Serum-Mediated Cleavage of *Bacillus anthracis* Protective Antigen Is a Two-Step Process That Involves a Serum Carboxypeptidase

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ABSTRACT Much of our understanding of the activity of anthrax toxin is based on *in vitro* systems, which delineate the interaction between *Bacillus anthracis* toxins and the cell surface. However, these systems fail to account for the intimate association of *B. anthracis* with the circulatory system, including the contribution of serum proteins to the host response and processing of anthrax toxins. Using a variety of immunological techniques to inhibit serum processing of *B. anthracis* protective antigen (PA) along with mass spectrometry analysis, we demonstrate that serum digests PA via 2 distinct reactions. In the first reaction, serum cleaves PA83 into 2 fragments to produce PA63 and PA20 fragments, similarly to that observed following furin digestion. This is followed by carboxypeptidase-mediated removal of the carboxy-terminal arginine and lysines from PA20.

IMPORTANCE Our findings identify a serum-mediated modification of PA20 that has not been previously described. These observations further imply that the processing of PA is more complex than currently thought. Additional study is needed to define the contribution of serum processing of PA to the host response and individual susceptibility to anthrax.

KEYWORDS anthrax, proteases, toxin

*Bacillus anthracis* is the causative agent of anthrax and is widely recognized for its potential use as an agent of bioterrorism. *B. anthracis* secretes 2 bipartite toxins, the lethal toxin and the edema toxin, that are essential for virulence. Both toxins require the protective antigen (PA) component to mediate cell entry. PA is, therefore, essential to the damaging effects of anthrax toxins, and PA-deficient mutants are avirulent (1).

The current paradigm of toxin pathogenesis posits that *B. anthracis* secretes the proform of PA (PA83), which binds to cell surface receptors (tumor endothelium marker-8 or capillary morphogenesis protein-2), where it undergoes cleavage by cell-associated furin into 2 fragments, PA20 and PA63. PA63 subsequently undergoes hexamerization to form a prepore structure that binds edema factor (EF) or lethal factor (LF) and is internalized. Understanding the mechanism by which anthrax toxin is processed is important because interference with the processing steps is the basis for the development of therapeutics, including furin inhibitors (2). In addition, antibodies (Abs) reactive to PA are protective in animal models of anthrax and one monoclonal antibody, raxibacumab, has been licensed for clinical use (3–5).

Much of our understanding about toxin processing in anthrax pathogenesis is based
on experiments using in vitro systems (reviewed in reference 6). Nonetheless, these models fail to take into account the role of host serum proteins as part of the host response to anthrax. During the course of anthrax, B. anthracis encounters serum proteins at multiple stages, including invasion of the lymphatic system and high-level bacteremia, which occurs in the context of sepsis. In late stages of experimental anthrax in macaques, for example, lethal toxin concentrations on the order of 10^10/μg/ml have been reported (7). The intimate association between B. anthracis and serum is further highlighted by the presence of pathogen-associated proteins that directly act on elements within the circulation. This includes enzymes that digest host hemoglobin and circulating lethal toxin, which interferes with neutrophil function (8, 9).

Several lines of evidence suggest that PA processing is more complex than is apparent from the current model. Anthrax toxin is released from B. anthracis in vesicles that contain all toxin components (10). Although these vesicles may be rapidly disrupted by serum albumin-releasing toxin components (11), they are also released intracellularly. In addition, PA circulating in the serum is found in animal models as a complex of PA63 bound to LF or EF rather than as intact PA83 (12). In fact, serum from humans and other species has been shown to contain proteolytic activity that digests PA in a manner similar to that seen with furin (13–15). Our previous studies suggest a correlation between serum-mediated digestion of PA and protection from the killing effects of lethal toxin in vitro (15). In the current work, we found that serum-mediated processing of PA is a 2-step reaction that involves carboxypeptidase (CP)-mediated truncation of the PA20 fragment.

RESULTS

Serum-mediated digestion of rPA. Serum treatment of recombinant PA83 (rPA83) produced 2 protein fragments, PA63 and a band that is slightly lower in molecular mass than PA20 (Fig. 1; lane 6). The larger protein is similar in size to the PA63 protein produced by furin digestion of rPA83. However, the smaller protein is smaller than the PA20 protein produced by furin digestion of rPA83 and is referred to as truncated PA20. Furthermore, serum treatment of rPA83 before or after furin digestion still produced this truncated fragment (Fig. 1, lanes 2 and 4). Heat inactivation of serum prevented this
truncation (Fig. 1; lanes 3 and 5), consistent with the idea that the enzyme responsible for truncation is heat labile.

**Inhibition of serum-mediated digestion of rPA.** To determine the precise site at which serum cleaves rPA, we attempted to inhibit serum-mediated cleavage using a library of overlapping peptides, which represent the PA sequence and antibodies that recognize various PA sites. Preincubation of rPA with the 19D2 monoclonal antibody (MAb), which recognizes an epitope immediately C terminal of the furin site (16), prevented rPA digestion by serum and furin. This inhibition of digestion was not seen with other PA-specific antibodies, including 7.5G, which recognizes domain 1 of PA83. Serum-mediated PA cleavage was also prevented by coincubation of serum with 3 overlapping peptides (D5, D6, and D7), which contain the furin digestion site, but not with other peptides (including D12, E1, and E2, which represent PA sequences approximately 30 amino acid [AA] residues C terminal to the furin site) (not shown).

Using chemical inhibitors while measuring PA63 formation, we found that the serine/cysteine protease antipain partially inhibited the formation of PA63. In contrast, none of the other tested protease inhibitors, including bestatin, chymostatin, E-64, leupeptin, pepstatin, phosphoramidon, Pefabloc SC, and aprotinin, prevented PA63 formation. As in previous studies, we found that EDTA was a potent inhibitor of serum-mediated digestion of PA. For furin inhibitor I, concentrations as low as 0.001 mg/ml resulted in complete inhibition of serum digestion, whereas for furin inhibitor II, concentrations as low as 0.010 mg/ml produced complete inhibition of digestion (Fig. 2).

**Truncated PA20 fragment.** To better identify the precise site of serum-mediated digestion of rPA, the truncated PA20 fragment produced by serum digestion was examined by mass spectrometry (MS). First, the intact-protein mass of this fragment was measured and the experimental mass determined by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI MS) to be 23,600 Da (Fig. S1). Furin cleaves at RXK/RR, which would correspond to a predicted molecular mass of 25,157 Da for rPA (Fig. 3; N terminus to RKKR), which represents a difference of 1,557 Da (a value far beyond the error of measurement). To determine the sequence of the truncated PA20 fragment, in-gel trypsin digestion was performed. The liquid chromatography-tandem mass spectrometry (LC-MS/MS) data identified the underlined tryptic peptides shown in Fig. 3 (identified peptides from this tryptic digest are listed in Table S1). The peptide sequence LLNES ... GFIK is too large for fragmentation on an LTQ (linear ion trap quadrupole) mass spectrometer and was not detected by MS/MS, but the +4, +5, +6, +7, and +8 charge states were detected (Fig. S2). The predicted protein mass from the N terminus to the last tryptic peptide identified is 23,213 Da and if the next 4 amino acids (SSNS) are included the predicted protein mass increases to 23,588 Da, a difference of 12 Da or 0.05% compared with the experimental intact-protein mass (23,600 Da). These findings are consistent with serum-mediated cleavage of the basic,
C-terminal arginine and lysine residues from the PA20 fragment produced by furin digestion, possibly followed by carboxypeptidase.

**Carboxypeptidase treatment of rPA.** Given these results, we sought to determine whether this truncated PA20 fragment could result from serum carboxypeptidase digestion of PA20. Carboxypeptidases are a family of enzymes that cleave residues from the C-terminal end of a protein. This includes a group of enzymes that cleave basic amino acid residues from the carboxy terminus. To determine if carboxypeptidase could produce a truncated PA20 fragment, we conducted studies with a pancreatic carboxypeptidase. The effects of carboxypeptidase B (CPB) treatment on furin-digested rPA were dose dependent. At higher concentrations (250 μg/ml) (Fig. 4, lane 5), multiple digestion fragments of PA were observed and PA20 reactivity was completely lost. A similar pattern was seen in the absence of furin and presumably relates to the presence of contaminating trypsin in this pancreatic preparation. In contrast, at lower concentrations of CPB (25 μg/ml) (Fig. 4, lane 4), treatment produced a truncated PA20 fragment that was similar in size to that observed with serum digestion of PA (Fig. 4, lane 1). Lower concentrations of CPB (2.5 μg/ml) had no effect on the size of furin-treated PA20 compared with the results seen with furin treatment alone.

**Inhibition of serum carboxypeptidase activity.** Next, we sought to determine whether the ability of serum to produce a truncated PA20 fragment could be inhibited...
by carboxypeptidase inhibitors. Both guanidinoethylmercaptosuccinic acid (GEMSA) and potato tuber extract (PTI) are potent competitive inhibitors of carboxypeptidase, though their inhibitory activity is not specific to any one class of carboxypeptidases. Addition of GEMSA (500 μg/ml) to serum prevented the formation of a truncated PA20 and resulted in a PA20 fragment that was more similar in size to that produced by furin digestion (Fig. 5). In contrast, no inhibition was seen with lower concentrations of GEMSA and for all concentrations of carboxypeptidase inhibitor (PTI) from potato tuber extract.

**DISCUSSION**

*B. anthracis* and the toxins that it secretes have an intimate association with the circulation and with serum over the course of infection. Our studies confirm earlier reports that both human and animal sera contain a furin-like enzyme, which digests PA to produce PA63 and PA20 fragments (13–15). In our own studies, this activity was associated with protection against lethal toxin in vitro (15). We now extend these findings to demonstrate that human serum contains a carboxypeptidase which further processes the PA20 fragment by removing the C-terminal basic amino acid residues, resulting in a truncated PA20 fragment. These findings contrast with the current model of anthrax toxin, which suggests that processing of PA occurs only at the cell surface, and provide additional evidence for the complexity of anthrax toxin mechanisms of action. However, we note that serum and cell surface PA processing are not mutually exclusive events.

PA20 has been detected in the blood of *B. anthracis*-infected animals, though its contribution to anthrax pathogenesis is unknown (17). Nonetheless, several lines of evidence suggest that it may play an active role in infection. For example, PA20 contains a PA14 domain that is conserved among bacterial toxins and appears to play a role in cell binding (18). Hammamieh et al. reported that exposure of human peripheral blood mononuclear cells to PA20 induced a variety of genes related to the inflammatory system and to cell migration and triggered apoptosis in these cells (17). Furthermore, PA20 has been reported to bind lethal factor (19). Although circumstantial, these findings are consistent with a role for PA20 in the pathogenesis of anthrax.

Serum is known to contain 2 carboxypeptidases, CP-N and CPB2 (which is also known as CPU, plasma carboxypeptidase B, and thrombin-activatable fibrinolysis inhibitor). Both carboxypeptidases cleave carboxy-terminal arginine and lysine residues from peptides/proteins and have been implicated in regulating inflammation through their effects on serum protein cascades, such as the complement anaphylatoxins and kinins (20). As members of the carboxypeptidase family, both CP-N and CPB2 contain a zinc-binding site that makes them susceptible to inhibition by metal chelators. CP-N is constitutively produced by the liver, with serum concentrations on the order of 30 μg/ml (21). In contrast, CPB2 must be activated by fibrin and, once activated, downregulates fibrinolysis by removing terminal lysines from fibrin and

**FIG 5** GEMSA, but not PTI, prevented truncation of PA20. In the presence of high concentrations of GEMSA (GEMSA1; 500 μg/ml), inhibition of serum-mediated truncation of PA20 was present. In contrast, PTI at concentrations as high as 1.25 mg/ml (PTI1) failed to inhibit serum-mediated truncation of PA20. Lower concentrations of GEMSA (50 and 5 μg/ml; GEMSA2 and GEMSA3) and PTI (PTI2; 125 μg/ml) did not prevent serum-mediated truncation of PA20. This experiment was done 3 times with similar results.
is present in serum concentrations on the order of 4 to 15.0 μg/ml (22, 23). Elevated levels of CPB2 have been found both in animal models of bacterial sepsis and in septic patients and have been hypothesized to play a role in the hypercoagulability associated with sepsis (24–26). Interestingly, both carboxypeptidases have been shown to inactivate complement anaphylatoxins (27, 28). Furthermore, both C3 and C5 have been implicated in the host response to anthrax (29, 30). Thus, PA20 may possibly alter anthrax pathogenesis by interfering with anaphylatoxin inactivation during anthrax-associated sepsis.

It is interesting that CP-N is more susceptible to inhibition by GEMSA whereas CPB2 is more susceptible to inhibition by potato carboxypeptidase inhibitor (31). Thus, our findings are consistent with the hypothesis that in vitro, CP-N is primarily responsible for the observed truncation of PA20. Nonetheless, the precise carboxypeptidase responsible for the truncation of PA20 in vivo (including during the sepsis of anthrax) is not known and it is likely that there is redundancy to the process. Of note, macrophages also express a membrane-associated carboxypeptidase (CP-M) that cleaves C-terminal lysines and arginine residues from proteins (32). It is, therefore, likely that a similar form of processing occurs at the surface of target cells.

In summary, we demonstrate that serum processing of PA is a 2-step process that involves a furin-like digestion of the PA83 component followed by truncation of the PA20 fragment by serum carboxypeptidases. The significance of these 2 serum-associated activities remains to be defined. On the basis of earlier studies that associated furin-like digestion with protection, we believe that this activity may in fact contribute to the host response to anthrax. This would be consistent with the close association of B. anthracis with the circulatory system. We also suggest that it is possible that the variations in these serum proteolytic activities contribute to differences in individual susceptibilities to anthrax. Additional studies examining gain and loss of function in the context of experimental infection may help further delineate the importance of these processes.

MATERIALS AND METHODS

PA. Recombinant PA83 (rPA) and its amino acid sequence were obtained from Wadsworth Laboratories, New York State Department of Health (Albany, NY).

Sera. Serum was obtained from laboratory volunteers and stored at −80°C with approval from the Committee of Clinical Investigations at Albert Einstein College of Medicine. In some experiments, pooled sera, processed to retain complement activity (Sigma, St. Louis, MO), was used. These commercial sera produced results comparable to those obtained with sera from human volunteers.

Antibodies and peptides. A library of 6 murine monoclonal antibodies (7.5G, 16A12, 10F4, 19D9, 20G7, and 2H9) that were previously generated and characterized was used both to define the digestion site and as detection reagents for immunoblot studies (33). Binding sites for these antibodies are provided in Table S2. A previously synthesized library of overlapping peptides which represents the PA sequence was used for inhibition studies (16).

Proteolytic digestion and fragment detection. Proteolytic digestion studies were performed as previously described (15). Briefly, rPA (2.5 μg) was incubated with 25 μl of serum, phosphate-buffered saline, or furin (In Vitrogen) (0.5 units) at 37°C for 30 to 60 min. In some experiments, serum was heat treated at 56°C for 30 min prior to incubation with toxin. In other experiments, protease inhibitors (see below) or peptides at a concentration of 5 μg/ml were added to serum prior to incubation with rPA. Digested rPA was separated by SDS-electrophoresis and transferred to a nitrocellulose membrane. Membranes were blocked with 5% milk and then incubated with primary antibody. The following MAbs were used to characterize rPA cleavage: 10F4 (IgG1) and 7.5G (IgG2b). All MAbs were used at a concentration of 0.25 μg/ml. Primary antibody was detected with horseradish peroxidase-labeled goat isotype-specific antibody at a dilution of 1:25,000. Proteins were visualized by development with an ECL chemiluminescence kit (Pierce, Rockford, IL).

Inhibition studies. (i) Peptides. Serum (24 μl) was incubated with individual biotinylated peptides, peptide mixtures, or phosphate-buffered saline (PBS) for 2 h at room temperature. These peptides were chosen from a library of peptides representing the entire length of rPA and were synthesized as 15-mer, overlapping by 10 residues (16). This serum peptide mixture was then incubated with 1.5 μg of rPA for 30 min at 37°C, and the resulting mixture was subjected to separation by SDS-PAGE and detection by Western blotting.

(ii) MAbs. PA (1.5 μg) was incubated with one of several PA-specific MAbs (2 μg) (33) for 10 min at room temperature. This mixture was then added to 24 μl of serum, incubated at 37°C for 20 min, and then subjected to SDS-electrophoresis and immunoblotting.

(iii) Protease inhibitors. A volume of 10 μl of sera was preincubated with 1 of 9 protease inhibitors included in a commercially available protease inhibitor set (Roche) for 30 min at 30°C. Individual
inhibitors (including antipain, bestatin, chymostatin, E-64, phosphoramidon, Pefabloc SC, and aprotinin) were reconstituted per the instructions of each manufacturer. Following this incubation, 1.5 μg of rPA was added to the mixture and incubated at 37°C for 1 h. Specific inhibition of furin activity was accomplished using furin inhibitor I (Cayman Chemical Company) and furin inhibitor II (Sigma). These compounds are selective competitive inhibitors of the proprotein convertases, including furin. Serum (12 μL) was incubated with furin inhibitors at room temperature for 10 min, after which rPA (1.5 μg) was added and the entire mixture incubated for an additional 1 h at 37°C.

(iv) Carboxypeptidase inhibition. For these experiments, sera were pretreated with a variety of inhibitors for 30 min prior to incubation with rPA. These inhibitors included the following: guanidinooethylmercaptosuccinic acid (GEMSA) (Santa Cruz Biotechnology) and carboxypeptidase inhibitor from potato tuber extract (Sigma). The serum-PA digestion mixture was separated by electrophoresis. PA15-like and truncated PA20 fragments were then detected with antibodies 10F4 and 19D2, respectively.

Mass spectrometry (MS). To isolate the truncated PA20 molecule, serum-digested rPA was incubated overnight at 4°C with 200 μL of protein G resin in binding buffer (20 mM Tris, 150 mM NaCl, pH 7.4) together with 50 μg of MAb 19D2. The resultant slurry was centrifuged for 2.5 min at 2,500 × g and the resin washed 5 times with binding buffer (Pierce). Following elution, the protein was separated in a nondenaturing gel and electroeluted for further analysis.

Mass spectrometry (MS) measurements and liquid chromatography (LC) separations were obtained on an LTQ (linear ion trap quadrupole) mass spectrometer (Thermo Scientific, San Jose, CA), an LC 3000 rapid-separation system (Dionex Corporation, Sunnyvale, CA) was used for processing of tryptic peptides, and an HP Agilent 1100 series system was used for intact-protein separation. For intact-protein molecular weight measurements of the electro-eluted protein, a C4 Vydac TP column (1 by 50 mm; 300 Å; 50 μL/min) was used. After desalting performed with 1% acetonitrile–0.1% aqueous formic acid (FA) for 2 min, the protein was eluted after increasing the level of acetonitrile to 55% acetonitrile–0.1% aqueous FA. The mass range from 600 to 1,800 m/z was acquired on the LTQ mass spectrometer, and the raw data were deconvoluted using MagTran (34) or ProMass (Thermo Fisher Scientific). Another aliquot of the electroeluted protein was separated on a one-dimensional (1D) SDS gel, and selected molecular weight bands were excised for in-gel tryptic digestion as described previously (35). After sample injection and LC peptide separation (using an acetonitrile gradient), the 10 most abundant ions obtained from the survey scan (300 to 1,600 m/z) were selected for fragmentation (MS/MS). Normalized collision energy of 35% and a 2 m/z isolation width were used for MS/MS. The MS/MS data were converted to a text file for peptide/protein identification using Mascot (Matrix Science, Inc.).

Carboxypeptidase-mediated digestion of PA. To determine whether carboxypeptidase digestion of furin-treated rPA could produce a fragment similar in size to that seen with serum digestion of rPA, experiments were done with carboxypeptidase B (CPB) (Sigma). For these experiments, rPA was treated with furin for 10 min at 30°C and the mixture was incubated with CPB at different concentrations at 37°C. Proteins were separated by SDS-PAGE and detected by immunoblotting as described above.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00091-18.

FIG S1, TIF file, 1.2 MB.
FIG S2, TIF file, 1.5 MB.
TABLE S1, TIF file, 1.8 MB.
TABLE S2, DOCX file, 0.04 MB.

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REFERENCES

1. Pezard C, Berche P, Mock M. 1991. Contribution of individual toxin components to virulence of Bacillus anthracis. Infect Immun 59:3472–3477.
2. Shilayev SA, Remacle AG, Ratnikov BI, Nelson NA, Savinov AY, Wei G, Bottini M, Rega MF, Parent A, Desjardins R, Fugere M, Day R, Sabet M, Pellecchia M, Liddington RC, Smith JW, Mustelin T, Guiney DG, Lebl M, Strongin AY. 2007. Targeting host cell furin proprotein convertases as a therapeutic strategy against bacterial toxins and viral pathogens. J Biol Chem 282:20847–20853. https://doi.org/10.1074/jbc.M703847200.
3. Albrecht MT, Li H, Williamson ED, LeBlut CS, Flick-Smith HC, Quinn CP, Westra H, Galloway D, Matzku M, Gasiorek H, Bailleul J. 2007. Human monoclonal antibodies against anthrax lethal factor and protective antigen act independently to protect against Bacillus anthracis infection and enhance endogenous immunity to anthrax. Infect Immun 75:5425–5433. https://doi.org/10.1128/IAI.00261-07.
4. Brossier F, Lévy M, Landler A, Lafaye P, Mock M. 2004. Functional analysis of Bacillus anthracis protective antigen by using neutralizing monoclonal antibodies. Infect Immun 72:6313–6317. https://doi.org/10.1128/IAI.72.11.6313-6317.2004.
5. Anonymous. 2013. Raxibacumab for anthrax. Med Lett Drugs Ther 55:27–28.
6. Moayeri M, Leppla SH, Vrentas C, Pomerantsev AP, Liu S. 2015. Anthrax protective antigen. Annu Rev Microbiol 69:185–208. https://doi.org/10.1146/annurev-micro-091014-104523.
7. Boyer AE, Gallegos-Candelaria M, Quinn CP, Woolfitt AR, Brumlow JO, Isbell...
K, Hoffmaster AR, Lins RC, Barr JR. 2015. High-sensitivity MALDI-TOF MS quantification of anthrax lethal toxin for diagnostics and evaluation of medical countermeasures. Anal Bioanal Chem 407:2847–2858. https://doi.org/10.1007/s00216-015-8509-5.

8. Tervillinger A, Swick MC, Pflughoft JK, Pomerantsev A, Lyons CR, Koehler TM, Maresso A. 2015. Bacillus anthracis overcomes an amino acid auxotrophy by cleaving host serum proteins. J Bacteriol 197:2400–2411. https://doi.org/10.1128/JB.00073-15.

9. Weiner ZP, Ernst SM, Boyer AE, Gallegos-CandelA M, Barr JR, Glomski LI. 2014. Circulating lethal toxin decreases the ability of neutrophils to respond to Bacillus anthracis. Cell Microbiol 16:504–518. https://doi.org/10.1111/cmi.12132.

10. Rivera J, Cordero RJ, Nakouzi AS, Frases S, Nicola A, Casadevall A. 2010. Bacillus anthracis produces membrane-derived vesicles containing biologically active toxins. Proc Natl Acad Sci U S A 107:19002–19007. https://doi.org/10.1073/pnas.1008843107.

11. Wolf JM, Rivera J, Casadevall A. 2012. Serum albumin disrupts Crypto-coccos neoformans and Bacillus anthracis extracellular vesicles. Cell Microbiol 14:762–773. https://doi.org/10.1111/j.1462-5822.2012.01757.x.

12. Panchal RG, Halverson KM, Ribot W, Lane D, Kenny T, Abshire TG, Ezzell JW, Hoover TA, Powell B, Little S, KaslanovicJ BV, Bavi S. 2005. Purified Bacillus anthracis lethal toxin complex formed in vitro and during infection exhibits functional and biological activity. J Biol Chem 280:10834–10839. https://doi.org/10.1074/jbc.M412210200.

13. Ezzell JW, Jr, Abshire TG. 1992. Serum protease cleavage of Bacillus anthracis protective antigen. J Gen Microbiol 138:543–549. https://doi.org/10.1099/00221287-138-3-543.

14. Mohammad M, Wiggins JF, Leppila SH. 2007. Anthrax protective antigen cleavage and clearance from the blood of mice and rats. Infect Immun 75:5175–5184. https://doi.org/10.1128/IAI.00719-07.

15. Goldman DL, Zeng W, Rivera J, Nakouzi A, Casadevall A. 2008. Human serum contains a protease that protects against cytotoxic activity of Bacillus anthracis lethal toxin in vitro. Clin Vaccine Immunol 15:970–973. https://doi.org/10.1128/CVI.00064-08.

16. Abboud N, De Jesus M, Nakouzi A, Cordero RJ, Pujato M, Fiser A, Rivera J, Casadevall A. 2009. Identification of linear epitopes in Bacillus anthracis protective antigen defines a neutralizing epitope in domain 1. J Immunol 182:25077–25086. https://doi.org/10.4049/jimmunol.0700611.

17. Hammanie R, Ribot WJ, Abshire TG, Jett M, Ezzell J. 2009. Activity of the Bacillus anthracis 20 kDa protective antigen component. BMC Infect Dis 9:124. https://doi.org/10.1186/1471-2334-8-124.

18. Rigden DJ, Galperin MY. 2004. The DxDxDG motif for calcium binding: multiple structural contexts and implications for evolution. J Mol Biol 343:971–984. https://doi.org/10.1016/j.jmb.2004.08.077.

19. Chvyorka I, Zhang XC, Terzyan S. 2007. Anthrax lethal toxin bound by neutralizing antibodies. J Biol Chem 282:25077–25086. https://doi.org/10.1074/jbc.M109.022061.

20. Djukanovic R, Wilson SJ, Kraft M, Jarjour NN, Steel M, Chung RF, Bao W, Fowler-Taylor A, Matthews J, Busse WW, Holgate ST, Fahy JV. 2004. Effects of treatment with anti-immunoglobulin E antibody omalizumab on airway inflammation in allergic asthma. Am J Respir Crit Care Med 170:583–593. https://doi.org/10.1164/rccm.200312-1615OC.

21. Skidgel RA, Erdos EG. 2007. Structure and function of human plasma carboxypeptidase N, the anaphylatoxin inactivator. Int Immunopharmacol 7:1688–1899. https://doi.org/10.1016/j.intimp.2007.07.014.

22. Mosnier LO, von dem Barne PA, Meijers JC, Bouma BN. 1998. Plasma TAFI levels influence the clot lysis time in healthy individuals in the presence of an intact intrinsic pathway of coagulation. Thromb Haemost 80:829–835. https://doi.org/10.1111/j.1538-7836.2008.01653.x.

23. Bajzar L, Nesheim ME, Tracy PB. 1996. The procoagulant effect of activated protein C in clots formed from plasma is TAFI-dependent. Blood 88:2093–2100.

24. Binette TM, Taylor FB, Jr, Peer G, Bajzar L. 2007. Thrombin-thrombo- modulin links coagulation and fibrinolysis: more than an in vitro phenomenon. Blood 110:3168–3175. https://doi.org/10.1182/blood-2007-03-078824.

25. Kim PY, Kim PY, Hoogendorn H, Giles AR, Nesheim ME. 2008. Activated thrombin-activatable fibrinolysis inhibitor is generated in vivo at levels that can substantially affect fibrinolysis in chimpanzees in response to thrombin generation. J Thromb Haemost 6:1600–1602. https://doi.org/10.1111/j.1538-7836.2008.01067.x.

26. Park R, Song J, An SS. 2010. Elevated levels of activated and inactivated thrombin-activatable fibrinolysis inhibitor in patients with sepsis. Korean J Hematol 45:264–268. https://doi.org/10.5045/kjh.2010.45.4.264.

27. Bokisch VA, Müller-Eberhard HJ. 1970. Anaphylatoxin inactivator of human plasma: its isolation and characterization as a carboxypeptidase. J Clin Invest 49:2427–2436. https://doi.org/10.1172/JCI106462.

28. Campbell WD, Lazoura E, Okada N, Okada H. 2002. Inactivation of C3a and C5a octapeptides by carboxypeptidase R and carboxypeptidase N. Microb Immunol 46:131–134. https://doi.org/10.1111/j.1348-0421.2002.tb02669.x.

29. Premanandan C, Storozuk CA, Bajzar L, Nemeth MD, Schlesinger LS, Phipps AJ. 2009. Complement protein C3 binding to Bacillus anthracis spores enhances phagocytosis by human macrophages. Microb Pathog 46:306–314. https://doi.org/10.1016/j.micpath.2009.03.004.

30. Harvill ET, Lee G, Gripee VK, Merkel TJ. 2005. Complement depletion renders C57BL/6 mice sensitive to the Bacillus anthracis Sterne strain. Infect Immun 73:4420–4422. https://doi.org/10.1128/IAI.73.7.4420-4422.2005.

31. Matthews KW, Mueller-Orritz SL, Wetsel RA. 2004. Carboxypeptidase N: a pleiotropic regulator of inflammation. Mol Immunol 40:785–793. https://doi.org/10.1016/j.molimm.2003.10.002.

32. Rehill M, Krause SW, Kreutz M, Andreessen R. 1995. Carboxypeptidase M is identical to the max1 antigen and its expression is associated with monocyte to macrophage differentiation. J Biol Chem 270:15644–15649. https://doi.org/10.1074/jbc.270.26.15644.

33. Rivera J, Nakouzi A, Abboud N, Revskaya E, Goldman D, Collier RJ, Dadachova E, Casadevall A. 2006. A monoclonal antibody to Bacillus anthracis protective antigen defines a neutralizing epitope in domain 1. Infect Immun 74:4149–4156. https://doi.org/10.1128/IAI.00150-06.

34. Zhang Z, Marshall AG. 1998. A universal algorithm for fast and automated charge state deconvolution of electrospray mass-to-charge ratio spectra. J Am Soc Mass Spectrom 9:225–233. https://doi.org/10.1016/S1044-0305(97)00284-5.

35. Xiao Y, Pollack D, Andrusier M, Levy A, Callaway M, Nieves E, Reddi P, Vogdner M. 2016. Identification of cell-specific targets of SUMOylation during mouse spermatogenesis. Reproduction 151:149–166. https://doi.org/10.1530/REP-15-0239.