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Both PA63 and PA83 are endocytosed within an anthrax protective antigen mixed heptamer: A putative mechanism to overcome a furin deficiency

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Abstract

Anthrax toxin consists of protective antigen (PA), and lethal (LF) and edema (EF) factors. A 83 kDa PA monomer (PA83) precursor binds to the cell receptor. Furin-like proprotein convertases (PCs) cleave PA83 to generate cell-bound 63 kDa protein (PA63). PA63 oligomerizes to form a ring-shaped heptamer that binds LF–EF and facilitates their entry into the cells. Several additional PCs, as opposed to furin alone, are capable of processing PA83. Following the incomplete processing of the available pool of PA83, the functional heptamer includes both PA83 and PA63. The available structures of the receptor–PA complex imply that the presence of either one or two molecules of PA83 will not impose structural limitations on the formation of the heptamer and the association of either the $(PA83)_1(PA63)_6$ or $(PA83)_2(PA63)_5$ heteroheptamer with LF–EF. Our data point to the intriguing mechanism of anthrax that appears to facilitate entry of the toxin into the cells which express limiting amounts of PCs and an incompletely processed PA83 pool.

Keywords: Anthrax; *Bacillus anthracis*; Protective antigen; Furin; Proprotein convertase; Endocytosis; Lethal toxin

Three proteins of anthrax toxin [protective antigen (PA), lethal (LF) and edema (EF) factors[1]] are responsible for the pathological symptoms of anthrax. During the process of intoxication, the 83 kDa PA monomer (PA83) binds to the cell surface receptor. The bound PA83 is then cleaved at the sequence Arg-Lys-Lys-Arg↓Ser by the cellular proprotein convertase (PC) furin [1]. This cleavage releases a 20-kDa N-terminal fragment and a cell-bound, C-terminal 63-kDa protein (PA63). The latter oligomerizes into a ring-shaped PA heptamer that exposes the binding sites for EF and LF [2,3]. The amino terminal ends of both EF and LF bind to PA63, while the respective C-terminal parts of EF and LF exhibit adenylate cyclase activity and proteinase activity, respectively. LF is a zinc-dependent metalloprotease which cleaves a specific bond at the amino terminal region of mitogen-activated protein kinase kinases (MAPKKs), destroying their ability to signal and, consequently, blocking the essential signal transduction pathways [4]. A complex formed by PA63 and either EF or LF is trafficked into the cell by receptor-mediated, clathrin-dependent, endocytosis [5]. Following binding with the receptor, receptor-mediated endocytosis, formation of the PA heptameric pore within the acidic lumen of the endosomal compartment

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[1] *Abbreviations used:* EF, edema factor; LFb, biotin-labeled lethal factor; PA83b and PA63b, the biotin-labeled protective antigen precursor and the mature, processed protective antigen, respectively; PC, proprotein convertase.

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and release into the cytosol, LF and EF attack their targets and produce their toxic effects.

There are two types of anthrax toxin receptor: a capillary morphogenesis protein 2 (CMG2) and an anthrax toxin receptor/tumor endothelial marker 8 (ATR/TEM8), both of which are type I transmembrane proteins with single membrane spanning domains. CMG2 and TEM8 are related to the von Willebrand factor A domains and to an α-integrin-like inserted (I) domain. CMG2 is likely to be expressed in most human tissues. In turn, the expression of TEM8 is restricted to tumor endothelium and cancer cells. A metal ion-dependent adhesion site within the receptor I domain is directly involved in the metal cation-dependent binding of the anthrax receptors with PA [6].

Seven related, albeit distinct, PCs have been identified and partially characterized in humans [7,8]. There is evidence that other members of the PC family, PC1 (also named PC3) and PACE4, in addition to furin, are also capable of processing PA83 [9,10]. The relative importance and potency of the individual furin-like PCs in the processing of PA83 was not studied in detail. The follow-up molecular events involved in the PA internalization, subcellular compartmentalization and stoichiometry of the PA–LF interactions are also not completely understood.

Here, we present evidence that several individual PCs efficiently substitute for furin in the processing of PA and that the internalized PA heptamer may include both the PA63 and the PA83 species. We suggest that these mechanisms are functionally relevant and important because they permit anthrax to widen the spectra of infected cell types and to overcome a potential deficiency in cell surface-associated activity of PCs.

Materials and methods

Antibodies, protein and cells

Reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated. PA83, PA63, the wild type LF and the catalytically inert E687C LF mutant [11] and goat antibody against PA were from List Biological Laboratories (Campbell, CA, USA). Murine anti-PA monoclonal antibody MAB8081 (clone BAP0101), anti-LF monoclonal antibody MAB8085 (clone BAL0105) and peptide hydroxamate inhibitor GM6001 were obtained from Chemicon (Temecula, CA, USA). Rabbit antibody against Rab5B was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Murine monoclonal antibodies against nucleoporin p62 (clone 63), murine monoclonal antibody (clone 515) to human CD44 and murine monoclonal antibody (clone 2297) to caveolin-1, were from Becton-Dickenson (San Diego, CA, USA). Anti-NH_2-terminal MEK1 (MAPKK1) rabbit antibody was from Upstate Biotechnology (Lake Placid, NY, USA). EZ-link sulfo-NHS-LC-biotin was purchased from Pierce (Rockford, IL, USA). Human U251 glioma cells were characterized earlier [12,13]. Murine macrophage-like RAW264.7 cells were obtained from ATCC (Manassas, VA, USA). Cells were routinely grown in DMEM supplemented with 10% FBS (Gibco, Carlsbad, CA, USA) and penicillin–streptomycin (50 IU/mL–50 μg/mL). A PA83-SNKE-deltaFF-E308D mutant (a nonfurin cleavage variant of PA in which the furin cleavage site was replaced by SNKE and which, in addition, lacks two proteolysis-sensitive sites due to the E308D and deltaFF mutations [14]) was a kind gift of Dr. Stephen Leppla (NIDCR, NIH, Bethesda, MD, USA). The fluorescence peptide substrate pyro-Glu-Arg-Thr-Lys-Arg-methyl-coumaryl-7-amide and the PC inhibitor decanoyl-Arg-Val-Lys-Arg-CH_2Cl(dec-RVKR-cmk) were obtained from Bachem Bioscience (King of Prussia, PA, USA).

Separation of the cytoplasmic-plasma membrane and plasma membrane-free protein fractions

Cells (90% confluent; 1×10^6) were co-incubated with nocodazole (2 μg/mL) and cytochalasin B (1 μg/mL) to destroy the cytoskeleton and to prevent contamination of the plasma membrane-free fraction with the membraneous and cytoskeletal materials. Cells were next extensively washed with PBS and lysed in 0.4 mL of 10 mM Hepes buffer, pH 7.0, supplemented with 5 mM $MgSO_4$, 5 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.5% NP-40, and 125 mM sucrose. The lysate was centrifuged for 10 min at 16,000*g* to isolate the supernatant, cytoplasmic fraction that contained, in addition, the plasma membrane fragments. To isolate the plasma membrane-free fraction, after cell lysis in 0.4 mL of 10 mM Hepes buffer, pH 7.0, supplemented with 5 mM $MgSO_4$, 5 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.5% NP-40, and 125 mM sucrose, the resulting lysates were diluted 10-fold by the same buffer. The diluted lysate was spun for 5 min at 1500*g*. The pellet was collected, washed with the lysis buffer, and the proteins were extracted for 1 h at 4 °C in 0.1 mL of 50 mM Tris–HCl buffer, pH 7.4, containing 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM sodium vanadate, and 1 mM sodium fluoride. The solubilized material (subsequently referred as the plasma membrane-free fraction) was separated from the insoluble pellet by centrifugation for 10 min at 16,000*g*. The plasma membrane-free fraction included the nuclei and the cytoplasmic material, but it was essentially free from the plasma membranes and the cytoskeleton. Protein concentration in the samples was routinely determined by colorimetric assay using Bradford reagent with BSA as the standard protein. The samples (30 μg each) were separated by SDS–PAGE in 10% polyacrylamide gel and analyzed by Western blotting with the MEK1, LF and PA antibodies. In addition, the samples were analyzed with the antibodies against nucleoporin (a nuclear marker), CD44 and caveolin (plasma membrane markers), and Rab5B (a marker of early endosomes) to verify the purity of the fractions and the absence of cross-contamination. In addition, the purity of the isolated samples was validated by DAPI staining followed by microscopy.