The Carboxyl-terminal End of Protective Antigen Is Required for Receptor Binding and Anthrax Toxin Activity*

(Received for publication, February 21, 1991)

Yogendra Singht, Kurt R. Klimpel, Conrad P. Quinata, Vijay K. Chaudhary, and Stephen H. Leppla†

From the Laboratory of Microbial Ecology, National Institute of Dental Research, and Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Anthrax toxin consists of three separate proteins produced by Bacillus anthracis: protective antigen (PA), lethal factor (LF), and edema factor (EF). Previous work showed that the process by which these proteins damage eukaryotic cells begins with binding of PA (83 kDa) to cell surface receptors. PA is then cleaved by a cell surface protease so as to expose a high-affinity binding site for LF or EF on the COOH-terminal, receptor-bound, 63-kilodalton fragment. In this report we more closely define a region of PA involved in receptor binding. The gene encoding PA was mutagenized so as to delete 3, 5, 7, 12, or 14 amino acids from the carboxyl terminus of the protein, and the truncated PA variants were purified from Bacillus subtilis or Escherichia coli. Deletion of 3, 5, or 7 amino acids reduced the binding of PA to cells and the subsequent toxicity of the PA-LF complex to J774A.1 cells and also the ability to cause EF binding to cells. Deletion of 12 or 14 amino acids completely eliminated all these activities. These results show that the carboxyl terminus comprises or is part of the receptor-binding domain of PA.

Bacillus anthracis secretes three proteins that are collectively designated anthrax toxin. These proteins are protective antigen (PA), lethal factor (LF), and edema factor (EF). Each protein lacks toxic activity when administered alone; instead, the proteins act in binary combinations to produce two toxic activities. Thus, PA with LF (designated "lethal toxin") causes death of experimental animals (8) and lysis of mouse and rat macrophages (9) and is considered to be responsible for the death of infected animals (2, 10). The intracellular mechanism for LF action is currently unknown.

The genes for each of the anthrax toxin components have been cloned and sequenced (11-15). The PA gene encodes a polypeptide of 735 amino acids. We have reported previously that PA binds to a specific cell surface receptor and is then cleaved by a cell-associated protease which releases an NH2-terminal 19-kDa fragment (16-18). Removal of this fragment creates a high-affinity binding site for LF and EF. PA can also be specifically cleaved in vitro at or near this site with trypsin, producing a 63-kDa fragment that retains bioactivity (16) and can insert in artificial lipid membranes (19). Thus PA appears to have at least three different functions: (i) receptor binding, (ii) LF or EF binding, and (iii) translocation of the toxic complex to the cytosol. The region involved in receptor binding has been studied in this paper by construction, expression, and characterization of PA proteins lacking 3-14 amino acids at the carboxyl terminus.

EXPERIMENTAL PROCEDURES

Reagents and General Procedures—Restriction endonucleases and DNA modification enzymes were purchased from Bethesda Research Labs, New England Biolabs, or Pharmacia LKB Biotechnology Inc. Low melting point agarose (Sea Plaque) was from FMC Corp. Oligonucleotides were synthesized on a PCR Mate (Applied Biosystems) and purified on oligonucleotide purification cartridges (Applied Biosystems). The polymerase chain reaction (PCR) was done using a DNA amplification reagent kit (GeneAmp) from Perkin-Elmer Cetus Instruments and a Perkin-Elmer Cetus Thermal Cycler. The amplification involved denaturation at 95 °C for 1 min, annealing at 37 °C for 2 min, and extension at 72 °C for 2 min, for a total of 30 cycles. A final extension was run at 72 °C for 7 min. DNA sequencing reactions were done using the T7 sequencing kit from Pharmacia, and DNA sequencing gels were made with Hydrolink gel solution from AT Biochem (Malvern, PA). [32P]Adenosine 45 Ci/mmol and [3H]labeled Bolton Hunter reagent (2000 Ci/mmol) were purchased from Amersham Corp. J774A.1 cells were obtained from the American Type Culture Collection. Chinese hamster ovary cells (CHO) were obtained from the laboratory of Dr. Michael Gottesman (National Cancer Institute, National Institutes of Health).

Plasmid Construction—The protective antigen gene (pag) was mutated by oligonucleotide-directed mutagenesis of two previously described plasmids, pYS2 and pYS5 (17). pYS2 contains a T7 promoter driving expression of pag without a signal peptide sequence. pYS5 is an Escherichia coli Bacillus subtilis shuttle vector containing the PA gene and additional 5' B. anthracis DNA that includes the pag promoter and signal peptide sequence.

Oligonucleotide-directed Mutagenesis—PA gene coding for proteins truncated at the carboxyl terminus were generated by PCR amplification of the native pag gene from a pYS5 template. A nonmutagenic oligonucleotide primer corresponding exactly to nucleotides 2942-2964 of pag (numbering system of Weikos et al. (20)), a region containing the Pat1 recognition site unique to pag and pYS5, was used for all amplification reactions. Mutagenic oligonucleotide primers were designed to be complementary to the intended new 3'
end of the PA coding sequence (near nucleotides 4054-4068) and to introduce two i-frame stop codons and a unique BamHI site. For example, the primer for deleting nucleotides 4087-4096 was 5'-CAC CTA GAA TTA CCT GGA TCC TAT TAG CCT TTG TTA GAA AAC-3', where the underlined nucleotides are the reverse complement 4086-4095. Amplified PCR products were digested with restriction endonucleases PstI and BamHI and gel-purified after electrophoresis in 1.2% low-melting point agarose gels. Plasmids pYS2 and pYS5 were digested with PstI and BamHI, purified by the same method, and ligated to the PCR generated mutant gene fragments. Mutant constructions were identified by restriction analysis and the mutations confirmed by dyeoxy sequencing of at least 200 nucleotides spanning the mutated region. PA deletion proteins were designated PA-732, PA-730, PA-728, PA-723, and PA-721, according to the number of residues remaining of the original 83-kDa proteins. PA-732, PA-730, and PA-728 were expressed from pYS2-derived shuttle vectors pYD15, pYD16, and pYD17 in B. subtilis DB104 (21), which lacks two major extracellular proteases. The longer deletions, proteins PA-723 and PA-721, were expressed in E. coli BL21(DE3) (22) from the pYS2-derived plasmids pYS18 and pYS10 (Table I).

Expression and Purification of PA—B. subtilis DB104 containing the pYS plasmids were grown at 37 °C in 500 ml of EMEM medium (17) supplemented with 5% heat-inactivated fetal bovine serum, 2% glucose, and 25 mM HEPES (binding medium). Approximately 1 mg of purified protein was determined by scanning the Coomassie-stained gel with a laser densitometer. The proteins were purified from 500-ml cultures.

Cell Culture Techniques and Cytotoxicity Measurements—The medium and growth conditions for J774A.1 cells have been described previously (24). The potencies of PA preparations were measured in vitro by the release of [14C]adenine from J774A.1 cells (26). Nucleotide pools in cells were labeled by incubation with 0.1 μCi/ml [14C]adenine in Dulbecco's modified Eagle's medium (DMEM) for 1 h at 37 °C. For in vivo labeling, the cells were then isolated by centrifugation at 10,000 g for 15 min at 4 °C. The cells from 1 liter were suspended in 50 ml of buffer (0.1 M sodium cacodylate, 1.5 mM EDTA to 10 mM, and 200 mM NaCl), filtered through a 0.45-μm membrane, and then sterically stabilized by filtration through a 0.05-μm membrane. The culture filtrate was then adsorbed on a 1.5-ml column of anti-PA monoclonal antibody 10G4 (23), and the column was washed with 50 ml of phosphate-buffered saline, and eluted with 2 ml sodium thiocyanate containing 20 mM HEPES, pH 7.4. Sodium thiocyanate was removed by passing the sample through a 10-ml column of Sephadex G-25 (Pharmacia PD-10) equilibrated with 10 mM HEPES, pH 7.4. Approximately 1 mg of purified protein from the PA mutants lacking 3, 5, or 7 amino acids were obtained from 500-ml cultures.

Attempts to purify the proteins lacking 12 or 14 amino acids from B. subtilis supernatants were unsuccessful because of extensive degradation. Therefore these two proteins were purified from supernatants of B. subtilis DB104 strains by immunoabsorbent chromatography with monoclonal antibody 10G4, as described previously (27). However, we obtained only about 2 μg of the purified proteins/ml of culture, compared with 20 μg/ml obtained from pYS5 making native PA. The low yield of these proteins may be due to their increased sensitivity to B. subtilis proteases. Electrophoresis (SDS-PAGE) of the purified proteins showed these proteins were at least 90% pure (Fig. 1). To analyze the relative potencies of the purified proteins, dose-response analyses were performed with J774A.1 cells, using release of preloaded [14C]adenine as a measure of toxicity (Table I). In the presence of a fixed concentration of LF (0.5 μg/ml), native PA at 0.1 μg/ml caused release of 80% of the adenine after 3 h (Fig. 2). The purified proteins lacking 3, 5, or 7 amino acids were also toxic to J774A.1 cells when added with LF, but the cytotoxic activity was decreased 10- to 20-fold compared with full-length PA. PA proteins with 12 or 14 amino acids deleted at the carboxyl terminus were not toxic to J774A.1 cells even at a 100-fold higher concentration (Fig. 2).

Because other data had suggested that the COOH-terminal region is required for receptor recognition, we considered it probable that the lower activity of the truncated PA proteins resulted from decreased receptor binding. To measure differences in the binding of truncated PA to cell surface receptors an assay was developed to measure competition between intact 125I-PA and the truncated PA proteins. Mutant proteins truncated by 3, 5, or 7 amino acids had a 2- to 10-
fold reduction in cell binding activity (Fig. 3), whereas binding activity was completely lost upon deletion of 12 or 14 amino acids. These results support the interpretation that the decreased toxicity of these truncated proteins is due to their failure to bind to receptors.

Because PA is a required component of both lethal toxin and edema toxin, it was expected that the truncated PA proteins would also fail to promote EF binding. To test this hypothesis, CHO cells were exposed for 12 h at 0 °C to EF in the presence of native or truncated PA proteins. The CHO cells were washed extensively with binding medium, lysed, and the adenylyl cyclase activity of the cell lysate was assayed (3). Because EF by itself is incapable of binding to the cell surface receptor (as confirmed in the controls shown), the amount of adenylyl cyclase activity is a direct measure of the ability of PA to promote binding of EF. The data show that PA proteins deleted by 3, 5, or 7 amino acids promoted binding of EF, whereas deletions lacking 12 or 14 amino acids did not (Table II).

The data presented above show that the integrity of the carboxyl terminus is required for binding of PA to its cell receptor. However, the data do not allow us to unequivocally state that the amino acids near the terminus interact directly with the receptor, because the effects observed would also be expected if the terminal residues were instead (or in addition) needed for maintenance of the conformation of a spatially distant protein surface that interacted with the receptor. Although the evidence available cannot rule out either of these interpretations, an analysis of the properties of the smaller deletions (3, 5, and 7 amino acids) does lead us to favor the view that the terminal 7 residues are directly involved in receptor interactions. The two larger deletions (12 and 14 amino acids) differ qualitatively, being totally inactive (Fig. 2), and these will be discussed separately.

All of the decrease in activity of the smaller deletions can be attributed to their lowered affinity for receptor. Thus, the 2- to 10-fold decrease in receptor-binding affinity implied by the competitive binding data (Fig. 3) is sufficient to explain the similar losses in toxicity (Fig. 2). The smaller decreases (1- to 2-fold) observed in the assay for EF binding (Table II) can be attributed to the use of the mutated PA proteins at a single high concentration, 1.0 µg/ml. Thus, although the 3-, 5-, and 7-amino acid deletions have 10-fold lower affinity for receptor, their use at 1.0 µg/ml will lead to saturation of the receptor. The fact that EF binding under these conditions nearly equals that with native PA shows that the mutant PA proteins are still capable of being nicked and of then binding EF with nearly normal efficiency. Further evidence that these proteins retain native conformation are their successful purification from B. subtilis culture supernatants in an unnicked state (Fig. 1), implying resistance to bacterial proteases, and studies (data not shown) in which it was found that the shorter deletions can be activated with trypsin and then bind LF or EF, in the same manner as native PA.

![Fig. 1. Electrophoretic analysis of truncated PA proteins.](image)

A. Proteins (2.5 µg/well) were separated by 10% SDS-PAGE, and stained with Coomassie Brilliant Blue. Lane M, protein standards; lane 1, PA-735; lane 2, PA-732; lane 3, PA-730; lane 4, PA-728; lane 5, PA-723; lane 6, PA-721. B. Western blot of the purified PA proteins, developed with monoclonal antibody 3D11. Samples were the same as in A.

**Table 1**

| Description of mutant plasmids and activity of expressed PA proteins |
|---------------------------------------------------------------|
| **PA variants** | **Plasmids** | **Expression host** | **Carboxyl-terminal sequence** | **Cytotoxicity, LC<sub>50</sub>** |
|-----------------|--------------|---------------------|-------------------------------|-------------------------------|
| PA (PA-735)    | pYS2         | *E. coli*           | GIKKLIFSKKGYEIG              | 0.05*                        |
| PA (PA-735)    | pYS5         | *B. subtilis*       | GIKKLIFSKKGYEIG              | 0.05                         |
| PA-732         | pYD15        | *B. subtilis*       | GIKKLIFSKKGY                | 0.5                          |
| PA-730         | pYD16        | *B. subtilis*       | GIKKLIFSKK                  | 1.0                          |
| PA-728         | pYD17        | *B. subtilis*       | GIKKLIFS                    | 0.8                          |
| PA-723         | pYS18        | *E. coli*           | GIKK                        | >10                          |
| PA-721         | pYS10        | *E. coli*           | GIKK                        | >10                          |

* Mutant PA proteins were expressed either in B. subtilis or *E. coli* and purified on anti-PA monoclonal antibody columns. J774A.1 cells were exposed to LF (0.5 µg/ml) and increasing amounts of truncated PA proteins. After 3 h, [<sup>14</sup>C]adenine released into the medium was counted. LC<sub>50</sub> is the concentration of PA required to release 50% of the [<sup>14</sup>C]adenine into the medium.
Activities of Truncated Anthrax Protective Antigen Proteins

**Fig. 2.** Toxicity of truncated PA for J774A.1 cells. Cells (2–3 x 10⁶ in 1 ml) were cultured and labeled in 24-well tissue culture plates in DMEM containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). LF at a final concentration of 0.5 µg/ml and the indicated concentrations of PA or truncated PA was added to the cultures. After a 3-h incubation at 37 °C, toxicity was measured by counting [³¹]adenine released into the medium.

**Fig. 3.** Binding of truncated PA proteins to cell receptors. J774A.1 cells were cultured in 24-well tissue culture plates, and PA (0.1 µg/ml) mixed with several concentrations of truncated PA was added to the wells and incubated for 12 h at 4 °C. At the end of the experiment, cells were washed four times with cold medium containing 1% bovine serum albumin to remove nonspecifically bound PA. The cells were solubilized in 0.1 M NaOH and counted in a γ counter. Controls showed that more than 85% of binding was specific (i.e., was competed by a 100-fold excess of nonradioactive PA).

The properties of the larger deletions (12 and 14 amino acids) cannot be so clearly explained. These proteins would obviously share with the shorter deletions any decreases in receptor binding due to the loss of the terminal seven amino acids. However it appears that these longer deletions also have additional defects in function. These proteins are much more susceptible to proteolysis in bacterial extracts and supernatants, implying that they have a less compact structure. The total inability of these proteins to bind to receptor or to promote toxicity could then be due either to the loss of an extended conformational structure involved in receptor binding or to the absence of the particular amino acids that constituted, with the terminal 7 residues, a sequence-determined receptor ligand.

Some additional insights into the structure of the receptor-binding structures in PA may be inferred from the behavior of certain anti-PA monoclonal antibodies that neutralize anthrax toxin. Antibodies 3B6 and 14B7 (23) cannot bind to PA that is already bound to its cell receptor and neutralize PA only if incubated with it prior to addition to cells. Therefore the antibodies are considered to bind at or very close to the site on PA which binds to the cell receptor. Because these antibodies fail to react with PA on immunoblots from SDS gels, it appears that this site on PA consists of a conformationally determined structure rather than a sequence of contiguous amino acids.

Several precedents exist for the participation of the COOH-terminal regions of protein toxins in receptor binding. For example, early evidence that the receptor-binding domain of diphtheria toxin was near the carboxyl terminus (27) was recently refined by showing that the last 54 amino acids of the toxin, released as a peptide by cleavage with hydroxylamine, can block binding of the toxin to receptor (28). Similarly, the receptor-binding domain of the Clostridium perfringens enterotoxin was localized to the COOH-terminal half of the protein by analysis of a recombinant fragment (29) and subsequently was shown to reside in the last 31 amino acids by expression and testing of this small region (32).

The carboxyl termini of some protein toxins may have roles other than or in addition to receptor binding. For instance, it was noted that Pseudomonas exotoxin A and cholera toxin have COOH-terminal sequences that resemble the sequence Lys-Asp-Glu-Leu (22), shown to be required for retention of newly synthesized eukaryotic proteins in the endoplasmic reticulum (30, 31). In the case of exotoxin A, the Arg-Glu-Asp-Leu-Lys sequence is not involved in binding to the cell surface receptor, but is essential for correct intracellular trafficking. Although it is also possible that the COOH terminus of PA may play a role in determining the intracellular trafficking of the LF and EF toxin components, the data presented here show that one important function of the carboxyl terminus is to form part of the region required for recognition of the target cell surface receptor.

A complete understanding of the role of the COOH-terminal amino acids in PA will come only after an extensive mutational analysis of this region is completed. This should include use of substitutions as well as deletions. Also, if the interpretation presented above is correct, then it would be expected that synthetic peptides corresponding to the COOH terminus of PA might compete for receptor. However we have been discouraged from beginning such work by preliminary evidence that a purified fragment corresponding to residues

---

**TABLE II**

| PA variants (1.0 µg/ml) | EF (0.5 µg/ml) | EF bound ng/well |
|------------------------|---------------|-----------------|
| PA                     | EF            | 0.05 ± 0.009    |
| PA-725                 | EF            | 0.25 ± 0.014    |
| PA-722                 | EF            | 9.8 ± 0.44      |
| PA-720                 | EF            | 7.9 ± 1.3       |
| PA-730                 | EF            | 5.7 ± 0.57      |
| PA-728                 | EF            | 5.0 ± 0.16      |
| PA-723                 | EF            | 0.04 ± 0.005    |
| PA-721                 | EF            | 0.09 ± 0.005    |

*CHO cells were cultured in 12-well plates in EMEM containing nonessential amino acids and 10% fetal bovine serum. EF and the truncated PA proteins were added at the indicated final concentrations, and the cells were incubated for 12 h at 0 °C. Cells were washed to remove unbound proteins, solubilized in 0.1% Triton X-100, and the lysates assayed for adenyl cyclase activity. Each value is the average ± S.E. of three wells.

---

² Y. Singh and S. H. Leppla, unpublished data.
Activities of Truncated Anthrax Protective Antigen Proteins

624–735 does not compete with 125I-PA for binding to receptor.3

Acknowledgments—We thank Ray Fields for technical assistance, Stephen F. Little for monoclonal antibodies, and Jerry Keith for making this work possible.

REFERENCES

1. Beall, F. A., Taylor, M. J., and Thorne, C. B. (1962) J. Bacteriol. 83, 1274–1280
2. Stephen, J. (1986) Pharmacology of Bacterial Toxins (Dorner, F., and Drews, J., eds) pp. 381–395, Pergamon Press, Oxford
3. Leppla, S. H. (1988) Methods Enzymol. 165, 103–116
4. Smith, H., Keppie, J., and Stanley, J. L. (1955) Brit. J. Exp. Path. 36, 460–472
5. O’Brien, J., Friedlander, A., Dreier, T., Ezzell, J., and Leppla, S. (1985) Infect. Immun. 47, 306–310
6. Gordon, V. M., Leppla, S. H., and Hewlett, E. L. (1988) Infect. Immun. 56, 1066–1069
7. Leppla, S. H. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 3162–3166
8. Stanley, J. L., and Smith, H. (1961) J. Gen. Microbiol. 26, 49–66
9. Friedlander, A. M. (1986) J. Biol. Chem. 261, 7123–7126
10. Turnbull, P. (1990) Bacterial Diseases. Topics and Wilson’s Principles of Bacteriology, Virology and Immunity (Smith, G. R., and Esmon, C. R., eds) Vol. 3, pp. 364–377, Edward Arnold, Sevenoaks, Kent, United Kingdom
11. Vodkin, M. H., and Leppla, S. H. (1983) Cell 34, 693–697
12. Robertson, D. L., and Leppla, S. H. (1986) Gene (Amst.) 44, 71–78
13. Tippetts, M. T., and Robertson, D. L. (1988) J. Bacteriol. 170, 2263–2266
14. Robertson, D. L., Tippetts, M. T., and Leppla, S. H. (1988) Gene (Amst.) 73, 362–371

3 K. R. Klimpel and S. H. Leppla, unpublished studies.

15. Mock, M., Labruyere, E., Glaser, P., Danchin, A., and Ullmann, A. (1988) Gene (Amst.) 64, 277–284
16. Leppla, S. H., Friedlander, A. M., and Cora, E. (1988) Bacterial Protein Toxins (Fehrenbuch, F., Alouf, J. E., Falmagne, P., Goebel, W., Jeljaszewicz, J., Jurgen, D., and Rappoli, R., eds) pp. 111–112, Gustav Fischer, New York
17. Singh, Y., Chaudhary, V. K., and Leppla, S. H. (1989) J. Biol. Chem. 264, 19103–19107
18. Leppla, S. H., Friedlander, A. M., Singh, Y., Cora, E. M., and Bhatnagar, R. (1990) Salisbury Med. Bull. 68, (special suppl.) 41–43
19. Blaustein, R. O., Koehler, T. M., Collier, R. J., and Finkelstein, A. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 2209–2213
20. Walkos, S. L., Lowe, J. R., Edren-McCutchan, F., Vodkin, M., Leppla, S. H., and Schmidt, J. J. (1986) Gene (Amst.) 69, 287–300
21. Kawamura, F., and Doi, R. H. (1984) J. Bacteriol. 160, 442–444
22. Chaudhary, V. K., Jinno, Y., FitzGerald, D., and Pastan, I. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 308–312
23. Little, S. F., Leppla, S. H., and Cora, E. (1988) Infect. Immun. 56, 1807–1813
24. Bhatnagar, R., Singh, Y., Leppla, S. H., and Friedlander, A. M. (1989) Infect. Immun. 57, 2107–2114
25. Shrivati, V., and Krishna, G. (1985) Anal. Biochem. 147, 410–418
26. Singh, Y., Leppla, S. H., Bhatnagar, R., and Friedlander, A. M. (1989) J. Biol. Chem. 264, 11099–11102
27. Proia, R. L., Wray, S. K., Hart, D. A., and Eidels, L. (1980) J. Biol. Chem. 255, 12025–12033
28. Rolf, J. M., Gaedlin, H. M., and Eidels, L. (1990) J. Biol. Chem. 265, 7331–7337
29. Hannan, P. C., Wnek, A. P., and McClane, B. A. (1989) J. Bacteriol. 171, 6815–6820
30. Munro, S., and Pelham, H. R. B. (1987) Cell 48, 899–907
31. Vaux, D., Tooze, J., and Fuller, S. (1990) Nature 345, 495–502
32. Hannan, P. C., and McClane, B. A. (1990) Abstracts of the 90th Annual Meeting of the American Society for Microbiology, May 13–17, 1990, Anaheim, CA, Abstr. B-310