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Biochemistry

Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin

(*Bacillus anthracis*/protein processing/paired basic residues/mutagenesis/consensus sequence)

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ABSTRACT **Proteolytic cleavage of the protective antigen (PA) protein of anthrax toxin at residues 164–167 is necessary for toxic activity. Cleavage by a cellular protease at this sequence, Arg-Lys-Lys-Arg, normally follows binding of PA to a cell surface receptor. We attempted to identify this protease by determining its sequence specificity and catalytic properties. Semi-random cassette mutagenesis was used to generate mutants with replacements of residues 164–167 by Arg, Lys, Ser, or Asn. Analysis of 19 mutant proteins suggested that lethal factor-dependent toxicity required the sequence Arg-Xaa-Xaa-Arg. Based on these data, three additional mutants were constructed with the sequences Ala-Lys-Lys-Arg, Arg-Lys-Lys-Ala, and Arg-Ala-Ala-Arg. Of these mutant proteins, Arg-Ala-Ala-Arg was toxic, confirming that the cellular protease can recognize the sequence Arg-Xaa-Xaa-Arg. The mutant containing the sequence Ala-Lys-Lys-Arg was also toxic but required >13 times more protein to produce equivalent toxicity. This sequence specificity is similar to that of the ubiquitous subtilisin-like protease furin, which is involved in processing of precursors of certain receptors and growth factors. Therefore we tested whether a recombinant soluble furin would cleave PA. This furin derivative efficiently cleaved native PA and the Arg-Ala-Ala-Arg mutant but not the nontoxic PA mutants. In addition, previously identified inhibitors of furin blocked cleavage of receptor-bound PA. These data imply that furin is the cellular protease that activates PA, and that nearly all cell types contain at least a small amount of furin exposed on their cell surface.**

Many bacterial protein toxins require proteolytic cleavage for maximal toxicity. Examples include diphtheria toxin, *Pseudomonas* exotoxin A, tetanus toxin, the enterotoxins of *Escherichia coli* and *Vibrio cholerae*, and the neurotoxins and C2 toxin of *Clostridium botulinum* (1). The proteases necessary for activation of the toxins might be of either bacterial or host origin. Diphtheria toxin provides an example where an obligatory involvement of eukaryotic proteases is suspected (2, 3). Diphtheria toxin can be obtained in both an intact, unactivated form and a proteolytically nicked form having the A and B chains linked by a disulfide bond, and these forms are equally active on cultured cells (4). Since the ADP-ribosylation activity of the A chain requires cleavage from the B chain, it follows that cellular proteases must perform this cleavage at some stage during internalization or transport to the cytosol. The protease involved in this cleavage has not been identified. As discussed below, prior work showed that anthrax toxin also requires proteolytic activation. The studies described here were directed toward identifying the cellular protease that activates anthrax toxin.

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Bacillus anthracis, the causative agent of anthrax, produces three proteins which when combined appropriately form two potent toxins. Protective antigen (PA, 82,684 Da) and edema factor (EF, 89,840 Da) combine to form edema toxin (ET), whereas PA and lethal factor (LF, 90,237 Da) combine to form lethal toxin (LT) (5–8). ET and LT are known collectively as anthrax toxin and each conforms to the AB toxin model, with PA providing the target-cell-binding function and EF or LF acting as the effector or catalytic moiety (9). EF is an adenylate cyclase which requires calmodulin for catalytic activity (10). LF is presumed to have an enzymatic function, although no such activity has yet been identified. PA is capable of binding to the surface of many types of cells (11, 12). After PA binds to a receptor on the surface of susceptible cells, it is cleaved by a cell surface protease to produce two fragments, an amino-terminal 20-kDa fragment that is released from the receptor/PA complex, and a carboxyl-terminal 63-kDa fragment (PA63) that remains bound to the cell surface (11, 13). This cleavage occurs near residues 164–167, Arg-Lys-Lys-Arg. Specific cleavage confined to this site can also be obtained by treatment with low concentrations of proteases (13–15). Only after cleavage is PA able to bind either EF or LF to form either ET or LT (14). The cleavage of PA by the cellular protease or by trypsin requires this site, because deletion of residues 163–168 yields a PA molecule which is not cleaved after binding to a cell surface or after treatment with trypsin and which therefore is not toxic when administered with EF or LF (14). The PA63 produced by cleavage is able to form ion-selective pores in lipid membranes, and these are believed to be involved in internalization of LF and EF (15, 16).

By systematic substitution of PA residues 164–167, we defined a consensus target sequence for the cellular protease and found it to closely resemble the sequence recognized by furin, the protease recently identified as responsible for processing precursors of certain receptors and growth factors. Furthermore, we found that substances which blocked furin activity inhibited cleavage of PA bound to the surface of L6 rat myoblasts.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and General Procedures. Procedures for growth of *E. coli*, purification of DNA, restriction enzyme digestion and ligations, and gel electrophoresis of DNA were those of Sambrook *et al.* (17). Construction of shuttle vectors containing the gene for PA (pYS5) for PA or the gene with amino acids 163–168 deleted (pYS6) and

Abbreviations: PA, protective antigen; PA63, 63-kDa carboxyl-terminal fragment of PA; LF, lethal factor; EF, edema factor; LT, lethal toxin; ET, edema toxin.

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assessment of toxin activity in rats were described previously (14). *B. anthracis* UM23C1-1 (pXO1$^-$, pXO2$^-$) was transformed by electroporation (18).

Cassette Mutagenesis. Two deoxyoligonucleotides were synthesized for cassette construction. Oligonucleotide 1 is 5'-CAA-AAA-TCT-TCG-AAT-TCA-ARN-ARN-ARN-ARN-TCG-ACG-TCT-GCA-GGA-CCT-ACG-GTT-3' and primer 1 is 5'-AAC-CGT-AGG-TCC-TGC-AGA-CGT-CGA-3', where R is a 1:1 mixture of A and G, and N is a 1:1:1:1 mixture of A, C, G, and T. Primer 1 was annealed to oligonucleotide 1 and extended across the region containing degenerate nucleotide additions. Annealing formed a *Ppu*MI site on the 3' end of the molecule. The 5' end of the molecule was trimmed with *Bst*BI to produce the cassette. Both strands of each cassette for site-specific mutants PA31, PA32, and PA33 were synthesized individually and annealed prior to ligation with vector fragments. The mutagenic cassettes were ligated with the *Bam*HI–*Bst*BI fragment from pYS5 and the *Ppu*MI–*Bam*HI fragment from pYS6. Plasmids shown to have correct restriction maps were transformed into the *E. coli dam*$^-$*dcm*$^-$ strain GM2163. Unmethylated plasmid DNA was purified from each mutant and used to transform *B. anthracis*.

PA Preparation. Transformed *B. anthracis* expressing mutated PA proteins were grown in dialyzed FA medium (14) with cyclodextrin (0.5 mg/ml) and neomycin (20 μg/ml) for 16 hr at 200 rpm and 37°C. PA was purified by precipitation with ammonium sulfate (0.47 g/ml of supernatant) from 200 ml of filter-sterilized culture supernatant. The precipitated proteins were centrifuged at 8000 × *g* for 30 min, dissolved in 10 ml of TE (10 mM Tris/1 mM EDTA, pH 8.0), and dialyzed overnight against 2 liters of TE. The dialyzed crude protein was purified by chromatography on a Mono Q column (Pharmacia) using a gradient of 0–1 M NaCl in 25 mM ethanolamine at pH 9.0.

Cell Culture and Cytotoxicity Assay. Mutated PA proteins were assayed for functional activity in the macrophage lysis assay (18, 19) in the presence of LF (0.25 μg/ml). Native PA (0.1–50 μg/ml) served as a control. RAW264.7 or J774A.1 cells were used as indicated.

Enzymatic Digestion of PA. A recombinant truncated, soluble form of human furin (residues 1–713) was purified from the supernatant of BSC-40 cells to ≈70% homogeneity (20). PA samples (10 μg) purified to near homogeneity by Mono Q chromatography were incubated in a volume of 20 μl with purified furin in 100 mM NaCl/1 mM EDTA/1 mM EGTA/1 mM $MgCl_2$/3 mM $CaCl_2$/50 mM Hepes, pH 7.3. Trypsin digestions were as described by Singh *et al.* (14).

Cell-Surface Cleavage of PA. Rat myoblast L6 cells (ATCC CRL 1458) in 24-well plates were fixed by washing twice with 10 mM Hepes, pH 7.3/125 mM NaCl/1 mM $MgCl_2$/1 mM $CaCl_2$, and then incubating for 30 min at room temperature in the same buffer containing 10 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and 30 mM *N*-hydroxysuccinimide (21). The fixation was terminated by incubation in protein-free minimum essential medium (GIBCO/BRL) containing 25 mM Hepes, pH 7.5. PA was radiolabeled with [^{125}I]Bolton–Hunter reagent (Amersham) to a specific activity of about 5 × 10^6 cpm/μg. To measure cell-surface nicking of PA, the fixed cell monolayers were washed twice with 2.0 ml of binding buffer [100 mM NaCl/5 mM $MgCl_2$/1 mM EDTA/1 mM EGTA/25 mM Hepes, pH 7.3, containing bovine serum albumin (200 μg/ml) and gentamicin (50 μg/ml)] and incubated 16–20 hr with 0.2 ml of binding buffer supplemented with cations or protease inhibitors as indicated plus ^{125}I-PA (0.50 μg/ml). After incubation, the monolayers were washed four times with Hanks' balanced salt solution and lysed by sequential additions of 100 μl of 0.2% SDS, 10 μl of 1% (vol/vol) Triton X-100, 10 μl of DNase I (10 μg/ml), and 20 μl of 1% SDS, at intervals of 1–2 min. The lysate was transferred to a microcentrifuge tube and heated at 95°C for 5 min, ^{125}I was determined, and portions were electrophoresed in SDS/polyacrylamide gels for analysis by autoradiography.

RESULTS

Cassette mutagenesis was used to randomly replace residues 164–167 of PA with Ser, Asn, Lys, or Arg. Plasmids which were successfully transformed into *B. anthracis* were sequenced across the region containing the mutagenic cassette, yielding the deduced amino acid sequences given in Table 1. Nineteen mutants encoding unique amino acid sequences were found.

The mutagenesis strategy was expected to produce mutants in which each of four residues, Arg, Asn, Lys, or Ser, was equally represented in residues 164–167. Analysis of the 76 altered residues from the 19 mutants showed an apparent 2.4-fold bias for Arg and Ser over Asn and Lys. Additionally, sequence analysis showed six instances of unexpected residues (Glu, Gly, or Thr) which may have been generated by misincorporation of nucleotides during extension of the primer.

Supernatants of each of the transformed bacilli were assayed for biological activity in the presence of LF with and without trypsin treatment (Table 1). Supernatants of three transformants, PA30-1, PA30-7, and PA30-41, had activity without trypsin treatment. Twelve clones produced supernatants which were active only if they were first treated with trypsin. Four supernatants, PA30-5, PA30-13, PA30-17, and PA30-35, were not active under either condition. Mutants PA30-17 and PA30-35 made PA which was unstable and degraded rapidly (data not shown).

Examination of the sequences that were recognized by the cell surface protease suggested that the arginine residues at both positions 164 and 167 were needed for cleavage and

Table 1. PA cassette mutant sequences and biological activity with and without trypsin treatment

Mutant	Sequence at residues 164–167	Toxicity of mutant	
		Untreated	After trypsin
Wild type	Arg-Lys-Lys-Arg	+	+
PA30-1	Arg-Ser-Arg-Arg	+	+
PA30-7	Arg-Ser-Lys-Arg	+	+
PA30-41	Arg-Arg-Arg-Arg	+	+
PA30-29	Lys-Arg-Asn-Arg	−	+
PA30-14	Ser-Thr-Arg-Arg	−	+
PA30-4	Ser-Asn-Ser-Arg	−	+
PA30-31	Ser-Ser-Ser-Arg	−	+
PA30-10	Asn-Ser-Asn-Lys	−	+
PA30-28	Arg-Arg-Arg-Ser	−	+
PA30-18	Arg-Arg-Ser-Ser	−	+
PA30-62	Arg-Lys-Ser-Ser	−	+
PA30-19	Ser-Lys-Lys-Ser	−	+
PA30-42	Ser-Lys-Ser-Ser	−	+
PA30-2	Asn-Arg-Glu-Ser	−	+
PA30-21	Lys-Arg-Asn-Asn	−	+
PA30-5	Ser-Asn-Ser-Ser	−	−
PA30-13	Ser-Asn-Lys-Glu	−	−
PA30-17	Arg-Glu-Gly-Gly	−	−
PA30-35	Ser-Asn-Lys-Ser	−	−
PA31	Ala-Lys-Lys-Arg	+/−	+
PA32	Arg-Lys-Lys-Ala	−	+
PA33	Arg-Ala-Ala-Arg	+	+

Plasmid DNAs from 19 cassette mutants were sequenced. Mutated proteins, expressed in *B. anthracis* and secreted into the medium, were added with LF to J774A.1 macrophages in culture. Toxicity was determined by visual inspection after 18 hr. Mutants are arranged according to their activity and sequence.

activation whereas the lysine residues at positions 165 and 166 were not absolutely required. Therefore, three specific constructs were prepared so as to measure the role of each residue: PA31 (Ala-Lys-Lys-Arg), PA32 (Arg-Lys-Lys-Ala), and PA33 (Arg-Ala-Ala-Arg). When supernatants were tested in combination with LF, PA33 was toxic for J774A.1 cells; PA31 was less toxic, and PA32 was not toxic (Table 1). PA31, PA32, and PA33 were purified to near homogeneity and used in the experiments described below.

When a constant amount (250 ng/ml) of PA or mutated PA was added with LF to RAW264.7 macrophages, each protein killed at a different rate. Native PA killed 50% of the cells by 100 min, PA33 killed 50% of the cells by 210 min, PA31 required 300 min to kill 50% of the cells, and PA32 was not toxic (Fig. 1*A*). Lysis of the cells was both dose- and time-dependent (Fig. 1 *B* and *C*). During a 2.5-hr incubation of RAW264.7 cells with the toxin, the effective concentration required to kill 50% of the cells (EC_{50}) was 45 ng/ml for PA, 600 ng/ml for PA33, and >10 μg/ml for PA31. When incubation was extended to 4.5 hr, all the proteins except PA32 had nearly equal potency.

These data show that cleavage of PA by the cell surface protease requires Arg at position 167 and that cleavage is greatly enhanced by the presence of Arg at position 164. Therefore the optimum recognition sequence can be represented as Arg-Xaa-Xaa-Arg, where Xaa represents any of several amino acids examined thus far. This sequence was found to be very similar to that preferred by the cellular protease furin (22, 23). Purified soluble furin was incubated with PA, PA31, PA32, and PA33 for various times. When visualized on Coomassie blue-stained polyacrylamide gels (Fig. 2), cleavage of native PA was 50% complete in <5 min. PA33 cleavage was not evident after 5 min and was 50% complete at 4.5 hr. PA31 did not appear to be cleaved at 5 min and was <10% cleaved after 4.5 hr (Fig. 2 and data not shown). PA32 was not cleaved at all. Further studies showed that mutants PA30-1, PA30-7, and PA30-41 were cleaved by furin, whereas mutants PA30-19, PA30-28, PA30-29, PA30-31, PA30-42, and PA30-62 were not (data not shown).

While the results described above implicate furin as the cell surface protease that activates PA, we sought further correlations to strengthen this argument. Furin has a strict requirement for Ca^{2+} and is quite resistant to a number of protease inhibitors, including inhibitors considered specific for serine proteases, such as phenylmethanesulfonyl fluoride (20). L6 cells were fixed by a gentle procedure optimized to preserve the activity of the PA receptor and the cell surface protease while maintaining the integrity of the cell membrane (S.H.L., unpublished studies). ^{125}I-PA incubated at room temperature with fixed L6 cells was bound and nicked (Fig. 3). A previous report showed that the activity of anthrax LT and the binding of PA are dependent on Ca^{2+} (24). When the fixed cells were washed with an EGTA-containing buffer to remove free Ca^{2+}, binding of PA was only about 10% of control levels (data not shown). Addition of 0.25 or 0.50 mM $CaCl_2$ allowed some binding but did not support cleavage of PA (Fig. 3, lanes 2 and 3). Addition of 1, 2, or 3 mM $CaCl_2$, yielding calculated free Ca^{2+} concentrations of 2, 195, and 1050 μM, respectively (lanes 4–6), led to both binding and nicking of PA, suggesting that the cellular protease requires Ca^{2+}. Efficient nicking occurred only at free Ca^{2+} concentrations ≥195 μM (lanes 5 and 6). Protease inhibitors such as aprotinin, pepstatin, phenylmethanesulfonyl fluoride and iodoacetamide, which were previously shown to be ineffective with furin, were similarly unable to block cell-surface cleavage of PA (data not shown). However, PA cleavage was largely blocked by 10 mM dithiothreitol (lane 7) and by 1 mM $ZnCl_2$ (lane 10) or 1 mM $HgCl_2$ (data not shown). The inhibition by dithiothreitol was readily reversed if the chemical was removed prior to addition of PA (lane 8). In addition,

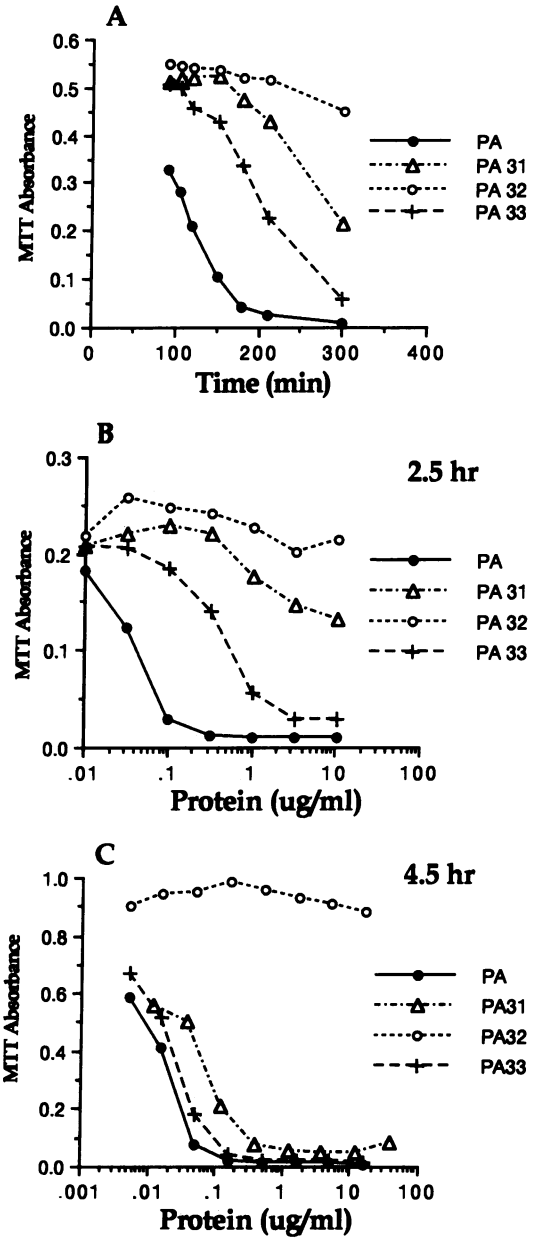

FIG. 1. Toxicity of mutated PA compared to that of native PA. (*A*) Native and mutated PA proteins (250 ng/ml) were assayed for functional activity on RAW264.7 cells in the presence of LF (250 ng/ml). (*B*) Mutated PA proteins were assayed for functional activity on RAW264.7 cells in the presence of LF (250 ng/ml) with native PA (20–5000 ng/ml) as a control for 2.5 hr. (*C*) Same as *B*, except cells were incubated for 4.5 hr. Cell viability was determined with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (18).

p-chloromercuribenzoate proved to be a potent inhibitor of cell-surface cleavage of PA (lane 9).

The chance that several different proteases in an infected animal are capable of activating PA has not been ruled out. It was possible then that the cell assay system would not accurately assess the requirements for PA activation in the host. We purified 2–4 mg of four mutated proteins to test in a rat lethality model. PA30-4, PA30-19, PA30-31, and PA30-42 were purified by anion-exchange chromatography and injected intravenously into Fischer 344 rats (14, 25). When 20-fold the normal lethal dose of PA (1.0 mg) was administered with LF (20 μg), no adverse reaction was noted for any of the mutated proteins. When 60 μg of each mutated

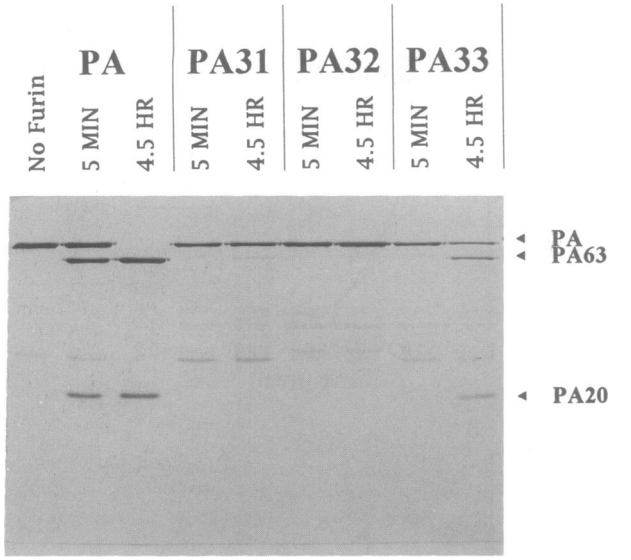

FIG. 2. Cleavage of PA and mutated PA by furin. Purified proteins were incubated with furin (5 μg/ml) at ambient temperature. Samples were removed from the reaction tube and the reaction was stopped by addition of $ZnCl_2$ to 2.5 mM. After heating to 95°C for 5 min, samples were electrophoresed in 12.5% SDS/polyacrylamide gels and stained with Coomassie blue dye.

protein was activated with trypsin prior to injection, the time to death was identical to that for native PA.

DISCUSSION

The anthrax toxins absolutely require that PA be proteolytically activated (14). Cleavage of PA near residues 164–167 must occur in order for PA to bind LF or EF to form LT or

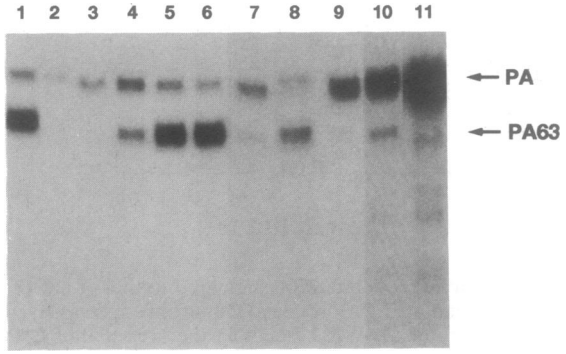

FIG. 3. Binding and cleavage of ^{125}I-PA on the surface of fixed L6 cells. Fixed L6 cells were incubated in 200 μl of binding buffer [100 mM NaCl/5 mM $MgCl_2$/1 mM EDTA/1 mM EGTA/25 mM Hepes, pH 7.3, with bovine serum albumin (200 μg/ml) and gentamicin (50 μg/ml)] with the addition of various potential inhibitors for 16 hr. Final concentrations of each addition were as follows: lane 1, 3.0 mM $CaCl_2$; lane 2, 0.25 mM $CaCl_2$; lane 3, 0.5 mM $CaCl_2$; lane 4, 1.0 mM $CaCl_2$; lane 5, 2.0 mM $CaCl_2$; lane 6, 4.0 mM $CaCl_2$; lane 7, 10 mM dithiothreitol/3.0 mM $CaCl_2$; lane 8, incubation in 10 mM dithiothreitol/3.0 mM $CaCl_2$ for 30 min, two washes with binding buffer containing 3.0 mM $CaCl_2$, and incubation for 16 hr; lane 9, 0.7 mM *p*-chloromercuribenzoate/3.0 mM $CaCl_2$; lane 10, 1.0 mM $ZnCl_2$/3.0 mM $CaCl_2$; lane 11, 1.0 mM $ZnCl_2$ only. After incubation, fixed cells were washed twice with 1.0 ml of binding buffer containing 3.0 mM $CaCl_2$ and then solubilized. Total proteins including bound PA were subjected to SDS/PAGE. Gels were dried and exposed to Kodak XAR5 film at −70°C for 24–48 hr. PA and PA63 indicate the positions of the native and cleaved PA molecules, respectively. All samples were from a single experiment. The autoradiographs of individual lanes were digitized with a Microtech scanner and assembled on an Apple Macintosh II fx computer.

ET. During intoxication of cultured cells, and probably also *in vivo*, a cellular protease performs this cleavage after PA binds to its cell surface receptor. Our objective in this work was to identify the activating protease by characterizing its sequence specificity and catalytic properties. We constructed semi-random and, subsequently, site-specific mutations at residues 164–167 of PA. The relative toxicity of these mutants showed the sequence specificity of the cellular protease to be Arg-Lys-Lys-Arg > Arg-Xaa-Xaa-Arg >> Xaa-Lys-Lys-Arg. PA32 (Arg-Lys-Lys-Ala) was not cleaved by, and therefore was not toxic to, RAW264.7 or J774A.1 cells. The slower killing and the increased EC_{50} for certain mutated PA proteins demonstrated that Arg at position 167 is essential for cleavage by the protease and that cleavage is greatly enhanced if there is also Arg at position 164. It appears that Arg or Lys at the intervening positions enhances the rate of cleavage but neither is essential.

Trypsin is capable of cleaving proteins adjacent to Arg and Lys residues and therefore was expected to activate any mutant PA that contained at least one of these residues in positions 164–167 (14). Trypsin did activate most of the mutants generated in this study, including PA31, PA32, and PA33 (Table 1). PA30-13, PA30-17, and PA30-35 were exceptions in which crude supernatants were not activated by trypsin treatment. PA30-17 and PA30-35 were consistently degraded in the culture supernatant and were probably inactivated before trypsin treatment. The unexpected resistance of PA30-13 to cleavage may be due to the presence of Glu adjacent to Lys.

When the mutated PA proteins are cleaved by trypsin, some of the resulting active PA63 peptides have extensions of one (PA30-19 and PA30-28) or two (PA30-18, PA30-2, PA30-21, PA30-42, and PA30-62) amino acids compared with the PA63 produced by cleavage at residue 167. These data demonstrate that while PA must contain a specific sequence to be recognized by the cellular protease, once cleaved, PA63 will tolerate alterations at the amino terminus.

The amino acid pattern required for PA cleavage appeared to be very similar to the sequence recognized by furin, an endoprotease (22, 23). Furin belongs to the family of eukaryotic, subtilisin-like proteases that includes the yeast enzyme KEX2 and the mammalian prohormone convertase enzymes PC1/PC3 and PC2 (26). Originally, these proteases were thought to cleave on the carboxyl side of paired basic residues as an essential posttranslational processing event in the biosynthesis of certain secreted hormones and cell surface proteins. However, a comparison of amino acid sequences of probable furin substrates led to the proposal that the preferred recognition site for furin is Arg-Xaa-(Arg/Lys)-Arg, with cleavage occurring at the carboxyl-terminal end of this sequence (27). While many probable furin substrates contain this motif, no direct evidence has been available to prove that furin requires either the −4 Arg or the −2 Arg/Lys.

To directly examine the sequence specificity of furin and to correlate its specificity with that of the cellular activating protease, we treated PA and mutated PA with furin. Cleavage of native PA was very rapid, and comparison to the mutated PA showed an *in vitro* furin sequence specificity of Arg-Lys-Lys-Arg > Arg-Ala-Ala-Arg >> Ala-Lys-Lys-Arg (Fig. 2). The mutants which were most active, PA33, PA30-1, PA30-7, and PA30-41, were cleaved by furin, whereas the inactive mutants PA32, PA30-19, PA30-28, PA30-29, PA30-31, PA30-42, and PA30-62 were not cleaved by furin. This demonstrates that furin requires a −4 Arg, is less dependent on the −2 Arg/Lys residue, and is unable to cleave Arg-Lys-Lys-Ala. In these respects furin is indistinguishable from the cellular PA-activating protease.

In studies reported elsewhere, we have shown that furin is inhibited by EDTA, EGTA, Hg^{2+}, Zn^{2+}, and dithiothreitol (20). We tested these and other potential inhibitors for their

ability to prevent cleavage of ^{125}I-PA bound on the surface of fixed L6 cells. In the absence of Ca^{2+}, PA did not bind to cells, consistent with the known involvement of Ca^{2+} in binding and toxicity (24). Low levels of binding were supported by addition of 0.25 mM $CaCl_2$, whereas efficient cleavage of PA required addition of 2.0 mM $CaCl_2$ (Fig. 3, lane 5), corresponding to a calculated free Ca^{2+} concentration of 195 μM. This concentration agrees well with the value of 200 μM free Ca^{2+} found to give half-maximal activity of purified furin (20). Interestingly, other metals (Zn^{2+}, Co^{2+}, and Cu^{2+}) promoted binding of PA to cells (Fig. 3, lane 11 and data not shown) but did not support cleavage. In all cases, the inhibitors which prevented cleavage of PA by furin prevented cleavage of PA on the cell surface. This suggests that furin is the cellular protease which cleaves PA.

The cleavage assays using either native cells at 4°C (14) or chemically fixed cells (as in Fig. 3) demonstrate that PA can be nicked on the cell surface. We have presented evidence here that indicates this cleavage is furin-mediated. The cleavage of PA in this assay is rather slow, requiring many hours to approach completion. This suggests that there may be only small amounts of furin on the cell surface or that the furin does not readily come into contact with the receptor-bound PA. Furin has been shown by immunochemical methods to be associated predominantly with the Golgi apparatus inside cells (28). Therefore, the implication from our data that some furin is exposed on the cell surface merits further study. Our data do not deal directly with the possible proximity of the PA receptor and furin. The receptor for PA has been partially characterized (7, 12). Chemical crosslinking of ^{125}I-PA to cells identified a 170-kDa band on SDS/polyacrylamide gels (12), indicating linkage of PA to a protein of about 90 kDa. This size is close to that predicted for mature furin (22), suggesting that furin may be both the receptor for PA as well as its activator. Alternatively, the crosslinking may have occurred with the protease and not the actual receptor.

The rates of PA cleavage at 5°C and on fixed cells ($t_{1/2}$ > 4 hr) are very slow compared to the rapid toxicity seen in LT-treated macrophages (lysis at 90–120 min) and to the rapid internalization of many receptor-bound proteins ($t_{1/2} \approx$ 10 min). Therefore it is possible that the required cleavage may actually occur after endocytosis of receptor-bound PA. In endosomes, PA cleavage together with the low pH would facilitate insertion into membranes (15, 16), and the embedded PA63 fragment might then recycle with membrane and/or receptor to the surface to bind and internalize LF in a subsequent round of endocytosis. Regardless of whether PA cleavage occurs principally on the surface or during membrane or receptor cycling, the sequence specificity shown in Fig. 1 argues that furin is probably responsible for most of the nicking.

PA31 (Ala-Lys-Lys-Arg) is cleaved very slowly by purified furin, at a rate < 1% that of native PA (Fig. 2). Similarly, the toxicity of PA31 for cells is about 100-fold lower when measured in short-term assays (Fig. 1*B*). Although PA31 does not contain the −4 Arg of the proposed furin consensus sequence Arg-Xaa-(Arg/Lys)-Arg, these low levels of activity might still be due to furin action. Alternatively, the very slow *in vitro* cleavage of PA31 (Fig. 2) might be due to proteases other than furin and perhaps occur during cycling of the receptor-bound PA as proposed above. These possibilities cannot be distinguished with the data currently available.

Other bacterial protein toxins may also utilize furin for activation. *Pseudomonas* exotoxin A has a putative furin site, Arg-Ser-Lys-Arg, and cleavage at the carboxyl terminus of this sequence is needed for activation of the toxin (29). Similarly, diphtheria toxin has a sequence at residues 190–193, Arg-Val-Arg-Arg, which must be cleaved for the A fragment to gain ADP-ribosylation activity. It has been suggested that urokinase performs this cleavage (3). This sequence is predicted to be recognized by furin as well, and we have in fact observed that diphtheria toxin is efficiently cleaved by furin under the same conditions as used here for cleavage of PA.[§] Thus it is possible that other pathogens in addition to *B. anthracis* have evolved to utilize furin or related eukaryotic proteases to activate their toxic proteins.

[§]Klimpel, K. R., Molloy, S. M., Bresnahan, P. A., Thomas, G. & Leppla, S. H., Annual Meeting of the American Society for Microbiology, May 26–30, 1992, New Orleans, Abstr. B-32.

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