Identification of Amino Acid Residues of Anthrax Protective Antigen Involved in Binding with Lethal Factor

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Protective antigen (PA) and lethal factor (LF) are the two components of anthrax lethal toxin. PA is responsible for the translocation of LF to the cytosol. The binding of LF to cell surface receptor-bound PA is a prerequisite for the formation of lethal toxin. It has been hypothesized that hydrophobic residues P184, L187, F202, L203, P205, I207, I210, W226, and F236 of domain 1b of PA play an important role in the binding of PA to LF. These residues are normally buried in the 83-kDa version of PA, PA83, as determined by the crystal structure of PA. However, they become exposed due to the conformational change brought about by the cleavage of PA83 to PA63 by a cell surface protease. Mutation of the above-mentioned residues to alanine resulted in mutant proteins that were able to bind to the cell surface receptors and also to be specifically cleaved by the cellular proteases. All the mutant proteins except the F202A, L203A, P205A, and I207A mutants were able to bind to LF and were also toxic to macrophage cells in combination with LF. It was concluded that residues 202, 203, 205, and 207 of PA are essential for the binding of LF to PA.

The primary virulence factor in anthrax pathogenesis is the three-protein exotoxin of *Bacillus anthracis*. It consists of protective antigen (PA; 83 kDa), which is responsible for the translocation of the other two proteins to the cytosol, lethal factor (LF; 90 kDa), which is a zinc metalloprotease (17), and edema factor (EF; 89 kDa), which is a calmodulin-dependent adenylate cyclase (14, 18, 19). None of the proteins is individually toxic. PA combines with LF to form lethal toxin and combines with EF to form edema toxin.

Anthrax intoxication of cells follows a series of steps. One of the first steps is the proteolytic activation of the 83-kDa version of PA, PA83, to PA63 by a cell surface protease after it binds to the anthrax toxin cell surface receptor (1, 8, 9, 28). The cleaved-off N-terminal fragment of PA has no further role in the intoxication process. This cleavage exposes a high-affinity site on PA to which LF and EF can bind competitively (21). Upon exposure to acidic pH after receptor-mediated endocytosis, PA63 heptamerizes and inserts itself into the membrane as a pore and also translocates EF and LF into the cytosol (22, 33). The exact mechanism by which PA allows LF and EF to pass into the cytosol remains a mystery. A prerequisite to this process is the binding of LF or EF to PA. The crystal structure of PA is known, and therefore it is possible to predict the residues of PA that might play an important role in the binding of LF or EF to PA (26).

PA has four domains, which are organized primarily into antiparallel beta sheets with only a few short helices of less than four turns. It is known that domain 1 is responsible for binding to LF and EF during the anthrax intoxication process (25, 26). A protease cleavage site divides domain 1 into the two subdomains, PA20 or subdomain 1a (residues 1 to 167) and subdomain 1b (residues 168 to 249). The removal of PA20 not

only disrupts hydrogen bonding and side chain interactions between the two subdomains but also causes several residues to become exposed, resulting in the creation of a large hydrophobic surface on the rest of domain 1b. This surface is in the form of a large, flat, hydrophobic patch on the top of the heptamer (6, 26).

Domains are known to represent substructures with specific functional properties, in the sense that spatially separated parts of a large polypeptide chain form compact entities with well-defined properties for binding to ligands such as substrates, coenzymes, effectors, etc. (16). The different domains of proteins are usually involved in different functions. Mutagenesis of specific residues in a domain can be successfully utilized to predict its function. Since it has been hypothesized that domain 1b of anthrax PA plays an important role in the binding of LF to PA, the present study aimed to mutagenize this domain in order to understand the role of domain 1b in the intoxication process of *B. anthracis* exotoxin and to define the role of hydrophobic residues P184, L187, F202, L203, P205, I207, I210, W226, and F236 in this domain in the binding of PA to LF. These are the residues that are normally buried in PA83 but that become exposed due to the conformational change brought about by the proteolytic activation of PA83 to PA63. The hydrophobic residues of most of the soluble proteins are buried inside the core, whereas the hydrophilic residues are exposed on the outer side. Hydrophobic residues present on the surfaces of most proteins are known to play a specific functional role such as interaction with another protein, ligand, or receptor. Since the presence of hydrophobic residues on the surface compromises the structural stability of the protein to a certain extent, it is interesting to investigate the functional role of these specific hydrophobic residues displayed on the surface of proteolytically activated PA.

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MATERIALS AND METHODS

Reagents and supplies. Agarose (SeaKem GTG) was from FMC Corp. Acrylamide, ampicillin, bovine serum albumin (BSA), Coomassie brilliant blue R-250,

TABLE 1. Sequences of the primers used for long PCR

Mutation or primer type	Primer sequence ^a (5'-3')
I210A	CTT TTC ATG <u>AGC</u> ATT AGA AAT CCA
I207A	CTT TTC ATG AAT ATT AGA <u>AGC</u> CCA TGG
P205A	CTT TTC ATG AAT ATT AGA AAT CCA <u>TGC</u> TGA AAG
L203A	CTT TTC ATG AAT ATT AGA AAT CCA TGG TGA <u>AGC</u> AAA AGT
F202A	CTT TTC ATG AAT ATT AGA AAT CCA TGG TGA AAG <u>AGC</u> ATG TCT
F184A	TTT GGT TAA CCC TTT CTT TTC ATG AAT ATT AGA AAT CCA TGG TGA AAG AAA AGT TCT TTT ATT TTT GAC ATC AAC CGT ATA TCC TTC TAC CTC TAA TGA ATC <u>AGC</u> GAT TCC
L187A	TTT GGT TAA CCC TTT CTT TTC ATG AAT ATT AGA AAT CCA TGG TGA AAG AAA AGT TCT TTT ATT TTT GAC ATC AAC CGT ATA TCC TTC TAC CTC <u>TGC</u> TGA ATC
Forward	GGA TTT CTA ATA TTC ATG AAA AGA AAG GAT TAA CCA AAT ATA AAT CAT CTC CTG AAA AAT GGA GCA CGG CTT CTG ATC C
W226A	GGA TTT CTA ATA TTC ATG AAA AGA AAG GAT TAA CCA AAT ATA AAT CAT CTC CTG AAA AAG <u>CGA</u> GCA CGG CTT CTG ATC C
F236A	GGA TTT CTA ATA TTC ATG AAA AGA AAG GAT TAA CCA AAT ATA AAT CAT CTC CTG AAA AAT GGA GCA CGG CTT CTG ATC CGT ACA GTG ATG <u>CCG</u> AAA AGG TT
Reverse	CTT TTC ATG AAT ATT AGA AAT CCA TGG TGA AAG AAA AGT

^a Mutation point is underlined.

calcium chloride, glycine, glutamine, glycerol, glucose, disodium hydrogen phosphate, sodium dihydrogen phosphate, potassium acetate, HEPES, phenylmethylsulfonyl fluoride, sodium dodecyl sulfate (SDS), sodium acetate, sodium chloride, sodium hydroxide, sodium bicarbonate, penicillin, streptomycin, imidazole, diaminobenzidine, Tris, Tween 20, protein determination dye, and other chemicals were purchased from U.S. Biochemicals (Cleveland, Ohio). Nitrocellulose membranes and radioactive chemicals such as ³⁵S-dATP and ¹²⁵I-Na were purchased from Amersham Pharmacia Biotech (Piscataway, N.J.). Ni-nitrilotriacetic acid (NTA) agarose was purchased from Qiagen (Valencia, Calif.). Cell culture plasticware was obtained from Corning (Acton, Mass.). Fetal calf serum, RPMI 1640, trypsin, 3-(4,5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide (MTT), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid (CHAPS), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.). Medium components for bacterial growth were purchased from Hi-Media Laboratories (Bombay, India). Prestained protein molecular weight markers were purchased from Bio-Rad Laboratories (Hercules, Calif.). PA and LF purified from *B. anthracis* was a generous gift from Stephen H. Leppla (National Institute of Dental and Craniofacial Research, National Institutes of Health).

DNA-modifying enzymes and reagents. The enzymes and chemicals used for DNA manipulation were purchased from Invitrogen (Carlsbad, Calif.), Roche Diagnostics Corporation (Indianapolis, Ind.), Amersham Inc., and New England Biolabs (Beverly, Mass.). The oligonucleotides were obtained from Monica Talmor, Critical Technologies for Molecular Medicine, Yale University Medical School. The PCR was performed on a Perkin-Elmer thermal cycler by using a DNA amplification kit from Perkin-Elmer. Expression vector pQE30 was obtained from Qiagen.

Bacterial strains and cell lines. *Escherichia coli* strain DH5 α and macrophage-like cell line RAW 264.7 were obtained from the American Type Culture Collection (Manassas, Va.).

Construction of the mutants using site-directed mutagenesis. The PA gene was mutagenized by using nine different mutagenic primers spanning the desired mutation point. All the mutations led to the change of the specified amino acids to alanine. Two oligonucleotides were used to prime DNA synthesis by the high-fidelity *Pfu* polymerase on the denatured plasmid template (3, 4). The two oligonucleotides both contained the desired mutation and annealed to the complementary strands. Entire lengths of both the strands of the plasmid DNA were

amplified in a linear fashion during several rounds of thermal cycling, generating a mutant plasmid with staggered nicks on the opposite strands (7, 13). The sequences of the primers are shown in Table 1.

PCR was performed in a 20- μ l reaction mixture by using a DNA thermal cycler (Perkin-Elmer) with 0.2-ml thin-wall tubes. The reaction mixture consisted of 10 ng of pMW1 template DNA (12), 0.2 mM (each) deoxynucleoside triphosphate (Amersham Inc.), 0.1 nmol of each oligonucleotide, 2 μ l of *Pfu* DNA polymerase buffer (10 $\times$), and 0.3 U of *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) (27). The product of the amplification was treated with *DpnI*, which specifically cleaves fully methylated G^{me}ATC sequences (32). *DpnI*-resistant molecules were recovered by transformation of the DNA into *E. coli* DH5 α cells (2) and selection on ampicillin plates. The plasmids were screened further by restriction analysis with *KpnI* and *BamHI* restriction endonucleases. The mutations were confirmed by cycle sequencing (Perkin-Elmer cycle sequencing kit).

Expression and purification of the mutant proteins. *E. coli* DH5 α cells harboring the mutant plasmids were grown on Luria-Bertani (LB) media containing 100 μ g of ampicillin/ml at 37°C and 250 rpm overnight (O/N). These O/N seed culture cells (5 ml) were diluted in shake flasks containing 100 ml of LB medium and incubated at 37°C and 250 rpm, and O/N culture aliquots were collected and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and also electroblotted.

The mutant PA proteins were purified by Ni-NTA metal chelate affinity chromatography under denaturing conditions as follows (10). The pellets from 1-liter cultures were resuspended in 25 ml of denaturing buffer containing 100 mM sodium phosphate buffer, 300 mM sodium chloride, and 8 M urea (pH 8.0). The resuspended pellets were incubated at 37°C for 60 min on a rotary shaker. The lysates were centrifuged for 30 min at room temperature, and the supernatants were mixed with a 50% Ni-NTA slurry. The slurry was packed into separate columns and allowed to settle. The Ni-NTA matrix was washed with 50 ml of denaturing buffer containing 8 M urea, followed by on-column renaturation of the proteins by using a gradient of 8 to 0 M urea. The mutant proteins were eluted with 250 mM imidazole chloride in elution buffer (100 mM sodium phosphate [pH 8.0], 250 mM imidazole, 300 mM sodium chloride). Fractions containing the protein were collected, pooled, and dialyzed against 10 mM HEPES buffer containing 50 mM NaCl and stored frozen at -70°C in aliquots. The purified mutant proteins were estimated by Bradford's method, subjected to SDS-PAGE, and electroblotted.

Iodination of PA mutants and LF. PA mutants and native LF (purified from *B. anthracis*) were iodinated by the chloramine T method of Hunter and Greenwood (15). In brief, the protein (50 μ g) was mixed gently with 100 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 5 mg of chloramine T. Sodium [¹²⁵I]iodide (1 mCi) was added to the mixture, and the mixture was incubated on ice for 5 min. The reaction was terminated by adding 10 mg of sodium metabisulfite in 0.1 M sodium phosphate buffer, pH 7.0. The labeled protein was separated from free iodine by passing the reaction mixture on a gel filtration (Sephadex G-25) column preequilibrated with phosphate-buffered saline (PBS). Nonspecific binding of the iodinated protein was minimized by washing the column with 1% BSA in PBS. Fractions of 0.5 ml were collected and monitored with a gamma counter. Fractions showing the first peak, representing the iodinated protein, were pooled. The labeled protein was stored at 4°C.

Binding of PA protein to cell surface receptors. The binding of the PA protein to cell surface receptors was carried out in 24-well plates by using a constant amount of radioiodinated mutant PA (1 μ g). PA from pMW1 was used as the positive control. RAW 264.7 cells were washed twice with cold Hanks balanced salt solution (HBSS) for 5 min each time and then placed on ice. The medium was replaced with cold binding medium (minimum essential Eagle's medium with Earle's salts without sodium bicarbonate containing 1% BSA and 25 mM HEPES, pH 7.4). The cells were incubated with 1 μ g of each iodinated mutant PA at 4°C for 3 h and then washed with cold HBSS. The cells were dissolved in 0.1 N NaOH, and radioactivity was measured with a gamma counter.

Proteolytic cleavage of mutant PA proteins in solution. The mutant PA proteins were tested for susceptibility to cleavage by trypsin. The proteins (1.0 mg/ml) were incubated with trypsin (1 ng/ μ g of protein) for 30 min at room temperature in 25 mM HEPES-1 mM CaCl₂-0.5 mM EDTA, pH 7.5. The digestion reactions were stopped by adding phenylmethylsulfonyl fluoride to a final concentration of 1 mM. Samples were visualized on an SDS-12% PAGE gel.

Binding of nicked PA mutants to LF in solution. Trypsin-nicked mutant proteins (1.0 mg/ml) (W226 and L203 W226 mutants were prone to degradation and were taken at a concentration of 0.1 mg/ml) were incubated with LF (1.0 mg/ml) in 25 mM Tris, pH 9.0, containing 2 mg of CHAPS/ml for 15 min at room temperature. Samples were applied to a nondenaturing 4.5% polyacrylamide gel.

Binding of nicked mutant proteins to LF on cells. RAW 264.7 cells were washed twice with cold HBSS for 5 min each time and then placed on ice. The

FIG. 1. (A) Electrophoretic analysis showing protein purification of PA from *E. coli* cells expressing mutant PA proteins. Proteins were separated by SDS-12% PAGE and stained with Coomassie blue. (B) Western blot of the Ni-NTA-purified mutant PA proteins, developed with a rabbit polyclonal antibody against PA. STD. PA, PA purified from *B. anthracis*.

medium was replaced with cold binding medium (minimum essential Eagle's medium with Earle's salts without sodium bicarbonate containing 1% BSA and 25 mM HEPES, pH 7.4). The cells were incubated with 1 μ g of each mutant PA at 4°C for 3 h and then washed with cold HBSS followed by an O/N incubation with 1 μ g of radioiodinated LF at 4°C. The cells were washed with cold HBSS and then dissolved in 0.1 N NaOH, and radioactivity was measured with a gamma counter.

Cell culture and cytotoxicity assay. For the biological assay, a 96-well culture plate with ~90% cell density was prepared. PA mutants were added in various concentrations with LF (1 μ g/ml), and the plate was incubated for 3 h at 37°C. PA from pMW1 along with LF was used as a positive control. Cell viability was determined by using MTT dye. MTT dissolved in RPMI 1640 was added to each well at a final concentration of 0.5 mg/ml, and the plate was incubated for another 45 min at 37°C to allow uptake and oxidation of the dye by viable cells. The medium was replaced by 0.5% (wt/vol) SDS-25 mM HCl in 90% isopropyl alcohol, and the plate was vortexed. The absorption was read at 540 nm by using a microplate reader (Bio-Rad).

RESULTS

Construction of the mutants by site-directed mutagenesis.

To elucidate the importance of residues P184, L187, F202, L203, P205, I207, I210, W226, and F236 of domain 1b of PA in binding to LF, these residues were changed to alanine by site-directed mutagenesis using long PCR on the pMW1 plasmid (12). Template plasmid pMW1 contains the entire PA gene cloned into expression vector pQE30 and expresses PA as a recombinant six-histidine-tagged fusion protein that is biologically and functionally similar to PA from *B. anthracis* (12). The change to alanine ensures that the hydrophobic character of these residues remains unchanged, with only a truncation of

the side chains of the residues being studied, so that there is minimal resulting change in the native protein conformation. The mutations were confirmed by cycle sequencing the region of interest.

Expression of PA gene mutants in *E. coli* DH5 α cells. To check the expression of the mutant proteins from the recombinant mutant plasmids in *E. coli* DH5 α cells, the cultures were grown O/N and the protein profile was studied. Expression was established by SDS-PAGE and Western blot analysis. The mutant proteins were expressed simultaneously with the culture growth and could be seen on the SDS-PAGE gel as an 83-kDa band corresponding to the standard PA. W226A and L203A W226A mutants were found to express the protein at lower levels than the other mutants (data not shown), and they were therefore concentrated and used for selected experiments.

Purification of the mutant proteins. The mutant proteins were present as inclusion bodies inside the cells. The recombinant PA from pMW1 and mutant PA proteins were purified from inclusion bodies under denaturing conditions using 8 M urea along with Ni-NTA affinity chromatography. Protein renaturation and refolding on the Ni-NTA column itself were carried out prior to elution. The proteins obtained after affinity chromatography were more than 95% pure (Fig. 1A). Mutant protein yields of ~32 to 37 mg/liter were obtained. The purified proteins were electroblotted, and the Western blot was developed with anti-PA antibodies raised against PA purified from *B. anthracis*. (Fig. 1B).

TABLE 2. Binding of mutant proteins to cell surface receptors^a

Protein	Total cpm $\pm$ SE	cpm/ μ g of protein (10 ⁷)	PA in total cell protein ^b (ng)	% Receptor binding ^c
I210A	82,806 $\pm$ 1,500	1.24	6.68	95.6
I207A	81,980 $\pm$ 1,872	1.23	6.67	95.4
P205A	75,549 $\pm$ 1,922	1.15	6.57	94.1
L203A	85,666 $\pm$ 1,277	1.28	6.69	95.8
F202A	78,192 $\pm$ 1,455	1.08	7.24	103.7
F184A	81,538 $\pm$ 2,769	1.21	6.74	96.5
L187A	79,956 $\pm$ 1,550	1.20	6.66	95.4
W226A	82,854 $\pm$ 1,380	1.19	6.96	99.7
F236A	81,354 $\pm$ 2,976	1.27	6.41	91.7
L203A W226A	78,989 $\pm$ 1,355	1.08	7.31	104.7
P205A W226A F236A	77,540 $\pm$ 1,882	1.09	7.11	101.9
PA from pMW1	78,922 $\pm$ 2,636	1.13	6.98	100.0

^a RAW 264.7 cells were incubated with 1 μ g of radioiodinated mutant and native PA for 3 h at 4°C.

^b Protein content of the cells per well was 0.91 $\pm$ 0.05 mg as determined by Lowry's method. All the experiments were done in triplicate.

^c Percent binding of the mutant proteins to the cellular receptors in comparison with that for pMW1 PA.

Binding of mutant PA proteins to cell surface receptors. Receptor binding of mutants and that of native PA were compared by radiolabeling the proteins and allowing them to bind to cell surface receptors present on RAW 264.7 cells. The total radioactivity bound to the cell surface was measured. The total cell protein was found to be 0.91 $\pm$ 0.05 mg. The cells were also incubated with a 100-fold excess of cold PA from pMW1 along with radiolabeled PA, which resulted in less than 5% ($\sim$ 365 cpm) of the counts of the radiolabeled PA being detected. Incubation with similar amounts of BSA was not found to decrease the counts of the radiolabeled PA bound to the cells, demonstrating the specificity of the interaction of PA with its receptors. Mutant proteins were found to bind to the cell surface receptors in amounts comparable with those for native PA from pMW1, reflecting the fact that the mutants are also able to interact with the receptors in a specific manner (Table 2).

Proteolytic cleavage of mutant PA proteins in solution. The proteolytic cleavage of PA and mutant PA proteins was studied by treatment of PA and mutant PA proteins with trypsin and analysis of the proteolytically degraded products by SDS-12% PAGE. Trypsin treatment cleaved all the mutant PA proteins into two fragments of 63 and 20 kDa, similar to what was found for native PA (Fig. 2).

Binding of nicked PA mutants to LF in solution. Trypsin-nicked mutant proteins (1.0 mg/ml) were incubated with LF (1.0 mg/ml), and the samples were applied to a nondenaturing 4.5% polyacrylamide gel. The formation of the PA-LF complex was seen (10). The results indicated that I207A, P205A, L203A, and F202A mutants and the P205A W226A F236A triple mutant were almost unable to bind to LF. The ability of I210A, F184A, and L187A mutants to bind to LF appeared to be decreased. The F236A mutant protein appeared to be able to bind to LF to the same extent as native PA (Fig. 3).

Binding of mutant proteins to LF on cells. To check the binding of mutant proteins to LF in vivo, macrophage-like cell line RAW 264.7 was incubated with mutant proteins and further incubated with radioiodinated LF. The results obtained were similar to those obtained in vitro. It was observed that not all the mutant PA proteins were capable of binding LF. The I207A, P205A, L203A, and F202A mutants and the P205A

W226A F236A triple mutant were almost unable to bind to LF, with values ranging from 0.8 to 12.6% in comparison to that for PA from pMW1. The ability of the I210A, F184A, and L187A mutants to bind to LF was decreased to a range of 47.5 to 68.0%. W226A, F236A, and L203A W226A mutants were found to be able to bind to LF to almost the same extent as PA from pMW1 (Table 3).

Cell culture and cytotoxicity assay. I207A, P205A, L203A, and F202A mutants and the P205A W226A F236A triple mutant, in combination with LF, were completely nontoxic to RAW 264.7 cells, even at concentrations of 10 μ g/ml. I210A, F184A, and L187A mutants were found to be partially toxic at higher concentrations. Forty-five to 55% cell death was seen to occur at a protein concentration of 10 μ g/ml. W226A, F236A, and L203 W226 mutants were found to be as toxic as native PA in combination with LF (Fig. 4 and Table 4).

DISCUSSION

On the basis of the crystal structure, it was predicted that nine hydrophobic residues of domain 1b of PA, P184, L187, F202, L203, P205, I207, I210, W226, and F236, are present at the interface between PA and LF and are likely to be involved in the association of PA with LF (26). The N-terminal residues of LF involved in the binding to PA have already been reported by our laboratory (11), but the residues on PA that interact with these residues on LF have not been pinpointed (20). It is known that interfaces between associated proteins are poorer in polar charged residues than surfaces and are richer in hydrophobic residues. The interior of the interfaces appears to constitute a compromise between stabilization contributed by the hydrophobic effect on one hand and avoiding patches on the protein surfaces that are too hydrophobic on the other hand (30). We aimed to explore whether the hydrophobic effect that guides protein folding is also the main driving force for protein-protein association in the case of PA and LF. Protein association is an example of rigid association, where only six degrees of translational and rotational freedom are available to the folding polypeptide chains to achieve their favorable bound configuration. Due to the above constraints, when proteins interact with each other, the hydrophobic effect,

FIG. 2. Electrophoretic analysis showing trypsin nicking of the mutant proteins. Proteins were separated by SDS-12% PAGE and stained with Coomassie blue. STD. PA, PA purified from *B. anthracis*.

which is the dominant driving force, together with a hydrophilic interaction, determines the most favorable configuration. The geometric and electrostatic complementarity observed within interfaces has been the basis of many studies of the docking of two proteins of known structure (references 29 and 31 and references therein). It is widely accepted that conformational changes occur upon binding. The crystal structure of LF has been recently determined (24). For PA and LF, we can conclusively prove that some conformational changes occur upon association of the two only after the crystal structure of the PA-LF complex becomes available.

The above-mentioned residues of PA that are believed to be critical in PA-LF binding have been investigated via site-directed alanine cassette mutagenesis (5). Amino acid residues P184, L187, F202, L203, P205, I207, I210, W226, and F236 were each individually changed to alanine because the systematic replacement of charged amino acids with alanine residues eliminates side chains beyond the beta carbon and disrupts the functional interaction of the amino acids without changing the conformation of the main chain of the protein. Alanine also serves as a good replacement for both hydrophobic and hydrophilic residues without disrupting the protein structure. To distinguish the mutations that affect local structures from those that have profound and deleterious effects on the folding and stability of the entire protein, the mutant proteins were checked for their ability to be specifically cleaved with trypsin. Trypsin treatment cleaved all the mutant PA proteins into two fragments of 63 and 20 kDa, similar to native PA. Since mis-

FIG. 3. Native PAGE of binding of LF to mutant PA proteins in solution.

folding of a protein is known to expose buried or inaccessible protease sites, it can be inferred that the mutant proteins probably fold into native conformation.

Since residues P184, L187, F202, L203, P205, I207, I210, W226, and F236 were likely to play a role in the PA-LF binding, this function of PA, along with the other steps of the mechanism of action that occur before PA-LF binding, were investigated (Fig. 5). All the mutant proteins were able

TABLE 3. Binding of mutant proteins to radiolabeled LF on cells^a

Protein	Total cpm $\pm$ SE	LF in total cell protein (ng)	% Binding ^b
I210A	65,787 $\pm$ 1,775	3.59	68.0
I207A	5,654 $\pm$ 867	0.31	5.8
P205A	768 $\pm$ 47	0.04	0.8
L203A	12,234 $\pm$ 788	0.67	12.6
F202A	6,783 $\pm$ 355	0.37	7.0
F184A	45,989 $\pm$ 1,832	2.51	47.5
L187A	63,378 $\pm$ 2,456	3.46	65.5
W226A	96,630 $\pm$ 3,020	5.28	97.9
F236A	99,877 $\pm$ 2,287	5.46	103.2
L203A W226A	95,674 $\pm$ 1,567	5.23	98.9
P205A W226A F236A	457 $\pm$ 33	0.02	0.5
PA from pMW1	96,749 $\pm$ 1,564	5.29	100

^a RAW 264.7 cells were incubated with 1 μ g of mutant and native PA for 3 h at 4°C, followed by O/N incubation with 1 μ g of radioiodinated LF (1.83×10^7 cpm/ μ g) at 4°C. All the experiments were done in triplicate.

^b Percent binding of the mutant proteins to the cellular receptors in comparison with that for pMW1 PA.

FIG. 4. Cytotoxicity profile of the mutants on RAW 264.7 cells.

to bind the cell surface receptor and to be cleaved by trypsin, but the ability to bind to LF, both in solution and on macrophage cells, showed great variation. It was severely impaired for the F202A, L203A, P205A, and I207A mutants and the P205A W226A F236A triple mutant, with the binding of PA to LF on cells reduced to only 7.0, 12.6, 0.8, and 5.8%, respectively, of that for PA from pMW1. Possibly as a result of this inability to bind to LF, these mutants also show a loss of cytotoxic activity on macrophages in combination with LF, suggesting an important role for these residues in the biological activity of the lethal toxin. W226A and F236A mutants and the L203A W226A mutant are fully cytotoxic in combination with LF, with levels of binding that were 97.9, 103.2, and 98.9%, respectively, of those for PA from pMW1. This is probably because these residues are not involved in interaction with LF. P184A, L187A, I210A mutants were found to have a decreased ability to bind to LF, with levels of binding that were 47.5, 65.5, and 68.0%, respectively, of those for PA from pMW1, and were only toxic to macrophage cells at high concentrations, suggesting that they play a role, however insignificant, in the intoxication process by

being able to interact with LF. Among the individual mutants, the P205A mutant was fully nontoxic while the W226A and F236A mutants were fully toxic. The combination mutant is possibly nontoxic because residue P205 is indispensable in the binding of PA to LF. Replacement of this residue with alanine might also lead to a local destabilization of the domain structure, resulting in the inability of the other two residues, residues 226 and 236 (normally fully capable of binding to LF), to interact with residues of the PA binding domain of LF. Similarly, a combination of a mutation resulting in no toxicity, L203A, with a mutation resulting in full toxicity, W226A, was seen to result in a toxic mutant, implying that even in the absence of side chain interactions of L203 with LF residues, the other mutation somehow renders the molecule able to bind to LF.

An interesting pattern is seen to emerge when the results of this study (on the LF binding domain of PA) and the mutations in the PA binding domain of LF done in our laboratory (11) are analyzed together. The residues on PA most important in the binding to LF are 202, 203, 205, and 207. The residues on LF, which are crucial for interaction with the residues on PA are 148, 149, 151, and 153. For both PA and LF these key amino acids are close together on the same stretch. The latest structural features to emerge as having functional significance are the surfaces of proteins. The regions of protein interaction are nearly always correlated with the largest contiguously concave patch on the surface (23). It can be speculated that the residues 148, 149, 151, and 153 are close together and form a hydrophobic patch on a polypeptide loop that extends outwards from the surface of LF and that probably forms hydrophobic interactions with the surface on PA on which the side chains of residues 202, 203, 205, and 207 extend (Fig. 5). It can be concluded that, in the interaction of proteins PA and LF, it is this small stretch of residues that is critical. This opens up an entirely new area for the design of a peptide vaccine against PA. Peptides as short as the PA binding stretch on LF may be studied as effective therapeutic agents against anthrax, as they would be fully capable of blocking the action of the toxin (PA, to be specific). Alternately, the above-mentioned completely nontoxic PA mutants, which are unable to bind to LF, can by themselves be used as potential vaccine candidates in combination with both LF and EF. The crystal structure of LF has been determined recently (24). This would also make it possible to determine the exact side chain interactions between residues 148, 149, 151, and 153 on LF and residues 202, 203, 205, and 207 on PA. The side effects of the cell-free vaccines in use are mainly due to the presence of trace quantities of LF and EF, which cause toxicity in combination with PA. It has been seen that the toxin proteins are able to enhance the immunogenicity manifold in a synergistic manner when present together, in comparison to the immunity induced by the use of a single toxin component. An ideal vaccine should contain all the three toxin components together, but such a combination of toxin proteins would be lethal. The above-mentioned mutants may serve as potential vaccine candidates in combination with LF or EF since they would be completely nontoxic due to their inability to bind to each other but would be fully immunogenic due to the presence of all toxin components.

TABLE 4. Relative cytotoxicity profile of the mutants in terms of EC_{50} values

Protein	EC_{50}^a (μ g/ml)
P184A.....	>10
L187A.....	7
F202A.....	>10
L203A.....	>10
P205A.....	>10
I207A.....	>10
I210A.....	10
W226A.....	0.06
F236A.....	0.07
L203A W226A.....	0.06
P205A W226A F236A.....	>10
PA from pMW1.....	0.065

^a EC_{50} , concentration of PA required to kill 50% of RAW 264.7 cells in combination with LF (1 μ g/ml).

**THE EXPOSED
SURFACE OF PA WITH
WHICH THE SURFACE
OF LF MAY INTERACT
AFTER REMOVAL OF
PA20**

FIG. 5. Structure of domain 1. The furin cleavage site is shown along with the mutated residues.

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