




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 heparanization 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

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 Serum carboxypeptidases cleave anthrax toxin component and could help in host defense

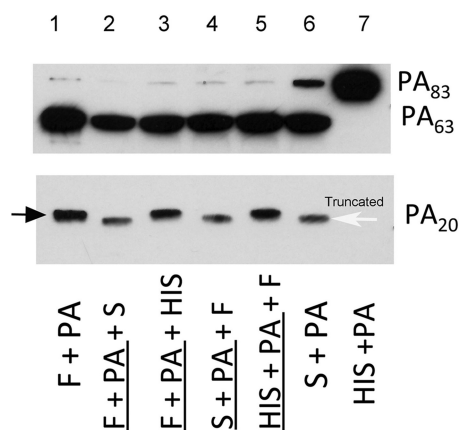


FIG 1 Serum-mediated digestion of rPA83 produces a truncated PA₂₀ fragment compared with furin-mediated digestion. Shown are the digestion fragments of PA₈₃ under conditions of incubation with furin (F; lane 1), serum (S; lane 6), or heat-inactivated serum (HIS; lane 7). Treatment of rPA83 with serum after and prior to furin digestion (lanes 2 and 4, respectively) produced a truncated PA₂₀ fragment, indicating that truncation is distinct from furin digestion. In contrast, incubation of furin-treated PA₈₃ with HIS (lanes 3 and 5) failed to result in PA₂₀ truncation, suggesting that this process is heat labile. For the purpose of the assay, serum was incubated with PA₆₃ for 1 h. MAb 10F4 (which recognizes domains 2 and 4 of PA₈₃) was used to detect the PA₆₃ fragment, while MAb 19D9 (which recognizes domain 1) was used to detect both the normal and truncated PA₂₀ fragments. The black arrow points to the normal PA₂₀ fragment, while the white arrow points to the truncated PA₂₀ fragment. This experiment was done 2 times with similar results. Underlining indicates preincubation.

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 $\mu\text{g}/\text{ml}$ 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

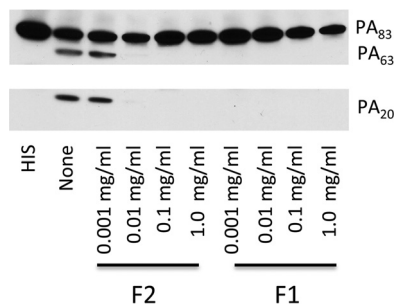


FIG 2 Furin inhibitors I and II prevent serum-mediated digestion of rPA₈₃. Heat-inactivated serum (HIS) had no effect on rPA. In the absence of inhibitor (none), PA₈₃, PA₆₃, and PA₂₀-like fragments were present. Both furin inhibitor I and furin inhibitor II (F1 and F2) prevented serum digestion of PA₈₃. PA₈₃ was incubated for the serum for 30 min. This experiment was repeated 3 times with similar results.

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 PA₈₃. In contrast, both competitive inhibitors of furin (I and II) prevented 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 LQ (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,

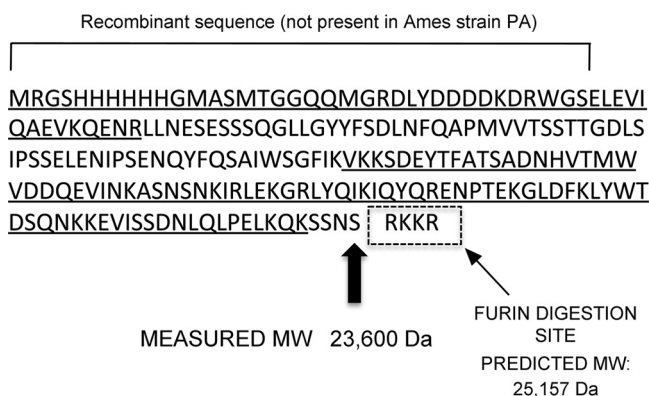


FIG 3 Mass spectrometry of the serum-truncated PA₂₀ fragment. The predicted size of the fragment to the SNSS amino acid sequence is 23,588 Da (thick arrow), a difference of 12 Da or 0.06% compared with the measured mass of 23,600 Da. In contrast, digestion at the furin consensus site should produce a PA₂₀ fragment with a mass of 25,157 Da. Underlined peptide sequences were detected by MS analysis. The dotted box represents the consensus recognition site for furin.

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 μ g/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 μ g/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 μ g/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

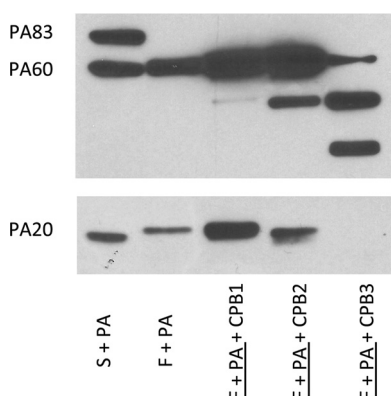


FIG 4 Carboxypeptidase B (CPB) treatment of furin-digested PA produces a truncated PA₂₀ fragment. Treatment of furin-digested rPA₈₃ with CPB from pig pancreas resulted in a dose-related truncation of the PA₂₀ fragment. This was most apparent for CPB2 (25 μ g/ml) compared to lower concentrations of CPB1 (2.5 μ g/ml). Incubation with higher CPB3 concentrations (250 μ g/ml) resulted in complete loss of PA₂₀ reactivity and the appearance of multiple digestion fragments. Underlining indicates preincubation.

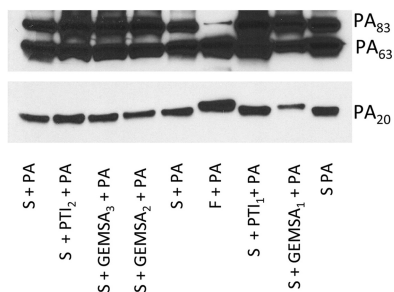


FIG 5 GEMSA, but not PTI, prevented truncation of PA₂₀. In the presence of high concentrations of GEMSA (GEMSA1; 500 μ g/ml), inhibition of serum-mediated truncation of PA₂₀ was present. In contrast, PTI at concentrations as high as 1.25 mg/ml (PTI1) failed to inhibit serum-mediated truncation of PA₂₀. Lower concentrations of GEMSA (50 and 5 μ g/ml; GEMSA2 and GEMSA3) and PTI (PTI2; 125 μ g/ml) did not prevent serum-mediated truncation of PA₂₀. This experiment was done 3 times with similar results.

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 μ g/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 μ g/ml (21). In contrast, CPB₂ must be activated by fibrin and, once activated, downregulates fibrinolysis by removing terminal lysines from fibrin and

is present in serum concentrations on the order of 4 to 15.0 $\mu\text{g/ml}$ (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 (Invitrogen) (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 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$. 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: guanidinoethylmercaptosuccinic acid (GEMSA) (Santa Cruz Biotechnology) and carboxypeptidase inhibitor from potato tuber extract (Sigma). The serum-PA digestion mixture was separated by electrophoresis. PA₆₃-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 $\times g$ 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 $\mu\text{l}/\text{min}$) 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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