A Dominant Negative Mutant of Bacillus anthracis Protective Antigen Inhibits Anthrax Toxin Action in Vivo*

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Yogendra Singh‡, Hemant Khanna§, Arun P. Chopra¶, and Varsha Mehra§
From the Centre for Biochemical Technology, Mall Road, Delhi-110007, India

PA63, a proteolytically activated 63-kDa form of anthrax protective antigen (PA), forms heptameric oligomers and has the ability to bind and translocate the catalytic moieties, lethal factor (LF), and edema factor (EF) into the cytosol of mammalian cells. Acidic pH triggers oligomerization and membrane insertion by PA63. A disordered amphipathic loop in domain II of PA (2β2–2β3 loop) is involved in membrane insertion by PA63. Because conditions required for membrane insertion coincide with those for oligomerization of PA63 in mammalian cells, residues constituting the 2β2–2β3 loop were replaced with the residues of the amphipathic membrane-inserting loop of its homologue iota-b toxin secreted by Clostridium perfringens. It was hypothesized that such a molecule might assemble into hetero-heptameric structures with wild-type PA ultimately leading to the inhibition of cellular intoxication. The mutation blocked the ability of PA to mediate membrane insertion and translocation of LF into the cytosol but had no effect on proteolytic activation, oligomerization, or binding LF. Moreover, an equimolar mixture of purified mutant PA (PA-I) and wild-type PA showed complete inhibition of toxin activity both in vitro on J774A.1 cells and in vivo in Fischer 344 rats thereby exhibiting a dominant negative effect. In addition, PA-I inhibited the channel-forming ability of wild-type PA on the plasma membrane of CHO-K1 cells thereby indicating protein-protein interactions between the two proteins resulting in the formation of mixed oligomers with defective functional activity. Our findings provide a basis for understanding the mechanism of translocation and exploring the possibility of the use of this PA molecule as a therapeutic agent against anthrax toxin action in vivo.

Bacillus anthracis, the etiologic agent of anthrax, is a potential agent of bioterrorism (1). The toxic action has been attributed to anthrax toxin produced by the bacterium. The anthrax toxin can be resolved into three distinct protein components: protective antigen (PA),1 lethal factor (LF), and edema factor (EF). The combination of EF and PA (an edema toxin) produces skin edema, whereas LF and PA (a lethal toxin) are lethal to animals (2). The three proteins are individually non-toxic (2). Whereas EF is a calcium- and calmodulin-dependent adenylate cyclase that acts by increasing the intracellular cAMP levels in eukaryotic cells (3), LF is a Zn2+-dependent metalloprotease (4) that leads to an increase in IL-1 and TNF-α production by susceptible cells (5) and cleaves several mitogen-activated protein kinase kinases (MKK 1, 2 and 3) (6–8).

According to the current model of anthrax toxin action, PA binds to an as yet unknown cell surface receptor and gets proteolytically activated by cell surface protease furin to PA63. This allows oligomerization and binding of LF/EF. The toxin complex is internalized by receptor-mediated endocytosis and is exposed to acidic pH inside the endosome. This change in pH triggers both membrane insertion by PA63 and translocation of LF/EF into the cytosol (recently reviewed in 9).

Membrane insertion and channel formation are brought about by a large 2β2–2β3 loop (amino acid residues 302–325) in the domain II of PA (10). The loop shows a conserved pattern of alternating hydrophilic and hydrophobic amino acid residues similar to that observed in Clostridium perfringens iota-b toxin and Staphylococcus aureus a-hemolysin (11). PA also has been shown to possess a high degree of homology with the iota-b toxin secreted by C. perfringens (12).

Translocation of LF or EF to the cytosol is believed to occur through a channel formed by insertion of heptameric PA63 into the membrane (11). The formation of ion-conductive channels by PA63 has been demonstrated in both artificial lipid membranes (13) and in CHO-K1 cells (14). Acidic pH triggers oligomerization, membrane insertion by PA63, and translocation of LF into the cytosol of mammalian cells (10, 15, 16). In this paper, we show that a mutant PA protein, in which amino acid residues comprising the 2β2–2β3 loop of PA (PA-I) were substituted with the residues of the amphipathic loop of the homologous iota-b toxin, is defective in its ability to insert into the membrane and completely inhibits the lethal effect of the wild-type toxin at equimolar concentrations.

EXPERIMENTAL PROCEDURES

Materials—Biochemicals and reagents were purchased from Sigma. Bacterial culture media were purchased from Difco Laboratories. The enzymes and chemicals for DNA manipulations were obtained from New England BioLabs. [3H]Leucine and [35S]methionine were obtained from Amersham Pharmacia Biotech.

Cell Culture—The Chinese Hamster Ovary cell line (CHO-K1) and J774A.1 macrophage cell line were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum and 50 µg/ml gentamicin sulfate (Life Technologies Inc.) at 37 °C in a CO2 incubator.

Plasmid Construction and Mutagenesis—Mutations in the PA gene were constructed in a previously described plasmid pYS3 (17). A non-mutagenic oligonucleotide primer corresponding to nucleotides 2169–2200 and spanning the unique HindIII site was used for polymerase chain reaction with a mutagenic primer corresponding to nucleotides 2785–2860 encompassing the unique PstI site and containing the de

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‡ To whom correspondence should be addressed: Center for Biochemical Technology, Mall Road, Delhi-110007, India. Tel.: 91-11-766 6156; Fax: 91-11-766 7471; E-mail: ysingh@cbt.res.in.
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1 The abbreviations used are: PA, protective antigen; LF, lethal factor; EF, edema factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAGE, polyacrylamide gel electrophoresis; LFn, N-terminal 254 amino acids of LF.
sired mutations at nucleotides 2792–2851 (nucleotide numbering is according to Ref. 18). The amplified polymerase chain reaction product was digested with PstI and HindIII and purified on a 1% low melting point agarose gel. The plasmid pYS5 was digested with the same enzymes, purified on agarose gel, and ligated to the mutant fragment. The DNA sequence of the mutant PA gene was verified by DNA sequencing of at least 200 base pairs spanning the mutated region.

Expression and Purification of PA—The plasmid carrying the desired sequence was transformed into E. coli dam dcm strain SCS110. Unmethylated plasmid DNA was purified and used to transform B. anthracis BH441. Wild-type and mutant PA proteins were purified from the cell supernatants of B. anthracis according to the method described earlier (16). Proteins were assayed using the Bio-Rad protein assay kit (Bio-Rad) according to the manufacturer’s instructions.

Cytotoxicity Assay—To study the cytotoxicity, varying concentrations of PA and its mutant protein were added to J774A.1 cells together with LF (1 μg/ml) and incubated for 3 h at 37 °C. At the end of the experiment, cell viability was determined using MTT assay (19).

Protein Synthesis Inhibition Assay—PA and its mutant protein were added to CHO-K1 cells in combination of LF fusion protein (LF(1–254)/TR-PE-(398–613)) (16) and incubated at 37 °C for 3 h. At the end of the incubation period, an aliquot of medium was removed, and released 86Rb⁺ was measured.

Inhibition of the channel-forming ability of wild-type PA by mutant PA protein was assayed by adding trypsin-nicked wild-type and mutant PA proteins for 2 h at 4 °C. The cells were then washed two times with cold phosphate-buffered saline to remove unbound PA and treated with isotonic buffer (20 mM MES-glucanate, 145 mM NaCl, pH 5.0 or 7.0) at 37 °C. At the end of the incubation period, an aliquot of medium was removed, and released 86Rb⁺ was measured.

In Vivo Translocation Assay and Electron Microscopy—An assay for measuring PA-mediated translocation of a labeled ligand into cells has been previously described (20). Briefly, CHO-K1 cells were chilled to 4 °C and then incubated with 2 μg/ml of trypsin-nicked wild-type PA or mutant PA proteins for 2 h. The cells were washed and incubated with in vitro transcribed and translated LFm (LF 1–254 amino acids) labeled with [35S]methionine for 1 h. After another washing step, the cells were either lysed with 100 μl of lysis buffer (20 mM sodium phosphate, pH 7.4, 10 mM EDTA, 1% Triton X-100) directly or incubated with isotonic buffer (20 mM MES-glucanate, 145 mM NaCl, pH 5.0 or 7.0) at 37 °C for 5 min, treated with Pronase E and then lysed. Proteins that translocated to the interior of the cells during the low pH pulse were protected from Pronase treatment. Cell-associated radioactivity was then measured in the samples. Percent translocation was calculated as: counts protected from Pronase/counts bound to cells × 100. Purified 63-kDa fragments of PA and mutant PA proteins (40 μg/ml) were adsorbed to a thin carbon film and negatively stained with 1% uranyl formate as described earlier (21).

RESULTS AND DISCUSSION

Prior work showed that proteolytic cleavage of PA at the sequence 164RKKR167 in solution or on the surface of mammalian cells results in the removal of the N-terminal 20-kDa fragment (PA20) that leads to heptamer formation (11). The heptamer has been assumed to insert into membranes at acidic pH (15). Acidic pH inside the endosome leads to insertion of PA63 into the membrane by forming a β-barrel composed of an
Dominant Negative Mutant of PA

**TABLE I**

<table>
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<th>Characteristics of mutant PA protein</th>
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<sup>a</sup> Sequence at 2β2–2β3 loop: 329TVGVSISAGYQNGFTGNITTSAG344.

<sup>b</sup> TTD is the time to death of Fischer 344 rats after administration of proteins.

<sup>c</sup> Toxicity was determined by dye oxidation assay (19) and inhibition of protein synthesis (16).

<sup>d</sup> PA and PA-I (1 mg/ml) were incubated with trypsin (1 µg/ml) for 30 min at 22 °C and analyzed on 10% SDS-PAGE followed by staining with Coomassie Blue. The extent of cleavage was estimated visually and compared to that of wild-type PA.

<sup>e</sup> Binding of LF to PA and PA-I was measured by incubating trypsin-nicked PA (2 µg/ml) and PA-I (2 µg/ml) with CHO-K1 cells at 4 °C for 2 h. Cells were then washed and incubated with 35S-labeled LFn for 1 h. After another washing step, cell-associated radioactivity was measured. Counts equivalent to 4500 dpm were taken as 100%.

<sup>f</sup> Trypsin-nicked PA and PA-I were incubated with CHO-K1 cells for 2 h at 4 °C. After washing, cells were treated with buffer of pH 5.0 and incubated at 37 °C for 30 min. Cells were then solubilized in SDS sample buffer, analyzed on 4–25% SDS-PAGE followed by immunoblotting, and detected using chemiluminescence detection kit.

amphipathic 2β2–2β3 loop consisting of an alternating arrangement of hydrophilic and hydrophobic amino acids (11).

Because acidic pH is necessary for both oligomerization and membrane insertion by PA63 (15), we investigated the functional significance of the correlation between the conditions required for both of the events to occur. Mutant PA protein (PA-I) was produced in which residues constituting the 2β2–2β3 loop were replaced with the corresponding residues of iota-b toxin, a closely related toxin secreted by C. perfringens (11). The typical yield of the proteins was 10 mg/l. PA-I did not aggregate in solution and behaved similar to wild-type PA.

Purified PA-I was tested for its ability to lyse J774A.1 macrophage cells in combination with LF. Whereas wild-type PA lysed 50% of the cells at a concentration of 0.04 µg/ml in 3 h (Fig. 2A), PA-I was completely non-toxic to J774A.1 cells at the highest concentration tested (100 µg/ml) (Fig. 2A). The data suggest that PA-I is inactive in exhibiting a lethal effect to macrophage cells as compared with wild-type PA. A more sensitive assay to measure the toxic activity of PA is to study the PA-dependent inhibition of protein synthesis in combination with LF-(1–254)/TR-PE-(398–613) (16). The fusion protein is comprised of the N-terminal 254 amino acids of LF (LF-(1–254); LFn) fused to the ADP-ribosylating domain of Pseudomonas aeruginosa exotoxin (16). Cytotoxicity in this assay is measured by the inhibition of protein synthesis catalyzed by Pseudomonas exotoxin and resulting from the ADP-ribosylation of elongation factor 2 (22). Whereas wild-type PA (0.1 µg/ml) showed 90% inhibition in protein synthesis when administered in combination with LF-(1–254)/TR-PE-(398–613) (1 ng/ml) to CHO-K1 cells as measured by the percent incorporation of [3H]leucine, no inhibition in protein synthesis was sensitive assay to measure the toxic activity of PA is to study the PA-dependent inhibition of protein synthesis in combination with LF-(1–254)/TR-PE-(398–613) (16). The fusion protein is comprised of the N-terminal 254 amino acids of LF (LF-(1–254); LFn) fused to the ADP-ribosylating domain of Pseudomonas aeruginosa exotoxin (16). Cytotoxicity in this assay is measured by the inhibition of protein synthesis catalyzed by Pseudomonas exotoxin and resulting from the ADP-ribosylation of elongation factor 2 (22). Whereas wild-type PA (0.1 µg/ml) showed 90% inhibition in protein synthesis when administered in combination with LF-(1–254)/TR-PE-(398–613) (1 ng/ml) to CHO-K1 cells as measured by the percent incorporation of [3H]leucine, no inhibition in protein synthesis was detected with PA-I when used even at a concentration of 100 µg/ml (Fig. 2B). The marked inhibition of the biological activity of the mutant PA protein confirmed the functional significance
cells were incubated with medium containing \[3H\]leucine (1 Ci/ml) for 2 h at 4 °C. After washing with phosphate-buffered saline, the cells were further incubated for 1 h with \[^{35}\text{S}\]labeled LFn at 4 °C. After another washing step, the pH of the medium was either maintained at 7.0 or lowered to 5.0. The cells were either lysed directly or treated with Pronase E to remove surface bound proteins. Cell-associated radioactivity was then counted. Results are expressed as percent \[^{35}\text{S}\]labeled LFn translocated of the total bound to cells.

**FIG. 5.** In vitro translocation assay. CHO-K1 cells were chilled in ice for 15 min and incubated with trypsin-nicked PA or PA-I for 2 h at 4 °C. After washing with phosphate-buffered saline, the cells were further incubated for 1 h with \[^{35}\text{S}\]labeled LFn at 4 °C. After another washing step, the pH of the medium was either maintained at 7.0 or lowered to 5.0. The cells were either lysed directly or treated with Pronase E to remove surface bound proteins. Cell-associated radioactivity was then counted. Results are expressed as percent \[^{35}\text{S}\]labeled LFn translocated of the total bound to cells.

**FIG. 6.** Inhibition of toxic activity of PA in combination with LF-(1–254)/TR-PE-(398–613). CHO-K1 cells were incubated with PA-I or PA-D mixed with varying concentrations of wild-type PA at 37 °C for 3 h in combination with LF-(1–254)/TR-PE-(398–613). At the end of 3 h, cells were incubated with medium containing \[^{3}H\]leucine (1 μCi/ml) for 1 h at 37 °C. At the end of the experiment, the amount of \[^{3}H\]leucine incorporated by viable cells in the absence of added proteins.

**FIG. 7.** Inhibition of channel-forming activity of PA by PA-I. CHO-K1 cells, preloaded with \[^{86}\text{Rb}\]^+, were incubated with trypsin-nicked PA or PA-I mixed in equimolar ratios at neutral pH for 2 h at 4 °C. After washing twice with cold phosphate-buffered saline, the cells were subjected to acidic pH shock as described under “Experimental Procedures.” The leakage of \[^{86}\text{Rb}\]^+ into the medium was then determined. Results are expressed as percent of \[^{86}\text{Rb}\]^+ associated with cells in the absence of added proteins.

of 2β2–2β3 loop of PA for the biological activity of anthrax toxin.

Several previous studies have shown that cleavage at the sequence RKKR167 by trypsin/furin is a prerequisite for anthrax toxin action (17) and leads to the formation of SDS-resistant oligomers by PA63 at acidic pH (15). Analysis of the mutant PA protein for sensitivity toward trypsin showed that the mutant was equally susceptible to trypsin as wild-type PA (Table I). The mutation introduced did not affect the ability of PA-I to bind LFn on cell surface (Table I) and, therefore, did not alter the ability of PA to bind to the receptor.

Purified PA63 forms SDS and boiling-resistant oligomers when exposed to acidic pH on mammalian cells (15). We, thus, determined whether PA-I retained the ability to form SDS-resistant oligomers in solution as well as when incubated with mammalian cells. PA-I was equally as effective in oligomerizing in solution as was wild-type PA (Table I). Electron microscopy data confirmed the formation of heptamers by PA-I as well as wild-type PA (Fig. 3, A and B). These results suggest that PA-I retained the ability to perform intermolecular interaction leading to oligomerization.

We next examined the ability of PA-I to insert into the plasma membrane of CHO-K1 cells at acidic pH. Membrane insertion was tested by measuring the ability of PA-I to form ion-conductive channels in the plasma membrane of mammalian cells. PA63 forms ion-conductive selective channels in artificial membranes and plasma membrane of mammalian at acidic pH (13, 14). Earlier studies have correlated the ability of PA to insert into membranes with the extent of \[^{86}\text{Rb}\]^+ released at acidic pH (16). Cells preloaded with \[^{86}\text{Rb}\]^+ were incubated with trypsin-nicked PA or PA-I at 4 °C and placed into acidic medium, and release of \[^{86}\text{Rb}\]^+ was measured. Incubation of trypsin-nicked wild-type PA (2 μg/ml) induced release of 70% \[^{86}\text{Rb}\]^+, whereas trypsin-nicked PA-I did not cause leakage of \[^{86}\text{Rb}\]^+ (Fig. 4). The result suggests that PA-I is unable to form ion-conductive channels and that integrity of the 2β2–2β3 loop is essential for proper membrane insertion by PA63.

To test the ability of PA-I to translocate LF into the cytosol of mammalian cells, we employed the previously described in vitro translocation assay (20) that uses the in vitro transcribed and translated LFn labeled with \[^{35}\text{S}\]methionine. We measured the ability of PA-I to translocate radiolabeled LFn across the plasma membrane of CHO-K1 cells upon treatment with low pH buffer. As shown in Fig. 5, whereas wild-type PA translocated 45% of the bound LFn at pH 5.0, PA-I showed no translocation of LFn. Insignificant translocation of LFn was observed with wild-type PA at pH 7.0 consistent with the earlier reports that showed that acidic pH is a prerequisite for the translocation event to occur (20). The results suggest that the mutant PA protein is inactive in translocation. The results confirm the previous propositions that membrane insertion by...
PA63 is a prerequisite for the translocation of LF into the cytosol but do not, in any way, suggest that LF passes through the lumen of the channel formed by the 2β2–2β3 loop.

We next investigated whether mixing of the mutant PA protein and wild-type PA at varying ratios resulted in alterations in the cytotoxic activity of the wild-type toxin. When the mutant and wild-type PA were present at equimolar concentrations, complete inhibition in protein synthesis of CHO-K1 cells was observed (Fig. 6). A significant inhibition could be detected when the ratio of PA-I to PA was 1:4. These data suggest that the PA-I inhibits wild-type PA-mediated cellular intoxication.

It has been reported previously that a mutant form of PA, in which the furin cleavage site 164RKKR167 was deleted (PA-D), blocked the anthrax toxin effect in vitro and in vivo (17). Because this molecule cannot be cleaved by trypsin or furin, does not form oligomers, and can only inhibit the action of wild-type PA by competing for cellular receptor, this molecule was employed as a control in our experiments to investigate whether inhibition in the cytotoxic activity was due to the competition for binding to the receptor. PA-D failed to inhibit the cytotoxic effect when mixed with wild-type PA in all the ratios tested (Fig. 6). It has been shown previously that PA-D, rather than wild-type PA, inhibits the lethal toxin activity when present at a 10-fold excess concentration (17). Typically, a substantial excess of mutant protein is required to inhibit the binding of an active ligand to cell surface receptors, and PA-I more likely inhibits the action of anthrax toxin by interacting with wild-type PA to form an inactive hetero-heptameric complex and thus, is a more potent inhibitor of anthrax toxin action. This model is consistent with the ability of purified PA-I to inhibit wild-type PA-mediated cytotoxic activity when the ratio of PA-I to PA is 1:4. Purification of active homoheptamers of PA and PA-I to homogeneity was not successful due to the presence of lower order oligomers as well.

To confirm the hypothesis that wild-type PA and PA-I might assemble to form non-functional oligomeric structures, the trypsin-nicked proteins (2 μg/ml each) were mixed together at neutral pH and incubated with CHO-K1 cells preloaded with 86Rb+ at 4 °C. After 2 h, the cells were washed to remove unbound proteins and incubated with isotonic buffer of pH 5.0, 5.5, or 7.0 for 30 min at 37 °C. Whereas wild-type PA released 62% of the radiolabel from cells, equimolar mixture containing PA and PA-I showed insignificant release of 86Rb+ (Fig. 7). The results suggest that there is complete inhibition of channel-forming ability of PA by PA-I. Indeed, the capacity of PA-I to dramatically alter the channel-forming ability of wild-type PA provides evidence that these two species can interact to form dysfunctional hetero-oligomeric structures. A dominant negative mutant of VacA toxin secreted by Helicobacter pylori has recently been reported that inhibits the vacuolating activity of wild-type toxin when present at a 5-fold-less concentration than wild-type VacA (23).

Identification of such a dominant negative inhibitor might be valuable for treatment of anthrax toxin action. Animal experiments were thus initiated to test the efficacy of PA-I to act as a dominant negative inhibitor of lethal toxin action in vivo. Whereas wild-type lethal (40 μg of PA + 8 μg of LF) resulted in the death of male Fischer 344 rats in ~60 min (Table I), a 1:1 mix containing wild-type PA and PA-I (40 μg of PA + 40 μg of PA-I + 8 μg LF) protected rats, and no symptoms were evident even after 48 h. Equimolar ratio of wild-type PA and PA-D resulted in the death of rats within 70 min (Table I). Taken together, these data confirm that PA-I can act as a dominant negative and a potent inhibitor of anthrax toxin action in vivo.

Use of B. anthracis as a bioweapon has become the bane of the defense establishments in various countries (1). Keeping in view the potent activity of PA-I both in vitro and in vivo it has the potential to be used as therapeutic agent for use in neutralizing anthrax toxin action in individuals infected with B. anthracis.

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