Functional Characterization of Protease-treated *Bacillus anthracis*
Protective Antigen*

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Characterization of the functional domains of *Bacillus anthracis* protective antigen (PA, 83-kDa), the common cellular binding molecule for both anthrax edema toxin and anthrax lethal toxin, is important for understanding the mechanism of entry and action of the anthrax toxins. In this study, we generated both biologically active (facilitates killing of *J774A.1* cells in combination with lethal factor, LF) and inactive preparations of PA by protease treatment. Limited proteolytic digestion of PA in vitro with trypsin generated a 20-kDa fragment and a biologically active 63-kDa fragment. In contrast, limited digestion of PA with chymotrypsin yielded a preparation containing 37- and 47-kDa fragments defective for biological activity. Treatment with both chymotrypsin and trypsin generated three major fragments, 20, "17," and 47 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This PA preparation was also biologically inactive. To investigate the nature of the defect resulting from chymotrypsin treatment, we assayed PA preparations for the ability to bind to the cellular receptor and to bind and internalize 125I-LF. All radiolabeled PA preparations bound with specificity to *J774A.1* cells and exhibited affinities similar to native 83-kDa PA. Once bound to the cell surface receptor, both trypsin-treated PA and chymotrypsin/trypsin-treated PA specifically bound 125I-LF with high affinity. Finally, these PA preparations delivered 125I-LF to a Proneas-resistant cellular compartment in a time- and temperature-dependent fashion. Thus, the biological defect exhibited by chymotrypsin-treated PA is not at the level of cell binding or internalization but at a step later, such as toxin routing or processing by *J774A.1* cells. These protease-treated preparations of PA should prove useful in both elucidating the intracellular processing of anthrax lethal toxin and determining the structure-function relationship of PA and LF.

*Bacillus anthracis* produces three protein toxin components: protective antigen (PA, 82.7 kDa), lethal factor (LF, 83.0 kDa), and edema factor (EF, 88.8 kDa) (1-4). PA binds with specificity to a surface receptor found on a number of cell types (5-9). Once bound, PA is cleaved at a trypsin-sensitive site by a cell surface protease. This cleavage generates a 63-kDa fragment which remains cell associated while the amino-terminal 20-kDa fragment is released. The newly exposed site on the 63-kDa PA can bind either EF or LF (3-5). The complex formed by EF bound to PA is known as edema toxin and has been shown to cause edema when injected into the skin of animals (10, 11). LF bound to 63-kDa PA produces a lethal toxin which causes death of experimental animals (12, 13) and lysis of mouse peritoneal macrophages (14). It has been shown that EF is an adenylate cyclase and in combination with PA dramatically increases cAMP levels of cells (15, 16). Whereas the intracellular target of edema toxin is known, little is known about the target or mechanism of action of lethal toxin (3, 4).

Both edema toxin and lethal toxin enter the cell by receptor-mediated endocytosis (3, 6). Incubation of sensitive macrophages with lethal toxin in medium containing lysosomotropic agents which raise the pH of intracellular vesicles, protected cells from killing by the toxin (14). This demonstrated that intracellular vesicle acidification was required for processing and/or targeting of the toxin. Delivery of LF directly into the cytosol of cells resulted in cell death (7). This suggested that the lethal toxin target was in the cytosol and that a translocation step was required to move the endocytosed toxin from a membrane-bound compartment to the cytosol. The intracellular sorting pathway and mechanism of translocation of LF is not yet defined.

The anthrax toxins resemble bacterial toxins of the A/B motif (17). Although the components are separate gene products, PA functions as the B component responsible for receptor binding, while EF and LF function as different A, or effector components (3, 4). Little is known about the structure-function relationship between LF and PA.

Previous studies showed that limited proteolysis of 83-kDa PA with trypsin yielded a 20- and 63-kDa molecule which retained biological activity (4). Cleavage of 83-kDa PA with chymotrypsin generated a 37- and 47-kDa molecule (2). In this report we characterized the interaction of protease-treated preparations of PA with target cells. We found whereas cleavage of PA at the trypsin site was absolutely required for generation of a biologically active molecule, chymotrypsin treatment resulted in PA preparations that were biologically inactive. We generated an additional protease-treated preparation of PA by sequentially treating with chymotrypsin, followed by trypsin. This dual-protease treatment resulted in a preparation containing a 47-, a "17," and a 20-kDa fragment and was also biologically inactive. In this study

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† The abbreviations used are: PA, protective antigen; LF, lethal factor; EF, edema factor; 83-kDa PA, non-cleaved (native) PA; T-PA, trypsin-treated PA; Ch-PA, chymotrypsin-treated PA; Ch/T-PA, chymotrypsin/trypsin-treated PA; BSA, bovine serum albumin; Hank’s balanced salt solution; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
we investigated whether the biologically inactive chymotrypsin-
treated PA preparations were defective for binding to the
acellular receptor or binding and internalization of 125I-LF.

**Materials and Methods**

**Reagents**—Trypsin type XIII, chymotrypsin type VII, soybean trypsin-chymotrypsin inhibitor (Bowman-Birk Inhibitor), and protease type XIV (Pronase E) from *Streptomyces griseus* were obtained through Sigma. PA and LF were purified as previously described (2). Iodination of PA was performed using 125I-Bolton-Hunter reagent (Amersham Corp.) (18). The specific activity of the 125I-PA ranged between 7.6 x 10^6 cpm/µg and 1.0 x 10^7 cpm/µg of PA. LF was iodinated using the chloramine-T method and specific activities of 125I-LF ranged from 2.0 x 10^6 cpm/µg to 3.4 x 10^6 cpm/µg (18).

Cells—J774A.1 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and grown in Dulbecco's medium containing 10% fetal calf serum (GIBCO), penicillin, and streptomycin (DF-10). Cells were passed by scraping and new cultures established from seed stocks every 40 passages. Cells were plated at least 24 h before use, and assays were performed when cell densities reached approximately 85-90% confluence.

**PA Protease Fragment Preparation**—Trypsin, Type XIII and chymotrypsin, Type VII were prepared as 5 mg/ml stock solutions in minimal essential medium containing 20 mM Hepes (MEM/Hepes) and stored at -20°C. Trypsin was diluted 1:100 in ice-cold phosphate-buffered saline (PBS) to a final working concentration of 50 µg/ml immediately before use. Chymotrypsin was diluted 1:10 to a final working concentration of 500 µg/ml as above. Soybean trypsin-chymotrypsin inhibitor (Bowman-Birk inhibitor) was prepared as a 10 mg/ml stock solution in MEM/Hepes and stored at -20°C. The inhibitor was not diluted before use. Limited digestion of PA was performed as follows. Trypsin-treated PA (T-PA) and chymotrypsin-treated PA (CH-PA) were prepared by placing 30-µl aliquots of unlabeled PA (starting concentrations between 0.5 and 3.0 mg/ml) on ice and treating with 10 µl of either trypsin (50 µg/ml) or chymotrypsin (500 µg/ml) for 5 min. The digestion was stopped by the addition of 100 µl of 0.1% SDS, radioactivity counted, and protein concentrations determined. T-PA and CH-PA were prepared by first digesting PA with chymotrypsin as above followed by treatment with 10 µl of trypsin (50 µg/ml) for an additional 5 min before stopping the reaction with soybean trypsin-chymotrypsin inhibitor. 25-µl aliquots of 125I-labeled PA containing 10 µg/ml bovine serum albumin (BSA) (starting concentrations between 0.05 and 0.07 µg/µl iodinated PA) were placed on ice and treated with 10 µl of trypsin or chymotrypsin for 5 min, as above. The reaction was either stopped by the addition of 5 µl of soybean trypsin-chymotrypsin inhibitor, or, alternatively, trypsin-treated PA was further treated for additional 5 min. After addition of 5 µl of 10 mg/ml inhibitor, all protease reaction volume samples were adjusted by the addition of PBS so that all preparations contained the same concentration of either unlabeled or iodinated PA. Gel samples of the fragment preparations were prepared in Laemmli denaturing sample buffer (19) and analyzed by SDS-PAGE and autoradiography. Preparations were aliquoted and stored at -70°C.

**Gel Electrophoresis and Analysis**—Protease-treated preparations of PA were analyzed on 16 cm, 12% SDS-PAGE. Gels were stained with Coomasie Brilliant Blue R-250, dried, and autoradiography performed on gels containing iodinated samples. Routinely, less than 5% of native 83-kDa PA remained in any preparation after protease treatment.

Amino-terminal sequencing of PA fragments was kindly performed by Mark Carter and Carolyn Deal (Water Reed Army Institute of Research, Washington, D.C.) Briefly, purified PA was digested with trypsin to generate a 16-kDa C-terminal amino-terminal peptide. Digests were separated on a 16-cm, 12% SDS-PAGE and electroblotted to a polyvinylidene difluoride membrane (Schleicher & Schuell). Protein bands were located by Ponceau S (Sigma) staining and the bands of interest cut out and sequenced on an Applied Biosystems 470 Sequencer (20).

**Internalization of PA**—J774A.1 cells were plated on 24-well dishes (Costar, Cambridge, MA) at least 24 h before use. PA fragment preparations and varying concentrations of LF were added to cells in 5 ml of medium in a CO2 incubator overnight. The extent of cell death was determined by lactic dehydrogenase release into the medium as described previously (14). Data are plotted as percent survival of the cells versus concentration of toxin.

**Binding and Internalization Assays**—Cells were plated in 6- or 24-well dishes (Costar, Cambridge, MA) for surface binding and cell surface protease cleavage experiments or in 4-well dishes (Nunc, Newbury Park, CA) for internalization experiments. For surface binding experiments, plates were placed on ice and medium changed to ice-cold MEM/Hepes. After 10 min of equilibration at 4°C, medium was changed to cold MEM/Hepes containing 7.0 mg/ml BSA, pH 7.4 (MEM/Hepes/BSA), for an additional 15 min. 125I-PA was added to cells in MEM/Hepes/BSA at concentrations indicated and allowed to bind at 4°C for 2-24 h. Monolayers were washed extensively in ice-cold Hank's balanced salt solution (HBSS), solubilized in 1.0 ml of 0.1% SDS, radioactivity counted, and protein concentrations determined. 125I-LF surface binding was performed similarly except that 125I-LF and unlabeled PA fragment preparations in MEM/Hepes/BSA were added simultaneously.

Non-specific binding of 125I-PA was determined by adding 100-500-fold excess unlabeled native 83-kDa PA in addition to 125I-PA. Non-specific binding of 125I-LF was determined by adding 1000-fold excess unlabeled LF to cells in the absence of unlabeled PA. (Nonspecific binding determined by this method yielded values similar to those measured in the presence of PA and 100-fold excess unlabeled LF.) Routinely non-specific binding for both ligands was less than 10% of total binding. Protein determinations were performed by the method of Lowry (21) on all samples and data expressed as counts/minute/microgram of cellular protein (cpm/µg).

Internalization of 125I-LF was performed as follows. Unlabeled PA fragment preparations and 125I-LF were allowed to bind to cells at 4°C for 4 h. Excess label was washed away with ice-cold HBSS and cells were shifted to 37°C by the addition of prewarmed MEM/Hepes/BSA and placement in a 37°C water bath. At the indicated times, plates were removed from the water bath and immediately washed with ice-cold HBSS. Surface-bound ligand was removed by a modified procedure of Sandvig et al. (22). Briefly, 0.25% Pronase E was prepared fresh in MEM/Hepes, pH 7.4, cooled to 4°C, and added to the cells in 1 ml at 4°C. Pronase was removed and the cell monolayer was washed with 1 ml of HBSS. (J774A.1 cells are resistant to release upon Pronase treatment.) This wash was pooled with the corresponding 1 ml of Pronase supernatant and radioactivity determined. Cells were solubilized in 1 ml of 0.1% SDS, radioactivity counted, and protein determinations performed as above. Surface-bound 125I-LF was released by Pronase stripping while internalized 125I-LF remained associated with the cell monolayer. Data are presented as percent internalized 125I-LF versus time at 37°C.

**Results**

The amino acid sequence of 83-kDa PA has been deduced from the nucleotide sequence of the protective antigen gene (23). Highly sensitive proteolytic cleavage sites exist within the sequence for both trypsin and chymotrypsin (4, 5). Under conditions of limited digestion, each of these proteases cleaves 83-kDa PA into two large fragments. Whereas cleavage at the major trypsin site is absolutely essential for biological activity, we found that chymotrypsin cleavage eliminated the biological activity of PA.

Limited digestion of PA with trypsin, chymotrypsin, or both proteases was performed as described under "Materials and Methods." Fragments generated were analyzed by SDS-PAGE (Fig. 1). Trypsin treatment of unlabeled 83-kDa PA (Fig. 1A) and 125I-labeled 83-kDa PA (Fig. 1B) resulted in preparations containing a 20- and a 60-kDa fragment (T-PA). Chymotrypsin treatment of 83-kDa PA yielded preparations containing a 37- and a 70-kDa fragment (CH-PA). Treatment of 83-kDa PA with both chymotrypsin and trypsin (CH/TPA) resulted in fragments with apparent molecular mass of 20, 24, and 47 kDa.

Previously, it was shown that limited trypsin digestion of 83-kDa PA generated an amino-terminal 20- and a 63-kDa fragment (18, 24). Limited chymotrypsin digestion yielded an amino-terminal 37-kDa fragment and a carboxyl-terminal 47-kDa fragment (2). Cleavage at both sites was expected to generate three fragments of molecular mass 20, 17, and 47 kDa. The size of the third fragment generated by dual protease treatment was 24 kDa rather than 17 kDa. Amino-terminal sequencing of both the 47- and 24-kDa fragments confirmed that the major trypsin cleavage occurred at the carboxyl side
Protease-treated Protective Antigen

**Fig. 1.** SDS-PAGE analysis of PA fragment preparations. An aliquot of each PA preparation was prepared for SDS-PAGE in Laemmli sample buffer. Panel A, samples of unlabeled PA preparations were run on 16 cm, 12% SDS-PAGE and protein bands visualized by Coomassie R-250 staining. Approximately 8 μg of protein were loaded per lane. Panel B, samples of 125I-PA preparations were run on 12% SDS-PAGE as above. The gels were stained with Coomassie R-250, dried, and autoradiography performed. Approximately 1000 cpm of each fragment preparation was loaded per lane. Samples were loaded in both panels as follows: lane a, 83-kDa PA; lane b, T-PA; lane c, Ch-PA; and lane d, Ch/T-PA. Molecular mass standards are indicated at the left of each panel and the major PA fragment bands are indicated at the right in kDa.

of arginine 167 and the major chymotrypsin cleavage occurred after phenylalanine 314 (data not shown). The protease cleavage sites and corresponding fragments are schematically represented in Fig. 2.

The amino-terminal sequence of the 24-kDa fragment corresponded to residues serine 168 through glycine 172 confirming that this fragment was actually a fragment that corresponded to the expected 17-kDa fragment. Therefore, we refer to this fragment as “17” kDa throughout this article. Although the reason for the decreased mobility of the “17”-kDa fragment has not yet been determined, treatment of PA with proteases in the reverse order, trypsin and then chymotrypsin, resulted in the same 20-, “17”-, and 47-kDa fragments as generated above (data not shown).

SDS-PAGE analysis of the fragment preparations revealed the presence of minor bands in addition to the major fragment bands generated. Additional bands were most apparent in the 125I-PA preparations in part, due to the fact that minor bands contaminating the 83-kDa stocks were labeled during iodination. Occasionally, a band with a mobility of approximately 48 kDa was observed in Ch-PA and Ch/T-PA preparations of 125I-labeled PA (Fig. 1B, lanes c and d). This fragment was most likely the result of cleavage of PA at a secondary chymotrypsin site, such as phenylalanine 324 or phenylalanine 727 (23).

Minor bands were also detectable in the Coomassie-stained SDS-PAGE analysis of unlabeled PA fragment preparations. The 46-kDa band observed in the 125I-PA analysis was occasionally present in chymotrypsin-treated preparations (Fig. 1A, lanes c and d). Secondary trypsin cleavage of the 20-kDa fragment at arginine 7 has been reported to occur and contribute to heterogeneity in the 20-kDa region of the gel (4). Amino-terminal sequencing of the 20-kDa fragment was not performed in this study to determine if this secondary cleavage occurred. However, the identity of this fragment was confirmed by immunoblot analysis using a mouse monoclonal antibody directed against purified 20 kDa (data not shown).

Finally, a 21.5-kDa band was present in all lanes of Fig. 1A. This band was soybean trypsin-chymotrypsin inhibitor used in the generation of the PA fragments. Regardless of the minor fragments present in the preparations of PA, or the presence of excess trypsin-chymotrypsin inhibitor, only the major PA fragments of interest became cell-associated (Fig. 5, to be addressed later). Thus, these fragment preparations were used throughout the study to assay binding, internalization, and biological activity without further treatment or purification.

Incubation of sensitive mouse peritoneal macrophages with PA and LF results in cell death (14). LF, in combination with PA, is also lytic for J774A.1 cells, a mouse macrophage-like cell line (8). Thus, J774A.1 cells provide a convenient system in which to assess the biological activity of toxin. To determine which fragment preparations of PA exhibited biological activity, we did the following. J774A.1 cells were incubated overnight at 37 °C with 1.0 μg/ml of the iodicated PA preparation and increasing amounts of LF (Fig. 3A). The 83-kDa PA preparation (containing trypsin-chymotrypsin inhibitor)
Precisely the same site as cell surface protease cleavage. Alternatively, formation of nonfunctional toxin complexes in the presence of T-PA in the presence of a constant amount of LF trypsin, the biological activity of the preparation was up to 10-fold less than that of the 83-kDa fragment (Fig. 3). Titration of T-PA in the presence of a constant amount of LF resulted in 50% cell death at approximately 0.06 μg/ml T-PA while 50% death occurred at approximately 0.02 μg/ml 83-kDa PA (Fig. 3B).

One explanation for the decreased biological activity of T-PA may be that in vitro protease cleavage does not occur at precisely the same site as cell surface protease cleavage. Alternatively, formation of nonfunctional toxin complexes in solution could account for the decreased activity of T-PA. Purified 63-kDa PA has been reported to form aggregates in solution (4). Although we have not detected aggregates in the T-PA preparations used in binding or biological activity assays, we cannot rule out the possibility that some aggregate formation of T-PA and LF occurs after addition to J774A.1 cells and incubation at 37 °C for several hours.

The biological activity of the T-PA appears to be due to the T-PA itself and not uncleaved 83-kDa PA remaining in the preparation. T-PA and Ch-PA preparations routinely contained less than 2 and 5% residual 83-kDa PA, respectively, as determined by densitometric analysis (data not shown). If the biological activity of the T-PA preparation was the result of residual 83-kDa protein, then activity would be detected with both T-PA and Ch-PA preparations (Fig. 3A) and be only about 2% of that observed with 83-kDa protein (Fig. 3B).

To determine if the biologically inactive preparations (Ch-PA and Ch/T-PA) bound to J774A.1 cells as well as the biologically active preparations (83 kDa and T-PA), the following experiments were performed. 125I-PA fragments were added to cells at various concentrations and allowed to bind for 24 h at 4 °C (Fig. 4). The Ch-PA (Fig. 4A) and Ch/T-PA (Fig. 4B) preparations exhibited the same half-maximal saturation binding as 83-kDa PA (Fig. 4A) and T-PA (Fig. 4B), approximately 1–2 nM. This was in close agreement with other estimations of affinity of 83-kDa PA binding to cells (5). Therefore, the defect in biological activity of chymotrypsin-treated T-PA was not due to altered binding of the protease-treated 125I-labeled PA preparations to the cell surface receptor.

The previous results demonstrated that the protease-treated 125I-PA preparations bound avidly to J774A.1 cell surface receptors. However, these experiments could not distinguish which fragments within each preparation were bound. Thus, we determined which of the 125I-labeled PA fragments were associated with cells by SDS-PAGE and autoradiography.

125I-PA preparations were incubated with J774A.1 cells at 4 °C. After 2 h of binding with 125I-labeled 83-kDa PA, the majority of the cell-associated radioactivity was still present as 83 kDa (Fig. 5, lane a). Note that the 63-kDa fragment was absent in 125I-labeled 83-kDa PA stock preparations (compare Fig. 1B, lane a with Fig. 5, lane a) and was not formed by incubation of the 83-kDa preparation under parallel conditions in the absence of cells (data not shown). Thus, the small amount of 63-kDa PA observed after the 2-h incubation was the result of in vivo cleavage of 83- to 63-kDa protein by the cell surface protease (discussed below).

Incubation of 125I-labeled T-PA with cells, resulted in cell association of the 63-kDa fragment but not the 20-kDa fragment (Fig. 5, lane c). This is consistent with the observation that the 20-kDa fragment dissociates from cell-bound 63-kDa protein after cleavage by the cell surface protease (5). Incubation of 125I-labeled Ch-PA with J774A.1 cells for 2 h at 4 °C, resulted in the cell association of both the 37- and 47-kDa fragments (Fig. 5, lane e). Incubation of cells with 125I-labeled Ch/T-PA preparations for 2 h at 4 °C resulted in cell-associated 47- and 17-kDa fragments (Fig. 5, lane g).

Specific binding of all the 125I-labeled T-PA preparations to cells could be inhibited by 100-fold excess unlabeled 83-kDa PA. Conversely, 100-fold excess unlabeled 47- to 37-kDa PA inhibited binding of 125I-labeled 83-kDa PA to cells by greater than 90%. In addition, the monoclonal antibody, 3B6, which has been shown to inhibit the binding of 125I-labeled 83-kDa PA to cells (25) also completely inhibited the cell binding of protease-treated Protective Antigen

Fig. 3. Biological activity of protease-treated PA preparations. Panel A, 1.0 μg/ml of each PA preparation and increasing concentrations (from 0.001 to 0.1 μg/ml) of LF were added simultaneously to J774A.1 cells in DF-10 medium. Panel B, 0.1 μg/ml of LF in the presence of increasing concentrations (0.0005–0.5 μg/ml) of the indicated PA preparations were added simultaneously to J774A.1 cells in DF-10 medium. In both experiments cells were incubated at 37 °C in a CO2 incubator for 20 h. Cell survival measured by lactic dehydrogenase release was determined as described under "Material and Methods." ○, 83-kDa PA; □, T-PA; △, Ch-PA; ◀, Ch/T-PA.

resulted in 50% cytolyis of cells when added in combination with LF at 0.002 μg/ml. This result was identical to that obtained with native 83-kDa PA that did not contain inhibitor (data not shown). The T-PA preparation, which was predominately 63 kDa with little or no 83-kDa PA (Fig. 1A), also exhibited lethal activity. However, in striking contrast, the Ch-PA and Ch/T-PA preparations were not biologically active (Fig. 3). Even when combined with high concentrations of LF (1.0 μg/ml), neither Ch-PA nor Ch/T-PA at 1.0 μg/ml resulted in J774A.1 cell lysis (data not shown).

Interestingly, even though T-PA was already cleaved by trypsin, the biological activity of the preparation was up to 10-fold less than that of the 83-kDa fragment (Fig. 3). Titration of T-PA in the presence of a constant amount of LF resulted in 50% cell death at approximately 0.06 μg/ml T-PA while 50% death occurred at approximately 0.02 μg/ml 83-kDa PA (Fig. 3B).

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all of the 125I-PA fragment preparations (data not shown).
Thus, specific cell association of these PA fragment preparations
appeared to be mediated by the same cell surface receptor as for native 83-kDa PA.

The association of the 37- and the "17"-kDa fragment with cells (Fig. 5) was due to noncovalent interactions of these fragments with the cell-bound 47-kDa fragment. Native-PAGE analysis of the Ch-PA and Ch/T-PA preparations suggested that both the 37- and "17"-kDa fragment remained noncovalently associated with the 47-kDa fragment in solution after in vitro proteolytic cleavage (data not shown).

Previously, it was reported that cell-bound 83-kDa PA was cleaved by a trypsin-like, cell surface protease (5). This cleavage exposed a binding site for LF and, hence, was required for biological activity. We investigated the possibility that Ch-PA is biologically inactive because it is not a substrate for cleavage by the cell surface protease. As a control, cleavage of 125I-labeled 83-kDa PA by the cell surface protease was also monitored. Cells were incubated with 125I-labeled 83-kDa PA or protease-treated 125I-labeled PA preparations for 2 h, washed, and then allowed to incubate an additional 14 h at 4 °C. Fig. 5, lanes a and b, demonstrate that 125I-labeled 83-kDa PA was a substrate for cleavage. After 2 h at 4 °C, approximately 15% of the cell-bound 125I-labeled 83-kDa PA was converted to 63-kDa PA by the cell surface protease (Fig. 5, lane a). However, after 16 h at 4 °C, approximately 65% of the cell-bound 125I-labeled 83-kDa PA was cleaved to 63 kDa (Fig. 5, lane b). The addition of trypsin-chymotrypsin inhibitor did not affect cleavage of cell-bound 125I-labeled 83-kDa PA as compared to 125I-labeled 83-kDa PA that did not contain the inhibitor (data not shown).

The cell surface protease also recognized the highly sensitive trypsin site contained within the 37-kDa fragment of the 125I-labeled Ch-PA preparation (Fig. 5, lanes e and f). Cleavage resulted in a time-dependent decrease in the amount of cell-associated 37-kDa protein and a concomitant increase in the cell-associated "17"-kDa fragment (Fig. 5, lanes e and f). However, the increase of cell-associated "17"-kDa fragment, even after a 16-h incubation was difficult to quantitate. Thus, the time-dependent decrease of the 37-kDa fragment was a good measure of the extent of cleavage of 125I-labeled Ch-PA by the cell surface protease. After 2 h at 4 °C, approximately 35% of the 125I-labeled Ch-PA preparation was cleaved to 37-kDa PA while after 16 h only about 15% of the Ch-PA was 37-kDa protein. As expected, neither 125I-labeled T-PA nor 125I-labeled Ch/T-PA, which were already cleaved at the trypsin site in vitro (Fig. 4, lanes c, d, g, and h), were substrates for the cell surface protease. Thus, the defect in biological activity exhibited by the Ch-PA preparation was not the result of inability
to be recognized as a substrate by the cell surface protease.

To determine if the biological defect exhibited by Ch-PA and Ch/T-PA was due to decreased binding of the effector molecule LF, we measured $^{125}$I-LF binding to cell-bound PA fragment preparations. Preliminary LF binding experiments revealed that all of the preparations could bind $^{125}$I-LF. The amount of $^{125}$I-LF radioactivity bound was dependent on the extent of cleavage that had occurred at the trypsin site. Cell-associated T-PA and Ch/T-PA preparations bound 3-6-fold more $^{125}$I-LF than cell-associated 83-kDa or Ch-PA preparations (data not shown). This is consistent with the fact that the in vitro trypsin treatment resulted in complete cleavage of 83- to 63-kDa protein (Fig. 5, lanes c and d) and 37- to 17-kDa protein (Fig. 5, lanes g and h) while even after 16 h at 4 °C, cell surface protease cleavage of the 83-kDa molecule (Fig. 5, lanes a and b) and the 37-kDa fragment (Fig. 5, lanes e and f) was incomplete. Since T-PA, which was biologically active and Ch/T-PA, which was biologically inactive, were both completely cleaved in vitro, we utilized these preparations to further analyze $^{125}$I-LF binding and internalization.

Cells were incubated with saturating amounts of T-PA or Ch/T-PA in the presence of increasing amounts of $^{125}$I-LF at 4 °C (Fig. 6). The concentration at which the half-maximal binding of $^{125}$I-LF binding occurred was similar, approximately 0.2-0.4 nM with both T-PA and Ch/T-PA. The inset of Fig. 6 shows these data plotted according to the method of Scatchard (26). $K_d$ values derived from these plots were 0.24 and 0.20 nM for binding of $^{125}$I-LF to T-PA and Ch/T-PA, respectively. Thus, the biologically inactive preparation Ch/T-PA bound $^{125}$I-LF with the same affinity as the biologically active T-PA preparation.

We tested whether T-PA and Ch/T-PA fragment preparations could mediate internalization of $^{125}$I-LF. Cells were incubated with saturating amounts of T-PA or Ch/T-PA and increasing amounts of $^{125}$I-LF for 4 h at 4 °C (Fig. 7). Cells were washed extensively and shifted to 37 °C. At the indicated times, cells were placed on ice, washed, and Pronase-treated to remove any remaining surface radioactivity. As shown in Fig. 7, internalization of prepbound $^{125}$I-LF at 37 °C was rapid with a half-time of less than 2.5 min. By 5 min at 37 °C, all of the $^{125}$I-LF bound by either T-PA or Ch/T-PA was Pronase-resistant. Pronase treatment of cells incubated with T-PA or Ch/T-PA fragments and $^{125}$I-LF at 4 °C only, released 90-95% of the cell-associated radioactivity at 4 °C. (Binding at zero time after 4 h at 4 °C ranged from 14,000 to 20,000 cpm/well in these experiments.)

**DISCUSSION**

PA is essential for cell binding and internalization of both anthrax edema toxin and anthrax lethal toxin (1, 3). Lethal factor has no biological activity in the absence of PA unless it is delivered directly into the cytosol of cells (7). Edema factor has been shown to be an adenylate cyclase and dramatically increases intracellular cAMP levels (11, 15). The intracellular target for lethal factor has not yet been identified. Similar to a number of other bacterial exotoxins (27), the anthrax toxins enter the cell by receptor-mediated endocytosis and require passage through an acidic compartment in order to elicit biological activity (14). The biochemical events involved in intracellular routing and translocation of the toxins are not well defined. Because of the critical role PA plays as the common "binding component" of the toxins, studies focusing on the structure and biological function of the PA molecule are important for elucidating the intracellular processing necessary for toxin action.
Activation of toxins such as diphtheria (28), shiga (29), and pseudomonas exotoxin A (30) is dependent on cleavage at a specific protease site. PA is similar to these bacterial exotoxins in that cleavage at a highly sensitive trypsin site is absolutely essential for binding of EF or LF and hence, biological activity of the toxins (5). In support of this, a recent study demonstrated that mutagenized PA, which did not contain this trypsin site, was not biologically active (18).

PA contains a second highly sensitive protease site (2, 4). Chymotrypsin cleavage at this site results in a defective PA molecule that does not facilitate cell killing when combined with LF (Fig. 3). In this study, we describe methods to generate both biologically active and biologically inactive protease-treated preparations of PA in vitro. We characterized these preparations for their ability to bind to the cell surface receptor, serve as substrates for the cell surface protease, bind LF, and mediate cellular internalization of LF.

The methods used to generate protease-treated PA preparations were simple and highly reproducible. Minor bands accounted for less than 5% of the total protein in each preparation. Limited digestion of 83-kDa PA with trypsin (T-PA) resulted in a 20- and 63-kDa fragment. Treatment with Ch-PA yielded a 37- and a 47-kDa fragment. Treatment with both proteases (Ch/T-PA) resulted in three major fragments, 20, "17", and 47 kDa. Neither the Ch-PA nor the Ch/T-PA preparations could facilitate killing of J774A.1 cells (Fig. 9).

SDS-PAGE analysis of cells after incubation with protease-treated 125I-labeled fragment preparations demonstrated that the 63-, 47-, and "17"-kDa fragments, but not the 20-kDa fragment, became cell-associated (Fig. 5). Recent work suggests that the receptor-binding domain within PA is localized to the carboxyl terminus of the molecule (31). A monoclonal antibody, 3B6, which is directed against 83-kDa PA, has been shown to inhibit the binding of 125I-labeled 83-kDa PA to cells (25). This monoclonal antibody also blocks cell binding of all of the 125I-labeled PA preparations described in this study (data not shown). In addition, 3B6 reacts with the 47-kDa fragment of PA in immunoblot assays (data not shown). These data support the hypothesis that the 47-kDa fragment becomes cell-associated (Fig. 5). Recent work suggests that the receptor-binding domain within PA is localized to the carboxyl terminus of the molecule (31). A monoclonal antibody, 3B6, which is directed against 83-kDa PA, has been shown to inhibit the binding of 125I-labeled 83-kDa PA to cells (25). This monoclonal antibody also blocks cell binding of all of the 125I-labeled PA preparations described in this study (data not shown). In addition, 3B6 reacts with the 47-kDa fragment of PA in immunoblot assays (data not shown). These data support the hypothesis that the 47-kDa fragment becomes cell-associated (Fig. 5).

We demonstrated that internalization of 125I-LF could be mediated by T-PA and Ch/T-PA preparations (Fig. 7). Internalization of 125I-LF was rapid. The rate of internalization was similar to other receptor-mediated endocytosed ligands (32). The half-time of internalization was 2.5 min with almost 100% of the 125I-LF surface counts internalized by 5 min when either T-PA or Ch/T-PA were tested. Hence, the defect in killing by chymotrypsin-treated PA was not the result of a defect in internalization of LF. It should be noted, however, that internalization of ligand is determined by "Pronase resistance" and does not exclude the possibility that Pronase resistance of toxin is not the result of endocytosis but insertion into the membrane. Preliminary subcellular fractionation experiments suggest that T-PA and Ch/T-PA mediate endocytosis of 125I-LF into intracellular, membrane-bound compartments.

The biological defect exhibited by chymotrypsin-treated PA appears to occur at a cellular step after binding and internalization of LF. It is possible that chymotrypsin cleavage of PA results in "misrouting" within the cell. If the interaction of PA with the receptor or the plasma membrane is altered after internalization due to chymotrypsin cleavage, the PA-LF complex may be handled differently by intracellular sorting mechanisms.

Biological activity of lethal toxin is dependent on intracellular vesicle acidification (14). The acidic environment of the endosome has been implicated in the correct processing and delivery of other endocytosed toxins such as diphtheria (28, 33) and Pseudomonas exotoxin A (34, 35). A recent study by Ogata et al. (30) demonstrated that proteolytic processing of Pseudomonas exotoxin A in an acidic endosome was necessary for the generation and translocation of the enzymatically active, carboxyl-terminal 37-kDa fragment. Mutant Pseudomonas exotoxins that were not properly cleaved and processed within the endosome were unable to kill cells. Thus, processing and translocation of biologically active toxin is not only dependent on functional endosomes but is also dependent on toxin structure.

Blaustein et al. (36) demonstrated that 63-kDa PA purified after chymotrypsin cleavage, but not the native 83-kDa molecule could form ion channels in artificial lipid bilayers. These observations support the hypothesis that PA is similar to other B components of toxins in that membrane insertion properties of PA may be essential for the delivery of LF to the cytosol. It is possible that chymotrypsin cleavage of PA results in a molecule which can no longer undergo conformational changes required for membrane insertion. An alternative possibility is that chymotrypsin-treated PA can insert into the membrane but cannot assume the proper conformation to facilitate translocation of LF into the cytosol.

The receptor-binding domain of PA appears to be located within the 47-kDa fragment of PA. If the "17"-kDa fragment contains the LF-binding domain and the membrane insertion/translocation domain is within the 47-kDa fragment, then these functional domains would no longer be covalently associated after in vitro chymotrypsin cleavage. Internalization of chymotrypsin-treated PA and LF into the acidic environment of the endosome could result in dissociation of the "17"-kDa-LF complex from the 47-kDa translocation domain and preclude entry of LF into the cytosol. Thus, the defect exhibited by chymotrypsin-treated PA could be the result of acid-induced dissociation of the functional domains of the toxin complex.

Fragmentation of protein toxins by proteolysis or chemical treatment has proven useful in identifying antigenic and functional domains of diphtheria toxin (37) and Pseudomonas exotoxin A (38). PA fragment preparations will be useful in identifying major antigenic epitopes and functional domains of PA. Because the PA gene has been cloned (39, 40), the information derived from studies utilizing protease fragments will aid in design of molecular clones of specific domains of PA for further study.

We have exploited highly sensitive protease sites within B. anthracis protective antigen to generate preparations of protease-treated PA with differential biological activity. Whereas limited digestion with trypsin yielded biologically active PA preparations, chymotrypsin cleavage yielded PA preparations which were biologically inactive when combined with LF. Further studies utilizing these preparations will focus on determining which PA domains are involved in binding, internalization, and translocation of LF, and on characterizing the intracellular compartments involved in sorting and translocation of anthrax toxin.

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