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Proteolytic activation of receptor-bound anthrax protective antigen on macrophages promotes its internalization

Kathryn E. Beauregard,[1,2†] R. John Collier[1] and Joel A. Swanson[2*]
[1]*Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115, USA.*
[2]*Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109–0620, USA.*

Summary

Immunofluorescence and other methods have been used to probe the self-assembly and internalization of the binary toxin, anthrax lethal toxin (LeTx), in primary murine macrophages. Proteolytic activation of protective antigen (PA; 83 kDa, the B moiety of the toxin) by furin was the rate-limiting step in internalization of LeTx and promoted clearance of PA from the cell surface. A furin-resistant form of PA remained at the cell surface for at least 90 min. Oligomerization of receptor-bound PA63, the 63 kDa active fragment of PA, was manifested by its conversion to a pronase-resistant state, characteristic of the heptameric prepore form in solution. That oligomerization of PA63 triggers toxin internalization is supported by the observation that PA20, the complementary 20 kDa fragment of PA, inhibited clearance of nicked PA. The PA63 prepore, with or without lethal factor (LF), cleared slowly from the cell surface. These studies show that proteolytic cleavage of PA, in addition to permitting oligomerization and LF binding, also promotes internalization of the protein. The relatively long period of activation and internalization of PA at the cell surface may reflect adaptation of this binary toxin that maximizes self-assembly.

Introduction

Lethal toxin (LeTx) from *Bacillus anthracis* kills macrophages (Friedlander, 1986) and its ability to do so is linked to the lethal nature of anthrax infections (Hanna *et al*., 1993). The enzymatically active moiety of LeTx, lethal factor (LF, 89 kDa), is a zinc-dependent protease, which has been shown to cleave MAP-kinase-kinase (Duesbery *et al*., 1998). However, it remains uncertain if, or how, the toxic effects derive from this activity. LeTx is a member of the A-B family of toxins (Gill, 1978), which are bipartite entities containing a B moiety that binds cellular receptors and delivers an enzymatically active A moiety to the cytosol of affected cells. LF, the A moiety of LeTx, and protective antigen (PA, 83 kDa), the B moiety, are released from the bacteria as separate polypeptides that self-assemble into a complex at the surface of affected mammalian cells (Leppla, 1991). PA also serves as the delivery vehicle for an alternate A moiety from *B. anthracis*, an adenylate cyclase termed edema factor (EF; Leppla, 1982).

Native PA must be proteolytically activated in order to bind to LF or EF. After binding to its receptor at the cell surface, PA is cleaved by furin or a furin-like protease into two fragments: a 63 kDa carboxy terminal fragment (PA63), which remains bound to the receptor, and a 20 kDa fragment (PA20), which is released from the cell (Singh *et al*., 1989; Klimpel *et al*., 1992). PA may be activated in solution by treatment with trypsin; upon removal of PA20, PA63 spontaneously oligomerizes forming a ring-shaped heptamer. This oligomer, termed the prepore, has also been assumed to form at the cell surface following cleavage of PA. The PA63 prepore (and perhaps also the PA63 monomer) is capable of binding LF and EF competitively. The resulting hetero-oligomeric complex is taken up by receptor-mediated endocytosis (Gordon *et al*., 1988) and trafficked to a low-pH compartment, where LF and EF are translocated to the cytosol (Friedlander, 1986; Gordon *et al*., 1988). Acidic pH triggers translocation and is also correlated with the insertion of the PA63 prepore into membranes, resulting in its conversion to a pore form. LF and EF have been proposed to unfold and translocate across the membrane via the pore (Wang *et al*., 1996, 1997).

It is known that trafficking of LeTx through target cells occurs slowly. Menard *et al*. (1996) found that bafilomycin A_1, which prevents endosomal acidification, blocks LeTx action when added to J774 cells up to 60 min after LeTx. Persistence of a significant portion of LeTx in a bafilomycin A_1-sensitive compartment indicates that the

Received 24 November, 1999; revised 16 March, 2000; accepted 24 March, 2000. *For correspondence. E-mail jswan@umich.edu; Tel. (+734) 647 6339; Fax (+734) 764 3562. †Present address: American Journal of Human Genetics, Emory University, Atlanta, GA 30322, USA.

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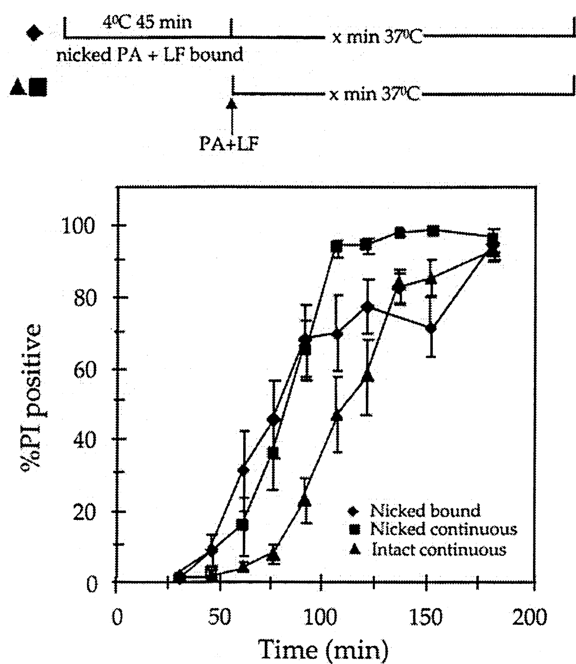


Fig. 1. Time course of LeTx action in bone marrow-derived macrophages. As diagrammed in the schematic above the graph, nicked PA (1 μg ml^{-1}) and LF (0.1 μg ml^{-1}) were bound to macrophages at 4°C. The cells were then washed, incubated at 37°C in RB-BSA for *x* min, stained with propidium iodide and fixed. Alternatively, PA (nicked or intact) and LF were added to macrophages at 37°C without a 4°C binding step, incubated for *x* min, then stained and fixed. At least 100 cells per coverslip were scored by fluorescence microscopy for the presence of stained nuclei.

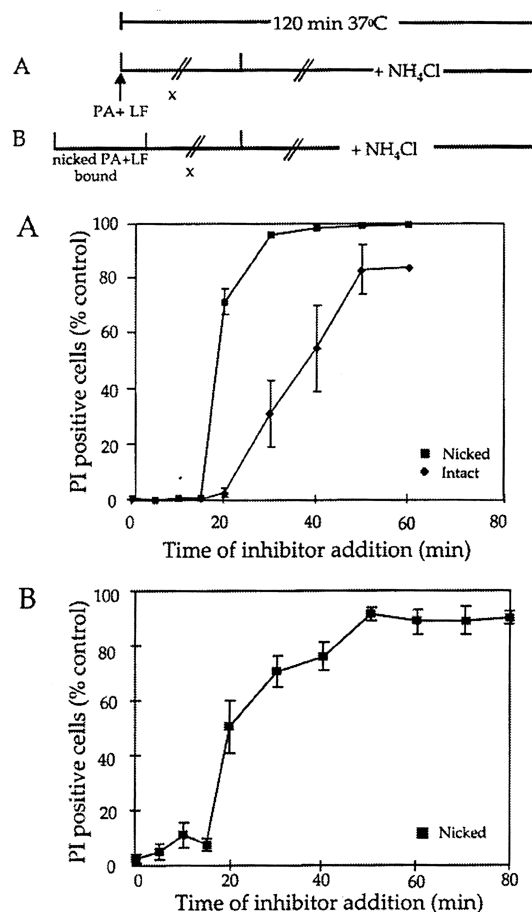


Fig. 2. Movement of LeTx to an NH_4Cl-insensitive compartment.
A. Toxicity of soluble LeTx. PA (nicked or intact; 1 μg ml^{-1}) and LF (0.1 μg ml^{-1}) were added to macrophages at 37°C in RB-BSA and incubated for different times before NH_4Cl was added to a final concentration of 10 mM. The total length of the incubation with LeTx was held constant at 2 h, after which the cells were stained with propidium iodide, fixed and scored for the presence of fluorescent nuclei.
B. Movement of prebound LeTx to the NH_4Cl-insensitive stage. Nicked PA and LF were bound to the cells for 30 min at 4°C as in Fig. 1. The cells were washed in RB then incubated for different times at 37°C before NH_4Cl was added. The cells were stained, fixed and scored as above. NH_4Cl alone did not kill macrophages under the conditions used in these assays.

toxin arrives slowly in the acidic compartment where LF translocation is triggered (Kochi *et al.*, 1994; Menard *et al.*, 1996). Such slow trafficking contrasts with other cell surface ligands, such as transferrin and epidermal growth factor, which are endocytosed within minutes (Watts, 1985; Hopkins *et al.*, 1990).

In the current study, we have sought a clearer understanding of assembly and trafficking of LeTx in macrophages. Using a variety of tools, we asked how the various steps in activation and self-assembly of the toxin affect internalization. The results provide insights into the adaptations that enable a binary toxin to assemble and enter cells.

Results

To follow the time course of killing of macrophages by LeTx, we first used an assay involving propidium iodide, a small, membrane-impermeant fluorescent dye that intercalates into DNA and thus labels the nuclei of cells in which the plasma membrane has been disrupted. By this measure, addition of intact PA and LF at standardized concentrations killed cells with a half-time of approximately 2 h at 37°C (Fig. 1). When PA that had been cleaved with trypsin (nicked PA) was substituted for intact PA, the half-time decreased to approximately 90 min. The half-time for killing remained approximately the same when cells were preincubated with nicked PA and LF for 45 min at 4°C, to allow binding and assembly of the toxin, before the 37°C incubation. These results indicate that the proteolytic activation step required approximately 30 min and was a rate-limiting event under the conditions used.

As a second means of examining the time course of LeTx action, we measured the progression of LeTx to a pH-insensitive state, using an assay based on the studies of Menard *et al.* (1996). Agents that raise vacuolar pH, such as bafilomycin A_1 and NH_4Cl, block LeTx activity by

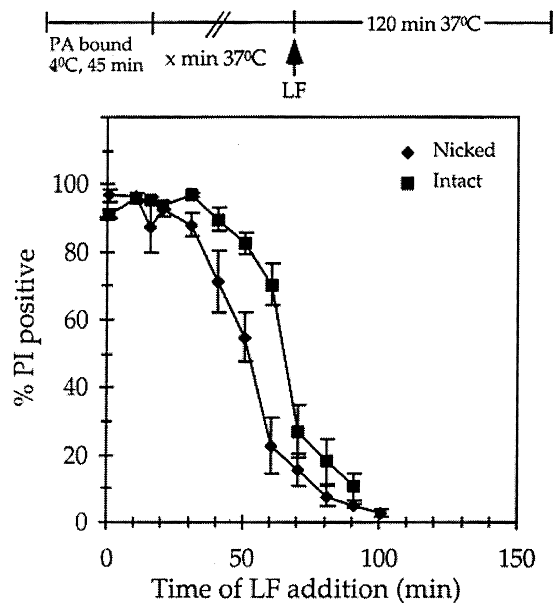


Fig. 3. Cell-associated PA remains accessible to LF for a prolonged period. PA (nicked or intact) was bound to macrophages at 4°C. The cells were washed then warmed for different periods before LF was added. Incubation with toxin continued for 2 h after LF addition. Cells were then stained, fixed and scored for propidium iodide labelling of nuclei.

preventing translocation (Friedlander, 1986; Milne *et al.*, 1994). NH_4Cl (10 mM) was added to macrophages at various intervals after PA (intact or nicked) and LF, and the percentage of dead cells was scored 2 h after toxin addition. As shown in Fig. 2A, the half-time for progression past the pH-sensitive stage was 40 min with intact PA and approximately 18 min with the nicked form (Fig. 2A). Results were almost identical ($t_{1/2} = 20$ min) with cells that had been preincubated with nicked PA and LF at 4°C (Fig. 2B). Experiments in which bafilomycin A_1 was used instead of NH_4Cl showed a similar temporal relationship between nicked and whole toxin, as did experiments in which the concentration of LF was 10-fold higher (data not shown). These results support the conclusion that proteolytic activation of PA is rate-limiting under the conditions employed, and show that approximately 20 min is required at 37°C for prebound, assembled toxin to reach a pH-insensitive state within the cell.

To probe the persistence of receptor-bound PA on the cell surface, we measured its accessibility to added LF as a function of time. Cells were preincubated with intact or nicked PA at 4°C, washed and moved to 37°C. LF was then added to samples at intervals and, after a further 2 h incubation, the percentage of propidium iodide-positive cells was measured. Given that LF cannot bind to cells in the absence of PA (Escuyer and Collier, 1991) we assumed that, at the low concentrations used, LF did not encounter PA anywhere other than at the cell surface. As shown in Fig. 3, nicked PA remained accessible for LF binding for approximately 45 min ($t_{1/2}$) and intact PA was accessible for longer, approximately 65 min. Thus, both nicked and intact PA persisted at the cell surface for long periods relative to ligands such as transferrin which are internalized within a few minutes of binding to macrophages (Racoosin and Swanson, 1992). Additionally, the

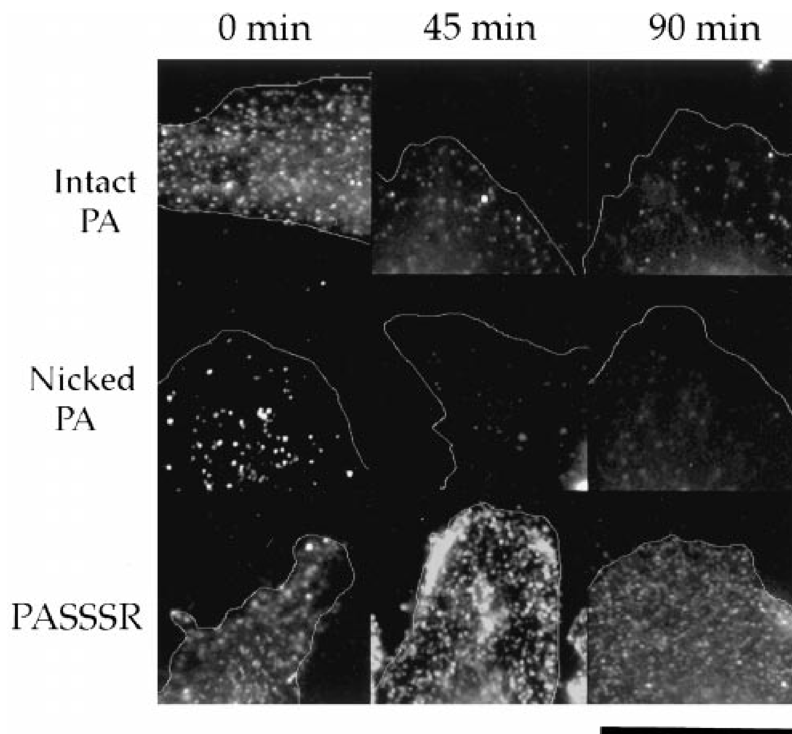


Fig. 4. PA is detectable at the surface of macrophages for over 45 min at 37°C and the clearance of PA from the cell surface is correlated with nicking. Intact PA, nicked PA, or PASSSR (1 μg ml^{-1}) was bound to macrophages at 4°C, excess protein was washed away with RB, then the cells were warmed to 37°C. After incubations for the times indicated, the macrophages were fixed and stained by immunofluorescence for PA without extraction. Shown are flat sections of macrophages stained in this manner. The white outlines were drawn to show the cell perimeters. The scale bar = 20 μM.

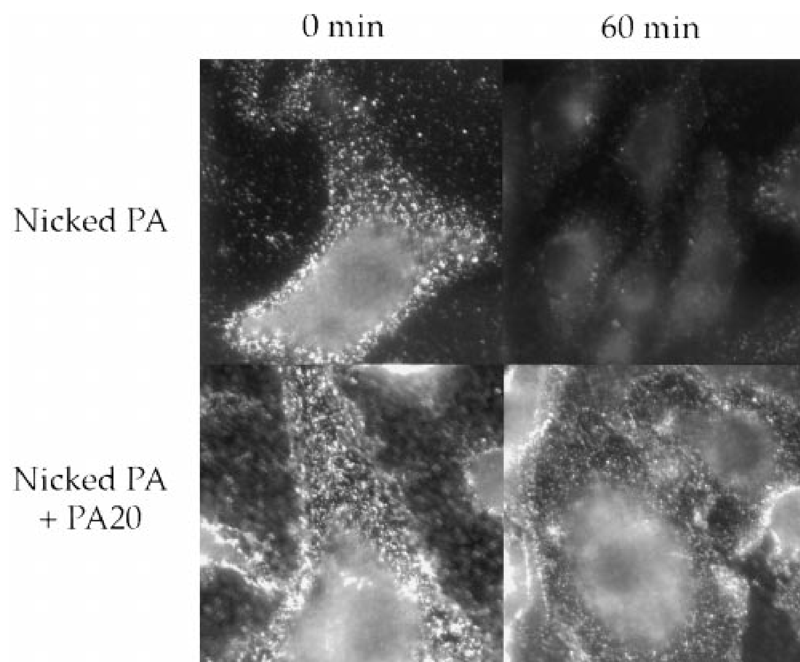


Fig. 5. Dissociation of PA20 facilitates clearance of PA from the cell surface. Nicked PA (1 μg ml^{-1}) was bound to macrophages in the presence or absence of 1.2 $\times$ 10^{-5} M PA20. Immunofluorescence for PA was performed on non-permeabilized cells following incubations of the cells at 37°C for 0 or 60 min. After 60 min at 37°C, nicked PA was largely cleared from the surface of the cells; this was inhibited by the presence of PA20.

different rates of LF delivery via intact and nicked PA indicated that PA was internalized more rapidly after being proteolytically activated.

To determine the distribution of PA on the cell surface and to follow its clearance from the cell surface, fixed but non-permeabilized macrophages were stained by immunofluorescence using anti-PA antibody. That labelling of non-permeabilized cells was selective for surface markers

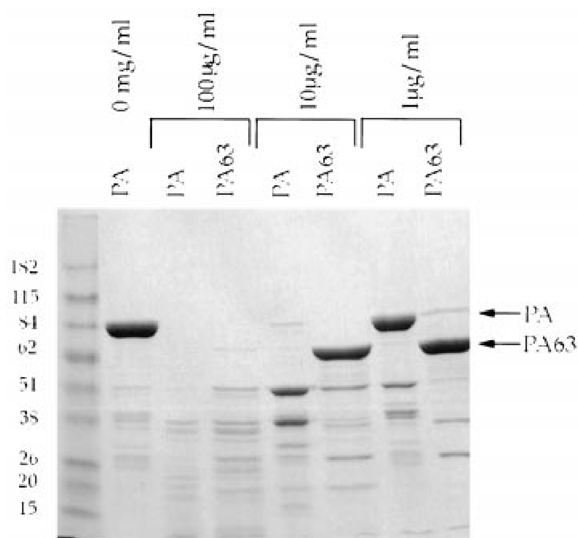
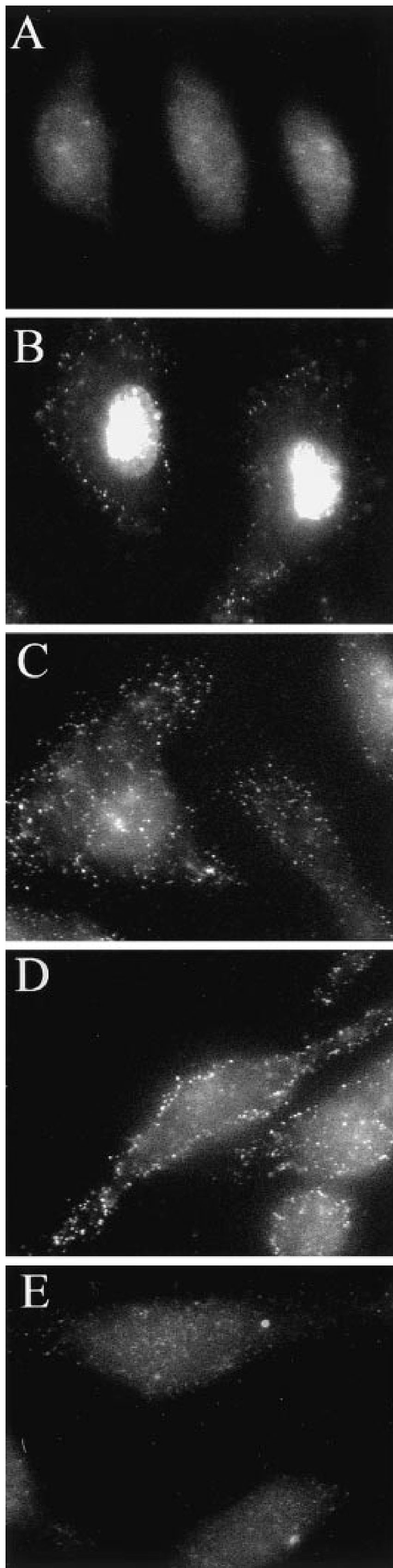


Fig. 6. Pronase resistance of PA in solution. Intact PA (83 kDa) and purified PA63 (both at 1 $\times$ 10^{-5} M) were incubated with pronase for 10 min at 4°C in 20 mM Tris pH 9. Pronase concentrations are indicated above the appropriate lanes. The products were run on 7.5% SDS–PAGE and stained with Coomassie blue. With 10 μg ml^{-1} pronase, intact PA was almost completely digested, while PA63 remained intact.

was confirmed in separate experiments by the absence of immunofluorescence signals for the intracellular proteins clathrin and LAMP-1 (data not shown). PA was bound to the cells at 4°C and the cultures were moved to 37°C to initiate endocytosis. Intact, wild-type PA gave a punctate pattern of staining that was broadly distributed over the cell surface. The magnitude of the staining diminished over time, but PA was readily detectable for at least 90 min (Fig. 4). With nicked PA, the staining decreased more rapidly and its punctate fluorescence had disappeared by 90 min. We also examined PASSSR, a mutant PA in which the furin site had been mutated from RKKR to SSSR, rendering the protein furin-insensitive (Gordon *et al.*, 1995; Ballard *et al.*, 1998). The pattern and magnitude of immunofluorescence with PASSSR were similar at time zero to those of intact wild-type PA and these parameters remained virtually unchanged over 90 min (Fig. 4). These results provide further indication that proteolytic activation of PA accelerates its internalization.

The most immediate effect of proteolytically activating PA is to permit its oligomerization. Removal of PA20 relieves a steric barrier to self-association, allowing PA63 to form the heptameric prepore. PA20 has a significant affinity for monomeric PA63, however, and oligomerization is therefore inhibited when the concentration of free PA20 is above a certain threshold. To determine whether the accelerated clearance of PA from the cell surface depended on oligomerization of PA63, we compared the clearance of nicked PA in the presence and absence of excess PA20 (1.2 $\times$ 10^{-5} M). In samples containing excess PA20, the cell surface PA signal remained virtually undiminished after 60 min at 37°C, whereas with nicked



PA alone the signal was greatly reduced over the same period (Fig. 5). Addition of LF (1×10^{-7} M) or LFn (1×10^{-6} M), the N-terminal PA63-binding domain of LF, instead of PA20 failed to preserve the PA signal. We conclude that oligomerization promotes endocytosis of PA63.

Further support for this conclusion came from studies of changes in pronase resistance of PA after binding to the cell surface. The PA63 prepore in solution was more resistant to pronase than intact PA (Fig. 6). Thus, when digested for 10 min at 4°C (pH 9.0) with 10 μg ml^{-1} pronase, intact PA was almost completely degraded, whereas PA63 remained largely intact. A study of differential sensitivity to pronase was therefore performed on macrophages using immunofluorescence to detect undegraded PA. First, we determined the concentration of pronase that was just sufficient to degrade intact PA (monomer) bound to macrophages at 4°C (Fig. 7A; 1 mg ml^{-1} pronase) and found that purified PA63 bound to cells was resistant to this concentration of pronase (data not shown). When cells that had been preincubated with intact PA at 4°C were warmed to 37°C, the protein became partially resistant to pronase within 5 min (Fig. 7B) and the level of resistance increased with time (Fig. 7C). Nicked PA that was bound to cells at 4°C and maintained at this temperature was pronase resistant (Fig. 7D), apparently reflecting rapid oligomerization of PA63. The furin-insensitive mutant, PASSSR, was pronase sensitive and did not develop significant resistance, even after long incubations at 37°C (Fig. 7E). These findings strongly support the notion that PA converts rapidly to a pronase-resistant oligomer at the cell surface following its nicking and the release of PA20.

Discussion

The results presented here provide insights into the events occurring at the cell surface when anthrax LeTx acts on murine macrophages. We found that intact receptor-bound PA was not rapidly internalized (as transferrin is, for example) and may not be internalized at all until the protein is proteolytically activated. Persistence of PA at the surface of primary macrophages

Fig. 7. PA nicking correlates with the development of pronase resistance. Various forms of PA were bound to cells at 4°C and incubated at 37°C for different times. The cells were moved back to 4°C and digested with pronase for 50 min PA was stained by immunofluorescence. Intact wild-type PA was completely digested by pronase when cells were kept at 4°C (A). Within 5 min at 37°C, resistance to pronase began to develop (B) and increased with time (C, 20 min 37°C). In contrast to A, nicked wild-type PA was pronase resistant at 4°C (D). In contrast to C, the furin-insensitive mutant PASSSR did not develop a high level of pronase resistance, even after 20 min at 37°C (E).

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agrees with the endocytic rate measured on RAW264.7 macrophage-like cells, where the half-time for PA endocytosis was found to be about 30–40 min and approximately 20% of the cell-bound PA remained at the cell surface after 90 min (Singh *et al*., 1999). These results probably reflect the intrinsic behaviour of the receptor, the identity of which is unknown. Evidence from cross-linking experiments suggests that the receptor is a protein of M_r 85–90 kDa (Escuyer and Collier, 1991) and the punctate pattern of immunofluorescence with anti-PA antibody implies localization to specific domains within the plasma membrane. The receptor has been found not to fractionate with detergent-insoluble caveolae-like membranes, as do cholera toxin receptors (Beauregard *et al*., 1999).

Once bound to its receptor, PA encounters furin or a related endogenous protease and is proteolytically activated. Consistent with the low concentration of such proteases at the plasma membrane (Molloy *et al*., 1994), the population of receptor-bound PA molecules is cleaved slowly over a period of 90 min or more. If the concentration of the proteases remains constant over this period, the cleavage is likely to be a first-order process with respect to PA.

As each PA molecule is cleaved, PA20 is released and PA63 is then able to oligomerize and to bind LF and EF. When PA20 is removed from nicked PA in solution (usually by ion-exchange chromatography) PA63 spontaneously oligomerizes to the heptameric prepore (Milne *et al*., 1994) and unless its lateral diffusion is constrained by interactions of the receptor, receptor-bound PA63 would also be expected to undergo heptamerization. Results presented here provide support for this concept. Thus, after binding to the cell surface, PA converted gradually from a protease-sensitive form to a more resistant form (Fig. 5), a change that is also characteristic of the PA63 heptamer in solution (Fig. 6). Additional evidence supporting prepore formation at the cell surface comes from the studies of Milne *et al*. (1994). A massive shift to the SDS-resistant pore form was observed when CHO-K1 cells that had been incubated with radiolabelled PA at 37°C were briefly treated at pH 5. Rapid formation of the pore in response to the pH pulse indicates that cell-bound, nicked PA is in a prepore form, poised to insert into the membrane.

The findings that proteolytic activation of PA promotes clearance from the cell surface and that PA20 blocks clearance of cell-bound, nicked PA could be readily explained if oligomerization of PA63 were the event triggering internalization. One model for the altered rate of PA internalization is that receptor cross-linking following PA oligomerization leads to endocytosis. Antibody cross-linking of some receptors, such as certain hormone receptors, has long been known to affect the rate of receptor internalization (King and Cuatrecasas, 1981). This has been observed with other receptors, such as GPI-anchored proteins, sphingomyelin and GM1, which cluster into membrane microdomains following cross-linking with antibodies or toxins (Mayor *et al*., 1994; Parton, 1994; Fujimoto, 1996).

The decrease in cell surface-associated PA upon warming from 4°C to 37°C could be attributable either to endocytosis or to dissociation of PA from its receptor. Endocytosis of some of the PA to acidic compartments was indicated by the cytotoxicity assay in which ammonium chloride could inhibit intoxication as late as 60 min after warming (Fig. 2). Moreover, the slow loss of LF-binding sites (nicked PA) from cell surfaces indicated the persistence of PA on cell surface receptors. Although dissociation of PA from receptors could explain the decline in cell surface labelling for intact and nicked PA (Figs 4 and 5), the persistence of the PASSSR mutant (Fig. 4) and of nicked PA in the presence of PA20 (Fig. 5) under identical circumstances argues against this.

Measurement of the period required for LeTx-mediated killing indicated a time window in which LeTx can function. Nicked PA bound to cells could deliver prebound LF past a pH-sensitive step within 20 min. However, some of the nicked PA persisted at the cell surface for nearly an hour, available for binding and transport of LF. This indicates that a small amount of LeTx delivery is apparently sufficient for toxicity, while the majority of toxin remains at the cell surface, capable of delivering LeTx at later times. This was supported by the immunofluorescence studies, which showed prolonged persistence of PA at the cell surface and very little co-localization with clathrin, transferrin or LAMP-1 (data not shown). Thus, we propose that PA is not internalized until it is processed by proteolysis at the cell surface. Processed PA is then internalized slowly, with or without bound LF, and these prolonged internalization kinetics allow delivery of LF into cytoplasm in as little as 20 min or as much as 1 h.

Our results differ somewhat from those obtained in J774 cells (Novak *et al*., 1992), where LF in the presence of nicked PA was found to be internalized within 5 min. The discrepancy between the two endocytic rates for LeTx could result from differences in cell type. It was not because of the presence of LF, as inclusion of LF or LFn in our experiments did not significantly alter the rate of clearance of PA from the cell surface. Slow proteolytic activation and clearance of whole PA from the cell surface favours the assembly of LeTx at the cell surface. A receptor has been chosen via evolution that exhibits a minimal amount of endocytosis (if any) in its putative monomeric form, allowing receptor-bound PA to persist and be slowly activated by proteolysis. Once activated, however, it is competent both to oligomerize, apparently permitting endocytosis, and to bind LF. Because LF must

bind to nicked PA for toxin assembly, successful intoxication reflects a race between proteolytic processing and the internalization step. Production of a sufficiently high concentration of LF, relative to PA, apparently permits this final assembly step to occur with acceptable efficiency.

Experimental procedures

Macrophages

Mouse bone marrow-derived macrophages were harvested from the femurs and tibias of female C3H/HeJ mice (Jackson Labs, Bar Harbor, ME) as previously described (Swanson, 1989). Six- to seven-day-old macrophage cultures were plated onto 13 mm glass coverslips (Goldseal, Fisher Scientific) in 24-well plates (Costar) at a density of 7.5×10^4 cells per coverslip for the immunofluorescence or killing assays**.** Prior to experiments, cells were incubated overnight in Dulbecco's modified eagle medium containing 10% heat-inactivated fetal bovine serum (Gibco BRL). Preparation of mouse bone marrow-derived macrophages used procedures approved by the University Committee on Use and Care of Animals (approval number 7500), which is accredited by the AAALAC.

Reagents

PA was purified from *Escherichia coli* as previously described (Beauregard *et al*., 1999). The gene for LF was amplified from the *B. anthracis* toxin plasmid pXO1 using the following primers: 5′-GATCGATCGGATCCGGCGGGCGGTCATGGT-3′ and 5′-GATCGATCGGATCCTCATTATGAGTTAATAAT-GAACTT-3′. The PCR products were cloned into the pET15b expression vector (Novagen) following restriction digests of both the vector and the insert with *Bam*HI. The ligation products were transformed into the *E. coli* expression strain BL21(DE3). Protein expression was induced and cell lysates were purified over a nickel column following the manufacturer's protocol (Novagen). The eluate fraction from the nickel column was dialysed overnight against 1 l of 20 mM Tris, pH 8.0, then the His-tagged protein was purified further using an anion-exchange column (Mono Q, Pharmacia).

The mutant PASSSR of PA has an alteration at the furin site (residues 164–167), changing the wild-type PA sequence, RKKR, to SSSR. This mutant has been previously described (Gordon *et al*., 1995; Beauregard *et al*., 1999). It was purified as above.

Anti-PA antibody was a polyclonal serum made in rabbits. The secondary antibody for PA immunofluorescence was Oregon green-labelled goat anti-rabbit, purchased from Molecular Probes. All other reagents were from Sigma unless otherwise specified.

Killing assay

Macrophages were washed three times with Ringer's buffer (RB; 155 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM NaH_2PO_4, 10 mM HEPES, 10 mM glucose, pH 7.2) containing 0.5 mg ml^{-1} BSA (RB-BSA) at 37°C before PA and LF were added to final concentrations of 1 $\mu g\ ml^{-1}$ and 0.1 $\mu g\ ml^{-1}$ respectively.

For assays where synchronous uptake of toxin was desired, PA was nicked *in vitro* with 10 $\mu g\ ml^{-1}$ trypsin for 5 min at 4°C. The reaction was stopped with 20 $\mu g\ ml^{-1}$ soybean trypsin inhibitor. Macrophages were chilled for 15 min on ice, then washed with RB. Nicked PA and LF (1 $\mu g\ ml^{-1}$ and 0.1 $\mu g\ ml^{-1}$ respectively) were then bound to cells in a solution of RB-BSA + 5% polyethylene glycol (20 000 MW) for 30 min at 4°C. Unbound toxin was washed away in three, 5 min washes in RB at 4°C. Unless otherwise indicated, cells were incubated with toxin for 2 h before determining the number of dead cells.

Cell viability was measured using the fluorescent dye propidium iodide, which labels nuclei of dead cells. Propidium iodide (7.5 $\mu g\ ml^{-1}$ in RB) was added to macrophages for 5 min at 37°C. The cells were then washed three times in warm RB before fixing for 20 min at 37°C in a fixative of 3.7% formaldehyde, 0.05% glutaraldehyde, 0.25 M sucrose, 1 mM EGTA, 0.5 mM EDTA, 20 mM HEPES, pH 7.4. The coverslips were washed three times in RB then mounted onto slides in a mounting medium of 90% glycerol, 10% phosphate buffer, and 1 mg ml^{-1} phenylenediamine. Approximately one hundred cells per coverslip were scored for nuclear propidium iodide staining using fluorescence microscopy with a Texas red filter set.

Immunofluorescence

PA was localized on surfaces of non-permeabilized cells, as well as within permeabilized cells, using immunofluorescence. PA or nicked PA was bound to macrophages at 4°C, as described above, and unbound protein was washed away using RB at 4°C (3×5 min washes). Cells were moved into RB-BSA at 37°C for various times before fixation; they were fixed at room temperature for 60 min in 4% paraformaldehyde in 40 mM HEPES, pH 7.2 containing 6.8% sucrose. Fixed cells were incubated for 10 min in PBS containing 0.25% NH_4Cl then 2×5 min in PBS containing 2% heat-inactivated goat serum (Sigma). The coverslips were incubated with primary antibody diluted in PBS-GS for 60 min at 37°C, washed 3×5 min in PBS-GS, incubated in secondary antibody under the same conditions, washed again, then mounted on slides.

Cells were viewed with a Zeiss Axioplan microscope and a Plan Apochromat lens (100×, 1.4 N.A., Carl Zeiss), using a fluorescein filter set. Digital images were collected using a cooled CCD camera (Quantix, Photometrics) and were manipulated in ADOBE PHOTOSHOP. The nuclei of macrophages in these experiments were autofluorescent to varying extents. This signal was not related to the presence or absence of toxin or antibody (data not shown).

Pronase digestion

For experiments in solution, 1×10^{-5} M PA or PA63 in 20 mM Tris pH 9.0 were incubated with various concentrations of pronase for 10 min at 4°C. Reducing protein sample buffer was added to the samples, which were then boiled for

5 min and loaded onto 7.5% SDS–polyacrylamide gels. Gels were stained with Coomassie blue.

For pronase digestion of cell surfaces, macrophages were incubated for 15 min at 4°C in RB before treatment with 1 mg ml^{-1} pronase (protease type XIV from *Streptomyces griseus*, Sigma) in RB for 50 min. The cells were then washed quickly three times in RB containing 1 mM PMSF and fixed for immunofluorescence. Pronase digestion under these conditions completely removed intact, wild-type PA from the surface of macrophages at 4°C, as determined in the immunofluorescence assay described above.

Acknowledgements

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