that site I is likely to be the predominant site mediating CD77 interaction [7], a projection supported by toxin variants harboring mutations at Asp17 and Phe30 [12–16]. In summary, residues found in loop regions are sites where random mutagenesis would lead to an altered receptor specificity. Toxin libraries were initially constructed with residues randomly mutated at only 9 positions, namely residues 15–19 (loop 1) and residues 30–33 (loop 2) (Figure 1).

Screening of SLT-1 libraries on cell lines resistant to the native toxin reveals the frequent occurrence of cytotoxic mutant toxins that do not target CD77

A degenerate SLT-1 library (60% doping) was screened for mutants able to kill human breast (CAMA-1; [17]) and prostate (PC-3) cell lines. These cell lines were selected based on two criteria: their resistance to the action of wild-type SLT-1 in cell cytotoxicity assays (Figure 3) and the absence of receptors for the native toxin on their surface as monitored by flow cytometry (Figure 4; left panels). The SLT-1 library produced an average amino acid substitution rate of close to nine out of nine residues in the target regions, resulting in a potential library diversity of up to 20^9 SLT mutants (Table 1). Searches through the library were conducted to identify cytotoxic mutants able to kill these two cell lines. A collection of 600 single clones was selected from the library. Briefly, individual colonies were picked from a library and their lysates screened on SLT-1-resistant cell lines. Several clones were typically able to delete >80% of cells in relation to

Figure 1



Backbone representations of Shiga toxin [**(a)**, side view] and its B subunit [**(b)**, bottom view] [6]. Shiga and Shiga-like toxin 1 (SLT-1) have identical B subunits and differ by a single amino acid substitution in their A chain. The catalytic A subunit [in magenta, (a)] has its C terminus inserted into a central hole formed by a pentamer of B subunits (in green). Residues located in two noncontiguous loop regions of the B subunit (in red, residues 15–19 and 30–33) implicated in defining the receptor specificity of the wild-type toxin SLT-1 were selected as target sites for random mutagenesis.

control wells containing viable cells (no toxin present). Plasmid DNA was recovered and sequenced from isolates that consistently killed such cells in cytotoxicity assays. A typical profile of cell cytotoxicity for a sample of 60 bacterial extracts (individual colonies labeled from 61 to 121) dispensed in wells containing viable PC-3 cells is presented in Figure 2.

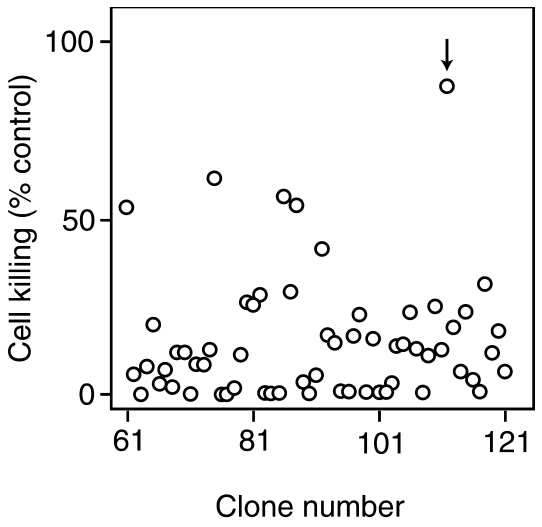
Table 1

Amino acid sequences of two mutated B subunit loop regions of SLT-1 variants exhibiting cytotoxic activity against CAMA-1 or PC-3 cells (clones recovered from SLT-1 library).

Clone number	Loop #1 (residues 15–19)	Loop #2 (residues 30–33)
Wild-type SLT-1	NDDDT	FTNR
Search through SLT-1 library/target cell line: CAMA-1		
122	CLLNG	YQEP
126	QGLQL	TLTG
142	TGATM	PTGI
241	FRPAG	LRCG
308	PYVFL	MVAN
324	KSMDQ	LSKW
715	QGEYG	IQER
823	MVQEK	SKKQ
824	DYFQT	RHYS
Search through SLT-1 library/target cell line: PC-3		
16	MVQEK	SKKQ
38	NYMTR	LAAI
43	IDEGS	TDYA
45	GDRME	RRQP
273	GDVTF	FMRQ
277	LAVAL	QPNL
296	LRYDK	PRES
297	YGKPA	HTPF

Cytotoxicity curves for toxin mutants derived from the SLT-1 library are presented in Figure 3. CD_{50} values ranging from 50 to 100 nM were calculated for these SLT-1 variants (variant 122, CD_{50} 80 nM ; variant 126, CD_{50} 100 nM ; and variant 824, CD_{50} 50 nM). The sequences of the mutated inserts present in the variants are presented in Table 1. No pattern of amino acid conservation was observed amongst the clones isolated from the library. Their toxicity was relatively modest in relation to the potency of the native toxin toward sensitive Vero cells (Figure 3a, CD_{50} 1.5 fM). A rationale for the lower toxicity of SLT-1 variants may be linked to a limited expression of most surface receptors targeted by mutant toxins or may reflect the rate of internalization and nature of the intracellular route taken by the targeted receptor. For instance, native SLT-1 binds to a glycolipid (CD77, Gb_3), which is internalized at a faster rate than most glycoproteins. In addition, CD77 allows for the retrograde transport of the toxin to the ER lumen, where the A chain is able to retrotranslocate across the ER membrane near ribosomes. Finally, isomers of this glycolipid define the routing of SLT-1 inside cells. More specifically, CD77 (Gb_3) isoforms associated with toxicity have typically short fatty acid tails (C16:0 and C18:0) [18, 19]. CAMA-1 cells eventually display some sensitivity toward the wild-type toxin (>5 μM).

Figure 2



Scatter plot displaying a representative set of data collected from testing 60 randomly selected toxin variants derived from the library for their ability to kill PC-3 cells. The relative cytotoxicity (open circles) of individual clones toward PC-3 cells (listed as clones 61 to 121 along the abscissa in this case) was calculated in relation to wells containing cells treated with extracts derived from bacteria untransformed with the library. Toxin variants corresponding to wells showing cytotoxicity >80% (arrow) were chosen for further analysis. The toxicity of cell extracts containing wild-type SLT-1 typically varied between 2% and 10% .

A significant proportion of clones examined in the SLT-1 library were found to contain mutations (including deletions) outside the target areas. A clone from SLT-1, clone 273, that was found to retain strong specificity for PC-3 cells in repeated assays was found, when sequenced, to have a deletion at the C terminus of its B subunit corresponding to residues 53–67 of the 69 amino acid-long chain. Upon examination of the crystal structure, this deleted region comprises of a largely exposed loop region (54–64) followed by a short C-terminal β strand (residues 65–68) that is contacting a strand (residues 9–14) located on the adjacent B subunit in the pentamer. Western blot analysis of toxin mutant 273 reveals the presence of both A and B subunits (see Supplementary material). Interestingly, mutations in the β sheet framework outside the CDR regions of antibodies have been shown to have a significant impact on the affinity of the antibody [20].

Flow cytometry reveals the presence of a new receptor on SLT-1-insensitive cells recognized by a mutant toxin

The binding of toxin variants derived from screening the SLT-1 library on PC-3 cells were monitored by flow cytometry using an anti-B chain antiserum recognizing a nonmutated region of the B subunit (residues 26–29; [21]). Three cell lines were monitored for the expression of receptors for wild-type and mutant toxins on their surface: CAMA-1 and PC-3 cells, which were resistant to wild-type SLT-1 and do not express its receptor, and Vero cells, which display CD77 on their surface and are sensitive to the action of the native toxin (Figures 3a and 4). Wild-type SLT-1 and the mutant 273 were the only toxins tested for which the binding to cells could be detected by flow cytometry (Figure 4). Cytometric profiles shown in Figure 4 (left panels) confirmed that CAMA-1 as well as PC-3 cells did not express SLT-1 receptors on their surfaces, in contrast to Vero cells, which were strongly positive for the binding of the native toxin. In comparison, histograms arising from the detection of mutant 273 (Figure 4; right side panels) illustrate the presence of a new surface receptor recognized by mutant 273 on both CAMA-1 and PC-3 cells. Interestingly, Vero cells weakly express this new receptor, a finding that may explain its weak sensitivity to the mutant toxin (Figure 3B).

Figure 3

Cytotoxicity curves showing the ability of wild-type SLT-1 and variants identified from the library to kill target cells. **(a)** Dose-dependent cytotoxicity of three SLT-1 variants and of wild-type SLT-1 screened against CAMA-1 cells: clone 122 (open squares), clone 126 (open triangles), clone 824 (open circles), and wild-type SLT-1 (closed squares). A curve showing the sensitivity of CD77-expressing Vero cells to wild-type SLT-1 (closed circles) is presented as a control. **(b)** Dose-dependent cytotoxicity of a SLT-1 variant (mutant 273) toward Vero cells (closed boxes), PC-3 cells (open squares), and CAMA-1 cells (open circles). Results are given as the percentage of viable cells in wells treated or not treated with a toxin. Values represent the average of experiments performed in triplicate.

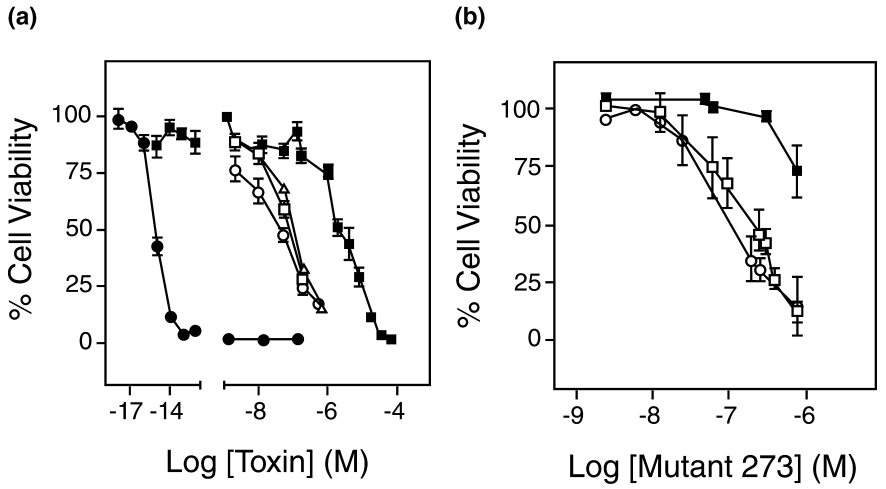
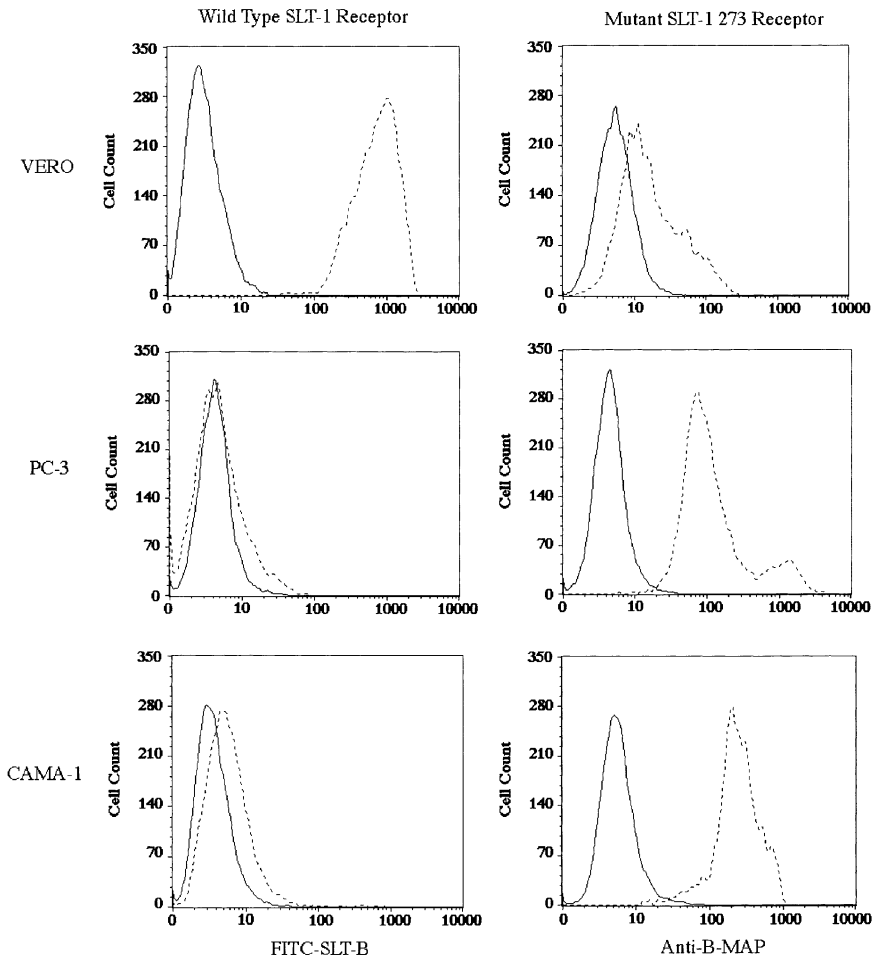


Figure 4



Detection of a cell surface receptor on PC-3 and CAMA-1 cells by a SLT-1 variant (mutant 273) that is distinct from the receptor recognized by wild-type SLT-1. Flow cytometric profiles of Vero, PC-3, and CAMA-1 cells either unstained (left side panels, solid lines) or stained with the fluorescein-labeled B subunit of wild-type SLT-1 (left side panels, dashed lines) are shown. Cytometric histograms on the right side highlight the same set of cells labeled with mutant SLT-1 273. The presence of bound mutant 273 was detected with a rabbit anti-MAP peptide antiserum directed at a nonmutated region of the B subunit followed by a fluorescein-labeled goat anti-rabbit Ig antibody. Profiles depict the fluorescence intensity distributions for cells treated (dashed lines) or not treated (solid lines) with the anti-MAP antiserum. Vero cells express the receptor for the native toxin, while PC-3 and CAMA-1 do not. A new surface receptor is targeted by mutant 273 on PC-3 and CAMA-1 cells.

Clues to the nature of the receptor identified by mutant 273

The receptor recognized by mutant 273 is not related to the native toxin receptor CD77 (Gb_3, globotriaosylceramide) or to Gb_4 (globotetraosylceramide), its closest structural homolog in the biosynthesis pathway of glycolipids. This conclusion is supported by the fact that the target cell lines have been grown and maintained at all times in the presence of the native toxin, a precaution to ensure that the native receptor would not be targeted on these cell lines. It has been well established that cells expressing even small levels of CD77 (Gb_3) remain very sensitive to the native toxin (CD_{50} values in the pM to fM range; Figure 3a; [17]). Furthermore, an average of nine mutations have been introduced in the receptor binding region of the B subunit, indicating that multiple positions defined by site-specific mutagenesis as essential for the recognition of either Gb_3 or Gb_4 [12–16] have been simultaneously altered, effectively destroying native receptor binding sites for either glycolipids [22]. Finally, the flow cytometry data presented in Figure 4 simply confirm results observed using the more stringent selection process based on cytotoxicity searches, where the strong selectivity pressure imposed on cells by the chronic presence of the native SLT-1 molecule would have deleted any cells expressing CD77 or internalized homologs. The disaccharide moiety Galα1–4Galβ represents the dominant feature of the receptor recognized by the native toxin [3, 22] and has been uniquely associated with glycolipid synthesis pathways [23] rather than glycoproteins. Western blot analyses performed on membranes derived from PC-3 cells using mutant 273 and detected using a second antibody directed at either the A or B chain, did not identify a membrane component acting as the receptor. These results would suggest that the putative receptor is a protein denatured under the conditions of SDS PAGE rather than a sugar component resembling Gal α1–4Galβ [3]. In summary, our data suggest that the new receptor recognized by mutant 273 is not CD77 (Gb_3) or Gb_4 but rather a new chemical entity that may be labile under denaturing conditions. A further characterization of this particular receptor is not presently essential, since our searches have

only covered a limited set on mutant clones (600 clones) and a narrow range of cell lines.

The scope of this study was to demonstrate that one could identify a collection of toxin mutants cytotoxic toward a relatively homogeneous cell population. Shiga-like toxin 1 was used as a molecular template in developing cytotoxic agents. The receptor binding portion of the toxin template could be altered, and the retained cytotoxic activity of each toxin variant could be exploited as a molecular search engine in designing, constructing, and screening combinatorial SLT protein libraries. Other related RIP structures could theoretically be used as templates. Using this novel concept, SLT-1 variants were identified that were able to kill cell lines insensitive to the wild-type toxin. Our findings suggest the use of toxin libraries as a strategy to define the surface of eukaryotic cells in terms of families of internalizable ligands. A major challenge will be to define useful families of SLT variants that can selectively target a given population of cells. Searches based on cytotoxicity assays are amenable to high throughput screening strategies and thus may allow a more profound exploration of SLT libraries to find such families of toxin mutants.

Supplementary material

All experimental procedures as well as a figure describing a Western blot analysis of wild-type SLT-1 and mutant 273 are available at http://images.cellpress.com/supmat.supmatin.htm/.

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