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Trp 346 and Leu 352 Residues in Protective Antigen Are Required for the Expression of Anthrax Lethal Toxin Activity

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The three separate proteins that make up anthrax toxin-protective antigen (PA), edema factor (EF) and lethal factor (LF) act in binary combinations to produce two distinct reactions in experimental animals: edema (PA+EF) and death (PA+LF). PA is believed to interact with a membrane receptor and, after proteolytic processing, to mediate endocytosis and subsequent translocation of EF or LF into the cytosol. Residues W346, M350, and L352 in loop 3 of domain 2 have been implicated to induce a conformational change when the pH is lowered from 7.4 to 6.5. Modification of the residues Trp (346), Met (350), and Leu (352) to alanine individually and all the three residues together to alanine residues resulted in the loss of cytotoxic activity in combination with LF. The mutant proteins were able to bind to the cell surface receptor, become cleaved by trypsin, bind LF, and oligomerize. These residues might play an important role in the membrane insertion of PA and/or translocation of LF/EF into the cytosol.

The pathogenesis of *Bacillus anthracis,* the causative agent of anthrax, depends on two important virulence factors: an antiphagocytic poly-D-glutamic acid and anthrax toxin which consists of Protective Antigen (PA), Lethal Factor (LF), Edema Factor (EF) proteins. PA (83 kDa) serves as the entry vehicle for either EF (89 kDa) or LF (90 kDa). Administration of PA and LF together (lethal toxin, LT) causes lethality (1), whereas PA together with EF (edema toxin) produces edema. EF has been shown to be a calcium and calmodulin-dependent adenylate cyclase (2). LF in cytosol acts by inhibiting MAP kinase signal transduction pathway (3).

PA binds with high affinity to a host cell surface receptor. Cleavage of the sequence $RKKR^{167}$ by a cell surface protease, furin (4) releases a N-terminal 20 kDa fragment, PA20 and exposes a high affinity binding site for LF or EF on the 63 kDa fragment, PA63. LF or EF then binds (competitively) to the receptor bound PA63 fragment, and the complex undergoes receptor mediated endocytosis (5). Acidification of the endocytic vesicle leads to insertion of PA63 into the cytosol, where these toxins exert their effects.

Both *in vitro* and during intoxication of mammalian cells (6) the PA63 fragments associate into an oligomer. At acidic pH PA63 inserts into membranes and forms ion conductive channels in both artificial lipid bilayers (7) and cells (8). The conditions required for PA63 oligomerisation correlate with those required for translocation of EF and LF across cellular membranes, suggesting that the oligomer may play a role in both activities. Two species of oligomeric PA exist: a water soluble form at neutral or basic pH and a membrane inserted form at acidic pH.

The crystal structure of PA has been determined at high resolution (9). The molecule comprises four domains organized mainly into antiparallel β-sheets with only a few short α-helices. Domain 2 (residues 259–487) has a β barrel core with modified Greek-key topology and elaborate excursions, including a large flexible loop between strands $2\beta_2$ and $2\beta_3$ that is implicated in membrane insertion (chymotrypsin sensitive loop) and a pH sensitive loop with its three hydrophobic residues Trp 346, Met 350 and Leu 352 (loop 3 between strands $2\beta_3$ and $2\beta_4$). Two crystal forms of PA, one obtained at pH 7.5 and the other at pH 6.0 have been studied (9). The largest structural difference occurs in residues 342–355 (loop 3) located at the base of the barrel formed by domain 2. This region contains three hydrophobic residues, a Trp, a Leu and a Met, in the sequence $x_4WxxxMxLx_3$, where x represents a hydrophilic residue. At pH 7.5 these three residues are buried within the core of the β barrel but become exposed at pH 6.0 in a disordered loop.

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TABLE 1

Sequence of Oligonucleotides Used for Constructing Mutants in pQE30

Primer 1	5′ CG GTC GCA AT**T GAT CA**T TCA CTA TCT CTA GCA GGG GAA AGA ACT GCG GCT GAA ACA ATG 3′
Primer 2	5′ CG GTC GCA AT**T GAT CA**T TCA CTA TCT CTA GCA GGG GAA AGA ACT TGG GCT GAA ACA GCG GGT TTA AAT ACC 3′
Primer 3	5′ CG GTC GCA AT**T GAT CA**T TCA CTA TCT CTA GCA GGG GAA AGA ACT TGG GCT GAA ACA ATG GGT GCA AAT ACC GCT GAT 3′
Primer 4	5′ CG GTC GCA AT**T GAT CA**T TCA CTA TCT CTA GCA GGG GAA AGA ACT GCG GCT GAA ACA GCG GGT GCA AAT ACC GCT GAT 3′
Reverse primer	5′ CCT AGA GGT ACC TTA TCC 3′.

Note. Restriction endonuclease sites are shown in bold and the mutations have been underlined.

The formation of a membrane inserted β barrel by the PA63 heptamer requires the chymotrypsin-sensitive loops of adjacent sub-units to be in close proximity. However the chymotrypsin-sensitive loops project outwards from the side of the water-soluble heptamer. Their assembly into a β barrel on the heptamer axis would be accomplished most simply if the Greek-key motif formed by the first four strands of domain 2 ($2\beta_1$ to $2\beta_4$) were to unfold, with strands $2\beta_2$ and $2\beta_3$ (chymotrypsin-sensitive loop) peeling away from the edge of the domain (9). This significant conformational change could be triggered by the drop in pH that is a prerequisite to channel formation (7, 8, 10). *In vivo,* channel formation by PA63 in planar lipid bilayers is accelerated rapidly when the pH is lowered from 7.4 to 6.5 (7, 10), implicating the titration of histidines. In addition, lowering the pH from 7.5 to 6.0 in PA crystals induces ordered residues at the bottom of domain 2 to form a disordered loop (loop 3) only 12 residues from loop 2. This order-to-disorder transition may resemble the initial conformational changes that occur in the heptamer during membrane insertion. The present study was undertaken to determine the importance of the residues Trp (346), Met (350) and Leu (352) in the intoxication process.

MATERIALS AND METHODS

Construction of mutants in which Trp (346), Met (350) and Leu (352) have been changed to Ala. PA gene was mutagenized using four different mutagenic primers (forward primers) corresponding to nucleotides 2882–2954 spanning a unique *Bcl*I site. Primer 1 contained mutations at nucleotides 2926–2928 encoding amino acid residue 346 (Trp). Primer 2 contained mutations at nucleotides 2938–2940 encoding amino acid residue 350 (Met). Primer 3 contained mutations at nucleotides 2944–2946 encoding amino acid residue 352 (Leu). Primer 4 contained mutations at all the above mentioned nucleotides. All the above mutations led to the change of the specified amino acids to alanine. The reverse oligonucleotide primer created a *Kpn*I site at the 3′ end. The sequence of the above-mentioned primers is given in Table 1.

PCR was done using the primers mentioned in Table 1 and pMW1 as template. Plasmid pMW1 (11) contains the PA gene cloned in plasmid pQE30 under the T5 promoter. PCR was performed in 100 μl reaction using DNA thermal cycler (Perkin Elmer) in 0.2 ml thin walled tubes. The reaction mix consisted of 50 ng of template DNA, 0.2 mM of each dNTP (Amersham Inc.), 0.1 nmol of each oligonucleotide, 10 μl of UlTma DNA polymerase buffer (10×) and 1 unit of UlTma DNA polymerase (Perkin Elmer).

The PCR reaction mix was fractionated by agarose gel electrophoresis. The fragment of the right size was excised from the agarose gel and was eluted from the gel slice using gel extraction kit (Qiagen). The purified PCR products and the plasmid pMW1 were digested with *Bcl*I and *Kpn*I. Ligation of the vector with the insert was carried out in 1:3 vector:insert molar ratio in the presence of 1 U of T4 DNA ligase (Stratagene) in 1× ligase buffer and incubated at 16°C for 16 h. Ligation reactions were set up for each of the different products.

The ligation mixtures were transformed into competent DH5αcells. The transformants were screened for the presence of the insert by minipreparations of plasmid DNA and restriction analysis. DNA was digested with *Bcl*I and *Kpn*I restriction enzymes. Restriction enzyme digestion mixtures were run on 1% agarose gel to check for the presence of insert. The mutations were confirmed by sequencing. Sequencing was carried out by chain termination DNA sequencing method (12) using T7 Sequenase Version 2.0 sequencing kit (United State Biochemical, Cleveland, Ohio).

Purification of PA and mutant PA proteins. Competent M15 (pREP4) cells transformed with DNA from positive clones were grown at 37°C in 100 ml of LB medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. When A_{600} = 0.8–1.0, IPTG was added to a final concentration of 0.5 mM. After 5 h of induction, cells were harvested by centrifugation at 4000*g* for 10 min. The pellet was resuspended in 10 ml of buffer containing 8 M urea, 0.1 M Na-phosphate, pH 7.8 and 300 mM NaCl. Cells were stirred at room temperature for 1 h. The lysate was centrifuged at 10,000*g* for 30 min at 4°C. The supernatant was mixed with 1 ml of 50% slurry of Ni-NTA resin and allowed to stir at room temperature for 1 h. The resin was loaded into an empty column. The column was washed with 5 column volumes of buffer containing 8 M urea, 0.1 M Na-phosphate, pH 7.8 and 300 mM NaCl. The resin was then washed with a gradient of 8 to 0 M urea in buffer containing 0.1 M Na-phosphate, pH 7.8 and 300 mM NaCl to facilitate the slow removal of urea. The recombinant protein was eluted with 250 mM Imidazole in 0.1 M Na-phosphate, pH 7.0 and 10% glycerol. The fractions were analyzed on SDS-PAGE and those containing the protein were pooled and dialyzed against 10 mM Hepes, pH 7.0 and stored in aliquots at −70°C.

Cell culture and cytotoxicity assay. Macrophage-like cell line J774A.1 was maintained in RPMI 1640 medium containing 10% heat inactivated FCS, 25 mM Hepes, 100 U/ml penicillin and 200 μg/ml streptomycin in a 95% humidified 5% CO_2 environment at 37°C. For biological assay of PA, a 96-well culture plate was prepared with 80–90% cell density. PA was added in varying concentrations with LF (1 μg/ml) and incubated for 3 h at 37°C. PA from *B. anthracis* was used as a positive control. Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) dye. MTT dissolved in RPMI was added in each well to a final concentration of 0.5 mg/ml and 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% (w/v) sodium dodecyl sulphate (SDS), 25 mM HCl in 90% isopropyl alcohol and the plate vortexed. The absorption at 620 nm was read using microplate reader (Bio-Rad).

Iodination of PA. PA was iodinated using Chloramine T method of Hunter and Greenwood (13). PA (50 μg) was mixed gently with 100 μl of 0.1 M sodium phosphate buffer pH 7.0 containing 5 μg of chloramine T. ^{125}I-sodium iodide (1 mCi) was added to the mixture and incubated on ice for 5 min. The reaction was terminated by adding 10 μg of sodium metabisulphite in 0.1 M sodium phosphate buffer pH 7.0. The labelled protein was separated from free iodine by passing the reaction mixture on a gel filtration (Sephadex G-25) column pre-equilibrated with 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 on Gamma counter. The first peak containing the iodinated protein was pooled together. The labelled protein was stored at 4°C.

Binding of PA and mutant proteins to cell surface receptor. The binding of mutant PA proteins to cell surface receptor was carried out in 24-well plates using constant amount of radioiodinated native PA (0.1 μg/ml). J774A.1 were washed twice with cold HBSS for 5 min each time and then placed on ice. The medium was replaced with cold binding medium (EMEM without sodium bicarbonate containing 1% bovine serum albumin and 25 mM Hepes, pH 7.4). The cells were incubated with 0.1 μg/ml of iodinated PA and varying concentrations of different mutant proteins at 4°C for 12 h and then washed with cold HBSS. The cells were dissolved in 0.1 N NaOH and radioactivity was measured in Gamma counter.

Proteolytic cleavage of PA and mutant proteins in solution. PA and its mutant proteins were tested for susceptibility to cleavage by trypsin. The proteins (1.0 mg/ml) were incubated with 1 μg/ml of trypsin for 30 min at room temperature in 25 mM Hepes, 1 mM $CaCl_2$, 0.5 mM EDTA pH 7.5. The digestion reaction was stopped by adding PMSF to a concentration of 1 mM. For SDS-PAGE, the samples were boiled in SDS sample buffer for 5 min and resolved on 12% SDS-PAGE.

Binding of LF to mutant PA proteins. Binding of LF to mutated PA proteins was studied in 24 well culture plates. CHO-K1 cells were washed twice with cold HBSS for 5 min each time and then placed on ice. The medium was replaced with a saturating concentration of PA and mutant PA proteins (1 μg/ml) in cold binding medium (EMEM without sodium bicarbonate 1% bovine serum albumin and 25 mM Hepes, pH 7.4) and incubated for 16 h at 4°C to saturate the receptors. Cells were then washed four times with cold HBSS to remove unbound PA and reincubated with a saturating concentration of iodinated LF (1 μg/ml) in binding medium for 5 h at 4°C. Cells with only PA and only LF were used as controls. Cells were then washed with cold HBSS to remove unbound LF, dissolved in 0.1 N NaOH, and radioactivity was counted.

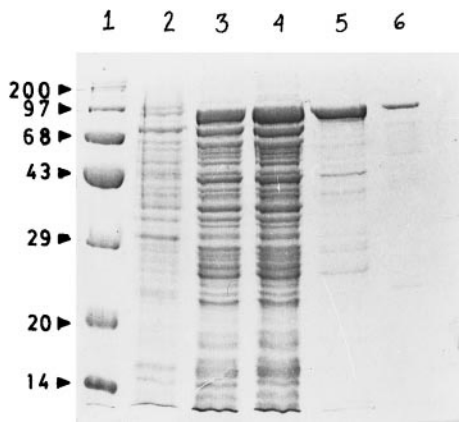

FIG. 1. SDS-PAGE protein profile from pQE30. Lane 1, Molecular weight marker; lane 2, uninduced M15 cells; lane 3, induced M15 cells; lane 4, cell lysate; lane 5, Ni-NTA purified protein; lane 6, standard PA.

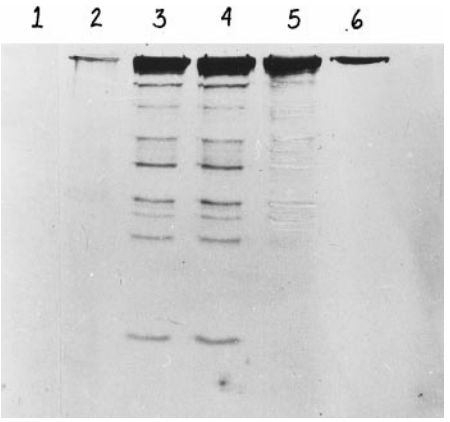

FIG. 2. Western blot of protein profile from pQE30. Lane 1, molecular weight marker; lane 2, uninduced M15 cells; lane 3, induced M15 cells; lane 4, cell lysate; lane 5, Ni-NTA purified protein; lane 6, standard PA.

Oligomerization of PA in solution. The analysis of PA63 oligomer (6) by SDS-PAGE was performed using a 3–12% linear gradient gel with the discontinuous buffer system of Laemmli (14). PA and mutant PA proteins (1 mg/ml) were digested with trypsin for 30 min at 25°C. The protein samples were brought to pH 5.0 by addition of 1 M Tris pH 5.0 to a final concentration of 100 mM. As indicated, the samples were boiled for 5 min in SDS sample buffer (0.0625 M Tris-Cl, 1.25% SDS, 2.5% β-mercaptoethanol and 5% glycerol, pH 6.8). The samples were then loaded on the gradient gel and silver stained.

RESULTS

Construction of Substituted Mutants of PA

In order to elucidate the importance of Trp (346), Met (350) and Leu (352) residues of domain 2 of protective antigen, these residues were altered to alanine using site-directed mutagenesis. Four different PCR products were obtained with mutations at different sites, namely Trp (346) → ala, Met (350) → ala, Leu (352) → ala, Trp (346) Met (350) Leu (352) → ala. Each of the digested PCR products was ligated with the digested vector and the resulting plasmids were designated as pW^{346}, pM^{350}, pL^{352}, and $pW^{346}M^{350}L^{352}$.

Expression and Purification of PA Mutants

Expression was established by SDS-PAGE and Western blot analysis. The Western blot revealed the presence of 83 kDa protein on probing with anti-PA antibodies (Figs. 1 and 2). The recombinant PA and mutant PA proteins were purified from inclusion bodies under denaturing conditions. Immobilizing one end of the protein during renaturation prevents intermolecular interactions that lead to aggregate formation.

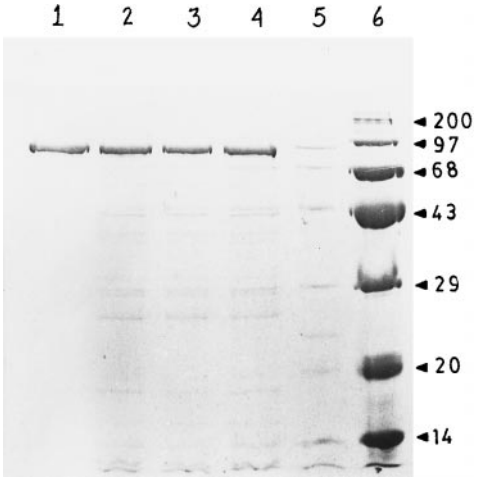

FIG. 3. Purification of *E. coli* expressed mutant PA proteins from pQE30-fractions on 12% SDS-PAGE. Lane 1, standard PA; lane 2, PA352; lane 3, PA350; lane 4, PA346; lane 5, PA346, 350, 352; lane 6, molecular weight marker.

The different proteins were designated as W^{346}, M^{350}, L^{352} and W^{346} M^{350} L^{352}. Protein $W^{346}M^{350}L^{352}$ was not expressed to a high level and the protein after Ni-NTA affinity chromatography was not found to be very pure (Figs. 3 and 4). Therefore this mutant protein could not be used for further studies.

Cell Culture and Cytotoxicity Assay

PA and mutant PA proteins were assayed for their functional activity by the J774A.1 macrophage lysis assay. It was observed that substitution of the methionine residue (350) did not alter the toxicity of PA in combination with LF as compared to the native PA. Substitution of tryptophan (346) and leucine (352) and all the three residues together with alanine altered the activity profile. It was observed that proteins W^{346}, L^{352} and $W^{346}M^{350}L^{352}$ were nontoxic in combination with LF up to concentrations as high as 2.5 μg/ml (Fig. 7).

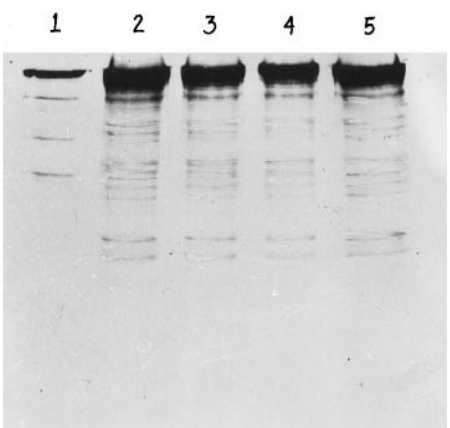

FIG. 4. Purification of *E. coli* expressed mutant PA proteins from pQE30-Western blot of the fractions. Lane 1, PA346, 350, 352; lane 2, PA346; lane 3, PA350; lane 4, PA352; lane 5, standard PA.

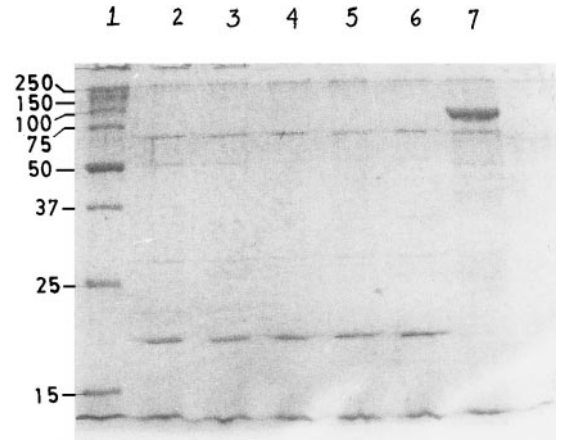

FIG. 5. Susceptibility of mutant PA proteins to trypsin. Lane 1, molecular weight marker; lane 2, PA352; lane 3, PA350; lane 4, PA346; lane 5, PA from *E. coli;* lane 6, PA from *B. anthracis;* lane 7, standard PA.

Binding of Mutant PA Proteins to the Cell Surface Receptors

The first step in cytotoxicity of the anthrax toxin proteins is receptor binding of PA. The mutant PA proteins were examined for their receptor binding activity in a competition assay with ^{125}I labelled PA. The results indicate that all the PA mutants competed with ^{125}I PA (Fig. 8).

Proteolytic Cleavage of PA and Mutant PA Proteins in Solution

The next step in cytotoxicity is proteolytic activation of PA by cell surface proteases. Proteolytic cleavage can also be achieved in solution by the treatment of PA with mild concentrations of trypsin which generates two fragments (63 and 20 kDa) as obtained by cleavage through cell surface proteases. The proteolytic cleavage of PA and mutant PA proteins was studied by treatment of PA and mutant PA proteins with trypsin and the products were analyzed on 12% SDS-PAGE. Trypsin treatment cleaved all the mutant PA proteins into two fragments of 63 kDa and 20 kDa similar to native PA (Fig. 5).

Binding of LF to Mutant PA Proteins

Trypsin cleaved PA has the ability to bind LF. The LF binding property of mutant PA proteins was examined by incubating CHO-K1 cells with mutant PA proteins (1 μg/ml) at 4°C for 16 h to saturate the receptors. It was observed that all the mutant PA proteins were capable of binding to LF (Table 2).

TABLE 2

Binding of Native PA and Mutant PA Proteins to LF

S. No.	Specific binding[a] of LF
Native PA	14,852 ± 184
M350	14,532 ± 197
W346	14,659 ± 152
L352	14,639 ± 178

[a] Difference in mean counts per minute (±standard error), of triplicate samples.

Oligomerization of PA and Mutant PA Proteins in Solution

Oligomerization was studied on 3–12% linear gradient gels. It was found that in all the mutants significant percentage of PA63 in solution was in the form of SDS-resistant oligomers (Fig. 6).

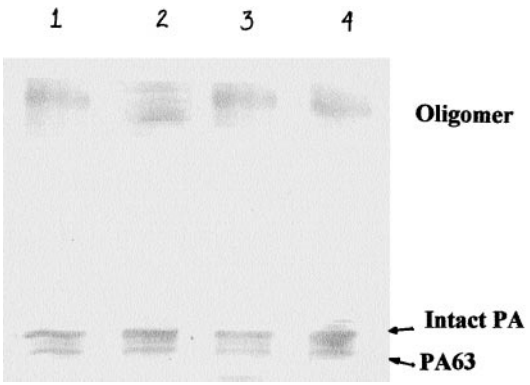

FIG. 6. Oligomerization of mutant PA proteins in solution. Lane 1, PA350; lane 2, PA346; lane 3, PA352; lane 4, native PA.

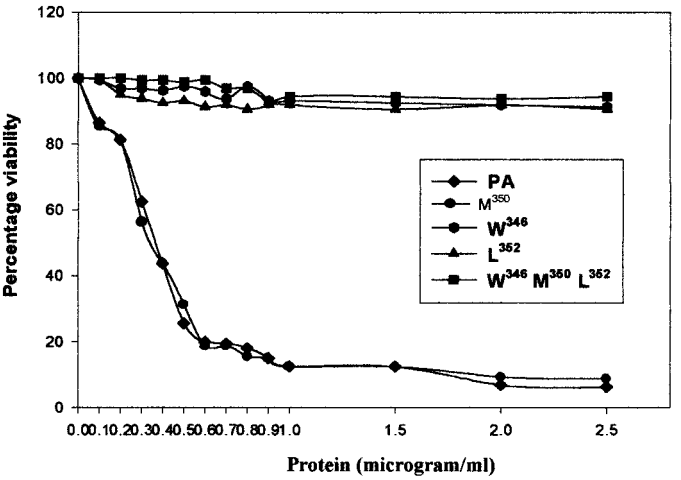

FIG. 7. Toxicity of PA and mutant PA proteins in combination with LF on J774A.1 cells.

DISCUSSION

Like many other protein toxins, anthrax toxin follows the 'A-B' model of toxins. The A subunit (lethal factor or edema factor) carries the enzymatic activity where as the B subunit (protective antigen) is implicated in binding to the target cell and in transport of the enzymatic A subunit into the cytoplasm. This inherent ability of toxin to interact with membranes in the absence of other proteins renders them a very interesting and simplified model system to study protein-membrane interactions.

Membrane insertion by the B subunit is one of the important steps in the process of intoxication. A common characteristic of the binding moieties of toxins is the ability to form ion-conductive channels across planar lipid bilayers. Bacterial channel forming toxins that employ closed β-barrels represent a growing family of proteins involved in bacterial pathogenesis. Members of this group include anthrax protective antigen (9), aerolysin (15), α-hemolysin of *Staphylococcus aureus* (16), *Clostridium septicum* α toxin (17), *Clostridium perfringens* β-toxin (18) and iota-β toxin (19), *Bacillus cereus* hemolysin-II (20) and vegetative insecticidal protein toxins (VIP) from *B. cereus* and *B. thuringiensis*. In the past few decades the elucidation of crystal structure of different toxins has thrown light on the structure function relationship. The domains within the proteins have been assigned for carrying out specific functions. Thus a change in the domain structure can lead to a loss in function. The crystal structure of the B moiety of anthrax toxin, the protective antigen has been determined at high resolution and has raised the prospect of understanding the anthrax toxin translocation process. The membrane insertion and translocation ability of anthrax toxin is governed by two factors (i) activation of protective antigen (B moiety) by cell surface proteases which lead to formation of a heptameric oligomer and (ii) acidic pH which induces a conformational change for membrane insertion.

The structure of PA has recently been solved at 2.1 A° resolution (9). PA is an elongated molecule, composed mainly of antiparallel β-sheet, which has been divided into four domains. Domain 2 has been implicated to play a role in oligomerization and membrane insertion. It has a β-barrel core with a modified Greek key topology and contains long loops. Pore forming bacterial toxins undergo a variety of conformational changes in the transition from a secreted hydrophilic protein to one capable of penetrating a hydrophobic membrane. Such transitions may involve the creation of entirely new surfaces. Hydrophobicity is thought to be the driving force for membrane insertion. It has been demonstrated that lowering the pH increases the hydrophobicity of PA and induces its oligomerization (6, 10). This suggests that upon pH lowering, some regions of the protein might be reorganized in order to

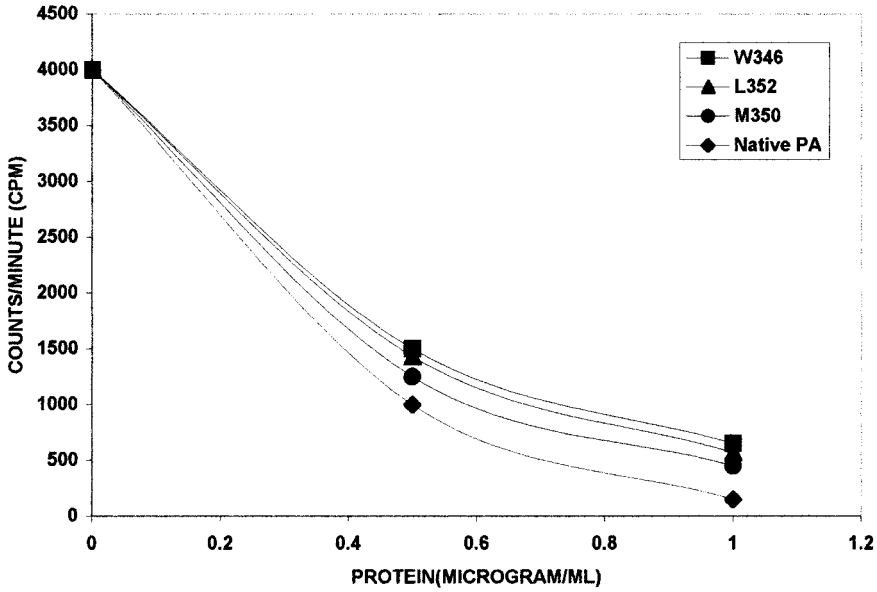

FIG. 8. Binding of PA mutants to cell surface receptors.

provide the right conformation to interact with the lipid bilayer which, in turn, may induce a modification of the secondary structure.

Two crystal forms of PA have been studied (9), one at pH 7.5 and the other at pH 6.0. The largest structural difference occurs in residues 342–355, located at the base of the barrel formed by domain 2. This region contains three hydrophobic residues, a Trp, a Leu and a Met, in the sequence $x_4W_{xxx}M_xL_x$ where x represents a hydrophilic residue. At pH 7.5, these three residues are buried within the core of the β-barrel but become exposed at pH 6.0 in a disordered loop. The corresponding sequence in iota Ib (x_4WxxxLxIx) displays a similar pattern of amphipathicity. The formation of a membrane inserted β-barrel by the PA63 heptamer would require the chymotrypsin sensitive loops of adjacent sub-units to be in close proximity. Their assembly into a β-barrel on the heptamer axis could be accomplished most simply if the Greek-key motif formed by the first four strands of domain 2 ($2\beta_1$–$2\beta_4$) were to unfold with strands $2\beta_2$ and $2\beta_3$ peeling away from the edge of the domain. *In vivo,* the trigger for membrane insertion is provided by acidification of the endosome. *In vitro,* channel formation by PA63 in planar lipid bilayers is accelerated rapidly when the pH is lowered from 7.4–6.5 implicating the titration of histidines. In addition, lowering the pH from 7.5–6.0 in PA crystals induces ordered residues at the bottom of domain 2 to form a disordered loop (loop 3) only 12 residues away from loop 2 (chymotrypsin sensitive loop). Thus a pH induced large scale conformational change is feasible in this region.

The amino acids W (346), M (350) and L (352) seem to play an important role in membrane insertion. Based on these observations, these amino acid residues were changed to alanine and the proteins were checked for their biological activity in terms of receptor binding, proteolytic cleavage, binding to LF and oligomerization. All these activities were performed by the mutant proteins and yet they were nontoxic in combination with LF in the macrophage lysis assay suggesting that a step after oligomerization probably membrane insertion was being affected. Change of methionine residue did not have any significant effect on the activity of PA.

Hydrophobic interactions are a major factor in stabilizing protein folded conformations. Hydrophobic residues have been implicated to play an important role in other channel forming toxins. In Staphylococcal α hemolysin, upon membrane binding tryptophan and tyrosine residues become more solvent exposed (16). In Diphtheria toxin, at low pH the B fragment opens up its structure to expose hydrophobic regions which become inserted into the membrane (21).

Change of methionine did not seem to have a major effect on the toxicity of PA as compared to tryptophan and leucine maybe because of its less hydrophobic character compared to the other two amino acids. As seen for other toxins, hydrophobic interactions play an important role in interaction with the membrane. The loss in the activity of the mutants W^{346}, L^{352} and $W^{346}M^{350}L^{352}$ may be attributed to a loss in the membrane insertion activity of PA. It is possible that the order-to-disorder transition resembling the initial conformational changes did not occur. These residues seem to play an important role in the biological activity of PA. We propose that the mutant PA proteins are defective in membrane insertion and translocation. These studies will help in better understanding of the membrane translocation process and may help in designing better vaccines.

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