Biochemical and Biophysical Research Communications **280,** 158–163 (2001)
doi:10.1006/bbrc.2000.4099, available online at http://www.idealibrary.com on IDEAL®

Involvement of Residues 147VYYEIGK153 in Binding of Lethal Factor to Protective Antigen of *Bacillus anthracis*

Pankaj Gupta,[1] Aparna Singh, Vibha Chauhan, and Rakesh Bhatnagar[2]
Centre for Biotechnology, Jawaharlal Nehru University, New Delhi 110067, India

Received November 20, 2000

Anthrax toxin is a complex of protective antigen (PA, 735 aa), lethal factor (LF, 776 aa), and edema factor (EF, 767 aa). PA binds to cell surface receptors and is cleaved by cell surface proteases into PA_{63}, while LF and EF compete for binding to PA_{63}. The PA_{63}–LF/EF complex is internalized into the cytosol and causes different pathogenic responses in animals and cultured cells. 1–300 amino acid residues of LF have been viewed as the region responsible for the high affinity binding of LF to PA. Amino acid analysis of LF and EF revealed a common stretch of 7 amino acids (147VYYEIGK153). In the present study, each amino acid of this stretch was replaced by alanine at a time. Y148A, Y149A, I151A, and K153A mutants were found to be deficient in their ability to lyse J774A.1 cells and their binding ability to PA_{63} was drastically reduced. We propose that these four amino acids play a crucial role in the process of binding of LF to PA_{63}.

Anthrax is a disease of herbivores caused by *Bacillus anthracis*, a Gram positive spore forming bacterium. Human infection is accidental and cases arise due to contact with the infected animals or animal products. Virulence of the bacterium is attributed to two factors, a poly D-glutamic acid capsule and a three component protein exotoxin. The genes coding for the toxin and the enzymes responsible for the capsule production are carried on *B. anthracis* plasmid pXO1 and pXO2 respectively (1, 2). The three proteins of the exotoxin are protective antigen (PA 83 kDa), lethal factor (LF 90 kDa), and edema factor (EF 89 kDa). None of the proteins are individually toxic but they combine pairwise to form lethal (PA + LF) and edema (PA + EF) toxins (3–5). PA acts as the common receptor binding moiety and it interacts with EF or LF to mediate their entry into the target cells (6, 7). PA binds to cell surface receptors where it is cleaved by furin like cellular proteases generating a cell bound, 63-kDa protein (PA_{63}) with a high affinity binding site for EF or LF (6). The complex is subsequently internalized by receptor-mediated endocytosis into endosomes (8, 9). Acidification of the endosomes brings about the oligomerization of PA_{63} and is believed to play a crucial role in the process of translocation of LF/EF in the cytosol (10). Mouse peritoneal macrophages and macrophage like cell lines such as J774A.1 and RAW 264.7, etc. are sensitive to anthrax lethal toxin (11–13). Protein synthesis and calcium is required for expression of anthrax lethal toxin activity in the macrophages (13, 14). Lethal toxin causes over production of certain cytokines such as IL-1β and TNF-α in its target cells (15). There is activation of phospholipase C and protein kinase C in macrophages during cytolysis (16). LF acts as an endopeptidase and cleaves the amino terminus of mitogen activated protein kinase kinases 1and 2 (MAPKKs) and inhibits the MAPK signal transduction pathway. However the exact mechanism of cell death is not yet established (17).

Amino acid analysis of LF and EF reveals an extensive homology in the residues 1–300 (18). Since both the proteins compete to bind to receptor bound, nicked PA and the intracellular actions and enzymatic activities of these two proteins are different, the regions of homology have been viewed as the regions responsible for high affinity binding to PA (18–20). Further insight into the amino acid sequence comparison of LF^{1-250} and EF^{1-250} using BESTFIT program of GCG revealed a stretch of 7 amino acids 147VYYEIGK153 which is present in lethal factor as well as edema factor. The aim of the study was to determine the importance of these seven residues in the process of binding of LF to PA.

MATERIALS AND METHODS

Reagents and supplies. The enzymes and chemicals used for DNA manipulation were purchased from Life Technologies (USA);

[1] Present address: Dept. of Biological Sciences, Columbia University, New York, NY.
[2] To whom correspondence should be addressed. Fax: (91) 11-6165886/6198234/6169962. E-mail: rakesh@jnuniv.ernet.in or rakbhat@hotmail.com.

0006-291X/01 $35.00

TABLE 1

Sequence of the Oligonucleotides Used for Creating Mutants

Oligonucleotide	Construct	Protein
5′ GTA GAA GGT ACC GAA AAG GCA CTG AAC GCT TAT TAT 3′	pPG-LF3	V^{147A}
5′ GTA GAA GGT ACC GAA AAG GCA CTG AAC GTT GCT TAT 3′	pPG-LF4	Y^{148A}
5′ GTA GAA GGT ACC GAA AAG GCA CTG AAC GTT TAT GCT GAA 3′	pPG-LF5	Y^{149A}
5′ GTA GAA GGT ACC GAA AAG GCA CTG AAC GTT TAT TAT GCA ATA 3′	pPG-LF6	E^{150A}
5′ GTA GAA GGT ACC GAA AAG GCA CTG AAC GTT TAT TAT GAA GCA GGT 3′	pPG-LF7	I^{151A}
5′ GTA GAA GGT ACC GAA AAG GCA CTG AAC GTT TAT TAT GAA ATA GCA AAG 3′	pPG-LF8	G^{152A}
5′ GTA GAA GGT ACC GAA AAG GCA CTG AAC GTT TAT TAT GAA ATA GGT GCA ATA 3′	pPG-LF9	K^{153A}
5′ GTA GAA GGT ACC GAA AAG GCA CTG AAC ATA TTA TCA 3′	pPG-LF10	LF^{del}

Note. Sequence of the reverse oligonucleotide used is 5′ GAA AAT TTT TAA TAG TCG ACT TAT GAG 3′.

Boehringer Mannheim (Germany); Amersham Inc. (UK); and New England Biolabs (USA). The oligonucleotides were obtained from Monica Talmor (Critical Technologies for Molecular Medicine, Yale University Medical School, USA). The PCR was performed on Perkin Elmer thermal cycler using DNA amplification kit from Perkin Elmer (USA). DNA purification kit, gel extraction kit, expression vector pQE30, *Escherichia coli* SG13009 cells and Ni-NTA agarose were obtained from Qiagen (Germany). Agarose (Sea Kem GTG) was from FMC Corp (USA). Mono-Q column were purchased from Pharmacia Biotech (Sweden). Cell culture plasticwares were obtained from Corning (USA). Fetal calf serum (FCS), RPMI 1640, Dulbecco's modified Eagle medium (DMEM), Hank's balanced salt solution (HBSS), trypsin, 3-(4,5-dimethylthiazol-2-yl),-5-diphenyltetrazolium bromide (MTT), 3-{(3-cholamidopropyl) dimethyl ammonio}-1-propanesulfonic acid (CHAPS), isopropyl-thio-β-D-galactopyranoside (IPTG), bovine serum albumin (BSA) and other chemicals were purchased from Sigma Chemical Co. (USA). J774A.1, a macrophage like cell line was obtained from ATCC (American type culture collection) (USA). Media components for bacterial growth were purchased from Hi-Media Laboratories (India).

Plasmid construction. A *Kpn*I site was constructed just upstream to the region of interest. To create a *Kpn*I restriction site, DNA fragments encoding LF^{1-143} and $LF^{138-776}$ were PCR amplified. The sequence of the primers used for the amplification of LF^{1-143} was 5′ GTA CAG GGA TCC GCG GGC GGT 3′ and 5′ CTT TTC GGT ACC TTC TAC ATA 3′ that added *Bam*HI and *Kpn*I restriction enzyme sites to the 5′ and 3′ ends of the PCR product. The oligos used for amplification of $LF^{138-776}$ were 5′ GTA GAA GGT ACC GAA AAG GCA 3′ and 5′ GAA AAT TTT TAA TAG TCG ACT TAT GAG 3′ which added *Kpn*I and *Sal*I sites to the flanking 5′ and 3′ ends of the PCR product. PCR was performed in a 100 μl reaction volume containing 100 ng of pXO1 (template), 0.25 μM (20–30 pmol of DNA) of each primer, 200 μM of each dNTPs, 1U of Pfu DNA polymerase and 10 μl of 10× buffer (supplied). Amplification was done with an initial hot start of 95°C for 5 min followed by 30 cycles of 94°C/30 s, 55°C/30 s, and 72°C/30 s, and a hold of 7 min at 72°C for LF^{1-143} while amplification of $LF^{138-776}$ involved 30 cycles of 94°C/1 min, 50°C/1 min and 72°C/2 min along with an initial denaturation at 94°C for 5 min and final extension at 72°C for 7 min. The PCR products were purified using a gel extraction kit (Qiagen) and cloned in pGEM-T vector (Promega) according to the manufacturer's instructions. White colonies were screened for the presence of insert by minipreparation of plasmid DNA and restriction analysis. Plasmid DNA was prepared from the clones containing LF^{1-143} and $LF^{138-776}$ DNA fragments in vector pGEM-T. About 50 μg of DNA from both constructs was digested with the restriction enzymes respective to the sites present at the 5′ and 3′ ends of the PCR product. The bands of desired size were excised from the gel and the DNA was eluted from the gel. The expression vector pQE30 was digested separately with the restriction enzymes *Bam*HI and *Sal*I. The digested vector DNA and the inserts were ligated. The transformants were screened for the presence of construct containing *Kpn*I restriction enzyme site upstream to the region of homology by restriction analysis. The resulting construct was designated as pPG-LF2.

To create mutations at the point of seven amino acid homology, i.e., 147VYYEIGK153 in the amino terminus of LF, LF gene extending from residues 138 to 776 was amplified using a primer containing *Kpn*I site at the 5′ end and mutation at the point of homology. In one of the forward oligonucleotide primers, the whole stretch of 21 nucleotides coding for seven amino acids was deleted. The backward oligonucleotide primer used in the PCR was complementary to the 3′ end of the LF gene followed by a *Sal*I site. The sequence of the oligonucleotide primers is given in the Table 1. The primers described in the table were used in eight different PCR reactions using pXO1 DNA as template to amplify DNA fragments having *Kpn*I and *Sal*I sites at the 5′ and 3′ ends respectively alongwith substitution/deletion at the point of homology. The reaction conditions used were similar to that used for amplification of $LF^{138-776}$ DNA. The purified PCR products were cloned in pGEM-T vector and transformed in competent *E. coli* DH5α cells. White colonies were screened for the presence of insert by minipreparation of plasmid DNA and restriction analysis. Plasmid DNA was prepared from the clones containing deleted and mutant $LF^{138-776}$ DNA fragments in vector pGEM-T. About 50 μg of DNA from all constructs was digested with the restriction enzymes *Kpn*I and *Sal*I. The bands of desired size were excised from the gel and the DNA was eluted from the gel. The construct pPG-LF2 was digested separately with the restriction enzymes *Kpn*I and *Sal*I and the backbone was eluted from the gel. The gel eluted mutants $LF^{138-776}$ were ligated to the *Kpn*I and *Sal*I sites of the construct pPG-LF2 to generate new constructs. The transformants were screened for the presence of construct containing *Kpn*I restriction site. Positive clones were confirmed by restriction analysis followed by sequencing using Sanger's dideoxy method.

Small scale expression of mutant LF in E. coli. To ascertain the expression of LF mutants, few colonies of different expression constructs were randomly selected and each colony was inoculated into a 5.0 ml LB medium containing 100 μg of ampicillin per ml and 25 μg of kanamycin per ml. Cultures were grown overnight at 37°C/250 rpm. 1.0 ml of the overnight grown culture was inoculated into a 10 ml LB flask containing 100 μg of ampicillin per ml and 25 μg of kanamycin per ml and grown at 37°C and 250 rpm. When A_{600} reached 0.7–0.9, cultures were induced with 1 mM IPTG and were further grown at 37–C and 250 rpm for 4–5 h. 1 ml of the culture from each sample was aliquoted, spun at 14,000 × rpm for 15 min. Twenty-five microliters of the supernatant of each sample was run on 12% SDS–PAGE. Proteins were either electro-blotted or visualized by Coomassie blue staining.

Purification of mutant/deleted LF proteins. The proteins were purified using metal chelate affinity chromatography followed by anion exchange chromatography on FPLC (21, 22) as follows. The pellet from 1 litre culture was resuspended in 25 ml of sonication

buffer. PMSF was added to a final concentration of 1 mM and sonicated at 4°C for 5 cycles. Lysate was centrifuged at 10,000*g* for 30 min and the supernatant was mixed with Ni-NTA slurry. Slurry was packed into a column and allowed to settle. Ni-NTA matrix was washed with wash buffer of pH 6.0 and the proteins were eluted with a gradient of 0 and 500 mM Imidazole chloride in elution buffer. Ten to fifteen microliters of each sample was analyzed on 12% SDS–PAGE. Fractions containing the protein were collected, pooled and dialyzed against $T_{10}E_5$ (Tris 10 mM and EDTA 5 mM pH 8.0) buffer. To further purify, protein was loaded onto an anion exchange Mono-Q column. Protein was eluted with a linear gradient of 0 and 1 M NaCl in $T_{10}E_5$ buffer. The purified proteins were dialyzed against 10 mM Hepes buffer containing 50 mM NaCl and stored frozen at −70°C in aliquots. The yield of different mutants was determined by Lowry's method (23).

Macrophage lysis assay. Different mutant LF proteins were assayed for their functional activity in the J774A.1 macrophage lysis assay, as described earlier (11, 14). Varying concentrations of LF mutants alongwith PA (1 μg/ml) were added to the cells. The native LF alongwith PA was kept as the positive control. After 3 h, viability was determined using MTT dye and the resulting precipitate was dissolved in a buffer containing 0.5% (w/v) sodium dodecyl sulfate, 25 mM HCl in 90% isopropyl alcohol. Absorption was read at 540 nm and percent viability was determined.

Receptor binding assay. The nontoxic LF mutants were checked for their ability to bind to receptor bound PA_{63} (11). The native LF was radiolabeled using chloramine T method and a competitive binding experiment was performed in presence of varying concentration of LF mutants. J774A.1 cells were plated in 12-well plates. The next day, plates containing J774A.1 cells were cooled at 4°C for 15 min. The medium was then replaced with cold binding medium (DMEM containing 1% BSA and 25 mM Hepes, pH 7.4) and PA (0.5 μg/ml). After 4 h, the cells were washed and binding media containing radiolabeled LF (0.1 μg/ml ^{125}I-LF, 8×10^6 cpm/μg) and different concentrations of different LF mutants (0.1 μg, 1 μg, 5 μg and 10 μg) were added in different wells and cells were incubated for 12 h at 4°C as previously described (11). The cells were washed four times with cold HBSS, solubilized in 0.5 ml of 100 mM NaOH and cell associated radioactivity was measured using a gamma counter. Nonspecific binding of ^{125}I-LF to cells was determined by incubating the cells with 100 fold excess of LF without PA. From the amount of radioactivity bound to cells, competitive binding of LF mutants to PA (% control) was determined.

Competitive binding of LF mutants to PA in solution. Radiolabeled ^{125}I-LF was used to study the binding of LF mutants to proteolytically cleaved PA. PA molecule was cleaved to PA_{63} by incubating with trypsin (1 ng/μg protein) for 30 min at 25°C in 25 mM Hepes, 1 mM $CaCl_2$ and 0.5 mM EDTA (11). Trypsin nicked PA (1 μg/μl) was incubated with LF mutants (1 μg/μl) and ^{125}I-LF (100,000 cpm) in 25 mM Tris pH 9.0, containing 2 mg/ml Chaps (3-{(3-cholamidopropyl) dimethyl ammonio}-propanesulfonic acid) for 15 min at room temperature. Samples were applied to non denaturing 4–15% polyacrylamide gradient Phast gels (Pharmacia LKB Biotechnology Ltd., native buffer strips). Gels were dried and autoradiographed.

RESULTS AND DISCUSSION

The importance of certain amino acid residues in a protein has been ascertained by site directed mutagenesis (24–28). Since the crystal structure of LF is not known, mutagenesis study is the only method to determine the importance of individual amino acid residues in the process of pathogenesis. Quinn *et al.* (20) performed functional mapping of LF by in frame insertion analysis and described the importance of its amino terminus and carboxyl terminus in the process of binding and catalysis respectively. However the exact residues responsible for the catalytic and binding activity were not known. Insertion analysis in the amino terminus of LF eliminated both toxicity and binding to PA. The residues 1 to 254 of LF are sufficient for binding to protective antigen component of the toxin (18, 19). Both the molecules (LF and EF) bind to PA, and they share a remarkable homology in the amino terminus that is believed to contain the PA binding domain. The maximal homology between LF and EF has been seen in a stretch of 7 amino acids (^{147}VYYEIGK^{153} of mature LF). In this study, attempts have been made to determine the role of these seven residues in the binding of LF to PA.

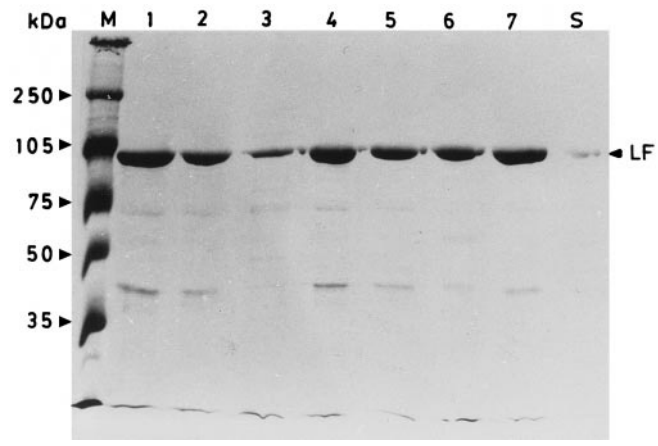


FIG. 1. Purified LF mutants from *E. coli.* The purified mutant proteins were analyzed on 12% SDS–PAGE and stained with Coomassie blue. Lanes 1–7 showing mutant proteins LF3, LF4, LF5, LF6, LF7, LF8, and LF9 respectively. Lane S, native LF and Lane M, molecular weight standards.

Creation of a KpnI Restriction Site in LF and Purification

The recombinant protein from the construct pPG-LF2 was purified using Ni-NTA affinity chromatography and anion exchange Mono-Q column on FPLC. The purified protein was checked for its ability to bind to trypsin nicked PA. The protein was also checked for its functional and biological activity by the macrophage lysis assay. It was found that protein from the construct pPG-LF2 could bind to trypsin nicked PA and the mobility of the PA-LF complex was retarded on nondenaturing PAGE (Fig. 1). The protein (LF^{KpnI}) along with PA was equally toxic to J774A.1 macrophages like the native LF.

Construction of the Mutants at the Point of Homology

The seven amino acids, i.e., residues ^{147}VYYEIGK^{153} of mature LF were substituted one by one with alanine to generate new constructs pPG-LF3–pPGLF10. In the

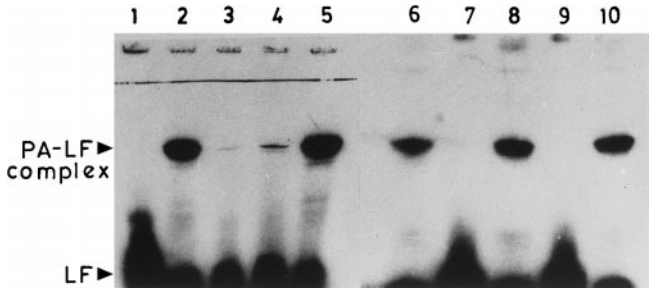


FIG. 2. Binding of LF2 to PA protein in solution. LF native from construct pPG-LF1 and LF2 prom the construct pPG-LF2 (1 μg) were incubated with trypsin nicked PA (1 μg) for 15 min and the samples were analyzed on a nondenaturing 8–25% Phast gradient gel. The gel was stained with Coomassie blue and dried Lane A, PA; Lane B, native LF; Lane C, PA nicked with trypsin incubated with native LF; Lane D, LF2; and Lane E, PA nicked with trypsin incubated with LF2.

constructs pPG-LF3 to pPG-LF9, one amino acid each of the 7 amino acids homology stretch was substituted with alanine while in the construct pPG-LF10, the whole 7 amino acid stretch was deleted. The different constructs were confirmed by the dideoxy sequencing of the region of interest (data not shown).

Expression and Purification of LF Mutants

Mutant LF proteins were purified using Ni-NTA affinity chromatography. The proteins were further purified by anion exchange Mono-Q column (Fig. 2). The yield of mutant proteins where one amino acid each was substituted with alanine were in between 1.5–2.0 mg per litre of the culture. Unfortunately the deletion mutant protein could not be purified as the protein got destabilized.

Toxicity of Mutant LF Proteins on J774A.1 Cells

The mutant LF proteins were tested for their ability to lyse J774A.1 cells, in the presence of 1 μg/ml PA. The toxicity results have been shown in the Fig. 3. Native LF lysed 50% of the cells at 0.030 μg/ml in 3 h while substitution of the residues by alanine in mutants V147A, E150A, and G152A had a negligible effect on toxicity. Mutants I151A and K153A were slightly toxic (at higher concentrations) while mutants Y148A and Y149A were nontoxic. It was observed that mutants V147A, E150A, and G152A were toxic and killed the J774A.1 macrophages like the native LF in 3–4 h. However, EC_{50} of E^{150} was 0.1 μg/ml which is 3 times more than the EC_{50} of native LF (0.030 μg/ml). The EC_{50} of V147A and G152A resembled EC_{50} of native LF. This is probably because in mutants V147A and G152A, valine and glycine is substituted with alanine resulting in minimal change in overall structure of the mutant proteins. The mutants Y148A, Y149A, I151A and K153A were nontoxic when used at a concentration of 1 μg/ml in macrophage lysis assay. EC_{50} of I151A and K153A was 4.5 and 4.0 μg/ml respectively. Mutants Y148A and Y149A (10 μg/ml) alongwith PA (1 μg/ml) were not toxic to macrophage cell line J774A.1 and the EC_{50} of both these mutants was >10 μg/ml.

Binding of LF Mutants to Receptor Bound PA

The mutant LF proteins were examined for their ability to bind to receptor bound PA_{63} in a competition assay. Native ^{125}I-LF (0.1 μg/ml) and various concentrations of cold native LF or mutant LF proteins Y148A, Y149A, I151A and K153A (0.1–10 μg/ml) were added after receptors on J774A.1 cells were saturated with PA (0.5 μg/ml) and incubated for 12 h at 4°C. The cell-associated radioactivity was counted and plotted against the concentration of the competitor protein (Fig. 4). PA binding (% control) of different LF mutants was determined (Table 2). The results indicate that the LF mutants Y148A and Y149A had less than 5% binding to PA (of control) while the mutants I151A and K153A binding to PA was 10 and 8%, respectively. PA binding to mutants E150A and G152A was found to be significantly reduced to 83 and 76%, respectively (Fig. 4).

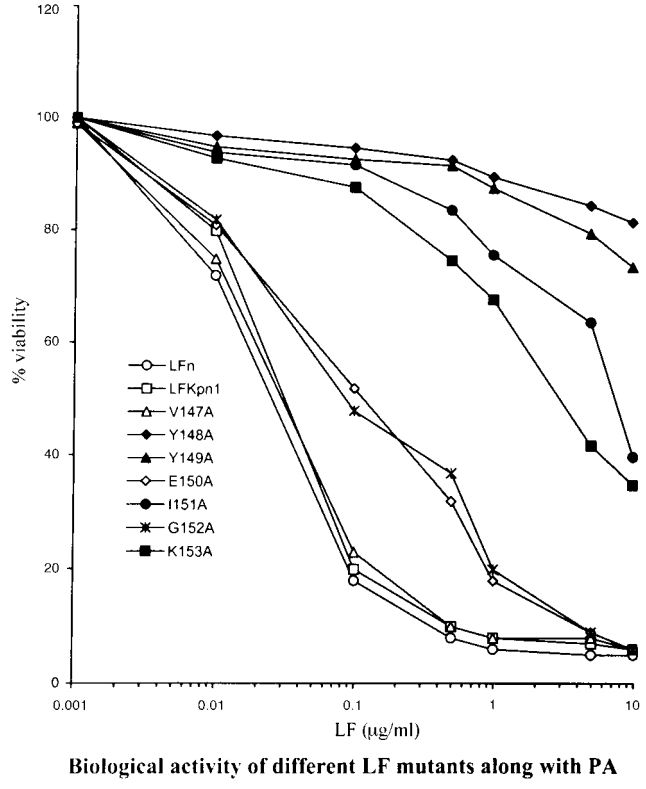
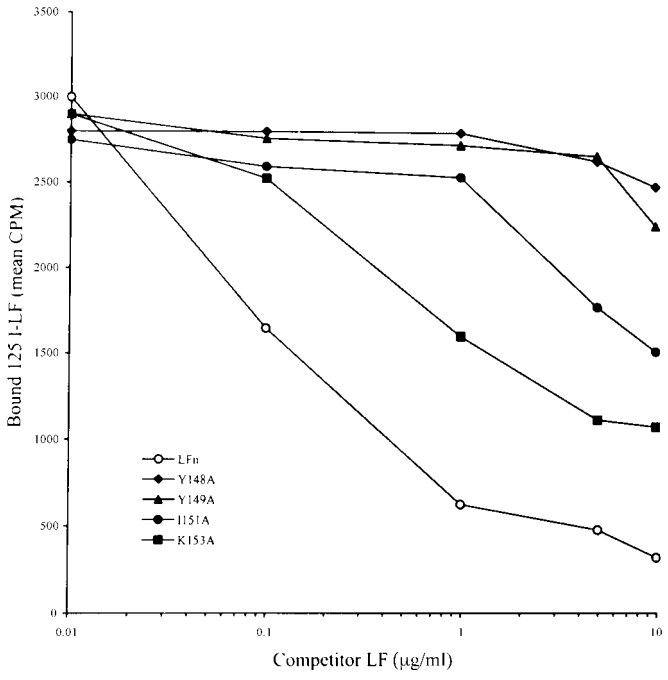


FIG. 3. Biological activity of LF mutants. J774A.1 cells were incubated with varying concentrations of different LF mutants separately in combination with PA (1 μg/ml) for 3 h at 37°C. Cell viability was determined by MTT.



Competitive binding of LF mutants to receptor bound PA

FIG. 4. Competitive binding of LF mutants to receptor bound PA. J774A.1 cells were incubated with native ^{125}I-LF (0.1 μg/ml) and various concentrations of cold native LF or mutant LF proteins LF4, LF5, LF7, and LF9 (0.1–10 μg/ml) after receptors on J774A.1 cells were saturated with PA (0.5 μg/ml) and incubated for 12 h at 4°C. Cells were washed with HBSS and solubilized in 100 mM NaOH. The cell associated radioactivity was counted and plotted against the concentration of the competitor protein.

Binding of LF to Trypsin Nicked PA in Solution

LF binds to trypsin cleaved PA and mobility of this PA_{63}.LF complex is retarded on nondenaturing gels. The ability of mutant LF proteins to bind to trypsin cleaved PA was also analyzed on nondenaturing gel. The ^{125}I-LF was used as a competitor in the process of binding of mutant LF proteins to PA_{63}. In presence of native LF, the binding of ^{125}I-LF to PA was reduced. Similarly in presence of mutants V147A, E150A, and G152A the binding of ^{125}I-LF to PA was inhibited while in the presence of Y148A, Y149A, I151A, and K153A the ^{125}I-LF could bind to PA_{63} (Fig. 5). This indicates that the mutants Y148A, Y149A, I151A, and K153A could not compete with ^{125}I-LF in binding to PA_{63} as they were defective in their binding ability.

The mutants Y148A and Y149A showed a remarkable reduction (~95%) in binding to PA. The mutants I151A and K153A also had significant reduction (~90%) in their ability to bind to PA. The mutants V147A, E150A and G152A showed an insignificant change in the binding to PA. This shows that out of the above mentioned seven residues Y148A, Y149A, I151A, and K153A play a crucial role in the interaction of LF to PA molecule. A change to the non-native residue alanine disrupts the interaction of these residues with PA_{63} resulting in significant reduction in their binding

TABLE 2

Potencies and % PA Binding of LF and Mutant LF Proteins for J774A.1 Cell Line

LF and mutant proteins	EC_{50} (μg/ml)[a]	% PA binding[b]
LFn	0.035	100
LF^{Kpn1}	0.040	100
V^{147A}	0.040	100
Y^{148A}	>10	<5
Y^{149A}	>10	<5
E^{150A}	0.1	83
I^{151A}	4.5	10
G^{152A}	0.1	76
K^{153A}	4.0	8

[a] EC_{50} is defined as the concentration of LF (μg/ml) along with PA (1 μg/ml) required to kill 50% of the J774A.1 cells. After 3 h of incubation, viability determined by MTT dye. The results represent the mean of three experiments.

[b] PA binding activity is expressed as {(native LF concentration for 50% binding/mutant LF concentration for 50% binding) × 100} as described under Materials and Methods.

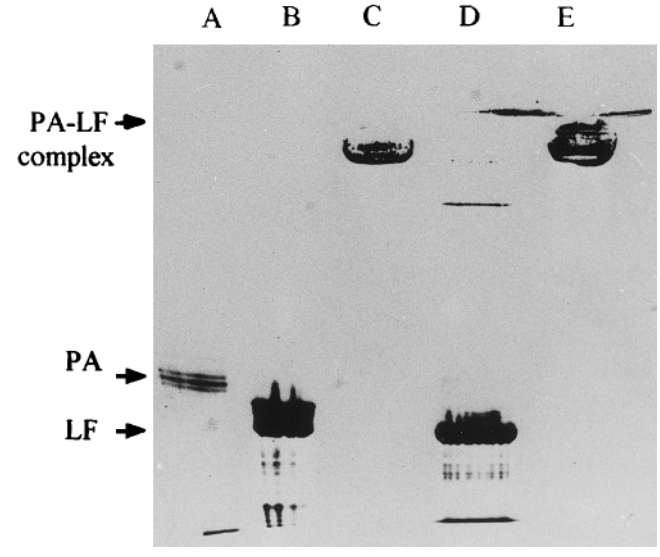


FIG. 5. Competitive binding of LF mutants in solution. Radiolabeled native LF was used to study the binding of LF mutants to proteolytically cleaved PA. Trypsin nicked PA (1 μg/μl) was incubated with LF mutants (1 μg/μl) and native ^{125}I-LF (100,000 cpm) in buffer for 15 min at room temperature. Samples were applied to nondenaturing 4–15% polyacrylamide gradient Phast gels (Pharmacia LKB Biotechnology Ltd., native buffer strips). Gels were autoradiographed. Lane 1, native radiolabeled LF only; lane 2, native radiolabeled LF+PA; lane 3, native radiolabeled LF + PA + LF native; lane 4, native radiolabeled LF + PA + LF3; lane 5, native radiolabeled LF + PA + LF4; lane 6, native radiolabeled LF + PA + LF5; lane 7, native radiolabeled LF + PA +LF6; lane 8, native radiolabeled LF + PA + LF7; lane 9, native radiolabeled LF + PA +LF8; and lane 10, native radiolabeled LF + PA +LF9.

to PA_{63} leading to a corresponding decrease in toxicity. We propose that these above-mentioned four residues play an important role in binding of LF to PA_{63} and a change of these residues to alanine might change the conformation of this domain of LF in such a way that interaction with PA is impaired.

REFERENCES

1. Green, B. D., Battisti, L., Koehler, T. M., Thorne, C. B., and Ivins, B. E. (1985) Demonstration of a capsule plasmid in *B. anthracis*. *Infect. Immun.* **49,** 291–297.
2. Mikesell, O. P., Ivins, B. E., Ristroph, J. D., and Dreir, T. M. (1983) Evidence for plasmid mediated toxin production in *B. anthracis*. *Infect. Immun.* **39,** 371–376.
3. Smith, H., and Keppie, J. (1954) Observations on experimental anthrax: Demonstration of a specific lethal factor produced in vivo by *Bacillus anthracis*. *Nature* **173,** 869–870.
4. Smith, H., Keppie, J., and Stanely, J. L. (1955) The chemical basis of virulence of *Bacillus anthracis* V. The specific toxin produced by *B. anthracis* in vivo. *Br. J. Exp. Pathol.* **36,** 460–472.
5. Stanley, J. L., and Smith, H. (1961) Purification of factor 1 and recognition of a third factor of anthrax toxin. *J. Gen. Microbiol.* **26,** 49–66.
6. Klimpel, R. K., Molloy, S. S., Thomas, G., and Leppla, S. H. (1992) Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin. *Proc. Natl. Acad. Sci. USA* **89,** 10277–10281.
7. Leppla, S. H. (1982) Anthrax toxin edema factor: A bacterial adenylate cyclase that increases cAMP conc. in eukaryotic cells. *Proc. Natl. Acad. Sci. USA* **79,** 3162–3166.
8. Leppla, S. H. (1991) The anthrax toxin complex. *In* Sourcebook of Bacterial Protein Toxins. (Alouf, J. E., and Freer, J. H. Eds.), pp. 277–302, Academic Press, London.
9. Singh, Y., Leppla, S. H., Bhatnagar, R., and Friedlander, A. M. (1989) Internalization and processing of B. anthracis letal toxin sensitive and resistant cells. *J. Biol. Chem.* **254,** 11099–11012.
10. Milne, J. C., Furlong, D., Hanna, P. C., Wall, J. S., and Collier, R. J. (1994) Anthrax protective antigen forms oligomers during intoxication of mammalian cells. *J. Biol. Chem.* **269,** 20607–20612.
11. Bhatnagar, R., Singh, Y., Leppla, S. H., and Friedlander, A. M. (1989) Calcium is required for the expression of anthrax lethal toxin activity in the macrophage like cell line J774A.1. *Infect. Immun.* **57,** 2107–2114.
12. Friedlander, A. M. (1986) Macrophages are sensitive to anthrax toxin through an acid dependent process. *J. Biol. Chem.* **261,** 7123–7126.
13. Friedlander, A. M., Bhatnagar, R., Leppla, S. H., and Singh, Y. (1993) Characterization of macrophage sensitivity and resistance to anthrax lethal toxin. *Infect. Immun.* **61,** 245–252.
14. Bhatnagar, R., and Friedlander, A. M. (1994) Protein synthesis is required for expression of anthrax lethal toxin cytotoxicity. *Infect. Immun.* **62,** 2958–2962.
15. Hanna, P. C., Kochi, S., and Collier, R. F. (1992) Biochemical and physiological changes induced by anthrax lethal toxin in J774A.1 macrophage like cells. *Mol. Biol. Cell* **3,** 1269–1277.
16. Bhatnagar, R., Ahuja, N., Goila, R., Batra, S., Waheed, S. M., and Gupta, P. (1999) Activation of Phospholipase C and Protein Kinase C is required for expression of anthrax toxin cytotoxicity in J774A.1 cells. *Cell. Signal.* **11**(2), 111–116.
17. Duesbery, N. S., Craig, P. W., Leppla, S. H., Gordon, V. M., Klimpel, K. R., Copeland, T. W., Ahn, N. G., Oskarsson, M. K., Fukasawa, K., Paull, K. D., and Woude, G. F. V. (1998) Proteolytic inactivation of MAP Kinase Kinase by anthrax lethal factor. *Science* **280,** 734–737.
18. Arora, N., and Leppla, S. H. (1993) Residues 1–254 of anthrax toxin lethal factor sufficient to cause cellular uptake of fused polypeptides. *J. Biol. Chem.* **268,** 3334–3341.
19. Arora, N., and Leppla, S. H. (1994) Fusions of anthrax toxin lethal factor with shiga toxin and diphtheria toxin enzymatic domains are toxic to mammalian cells. *Infect. Immun.* **62,** 4955–4961.
20. Quinn, P. C., Singh, Y., Klimpel, R. K., and Leppla, S. H. (1991) Functional mapping of anthrax toxin lethal factor by in frame insertion mutagenesis. *J. Biol. Chem.* **266,** 20124–20130.
21. Gupta, P., Batra, S., Chopra, A. P., Singh, Y., and Bhatnagar, R. (1998) Expression and purification of the recombinant lethal factor of *Bacillus anthracis*. *Infect Immun.* **66,** 862–865.
22. Gupta, P., Waheed, S. M., and Bhatnagar, R. (1999) Expression and purification of the recombinant Protective antigen of *B. anthracis*. *Protein Expr. Purif.* **16,** 369–76.
23. Lowry, O. H., Rosenbrough, N. J., Farr, L. A., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.
24. Ayvazian, L., Crenon, I., Hermoso, J., Pignol, D., Chapus, C., and Kerfelec, B. (1998) Role of Lys^{400}-Glu^{45} Ion pairing plays a crucial role in lipase/colipase catalysis. *J. Biol. Chem.* **273,** 33604–33609.
25. Henderson, L. M. (1998) Role of histidines identified by mutagenesis in the NADPH-oxidase associated H^+ channel. *J. Biol. Chem.* **273,** 33216–33223.
26. Malcom, B. A., Rosenberg, S., Corey, M. J., Allen, J. S., and Kirsch, J. (1989). Site directed mutagenesis of the catalytic residues Asp52 and Gly 35 of chicken egg white lysozyme. *Proc. Natl. Acad. Sci. USA* **86,** 133–137.
27. Matsumura, I., and Kirsch, J. (1996) Synergistic contributions of asparagine 46 and aspartate 52 to the catalytic mechanisms of chicken egg white lysozyme. *Biochemistry* **39,** 1890–96.
28. Zhang, Z. P., Gambone, C. J., Gabriel, J. L., Wolfgang, C. L., Soprano, K. J., and Soprano, D. R. (1998) Arg^{278} plays an important role in the binding of retionic acid by retionic acid receptor γ. *J. Biol. Chem.* **273,** 34016–34021.