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Screening of Peptides Bound to Anthrax Protective Antigen by Phage Display

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Abstract *Bacillus anthracis* is a causative agent of anthrax. Anthrax toxins are composed of a protective antigen (PA), lethal factor (LF), and edema factor (EF), in which the PA is a central mediator for the delivery of the two enzymatic moieties LF and EF. Therefore, the PA has been an attractive target in the prevention and vaccinization for anthrax toxin. Recently, it has been reported that the molecule consisting of multiple copies of PA-binding peptide, covalently linked to a flexible polymer backbone, blocked intoxification of anthrax toxin in an animal model. In the present study, we have screened novel diverse peptides that bind to PA with a high affinity (picomolar range) from an M13 peptide display library and characterized the binding regions of the peptides. Our works provide a basis to develop novel potent inhibitors or diagnostic probes with a diverse polyvalence.

Key words: Anthrax, protective antigen, PA-binding peptides, M13 phage display

Anthrax is a zoonotic disease whose etiologic agent is a Gram-positive sporulating bacterium, *Bacillus anthracis*. Although clinical anthrax is rare, there is growing concern over the potential use of *B. anthracis* in biological warfare and terrorism [6]. Virulence of the bacteria is due to two major antigens; antiphagocytic capsular antigen, which is unique among bacterial capsules consisting of poly-D-glutamic acid, and tripartite anthrax toxin. Anthrax toxins were composed of protective antigen (PA; 83 kDa), lethal factor (LF; 90 kDa), and edema factor (EF; 89 kDa), of which no single component is toxic but a combination of PA with either of LF or EF leads to pathogenesis in laboratory animals [4, 15 for reviews]. PA, the protein translocase component, binds to a cellular receptor and is cleaved to be activated by a furin family protease. The resulting C-terminal 63-kD receptor-bound fragment, PA63, self-assembles into the prepore, which is a ring-shaped homoheptamer [22]. The prepore then forms complexes with the two enzymatic components, LF and EF. These complexes are endocytosed and delivered to an acidic compartment [3]. There, the prepore undergoes an acidic pH-dependent conformational rearrangement [13] to form an ion-conducting, cation-selective, transmembrane pore [1], allowing bound LF and EF to translocate into the cytosol. Therefore, PA has been an attractive target for the prevention and therapeutics of anthrax. Although the bacteria can be eradicated from the host by treatment with antibiotics, such a therapy is of little value once symptoms have become evident, because of the continuing action of the toxin [8]. Thus, a specific inhibitor of the toxin's action might prove a valuable adjunct to antibiotic therapy.

Accumulating reports showed diverse approaches to inhibit the toxicity of anthrax based on the PA [2, 16, 24, 25]. It has been documented that molecules consisting of multiple copies of PA-binding peptide, covalently linked to a flexible polymer backbone, prevented assembly of the toxin complex *in vitro* and blocked toxin action in an animal model [16]. This method is shown to be an exceptionally useful approach when the molecular target is oligomeric. It may thus be applicable, for example, to the development of inhibitors of oligomeric microbial structures or to strategies for increasing the therapeutic potency of antitumor peptides [7, 9], growth factor receptor agonists [28], or molecules with related mechanisms of action.

Phage display of randomized peptide libraries has become one of the standard technologies for selecting peptides that target specific molecules. The principle of this technique is well-known and it is extensively used in various pharmaceutical biotechnologies [26]. Highly diverse libraries can be constructed

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by fusing a degenerate DNA fragment to one of the coat protein genes, which is encapsulated into the phage particles that display the encoded polypeptides on their surfaces. Library members with desired binding specificities can be isolated by binding to an immobilized receptor *in vitro*.

In this study, we isolated novel diverse peptides displaying on M13 bacteriophage that bind to PA83 with high affinity (picomolar range) from a phage display library. In addition, we addressed the binding regions of the peptides into two structural moieties of PA83, N-terminal PA20 and C-terminal PA63, respectively.

MATERIALS AND METHODS

Materials

E. coli strain BL21(DE3) was used as an expression host strain to produce anthrax protective antigen. Bacteria culture media, Bacto-tryptone, and Bacto-yeast extract, were purchased from Difco. Restriction enzyme, *Pfu* DNA polymerase, and T4 DNA ligase were from Promega. The M13 peptide library screening kit (Ph.D. phage display peptide library kit) was obtained from New England Biolabs. The other chemicals used, including isopropyl-1-thiogalactopyranoside (IPTG), were from Sigma.

Preparation of Protective Antigen

Construction and purification of recombinant PA (rPA83) were performed as described elsewhere [5, 17]. Briefly, the mature form of the PA gene (PA83) was subcloned into pET22b (Novagen) at BamHI and NotI sites (pET22-PA), in which target protein is expressed as a C-terminally 6-histidine (6His) fusion protein. The plasmid was transformed into BL21(DE3) and cells were induced to express the protein by addition of 0.5 mM IPTG at 18°C overnight. rPA83 was purified under denaturing condition as follows. Cell pellets were dissolved in buffer A (6 M guanidine hydrochloride, 20 mM Tris-HCl, 300 mM NaCl, 5 mM imidazole, 1 mM β-ME, pH 8.0) and soluble extracts were collected. The extracts were loaded onto a Ni-Sepharose column (Amersham Bioscience), which was pre-equilibrated with the same buffer. The column was washed with buffer B (6 M urea, 20 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole, 1 mM β-ME, pH 8.0) and renaturation of target protein was performed in on-column method by a decreasing concentration of urea. rPA83 was eluted by a 0.02–0.5 M linear gradient of imidazole and desalted in the buffer containing 20 mM Tris-HCl, pH 8.0. The eluate was further purified by a Mono-Q anion-exchange column. Fractions containing rPA83 were collected and extensively dialyzed against phosphate-buffered saline containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, at pH 7.4. Purified proteins showed a high homogeneity on 12% SDS-PAGE analysis.

Peptide Library Screening

The Ph.D.-12 Phage Display Peptide Library with a complexity of 2.7×10^9 was used for biopanning. Purified rPA was immobilized onto a Ni-Sorb plate (Qiagen) via its C-terminally fused 6His-tag, and wells were then covered by 2% BSA (2% bovine serum albumin, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20) for 1 h at room temperature. Wells were washed three times with the washing buffer W1 (50 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 0.1% Tween-20, and used for biopanning. An indicated amount of the phages was added to the well and incubated for 1 h at room temperature with gentle shaking. Wells were washed 10 times with the buffer W1 and bound phages were eluted in a chemical elution buffer (0.2 M glycine-HCl, pH 2.2, and 1 mg/ml BSA), which was collected and neutralized with the buffer C (1 M Tris-HCl, pH 9.1). At the following rounds of biopanning (from 2nd to 5th rounds), the concentrations of detergent or salt in washing buffer W1 were increased as follows: 0.3% (second round), 0.5% (third round), and 0.5% Tween-20 plus 500 mM NaCl (fourth and fifth rounds), respectively. At the final round, bound phages were eluted by addition of 10 molar excess of rPA83. Eluted phages at each round were infected into *E. coli* ER2738 cells to be amplified. After being cultured 5 h to propagate, the bacterial cells were removed by a centrifugation at 10,000 rpm for 10 min. The phages were then precipitated by adding PEG solution (20% (w/v) polyethylene glycol-8000, and 2.5 M NaCl) to the culture supernatant according to the manufacturer's guide. After centrifugation at 10,000 rpm for 20 min, phage pellets were resuspended in the buffer containing 50 mM Tris-HCl, pH 7.5, and 150 mM NaCl, and used for the next round of biopanning.

DNA Sequencing

The overnight culture (*E. coli* ER2738) was diluted 1:100 in LB and dispensed into 1 ml, into which individual phage displaying PA-binding peptide from a plaque was transferred. Cells were incubated at 37°C with shaking (200 rpm) for 5 h and then the supernatant containing the phages was harvested by a centrifugation. Two-hundred μl of PEG/NaCl (20% (w/v) polyethylene glycol-8000 and 2.5 M NaCl) solution was added to precipitate the phages, and the resulting pellet was thoroughly suspended in iodide buffer (containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 4 M NaI), followed by alcohol precipitation. Single-stranded phage DNA was recovered and dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). The amino acid sequence of the screened peptides was deduced from the genetic code information supplied by the manufacturer's guide (New England Biolabs), in which the randomized region of the library encodes all 20 amino acids with only 32 codons and this is aimed to increase the relative frequency of residues with a single codon, as well as removing 2 of

the 3 stop codons. The amber stop codon TAG is suppressed by glutamine (Q) in the strain *E. coli* ER2738.

Generation of PA63

PA63 was generated to estimate the binding map on the PA, as described elsewhere [13]. rPA83, which was immobilized on plate through its C-terminal 6His-tag, was treated with 1/50 molar trypsin and incubated for 1 h at room temperature with gentle shaking. The reaction was terminated by adding 100 molar excess soybean trypsin inhibitor and washed five times with a sufficient volume of TBST (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween-20). Cleavage of PA83 into PA20 (supernatant) and PA63 (well-bound) was confirmed by SDS/PAGE analysis.

Estimation of Binding Affinities on ELISA

The phages displaying PA-binding peptide were prepared as described above. The amplified phages were titered to determine the concentration of phage solution by counting of the number of plaques. To estimate the binding affinities of peptides, PAs (PA83 or PA63, 0.5 μg/well) were added to the individual wells of a polystyrene plate (SPL), in which the indicated concentration of the individual phages harboring binding peptide was added, and incubated for 1 h at room temperature. The wells were then washed five times with TBST buffer and probed with an anti-M13 antibody (mouse monoclonal antibody, 1:5,000 in TBST, 0.2 μg/ml, Amersham Bioscience) for 1 h at room temperature. After washing with TBST buffer five times, anti-mouse IgG-HRP conjugates were added and incubated for 30 min, followed by color-developing reaction with TMB substrate solution (3,3', 5,5'-tetramethylbenzidine (TMB)/H_2O_2, Chemicon). After 15 min, the reaction was terminated by the addition of 1 M H_2SO_4 and the optical densities were measured with a Biotrak multiwell plate reader (Amersham Bioscience) at 450 nm.

RESULTS

Expression and Purification of PA83

To screen the PA-binding peptides, we firstly constructed a bacterial overexpression system of rPA83 in pET22b vector, which produces the target protein as a C-terminally 6His-tag fusion protein. The protein expression was induced by addition of 0.5 mM IPTG at 18°C overnight. However, most of the target protein was present in the insoluble fraction. Induction temperature, IPTG concentration, and expression host were changed to find the optimum condition to increase protein solubility, but there was little improvement on protein solubility (data not shown). Therefore, we tried to purify rPA83 under the denaturing condition, as described elsewhere [5, 17]. Inclusion body was harvested after sonication and dissolved in the presence of 6 M guanidium-HCl. Dissolved extracts were loaded on a Ni-Sepharose column at room temperature and washed with the buffer containing 8 M urea. The protein was renatured in on-column method by slow decrease of the urea concentration and eluted by a 0–0.5 M linear gradient of imidazole (Fig. 1A). The eluate was further purified by a Mono-Q anion-exchange column chromatography (Fig. 1B). The purified protein showed a high homogeneity on 12% SDS-PAGE. Then, rPA83 was challenged with trypsin digestion to estimate the correct folding. It has been widely documented that only PA83 with a native structure cleaved into PA63 and

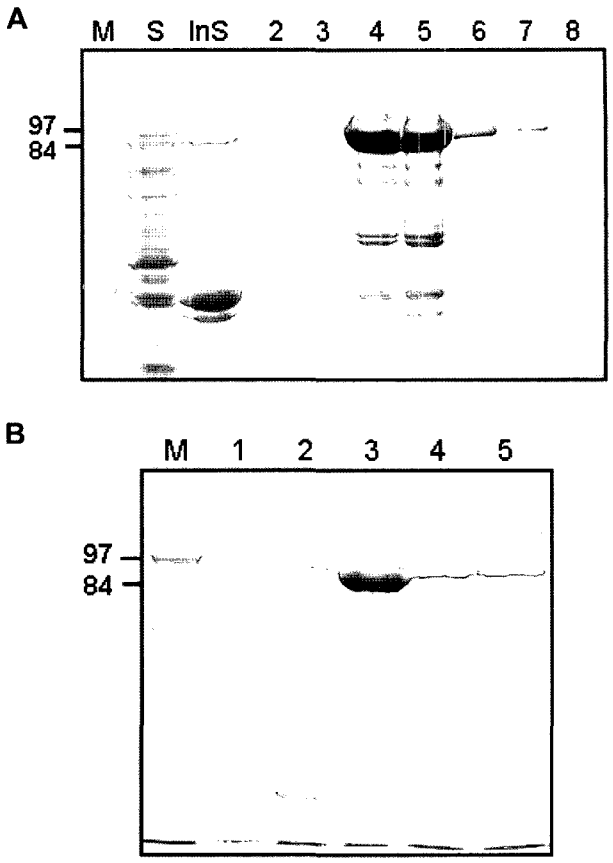

Fig. 1. Preparation of PA under the denaturing condition.
A. Cells harboring the expression vector, pET22b-PA, were induced to express the protein with 0.5 mM IPTG at 18°C overnight. Cells were harvested and lyzed by sonication (S). The resulting pellet, including the target protein as an inclusion body, was collected and dissolved in the buffer containing 6 M guanidium-HCl (InS), as described in Materials and Methods. Dissolved extracts were loaded onto a Ni-Sepharose column and the protein was renatured by a linear 6–0 M urea gradient (buffer: 20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole, and 1 mM β-ME), which took place over a period of 2 h in FPLC (Amersham Bioscience). The protein was eluted by a linear gradient of imidazole (20–500 mM) and fractions (2–8) were resolved on 12% SDS-PAGE. Fractions (4–5, ~200 mM imidazole) containing rPA83 were collected and desalted in the buffer containing 20 mM Tris-HCl, pH 8.0. **B.** The eluate from Ni-Sepharose was further purified on a Mono-Q anion-exchange column. The protein was eluted by a linear 0–0.5 M NaCl gradient and fractions (1–5) were resolved on 12% SDS-PAGE. The target protein (rPA83) was purified with high homogeneity in fraction #3. M, molecular weight marker.

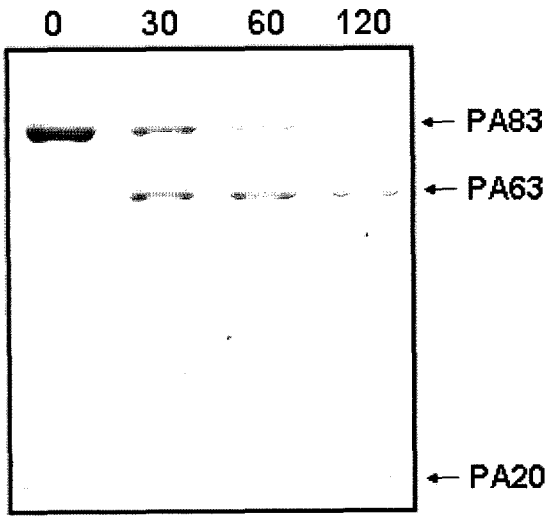

Fig. 2. Confirmation of protein refolding.
Prior to phage display screening, the purified PA83 was treated with trypsin to estimate its native structure from the purification in denaturing condition. Ten μg of PA83 was challenged with 1/50 molar trypsin at room temperature and samples were withdrawn at the indicated time periods (0, 30, 60, 120 min). The reaction was terminated by the addition of 100 molar excess soybean trypsin inhibitor, and cleavage of PA was monitored on 12% SDS-PAGE.

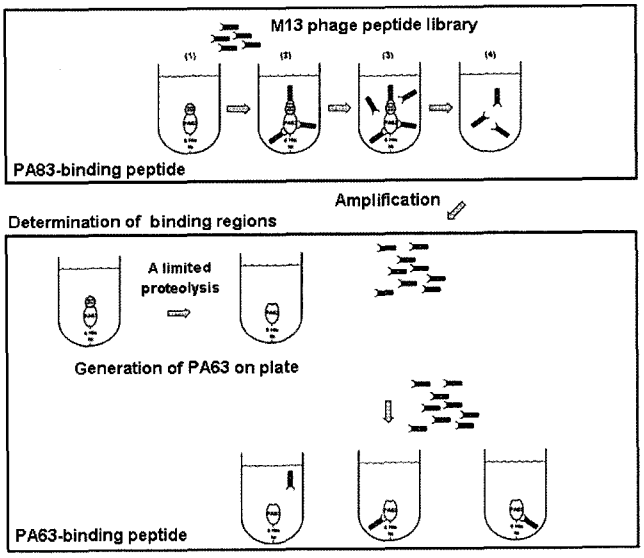

Fig. 3. Schematic diagram of the experimental approach for PA-binding peptide screening by a phage display (Upper box).
To screen a family of a phage displaying PA83-binding peptide, PA83 was immobilized onto the plate (Ni-Sorb, Qiagen) and the phage library was added. After 5 rounds of biopanning, the phages displaying PA-binding peptide with high affinity were collected and analyzed to determine the amino acid sequence corresponding to the peptide. (Lower box) The phages obtained were added to PA63 prepared as described in the text. While the phages preferable for PA20 are washed out from the well, the phages displaying PA63-binding peptide remain on the well with interaction with immobilized PA63, which can be monitored by ELISA using anti-M13 antibody, which specifically recognizes major coat protein gpVIII.

PA20 by a serine-like protease such as furin or trypsin. As shown in Fig. 2, treatment of trypsin produced PA63 and PA20 as expected, suggesting that the renaturation of protein was successfully performed.

Peptide Library Screening

To screen the peptides that specifically bind to PA, we performed M13 phage display. We designed the experiments to select the family of a phage displaying PA83-binding peptide and classify them into the binding region, PA20 and PA63, respectively (illustrated in Fig. 3). We firstly immobilized PA83 onto a plate (Ni-Sorb plate, Qiagen) and added the M13 phage libraries displaying random 12-

Table 1. PA-binding peptides displayed on M13 phage.

Number	Occurrences[a]	Peptide sequences[b]	Binding affinities[c] (K_d^{app}, pM)
P1	15	**L**(S/T)**HN**(Q/L)**T**(I/L)**QQ**(D/A)**SD**	97.81±21.3
P2	24	**HKHAHN**(Y/T)**RLPXS**	4.06±0.33
P3	11	TPYYWHHHHIPP	41.4±0.9
P4	10	QSPVNHHYHYHI	119.4±25.5
P5	9	(G/A)(H/L)KPQVHRHTH(I/L)	136.8±59.9
P6	16	LMPTPHHRLFPM	25.4±3.3
P7	7	ALHPHRTPHHHH	268.1±10.8
P8	8	NAYKHHHPPVFY	68.5±12.3

[a]After 5 round cycles, 100 plaques were selected and their genes corresponding to the displayed peptides at M13 minor coat protein gpIII analyzed. Phages were classified according to the deduced amino acids of the peptide using the BioEidt program and the number of phages in the same group was shown.
[b]Amino acid sequences of peptides were deduced from the manufacturer's genetic code information, in which the library region encodes all 20 amino acids with only 32 codons. The third (wobble) position of the codon is G or T. This increases the relative frequency of residues with a single codon, as well as removing 2 (TAA and TAG) of the 3 stop codons. The amber stop codon TAG is suppressed by glutamine (Q). Amino acid is shown as a single-letter representation (X, any amino acid) and degenerate amino acids in the peptide are represented in the parenthesis.
[c]Binding affinity was determined as described in Materials and Methods. rPA83 (0.5 mg) was immobilized onto individual wells of a polystyrene plate with a random orientation and each phage displaying the peptide was added. After extensive washing, the bound phage was estimated by an anti-M13 antibody, which specifically recognizes the major coat protein (gpVIII) of M13 phage. The value (apparent dissociation constant, K_d^{app}) was obtained in a binding-saturation curve fitting from three-independent experiments.

residue peptides fused to the N terminus of the pIII protein. After incubation, we washed the well with an increasing stringency, and then eluted the phages by addition of 0.2 M glycine-HCl, pH 2.2. At the final biopanning round, soluble PA83 was added, and phages that adsorbed to it were recovered. After five cycles of biopanning, we selected 100 plaques and determined DNA sequences corresponding to the displayed peptides. Single-strand M13 DNA was isolated and DNA sequencing was carried out. Based on the amino acid information deduced by the DNA sequence, the phages were categorized into 8 groups according to their amino acid similarity (Table 1).

Estimation of Binding Affinities

The selected phages displaying PA-binding peptide were further characterized to estimate their binding affinities on PA83. At first, the phages were amplified and purified. The concentration of each phage was determined by titering of serial diluents of the purified phage solution. To analyze the binding affinity, an enzyme-linked immunosorbent assay was performed. PA83 was immobilized onto a plate and then individual phage was added in a concentration-dependent manner. After 1 h of incubation at room temperature, the wells were extensively washed with W1 containing 0.5% Tween-20 and 0.5 M NaCl 10 times, followed by addition of anti-M13 antibody (0.2 μg/ml in TBST), which specifically recognizes the M13 coat protein product of gene VIII (gp8). After color-development reaction coupled with HRP-conjugates, the optical density was measured at 450 nm. As shown in Fig. 4, most of the phages had comparable binding affinities to PA83 in pico (10^{-12}) to nanomolar (10^{-9}) ranges. Specifically, phages containing P2 (HKHAHN(Y/T)RLPXS), or P6 (LMPTPHHRLFPM) showed most higher bindings on PA83 *in vitro*. The binding affinities of each peptide displayed on M13 phages are summarized in Table 1.

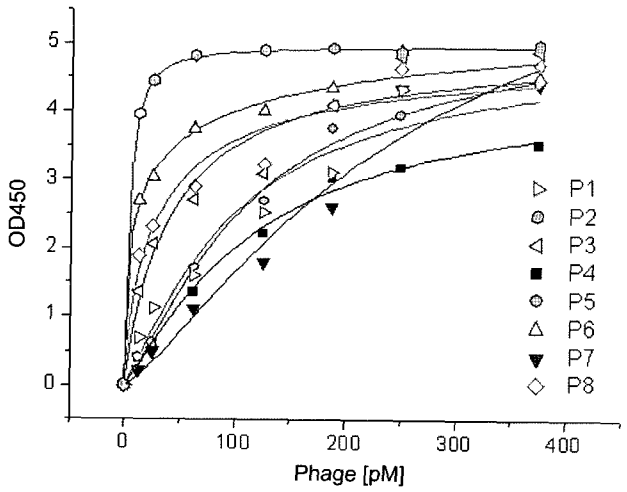

Fig. 4. Determination of binding affinities.

The binding affinities of individual phages encoding PA-binding peptide were determined by ELISA as described in Materials and Methods. The concentration of amplified phage solution was calculated as follows: Concentration=(the total number of phage in solution/6.02×10^{23})/ suspension volume. Total number of phage was determined by counting the number of plaque of individual phage on titering plate. The representative data was one of the three-independent experiments and the binding affinity, which is documented as an apparent K_d, was obtained from the non-linear fitting program for the saturation binding curve. The values for each phage were summarized on Table 1.

Determination of Binding Regions

Next, the phages were classified based on the binding region. PA83 is composed of 2 functional moieties, N-terminal PA20 and C-terminal PA63 [18]. During the toxification, PA83 binds to the receptor (ATR/CMG2) at the cell surface, where the protein undergoes cleavage by a furin-like protease into a functionally active C-terminal PA63 fragment. This cleavage of PA83 into two fragments enables the fragment that remains bound to the cell, PA63, to heptamerize and bind EF and/or LF with high affinity (K_d, ~1 nM) [14]. Therefore, it is of interest to identify the binding site of the PA-binding peptide, which provides information to develop a novel peptide inhibitor to compete with the binding regions of LF and/or EF, leading to the inhibition of toxin complex formation. To this end, rPA83 was immobilized onto a Ni-Sorb plate (Qiagen) in a defined orientation by the interaction between its C-terminal 6His-tag and Ni-ion coordinated well surface, and then PA63 was generated by a limited proteolysis with 1/50 molar excess trypsin. The selected phages (P1-P8) were added to the well and the binding was determined by ELISA, as described above. As shown in Fig. 5, phages containing P2, P3, P4, or P6 bound to PA63 and the others (P1, P5, P7, P8, and P9) had a

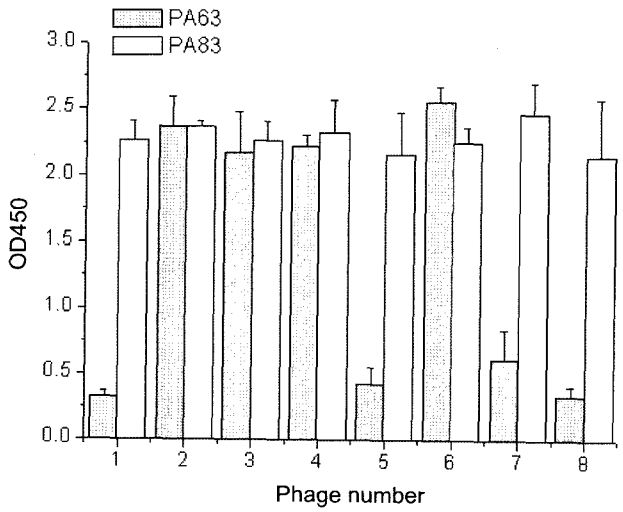

Fig. 5. Estimation of the interaction site of PA-binding peptides on PA.

The binding region of the phages on PA was determined as illustrated in Fig. 3. The binding of individual phages on PA63 was estimated by ELISA using an anti-M13 antibody. The representative data were from the average of four independent experiments.

preference to PA20. The binding affinities of the phages on the fragments (PA63) were nearly similar to those of the phages on the entire form of the PA (PA83).

DISCUSSION

Anthrax toxin is composed of three nontoxic proteins, which combine on eukaryotic cell surfaces to form toxic complexes. Among the three proteins, protective antigen (PA) is a central mediator of anthrax toxicity. After internalization of the toxin complex between PA and LF/EF by receptor (ATR/CMG2)-mediated endocytosis [22], the complexes are trafficked to the endosome. There, at low pH, the PA moiety inserts into the membrane and mediates translocation of EF and LF to the cytosol. This intoxification mechanism makes PA an attractive target for prevention and therapeutics of anthrax. In fact, the use of polyclonal antibodies against PA has been shown to give moderate protection against a lethal challenge with *B. anthracis* in guinea pigs [11] and is being further developed [9]. Moreover, in the past year, novel toxin inhibitors against PA have been described [15]. A synthetic inhibitor was one of the successful approaches, which was designed by selecting a peptide from a phage-display library that was able to compete with LF for its binding to the PA63 heptamer [16]. The authors isolated two related peptides (P1, HTSTYWWLDGAP, and P2, HQLPQYWWLSPG) that binds weakly to the heptameric cell-binding subunit of anthrax toxin and prevents the interaction between cell-binding and enzymatic moieties. Multiple copies of these peptides were grafted onto a polyacrylamide backbone, resulting in a polyvalent molecule that could prevent intoxication of rats challenged with purified LeTx. Polyvalent display greatly enhanced the biological efficacy of the peptide, probably because the resulting molecule could bind simultaneously to multiple sites of the heptamer. It should also be noted that the flexibility of the backbone permitted synthesis of the molecule without the knowledge for the binding site of the peptide on the heptamer. Therefore, it is of interest to find a number of peptides with various binding regions on target molecule, which can increase the efficiency of the polyvalent peptide inhibitor. That is, the polyvalent molecule harboring multiple copies of various peptides with diverse binding regions can bind and wrap the target more tightly to inhibit its biological activity.

In this study, we reported on novel peptides displaying on M13 bacteriophage that bind to various regions on PA83 with a high affinity (picomolar range) from a phage display library. Phage display was first developed with the *E. coli*-specific bacteriophage M13 [23, 27]. These include systems that utilize other *E. coli*-specific phages, such as λ-phage [21] and T4 phage [20], and also systems that use eukaryotic viruses [19]. The M13 phage particle consists of a single-stranded DNA core surrounded by a coat composed of five different proteins. The length of the filament is covered by several thousand copies of the major coat protein, gpVIII. Each end of the particle is capped by five copies each of two minor coat proteins, gpIII and gpVI at one end, and gpVII and gpIX at the other end [12 for review]. We screened the PA-binding peptides from the library with a 2.7×10^9 complexity, of which eight different peptides were found to be bound with PA83, the entire form of native PA (Table 1). ELISA showed that phages carrying these peptides specifically and tightly bound to PA83 with picomolar ranges (10^{-12} M). More importantly, these peptides had different binding regions on PA83: PA20 (P2-4 and P6) and PA63 (P1, P5, and P7-9). Considering that efficacy of polyvalent inhibitors depends on a number of binding sites, it is important to find the peptide with various binding sites on the target.

However, our research needs to further investigate the characteristics of the peptide. Since we have screened and analyzed the peptides displaying on M13 phages, their characteristics seem not to be in line with that of a peptide. The binding affinity of a single peptide to the target is relatively lower than that of antibody. However, the chemical and physical stability of a peptide makes it a more applicable form in clinical and engineering fields. Our observation might provide an opportunity to construct novel potent inhibitors or diagnostic probes with an increased polyvalency.

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