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Functional Characterization of Peptide-Based Anthrax Toxin Inhibitors

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Abstract: We have identified an optimized peptide inhibitor that can be used to develop potent anthrax toxin therapeutics. Anthrax toxin, an essential virulence factor of *Bacillus anthracis*, elicits many of the symptoms associated with the disease, and is responsible for death. The toxin is composed of a cell-binding component, protective antigen, and two enzymatic components, edema factor and lethal factor. The three proteins are secreted individually by the bacterium and then assemble into functional complexes on the surface of mammalian cells. These complexes are endocytosed, and the enzymatic components are translocated into the cytosol, where they exert their activities. We screened a phage display library for peptides that can bind the heptameric cell-binding subunit of anthrax toxin, and identified a novel peptide that can block toxin assembly. We made a series of mutant peptides and attached these peptides to polymer backbones to assess their inhibitory activities in vitro. This series of truncated peptide mutants was used to identify a minimal peptide sequence, TYWWLD, that can be used to develop potent polyvalent inhibitors of anthrax toxin.

Keywords: Phage display; *Bacillus anthracis*; polyvalent inhibitor; anthrax toxin

Introduction

Bacillus anthracis secretes a three-component toxin that assembles into complexes after the individual proteins are released by the bacterium.[1,2] Protective antigen (PA) is the toxin component that targets mammalian cells by binding to the cellular receptors ANTXR1 and ANTXR2.[3,4] PA is processed by proteases, either in the blood or on the cell surface,[5,6] into a 63 kDa fragment that can self-associate into ring-shaped heptamers,[7,8] referred to as $[PA_{63}]_7$ (Figure 1). Oligomerization of PA_{63} is a required step in the toxin assembly process because the binding sites for both enzy-

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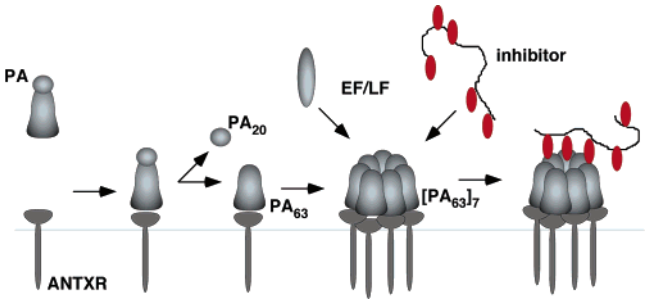

Figure 1. Inhibition of toxin assembly by a polyvalent inhibitor. PA binds ANTXR at the cell surface and is processed by a protease into PA_{20} and PA_{63} fragments. PA_{63} oligomerizes and binds either EF/LF or a peptide-based inhibitor.

matic moieties, edema factor (EF) and lethal factor (LF), extend across two adjacent PA_{63} monomers.[9,10] The enzymatic moieties compete for the same binding sites on $[PA_{63}]_7$ through amino-terminal domains that contain conserved residues that form a distinct binding surface.[11] Although $[PA_{63}]_7$ displays seven identical binding sites for EF and LF, steric hindrances between the enzymatic components restrict the number of ligands that can simultaneously bind $[PA_{63}]_7$ to three.[12]

Assembled toxin complexes are endocytosed through lipid rafts to an acidic compartment where unstructured loops of $[PA_{63}]_7$ insert into the membrane to form a water-filled channel.[13−15] This channel is likely a conduit through which EF and LF molecules are threaded out of the vesicle along a proton gradient.[16] On reaching the cyotosol, EF binds calmodulin and exhibits adenylate cyclase activity that can inhibit the phagocytic ability of neutrophils.[17,18] LF cleaves members of the mitogen activated protein kinase kinase family to reduce cytokine expression and to cause cytotoxicity of macrophages.[19−23] The consequence of these effects is to dampen the innate immune response to the infection, which facilitates bacterial survival and disease progression. In addition, the combination of PA and LF is sufficient to cause death of the host,[24,25] which might explain the significant mortality rate even among patients who receive antibiotics.[26] Without treatment, the mortality rate from inhalational anthrax is >85%, but even with the intensive treatment provided to the victims of the 2001 bioterrorism attacks, the mortality rate was 45%.[27] Thus, an inhibitor that targets the toxin would be a valuable addition to the current antibiotic therapy.[28]

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Mourez and co-workers identified a 12-mer peptide that bound $[PA_{63}]_7$ and competed with LF.[29] The inhibitory activity of the peptide was increased when it was attached to a polymeric scaffold to an extent that the polyvalent inhibitor protected rats that were challenged with lethal doses of toxin. The phage display library from which the peptide was isolated was incomplete because it is not possible to construct a 12-mer library with all of the possible amino acid combinations. Two of the isolated peptides (HTSTYWWLDGAP and HQLPQYWWLSPG) did, however, share the sequence YWWL, which suggested that these amino acids were primarily responsible for mediating the interactions of the peptides with $[PA_{63}]_7$. Here we screen a 7-mer phage display library for peptides that bind $[PA_{63}]_7$ and identify a new inhibitory peptide that also contains the YWWL sequence. We made a series of truncations and substitutions to identify the minimal sequence, TYWWLD, shared by the 7-mer and 12-mer peptides that confers maximal inhibitory activity.

Experimental Section

Phage Display. PA, $[PA_{63}]_7$, and LF were purified as described previously.[23,29] Maxisorp tubes (Nunc) were coated overnight at 4 °C with 2 μg of purified $[PA_{63}]_7$, and the tubes were subsequently blocked for 2 h at 37 °C with a solution of 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS). Approximately 1×10^{11} M13 bacteriophage from the Ph.D.-7 Phage Display Library (New England Biolabs) were suspended in binding buffer (PBS containing 0.1% Tween-20) and added to the tube for either 60 min (round 1), 30 min (round 2), or 5 min (round 3). The tube was washed extensively with binding buffer to remove unbound phage. A solution of 15 μg of PA in binding buffer was added to the tube for 60 min. Phage were then eluted with 40 μg of $[PA_{63}]_7$ either for 60 min (round 1) or for approximately 16 h (rounds 2 and 3). The isolated phage were amplified after rounds 1 and 2 to be subjected to additional selection. Phage that remained after 3 rounds of selection were purified.

Enzyme-Linked Immunosorbent Assay. PBS containing 1 μg of $[PA_{63}]_7$ or PA was added to individual wells of a 96-well Maxisorb plate (Nunc) and incubated overnight at 4 °C. Wells were blocked with a solution of 2% BSA in PBS for 2 h at 37 °C, and approximately 1×10^8 phage were added in the absence or presence of 0.05 mg/mL LF.

Table 1. Substitution Mutagenesis of an Inhibitory Peptide[a]

peptide	IC_{50} (M)
HTSTYWWLDGAPK	$1.1 \pm 0.3 \times 10^{-7}$
HTSTYWWLDGGGK	$2.9 \pm 1 \times 10^{-7}$
HTSTYWWLGGGGK	$1.3 \pm 0.3 \times 10^{-6}$

[a] The indicated peptides were attached to polyhydroxyethylacrylamide backbones, and the IC_{50}s of the resulting inhibitors were determined in a RAW264.7 cell cytotoxicity assay. The results of three independent experiments ± SEM are shown.

After washing with binding buffer, phage were detected with anti-M13 antibody coupled to horseradish peroxidase (Amersham Pharmacia) and 1 Step Turbo TMB-ELISA (Pierce), according to manufacturer's instructions.

Toxin Assembly Assay. CHO-K1 cells in 24-well plates were incubated with 2×10^{-8} M trypsin-nicked PA for 2 h on ice. The cells were washed twice with cold PBS and incubated with ^{35}S-labeled LF_N in either the absence or presence of peptide inhibitors for 1 h on ice. The cells were washed three times with cold PBS and lysed, and the radioactivity was measured by scintillation counting. Background was measured as the amount of ^{35}S-LF_N associated with cells in the absence of PA and was subtracted from values obtained in the presence of PA. The percentage inhibition was calculated by dividing the radioactivity of the peptide-containing samples by that of the sample without inibitor. The results are the mean ± SEM of three independent experiments.

Cytotoxicity Assay. RAW264.7 cells were plated in 96-well plates. The cells either were left untreated or were treated with 2×10^{-8} M PA and 1×10^{-11} M LF in the absence or presence of inhibitors. After an incubation period of 4 h, cell viability was assessed using the MTS assay (Promega). The IC_{50} of each inhibitor was determined using the software program Prism (GraphPad Software).

Synthesis of Monovalent and Polyvalent Inhibitors. Peptides were obtained from Genemed Synthesis Inc. The peptides were acetylated at their N-terminus and amidated at their C-terminus. These peptides were attached to an activated polymer, poly(*N*-acryloyloxysuccinimide), to form polyvalent inhibitors. The synthesis of the activated polymer is described elsewhere.[30] Peptides, predissolved in dimethylformamide, were added to the activated polymer in anhydrous dimethylformamide followed by the addition of triethylamine. After 12 h, the reaction was quenched using ethanolamine (for the inhibitors described in Table 1) or ammonium hydroxide to obtain polyvalent inhibitors. The polyvalent inhibitors were extensively dialyzed for 48 h and lyophilized. The polymers were characterized by ^{1}H nuclear

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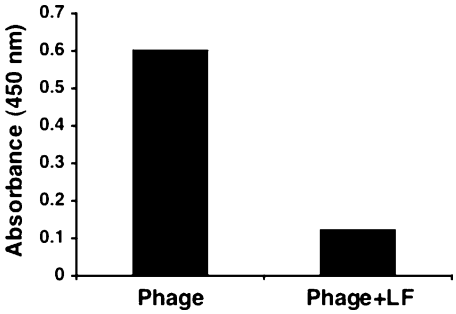

Figure 2. LF competes with phage for binding $[PA_{63}]_7$. Phage displaying HYTYWWLD were added alone or in combination with LF to wells containing $[PA_{63}]_7$. Unbound phage were washed away, and the remaining phage were detected using an anti-M13 phage antibody conjugated to horseradish peroxidase. Horseradish peroxidase activity was detected by adding of 3,3′,5,5′-tetramethylbenzidine and measuring absorbance at 450 nm.

magnetic resonance on a Varian 500 instrument. The peptide concentration was determined by the Edelhoch method.[31]

Results

We screened a 7-mer phage library using a previously described strategy to identify peptides that bind the surface of $[PA_{63}]_7$ that interacts with EF and LF.[29] Purified $[PA_{63}]_7$ was adsorbed to a plastic tube, and the pool of phage was added and then incubated overnight. Non-interacting phage were washed away, and then PA was added to elute phage that specifically bound PA. These phage were discarded because we were interested in isolating phage that bound to $[PA_{63}]_7$, but not to PA, so as to enrich for phage that bound to the surface of $[PA_{63}]_7$ that interacts with the enzymatic moieties (Figure 1). After the PA elution, we added $[PA_{63}]_7$ to the tube and collected phage that were released. These phage were amplified and subjected to two additional selection cycles. Individual phage that survived the selection procedure were subjected to an enzyme-linked immunosorbent assay (ELISA) using either PA or $[PA_{63}]_7$ as the ligand. Those phage that preferentially bound $[PA_{63}]_7$ were sequenced to identify the peptide that they displayed. Of the 10 independent clones that we sequenced, we found that each encoded the peptide HYTYWWL.

We performed an ELISA to determine if phage that displayed the HYTYWWL peptide competed with LF for binding $[PA_{63}]_7$ (Figure 2). Phage that displayed HYTYWWL were added alone or in combination with LF to wells containing adsorbed $[PA_{63}]_7$. After an incubation period, unbound phage were washed away and the remaining phage were detected with an antibody conjugated to horseradish peroxidase. LF competed with the phage for binding $[PA_{63}]_7$ (Figure 2), which indicated that the peptide that the phage displayed could form the basis of a toxin inhibitor.

Although this peptide and the previously identified 12-mer peptide share a five amino acid sequence, TYWWL, we noticed that an Asp residue was immediately carboxy-terminal to the 7-mer sequence in the region that links the peptide to the phage coat protein, which would extend the shared sequence to TYWWLD. The Asp residue arose from random mutation of one nucleotide in the DNA encoding the glycine linker. To determine whether the carboxy-terminal Asp residue was important for the inhibitory activity of the peptides, we compared the activities of a peptide that contained the Asp with one that lacked it. The peptides were synthesized with an additional Lys at the carboxy terminus, which allowed subsequent attachment to a polymeric backbone. PA was added to CHO-K1 cells at 4 °C to allow binding of PA and formation of $[PA_{63}]_7$ on the cell surface. The cells were then exposed to ^{35}S-labeled LF_N, the $[PA_{63}]_7$-binding domain of LF, in either the absence or presence of different concentrations of the peptides. Unbound ^{35}S-LF_N was washed away, and the remaining LF_N was measured in a scintillation counter. We found that the HYTYWWL(K) peptide had very little inhibitory activity compared to the peptides HYTYWWLD(K) or the 12-mer HTSTYWWLDGAP(K) (Figure 3). This would indicate that the Asp residue is important for binding $[PA_{63}]_7$.

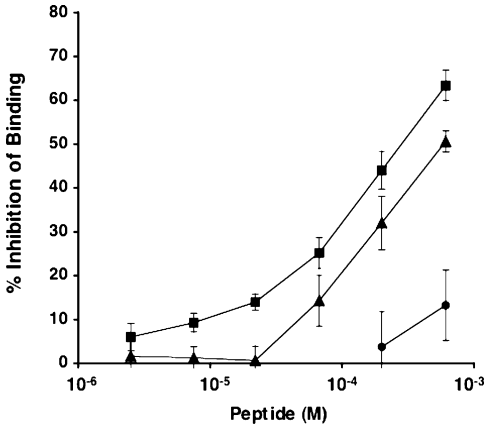

Figure 3. Inhibition of toxin assembly by peptides. CHO-K1 cells were incubated on ice with PA for 2 h, and unbound PA was then washed away with cold PBS. Cells were incubated with ^{35}S-LF_N in the presence of indicated concentrations of inhibitory peptides for 1 h, and the amount of ^{35}S-LF_N bound to $[PA_{63}]_7$ on the surface of the cells was measured by scintillation counting. The peptides used were HTSTYWWLDGAP(K) (squares), HYTYWWLD(K) (triangles), and HYTYWWL(K) (circles).

Because the shared sequence between the 12-mer and 7-mer peptides ended with the Asp residue, we hypothesized that the nature of the side chains carboxy-terminal to the Asp in the 12-mer would not influence the inhibitory activity of the peptides. Thus, we compared the inhibitory activities of HTSTYWWLDGAP(K), HTSTYWWLGGGG(K), and HTSTYWWLDGGG(K) and found that mutation of Ala and Pro to Gly residues did not impair the ability of the peptide to block the assembly of ^{35}S-LF_N (Figure 4). As expected, an additional Asp to Gly mutation did reduce the activity of the peptide.

We attached each of the three peptides to a polyhydroxyethylacrylamide backbone and compared the activities of the three polyvalent inhibitors. The mouse macrophage cell line

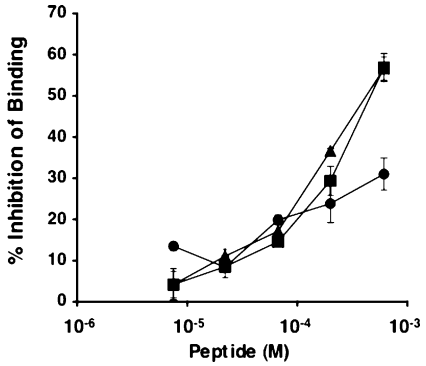

Figure 4. Inhibition of toxin assembly by peptides. The association of toxin was measured as the binding of ^{35}S-LF_N to $[PA_{63}]_7$ on the surface of CHO-K1 cells in the presence of indicated concentrations of peptides HTSTYWWLDGAP(K) (squares), HTSTYWWLDGGG(K) (triangles), and HTSTYWWLGGGG(K) (circles).

Table 2. Carboxy-Terminal Truncations of Inhibitory Peptides[a]

peptide	IC_{50} (M)
HTSTYWWLDGAPK	$1.8 \pm 0.8 \times 10^{-7}$
HTSTYWWLDK	$9 \pm 2 \times 10^{-8}$
HYTYWWLDK	$1.1 \pm 0.2 \times 10^{-7}$
HYTYWWLK	$1.9 \pm 0.2 \times 10^{-6}$

[a] The indicated peptides were attached to polyacrylamide backbones, and the IC_{50}s of the resulting inhibitors were determined in a RAW264.7 cell cytotoxicity assay. The results of three independent experiments ± SEM are shown.

RAW264.7 was treated with PA, LF, and various amounts of the polyvalent inhibitors, and cell viability was assessed using the MTS assay. We found that the IC_{50} for the polyvalent inhibitors displaying HTSTYWWLGGGG(K) was higher than for the inhibitors displaying either HTSTYWWLDGAP(K) or HTSTYWWDGGG(K), indicating that the Asp residue is required for maximal inhibitory activity (Table 1).

These data indicated that the side chains of residues carboxy-terminal to the Asp were not necessary for maximal inhibitory activity of the peptide. These residues might, however, form an important linker between the binding residues and the backbone. To address this possibility, we compared polyvalent inhibitors that were synthesized using either the 12-mer peptide, HTSTYWWLDGAP(K); a truncated version, HTSTYWWLD(K); the 7-mer peptide we identified, HYTYWWL(K); and the 7-mer with an additional Asp, HYTYWWLD(K). The IC_{50}s were determined using the RAW264.7 cell cytotoxicity assay (Table 2). We found that the IC_{50}s of these polyvalent inhibitors were very similar, except for the inhibitor that lacked the Asp residue. This result suggests that a linker region is not required between the binding residues and the backbone.

We hypothesized that the shared sequence between peptides HTSTYWWLDGAP and HYTYWWLD was both necessary and sufficient for maximal inhibitory activity. Thus, we compared the inhibitory activities in cytotoxicity assays of polyvalent inhibitors that displayed HTSTYWWLDGAP(K), TYWWLD(K), and YWWLD(K). The TYWWLD(K) sequence was sufficient to confer maximal activity, whereas the polyvalent inhibitor that displayed YWWLD(K) peptides had reduced activity compared to the other inhibitors (Table 3).

Table 3. Amino-Terminal Truncations of an Inhibitory Peptide[a]

peptide	IC_{50} (M)
HTSTYWWLDGAPK	$1.8 \pm 0.8 \times 10^{-7}$
TYWWLDK	$1.8 \pm 0.1 \times 10^{-7}$
YWWLDK	$1.2 \pm 0.3 \times 10^{-6}$

[a] The indicated peptides were attached to polyacrylamide backbones, and the IC_{50}s of the resulting inhibitors were determined in a RAW264.7 cell cytotoxicity assay. The results of three independent experiments ± SEM are shown.

Discussion

In this study we have identified the minimal peptide sequence required to maximally inhibit the assembly of LF to $[PA_{63}]_7$. We believe that the TYWWLD peptide will form the basis of novel anthrax toxin inhibitors, rather than the 12-mer peptide, because it is equally potent and its use would be cost-effective in large-scale syntheses required to stockpile anthrax therapeutics.

We screened a 7-mer phage display library to identify peptides that bound $[PA_{63}]_7$, but not PA. This strategy was previously devised to identify peptides from a 12-mer library that could block toxin assembly;[29] this procedure enriched for inhibitory peptides because the surface of $[PA_{63}]_7$ that binds the enzymatic moieties is not exposed in PA. An advantage of screening the 7-mer library is that it contains almost all of the possible amino acid combinations, whereas the 12-mer library contains only a small fraction. This screen yielded one peptide, HYTYWWL, that bound $[PA_{63}]_7$ and competed with LF. We also screened for phage that bound $[PA_{63}]_7$ and were eluted by LF (data not shown). This strategy was not as successful, and after several attempts we isolated only one clone that bound weakly to $[PA_{63}]_7$. Differences between the elution techniques may explain why this alternative procedure did not yield phage that bound tightly to the ligand binding site of $[PA_{63}]_7$. Elution of phage is based on shifting the equilibrium from phage bound to the adsorbed target to unbound phage. Soluble $[PA_{63}]_7$ elutes phage by providing a target that the phage bind after they dissociate from adsorbed $[PA_{63}]_7$, whereas the soluble LF elutes phage by blocking sites on adsorbed $[PA_{63}]_7$ to prevent rebinding of phage. LF, however, cannot block all of the potential binding sites on $[PA_{63}]_7$ because the maximum number of molecules of LF that can bind $[PA_{63}]_7$ is three.[12] Each LF molecule would block two adjacent monomers, but the seventh monomer would remain available for phage to bind. Thus, elution of phage by LF would be inefficient.

In previous work, the sequence YWWL was shared between two 12-mer peptides that were displayed by phage that bound $[PA_{63}]_7$,[29] so it was hypothesized that these amino

acids formed a binding site for $[PA_{63}]_7$. Glick et al. subsequently investigated the interactions of this tetrapeptide with the $[PA_{63}]_7$ structure using computational methods in an attempt to identify the binding site.[32] The phage peptide identified in our study also contained this tetrapeptide and, in addition, had two additional common amino acids that extended the shared sequence with the 12-mer to TYWWLD. We found that this six amino acid sequence was necessary and sufficient for potent inhibitory activity.

Hicks et al. used nuclear magnetic resonance to probe the structure of the HTSTYWWLDGAP peptide bound to $[PA_{63}]_7$.[33] They determined that residues 3 to 9 of this peptide (STYWWLD) maintain a helical conformation when the peptide binds $[PA_{63}]_7$, whereas the remaining N-terminal and C-terminal residues appear to be very flexible. They proposed that the N-terminal histidine residue plays a critical role in binding, even though it is more conformationally flexible than residues 3 to 9. Our data, however, indicates that neither this histidine residue nor the serine residue (which was found to maintain a helical conformation) contributes to the inhibitory activity of the peptide.

The peptides used in this study were synthesized with an additional carboxy-terminal Lys to facilitate coupling of the peptide to the backbone. We found that no additional amino acids were required to extend the TYWWLD sequence away from the backbone to allow the interaction with $[PA_{63}]_7$. This would imply that the backbone does not interfere with the peptide−$[PA_{63}]_7$ interaction.

The identification of determinants in an anthrax toxin inhibitory peptide has allowed us to synthesize a smaller peptide that has retained its potency. This will facilitate the characterization and development of clinically important compounds to treat anthrax infections.

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