Cell Surface Tumor Endothelium Marker 8 Cytoplasmic Tail-independent Anthrax Toxin Binding, Proteolytic Processing, Oligomer Formation, and Internalization*

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The interaction of anthrax toxin protective antigen (PA) and target cells was assessed, and the importance of the cytosolic domain of tumor endothelium marker 8 (TEM8) in its function as a cellular receptor for PA was evaluated. PA binding and proteolytic processing on the Chinese hamster ovary cell surface occurred rapidly, with both processes nearly reaching steady state in 5 min. Remarkably, the resulting PA63 fragment was present on the cell surface only as an oligomer, and furthermore, the oligomer was the only PA species internalized, suggesting that oligomerization of PA63 triggers receptor-mediated endocytosis. Following internalization, the PA63 oligomer was rapidly and irreversibly transformed to an SDS/heat-resistant form, in a process requiring an acidic compartment. This conformational change was functionally correlated with membrane insertion, channel formation, and translocation of lethal factor into the cytosol. To explore the role of the TEM8 cytosolic tail, a series of truncated TEM8 mutants was transfected into a PA receptor-deficient Chinese hamster ovary cell line. Interestingly, all of the cytosolic tail truncated TEM8 mutants functioned as PA receptors, as determined by PA binding, processing, oligomer formation, and translocation of an lethal factor fusion toxin into the cytosol. Moreover, cells transfected with a TEM8 construct truncated before the predicted transmembrane domain failed to bind PA, demonstrating that residues 321–343 are needed for cell surface anchoring. Further evidence that the cytosolic domain plays no essential role in anthrax toxin action was obtained by showing that TEM8 anchored by a glycosylphosphatidylinositol tail also functioned as a PA receptor.

Anthrax toxin, the major virulence factor of Bacillus anthracis, consists of three polypeptides: protective antigen (PA), lethal factor (LF), and edema factor (EF) (1, 2). These three proteins are individually non-toxic. To inactivate mammalian cells, PA binds to a ubiquitously expressed, recently identified cellular receptor, tumor endothelium marker 8 (TEM8) variant 2 (3), and is cleaved at the sequence RKKR in the cell surface by furin or furin-like proteases (4, 5). Proteolysis yields the amino-terminal 20-kDa fragment (PA20), which is released into the medium, and the carboxyl-terminal 63-kDa fragment (PA63), which remains bound to the receptor and self-associates to form a ring-shaped heptamer (6, 7). The heptamer binds up to 3 molecules of LF or EF (8, 9). The resulting oligomeric complex is then internalized into endosomes, where the decreased pH causes the PA63 heptamer to insert into the endosomal membrane and produce a channel through which LF and EF translocate to the cytosol (10). Therefore, PA is the central part of anthrax toxin, serving as the delivery vehicle for binding and translocation of LF and EF into the cytosol of the cells. The combination of PA plus LF kills animals (11, 12) and certain cells, including mouse macrophages (13, 14). LF is a zinc-dependent metalloprotease that cleaves several mitogen-activated protein kinase kinases (MAPKK) in their amino-terminal regions (15, 16). How this cleavage triggers the lethal effects of the toxin and whether there are additional cellular substrates remains unclear. EF is a calmodulin-dependent adenylate cyclase that elevates intracellular cAMP concentrations (17), thereby causing diverse effects in cells including the impairment of phagocytosis (18).

Previous studies on the interaction of PA with host cells have often used cytotoxicity assays to infer internalization mechanisms, or have used radiolabeled or chemically labeled PA that may behave differently due to modification. In the present studies, we directly assessed PA binding, proteolytic processing, and internalization by target cells using a highly sensitive and specific rabbit antiserum to PA. We found that following binding and processing by cell surface furin, the cleaved PA immediately forms the PA63 oligomer and that this oligomer is the only species of PA that is internalized. In addition, following internalization, the oligomer is quickly transformed into an SDS/heat-resistant form, a process coincident with insertion and channel formation in endosomal membranes. In related studies we extended the understanding of toxin internalization obtained in the recent breakthrough that identified TEM8 variant 2 as a PA receptor (3). Currently, there are three reported cDNAs that result from splicing variations in TEM8 (GenBank™ accession number NM_032208, NM_053034, and NM_18153). The physiological functions of these genes have not been studied. Beyond the fact that TEM8 variant 2 functions as a PA receptor, the only information available is that implicit in the initial identification that TEM8 expression is up-regulated in tumor endothelium (19, 20). Thus, it remains unknown whether other TEM8 variants can also function as PA receptors, and whether TEM8 has functions beyond binding PA in anthrax toxin action. To answer these questions, in the present work, we constructed a series of TEM8 truncated...
transfected them into a PA receptor-deficient Chinese hamster ovary (CHO) cell mutant, and found that all constructs having a membrane anchor functioned as PA receptors.

**EXPERIMENTAL PROCEDURES**

Reagents—Protein toxins produced as described previously included PA (21), PA-ΔFF PA with the EF-encoding leader peptide deleted (22), diphteria toxin (23), PA-U7 (a non-cleavable variant of PA with the furin site RKKR replaced by PAA) (24), and FP59, a recombinant fusion toxin consisting of anthrax toxin LF amino acids 1–254 (LFn) fused to the ADP-ribosylation domain of *Pseudomonas* exotoxin A (25). Rabbit anti-PA polyclonal antisem (number 5308) and LF polyclonal antiserum (number 5309) were prepared by immunization with recombinant LF and LF. Polyclonal antibody against the amino-terminal sequence of MAPKK1 (MEK1-NT) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Goat anti-rabbit IgG-HRP (sc2054) and goat antimouse IgG-HRP (sc2005) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Bafilomycin A1, saponin, and phosphatidylinositol-specific phospholipase C (FI-PLC) were purchased from Sigma.

**Cell Lines and Culture Media—**CHO cell clone 6 (CHO CL6) is a line reconstituted in this laboratory from CHO 10001, a subclone of CHO-S (26), which was obtained from Dr. Michael Gottesman (National Institutes of Health, Bethesda). CHO FD11, a furin-defective derivative of CHO CL6, was developed in our laboratory by chemical mutagenesis (27). CHO PR230 is a spontaneous PA receptor-deficient mutant derived from CHO WTP4, which is derived from thioguanine- and ouabain-resistant cell WTBl111 (28), which was derived from CHO-K1. All CHO cells were grown in a minimal essential medium supplemented with 5% fetal calf serum, 2 mM glutamine, 50 μg/ml gentamycin, and 25 mM HEPES.

**PA Binding and Internalization by CHO Cells—**PA binding was assessed at both 37 and 4 °C. Cells were grown in 24-well plates to confluence. Cells were incubated with 1 μg/ml PA for different lengths of time and then washed five times with Hank’s balanced salt solution (HBSS) (Biofluids, Rockville, MD). The cells were lysed in 100 μl of modified RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonlfuryl fluoride, 1 μg/ml each of aprotinin, leupeptin, and pepstatin). In measurements of PA internalization, the cells were first treated with 0.5 ml of 0.5 mg/ml trypsin in HBSS per well at 37 °C for 5 min to remove proteolytically the cell surface-bound PA, then washed, and the cell lysates were subjected to SDS-PAGE or native-PAGE for 10 min at room temperature in 1× native buffer (NOVEX) for native-PAGE.

The proteins were then transferred to nitrocellulose membranes, followed by Western blotting as described (29). PA was visualized by chemiluminescence using the West Pico Kit (Pierce). For the two-dimensional analysis, cell lysate was first separated on native-PAGE, and the gel strip was then sequentially equilibrated for 15 min each in Buffer I (125 mM Tris-HCl, pH 6.8, 1% SDS, 8.7% glycerol, 5 mM dithiothreitol) and Buffer II (125 mM Tris-HCl, pH 6.8, 1% SDS, 8.7% glycerol, 2% iodoacetamide) and subjected to SDS-PAGE, followed by Western blotting as described above.

**LF Translocation and MAPKK1 Cleavage Assay—**In measurements of LF translocation, CHO CL6 cells grown in 24-well plates were incubated with 1 μg/ml LF along with 1 μg/ml PA or PA-Δ FF for 1 h at 37 °C, washed once with HBSS, and treated with 0.5 ml 0.5 mg/ml trypsin in HBSS per well at 37 °C for 5 min to remove proteolytically the cell surface-bound toxin. The cells were then washed and permeabilized by saponin to allow efflux of cytosol as described (30). Briefly, the cells were resuspended and incubated in 100 μl of phosphate-buffered saline (PBS) containing 50 μg/ml saponin, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of aprotinin, leupeptin, and pepstatin for 30 min at 4 °C. The soluble fraction was separated from the particulate fraction by centrifugation at 15,000 × g for 5 min at 4 °C. The pellet was washed in HBSS and solubilized in RIPA lysis buffer. The samples from the soluble and pellet fractions were analyzed by native-PAGE followed by Western blotting using LF antisem (number 5309). In measurements of MAPKK1 cleavage by LF, CHO CL6 cells grown in 24-well plate were incubated with 1 μg/ml LF along with 1 μg/ml PA or PA-Δ FF for 1 h at 37 °C, washed, lysed, and analyzed by SDS-PAGE followed by Western blotting using an antibody against the amino-terminal sequence of MAPKK1 (MEK1-NT).
adding 50 μl of 2.5 mg/ml 3′,4′,5′-trimethoxybenzyl-2′-5′-diphényl-2,4,5-tetrazolium bromide (MTT) in a-essential minimal medium. The cells were incubated with MTT for 45 min at 37 °C; the medium was removed, and the blue pigment produced by viable cells was solubilized with 100 μl/well of 0.5% (w/v) SDS, 25 mM HCl, in 90% (v/v) isopropyl alcohol. The plates were vortexed, and the oxidized MTT was measured as A570 using a microplate reader.

**RNA Isolation and Northern Hybridization—**Total RNA was isolated from exponentially growing CHO cells by using TRIzol Reagent (Invitrogen), separated on 1.0% agarose, 6.66% formaldehyde gels and then transferred onto nylon membranes (Immobilon-N, Millipore). Membranes were hybridized with a 32P-labeled 1.0-kb CHO TEM8 cDNA fragment isolated by reverse transcriptase-PCR by using 5′/H11032 primer TTCTGCCAGGAGGAGACACTTACATGC, and 3′/H11032 primer CCCACAAGGCATCGAGTTTTCCCTT. DNA sequencing analysis in vitro

We used a high titered polyclonal anti-PA serum (number 5308) to assess PA binding and its subsequent processing and internalization by CHO cells. Analysis of unmodified PA eliminated concerns that radiolabeled or chemically labeled PA might behave differently from native PA due to the modification. The results showed that PA bound to CHO CL6 cells and was rapidly cleaved to PA63. More than half the PA bound was cleaved to PA63 within 5 min (Fig. 1A, CL6 lanes). Because furin is the major cell surface protease that cleaves PA (4, 5), we compared processing of PA by CHO FD11, a derivative of CHO CL6 cells that lacks furin (27). The FD11 cells bound PA efficiently, but cleavage to PA63 was very slow (Fig. 1A, FD11 lanes). In addition to intact PA and PA63, another PA species revealed on SDS-PAGE was an SDS/heat-resistant PA63 oligomer that migrated very slowly (Fig. 1A). Because the formation of the PA63 oligomer requires proteolysis, the PA63 oligomer was hardly detected in cell lysates from FD11 (Fig. 1A). The cell lysates were further analyzed by native-PAGE. The FD11 cell lysates contained mainly the intact PA (Fig. 1B, FD11 lanes), as expected, whereas the lysates from CHO CL6 cells contained intact PA as well as two higher order PA63 oligomers, but no monomeric PA63 (Fig. 1B, CL6 lanes). When the binding assay was performed by using trypsin-nicked PA in which the furin site was pre-cleaved by limited trypsin digestion (22), as expected the major PA species detected were the oligomers, and just a negligible amount of monomer PA was shown (Fig. 1C). The nature of these two distinct PA63 oligomers is unclear. The faster migrating species, termed oligomer A in this study (Fig. 1, B and C), is probably free PA63 heptamer, whereas the more slowly migrating species, termed oligomer B (Fig. 1, B and C), may be a complex of the PA63 heptamer with cellular components such as the PA receptor or detergent-resistant membrane structures. When these oligomeric species (in Fig. 1C, lane 1h) were subjected to second dimension SDS-PAGE, interestingly, oligomer A dissociated to PA63 monomer, whereas oligomer B turned out to be the mixture of both SDS-sensitive and -resistant oligomers (Fig. 1D). Together these results showed not only that bound PA is rapidly cleaved by furin but also that the resulting PA63 monomer very rapidly oligomerizes. Thus, the cell surface-associated PA63 mimics the behavior of PA63 produced in vitro, which forms the heptamer in neutral aqueous solutions (21). These heptamers have been visualized previously by electron microscopy (5), x-ray diffraction (6), and electron microscopy. Absence of PA63 monomer further indicated that oligomerization of PA63 is effectively irreversible. PA63 detected by SDS-PAGE (Fig. 1A) evidently resulted from the resolution of PA63 oligomer by boiling in SDS loading buffer.

**Interaction of Anthrax Toxin and Cell**

**RESULTS**

**PA63 Produced from PA by Cell Surface Proteolytic Cleavage Rapidly Forms Oligomers**—We used a high titered polyclonal anti-PA serum (number 5308) to assess PA binding and its subsequent processing and internalization by CHO cells. Analysis of unmodified PA eliminated concerns that radiolabeled or chemically labeled PA might behave differently from native PA due to the modification. The results showed that PA bound to CHO CL6 cells and was rapidly cleaved to PA63. More than half the PA bound was cleaved to PA63 within 5 min (Fig. 1A, CL6 lanes). Because furin is the major cell surface protease that cleaves PA (4, 5), we compared processing of PA by CHO FD11, a derivative of CHO CL6 cells that lacks furin (27). The FD11 cells bound PA efficiently, but cleavage to PA63 was very slow (Fig. 1A, FD11 lanes). In addition to intact PA and PA63, another PA species revealed on SDS-PAGE was an SDS/heat-resistant PA63 oligomer that migrated very slowly (Fig. 1A). Because the formation of the PA63 oligomer requires proteolysis, the PA63 oligomer was hardly detected in cell lysates from FD11 (Fig. 1A). The cell lysates were further analyzed by native-PAGE. The FD11 cell lysates contained mainly the intact PA (Fig. 1B, FD11 lanes), as expected, whereas the lysates from CHO CL6 cells contained intact PA as well as two higher order PA63 oligomers, but no monomeric PA63 (Fig. 1B, CL6 lanes). When the binding assay was performed by using trypsin-nicked PA in which the furin site was pre-cleaved by limited trypsin digestion (22), as expected the major PA species detected were the oligomers, and just a negligible amount of monomer PA was shown (Fig. 1C). The nature of these two distinct PA63 oligomers is unclear. The faster migrating species, termed oligomer A in this study (Fig. 1, B and C), is probably free PA63 heptamer, whereas the more slowly migrating species, termed oligomer B (Fig. 1, B and C), may be a complex of the PA63 heptamer with cellular components such as the PA receptor or detergent-resistant membrane structures. When these oligomeric species (in Fig. 1C, lane 1h) were subjected to second dimension SDS-PAGE, interestingly, oligomer A dissociated to PA63 monomer, whereas oligomer B turned out to be the mixture of both SDS-sensitive and -resistant oligomers (Fig. 1D). Together these results showed not only that bound PA is rapidly cleaved by furin but also that the resulting PA63 monomer very rapidly oligomerizes. Thus, the cell surface-associated PA63 mimics the behavior of PA63 produced in vitro, which forms the heptamer in neutral aqueous solutions (21). These heptamers have been visualized previously by electron microscopy (6), x-ray diffraction (7), and electron microscopy. Absence of PA63 monomer further indicated that oligomerization of PA63 is effectively irreversible. PA63 detected by SDS-PAGE (Fig. 1A) evidently resulted from the resolution of PA63 oligomer by boiling in SDS loading buffer.

**PA63 Oligomer Is the Only PA Species That Is Internalized**—The data above indicated that cells exposed to PA contain intact PA and PA63 oligomers on their surface (Fig. 1B). To explore whether these PA species are equally internalized, we performed a PA trypsin protection assay. After incubation with PA at 4 or 37 °C, cells were treated with trypsin to remove the cell surface-bound PA, allowing identification of those materials internalized by endocytosis. Remarkably, PA63 oligomer constituted the major protected PA species at 37 °C (Fig. 1, A and B, CL6 lanes), indicating that the PA63 oligomer was the only form of PA to be internalized. Also present were small amounts of a PA fragment, probably the carboxy-terminal 47-kDa receptor-binding portion remaining bound to receptor after incomplete cleavage by trypsin (33). Endocytosis is tem-
The presence of bafilomycin A1 (0.2 μg/ml) for 30 min and then decreased with time, indicating that the SDS/heat-resistant PA63 oligomer remains at 37 °C for 30 min and then decreased with time.

FIG. 2. SDS/heat-resistant PA63 oligomer forms in acidic endocytic compartments. A, PA63 oligomer forms at both 37 and 4 °C. CHO CL6 cells were incubated with 1 μg/ml PA at 4 or 37 °C for 1 h, washed, and lysed. The cell lysates were subjected to native-PAGE, followed by Western blotting using PA antisera (number 5308). B, SDS/heat-resistant PA63 oligomer formation requires internalization and endosome acidification. CHO CL6 cells were incubated with PA (1 μg/ml) at 4 °C for 3 h, washed, and changed to fresh medium. The cells were shifted to 37 °C in the absence or presence of bafilomycin A1 (0.2 μg/ml) for various lengths of time and lysed. The cell lysates were analyzed as in Fig. 1A.

perature-dependent (10), and therefore all surface-bound PA should be removed by trypsin from cells incubated at 4 °C. Thus, in a control for the previous experiment, we showed that trypsin removed all the cell-associated PA (Fig. 1E), with the exception of the 47-kDa fragment mentioned above. Further evidence that intact, monomeric PA is not internalized into cells was obtained using PA-U7, an uncleavable variant of PA that can bind but cannot be proteolytically activated by cellular furin (24). This PA mutant was not internalized to a trypsin-resistant site even when incubated with cells at 37 °C for 4 h (Fig. 1F). These results demonstrated that the proteolytic cleavage of receptor-bound PA is an absolute prerequisite not only for the biochemical property of self-assembly but also for its subsequent biological activity of undergoing endocytosis.

Oligomeric PA63 Endocytosed to Acidic Compartments Is Rapidly Transformed to an SDS/Heat-Resistant Form—Previous studies showed that in solution, purified PA63 can form two types of oligomers, an SDS-sensitive type, which forms at neutral pH and can be resolved into PA63 monomer by SDS, and the SDS-resistant type, which forms at acidic pH and persists in the presence of SDS (34). This suggested that the SDS/heat-resistant oligomer shown above (Fig. 1A) may be the counterpart of this SDS-resistant oligomer formed in acidic solution, and therefore may be produced following endocytosis and delivery to acidic compartments. In fact, when CHO CL6 cells were incubated with PA, the SDS/heat-resistant oligomer was formed at 37 °C (Fig. 1A, CL6 lanes) but not at 4 °C, a temperature at which endocytosis does not occur (Fig. 1E, 1h lane). Moreover, native-PAGE analysis revealed that the PA63 oligomer A formed at both 4 and 37 °C (Fig. 2A). Based on these observations we hypothesized that the PA63 oligomer formed on the cell surface encounters a progressively more acidic environment along the endocytic pathway and undergoes conformational changes and membrane insertion at acidic pH that renders it resistant to SDS/heat. To verify this hypothesis, we incubated CHO CL6 cells with PA at 4 °C, washed, and then shifted to 37 °C for various lengths of time in the absence or presence of bafilomycin A1, a potent and specific inhibitor of the vacuolar (H+)-ATPase proton pumps that maintain the pH gradients of acidic compartments (35, 36). We showed that following 3 h of incubation at 4 °C, little, if any, SDS/heat-resistant oligomer formed (Fig. 2B, -Bafilomycin, 0 lane), although a significant amount of oligomer was present when lysates were analyzed by native gels (data not shown). However, the resistant oligomer formed efficiently in a time-dependent manner when the cells were shifted to 37 °C following binding at 4 °C (Fig. 2B, -Bafilomycin lanes). Moreover, the amount of resistant oligomer quickly declined after peaking at 30–40 min of incubation at 37 °C, and less than half remained after 60 min (Fig. 2B, -Bafilomycin lanes). In contrast, formation of the SDS/heat-resistant PA63 oligomer was potently inhibited when bafilomycin A1 was included at the time when the cells were shifted to 37 °C (Fig. 2B, +Bafilomycin lanes), indicating that the SDS/heat-resistant PA63 oligomer is produced within acidic compartments. Because the resistant oligomer began to appear as early as 5 min after shifting to 37 °C, it likely formed in early endosomes, the first compartments along the endocytic pathway that have a significantly acidic pH. Because only trace amounts of monomeric PA63 were detected in the trypsinized samples shown above (Fig. 1A, trypsin +lanes), we conclude that the majority of internalized PA63 oligomer is rapidly transformed to the SDS/heat-resistant form in endosomes following internalization.

The chymotrypsin-sensitive loop in domain 2 is involved in membrane insertion (7, 22, 37). We used the PA protein altered in this loop to show that formation of the SDS/heat-resistant oligomer requires membrane insertion. The mutated PA protein, PAΔFF, with H11001 and F134 at the tip of the chymotrypsin-sensitive loop deleted (22), was bound, processed, and internalized normally (Fig. 3A), but its transformation into the SDS/heat-resistant form in acidic compartments was greatly decreased (Fig. 3B). This in turn made the mutated PA unable to translocate LF into the cytosol of CHO CL6 cells, observed both biochemically (Fig. 3C) and through the failure to cause MAPKK1 cleavage (Fig. 3D). These data show that membrane insertion and channel formation by the chymotrypsin-sensitive loop is the basis of the conformational changes that render the oligomer resistant to boiling in SDS sample buffer.

In the experiment using bafilomycin, we noted that the small amount of resistant oligomer that appeared late in the presence of bafilomycin A1 persisted for at least 120 min (Fig. 2B, +Bafilomycin lanes), suggesting both its formation and degradation were arrested by bafilomycin A1. We therefore assessed the oligomer degradation more systematically. The acidic pH of early endosomes is important for sorting endocytosed material, influencing whether materials are recycled to the plasma membrane, or transported to lysosomes for degradation. The SDS/heat-resistant oligomer is likely transported to lysosomes for degradation. To examine oligomer stability, cells were incubated with PA for 1 h at 37 °C and then washed to remove PA. At this point, all the compartments in the endocytic pathway, including cell surface, endosomes, and lysosomes, should be loaded with the corresponding PA species. During a subsequent incubation at 37 °C in the absence of bafilomycin A1, it was found that the SDS/heat-resistant PA63 oligomer remained at the same level for 30 min and then decreased with time, completely disappearing after 180 min (Fig. 4, -Bafilomycin lanes). In contrast, oligomer degradation was completely inhibited in the presence of bafilomycin A1 (Fig. 4, +Bafilomycin lanes). Bafilomycin A1 probably arrested the degradation of the SDS/heat-resistant PA63 oligomer by inhibiting its transport to lysosomes and also by decreasing the activity of lysosomal...
proteases that prefer an acidic environment (38). The stability of the SDS/heat-resistant PA63 oligomer in cells treated with bafilomycin A1 also suggests that the acid-induced conformational change is effectively irreversible, because elevating the pH of endocytic compartments does not produce monomeric PA63.

TEM8-mediated PA Binding, Proteolytic Processing, and Endocytosis Are Independent of Its Cytoplasmic Sequence—An important advance in understanding anthrax toxin action was the recent demonstration that TEM8 variant 2 (368 residues, designated ATR) acts as a PA receptor (3). However, no information is available regarding the ability of other TEM8 gene products to serve as PA receptors. TEM8-related proteins produced through alternative splicing also include TEM8 variant 1 (564 residues) and variant 3 (333 residues) (Fig. 5A). TEM8 variants 1 and 2 contain a putative 23-residue transmembrane region (residues 321–343), whereas TEM8 variant 3 lacks this sequence and instead has a unique 15-residue carboxyl-terminal sequence whose properties suggest that the protein will be released as a soluble form that would not function as a receptor. TEM8 variant 2 (ATR) has a very short putative cytoplasmic tail (25 residues) with a ESEEEDDD acidic cluster whose action is required for PA activation (3). The TEM8 variant 1 cytoplasmic region (221 residues) includes a more extended acidic cluster360EESEEEDDD368, a proline-rich region (34 prolines within the last 57 residues), and 14 potential phosphorylation sites.

To explore the roles of the various TEM8 variants as PA receptors and experimentally evaluate the roles of the motifs in the cytoplasmic region of TEM8 in PA binding, proteolytic processing, and internalization, we constructed eight variants of human TEM8 (Fig. 5A). The corresponding cDNA fragments were cloned into the bicistronic mammalian expression vector pIRESHyg2b. The resulting expression plasmids were transfected into PR230, a spontaneous CHO cell PA receptor-deficient mutant. In contrast to its parental CHO cell line WTP4, PR230 is specifically defective in PA binding and thus is resistant to PA plus FP59 but sensitive to diphtheria toxin and Pseudomonas exotoxin A (data not shown). Stably transfected cells were established by hygromycin B selection for 2 weeks, and the hygromycin-resistant colonies were either isolated individually or pooled. We found that >80% of the hygromycin-resistant clones expressed the transfected genes (data not shown). Therefore, in addition to analyzing representative isolated clones from selected TEM8 variant constructs (Fig. 5, B

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**Fig. 3.** PA-ΔFF has decreased ability to form SDS/heat-resistant oligomer and to translocate LF into the cell cytosol. A and B, PA-ΔFF fails to produce SDS/heat-resistant oligomer formation. CHO CL6 cells were incubated with 1 μg/ml PA or PA-ΔFF for 1 h at 37 °C and treated as in Fig. 1, and cell lysates were separated by SDS-PAGE (A) or native-PAGE (B) for Western blotting using PA antiserum (number 5308). C and D, PA-ΔFF fails to produce SDS/heat-resistant oligomer formation. CHO CL6 cells were incubated with 1 μg/ml PA or PA-ΔFF plus 1 μg/ml LF for 1 h at 37 °C; the cells were then trypsinized, permeabilized by saponin, and separated into cell pellet and supernatant cytosol fractions as described under “Experimental Procedures.” The cell pellet lysates (p) and the cytosol (c) were separated by native-PAGE followed by Western blotting using LF antiserum (number 5309) (C). CHO CL6 cells were incubated with 1 μg/ml PA or PA-ΔFF plus 1 μg/ml LF for 1 h at 37 °C; the cell lysates were then separated by SDS-PAGE followed by Western blotting using a antibody that detects the amino-terminal sequence of intact but not cleaved MAPKK1 (MEK1-NT) (D).

**Fig. 4.** Bafilomycin A1 inhibits PA63 oligomer degradation. CHO CL6 cells were incubated with 1 μg/ml PA for 1 h at 37 °C, washed, and changed to fresh medium without (−) or with (+) bafilomycin A1 (0.2 μM) for different lengths of time. The cells were then washed and lysates analyzed as in Fig. 1A.

- Bafilomycin
  - Time(min)
  - 0 10 30 60 90 120 180
  - Oligomer
  - PA
  - PA63

+ Bafilomycin
  - Time(min)
  - 0 10 30 60 90 120 180
  - Oligomer
  - PA
  - PA63
and C), we fully analyzed pools of transfectants to obtain results characteristic of average expression levels (Fig. 5, D and E). Remarkably, all the TEM8 variants or truncated mutants containing the putative transmembrane domain and extracellular region, including TEM8 variant 1, TEM8–115 aa, TEM8–16 aa, and TEM8–0 aa, were functional PA receptors (Fig. 5). These transfected PR230 cells regained the ability to bind and proteolytically process PA, leading to the internalization and conversion of the PA63 oligomer into the SDS/heat-resistant form to an extent matching that of the parental WTP4 cells (Fig. 5, B and D). Moreover, these transfected cells became sensitive to killing by PA plus FP59 (Fig. 5, C and E).

Like CHO CL6 cells (Figs. 1–4), WTP4 cells can only internalize the oligomeric form of PA63 (Fig. 7), as demonstrated by trypsin treatment method. We found that constructs with no cytosolic tail (TEM8–0 aa, Fig. 7, middle panel) or with a GPI anchor (TEM8-GPI, Fig. 7, right panel) had the same ability to internalize the oligomeric PA but not the monomer PA (Fig. 7).

Northern blot analysis revealed that CHO WTP4 cells express one major TEM8 transcript, one having a size slightly
PA receptors.

PA receptors.

surviving from PA receptor-deficient CHO mutants obtained in this laboratory by virus insertional mutagenesis. All the PA receptor-deficient CHO mutants analyzed so far by cell fusion belong to a single genetic complementation group, and their phenotype is re-

Surprisingly, we did not find any monomeric form of PA inside the cells, and the PA63 oligomer was the only form of PA internalized. These results are consistent with the previous report (41) that the proteolytic activation of PA on macro-

We further assessed the role that the acidic pH of intracellular compartments plays in the formation and degradation of the PA oligomers. PA could be processed to form an SDS/heat-sensitive PA63 oligomer on the cell surface at 4 °C, a tempera-

because bafilomycin A1 could not affect the resistant oligomer degradation which otherwise is complete within 3 h, suggesting that the degradation takes place in a low pH compartment, probably the lysosome.

Because the PA63 band formed by dissociation of the SDS/heat-sensitive oligomer was barely detectable inside cells (Fig. 1A, trypsin + lanes), we concluded that the majority of the internalized PA63 oligomer rapidly transformed to the SDS/heat-resistant form in the acidic environment in endosomes following the endocytosis. Therefore, the PA63 SDS/heat-sensitive oligomer, the source of the PA63 band in the SDS gels, mainly exists on the cell surface. Thus, the progressive decrease of PA63 on the SDS gel (Fig. 4, –Bafilomycin lanes)
reflects the endocytic process. The clearance of PA63 (Fig. 4, —Bafilomycin a lanes) required about 2 h, indicating that the endocytic process is a slow and rate-limiting step in toxin internalization.

Taken together, the data show that the anthrax toxin system has evolved to deliver efficiently LF and EF to the cytosol. PA is retained on cell surface receptors until it is cleaved and converted to the oligomer, the first species able to bind LF and EF (9). Endocytosis, initiated after oligomerization, is relatively slow compared with furin cleavage, so that the PA oligomer remains on the surface for a sufficient time to allow LF (or FP59) and EF to bind. This mechanism makes it probable that the internalized PA oligomers are loaded with at least one molecule of the enzymatic moieties before entry. This may explain why CHO cells are very sensitive to PA, having an EC₅₀ only 1–2 ng/ml of PA in the presence of FP59.

The other objectives of this work were to determine whether TEM8 variants 1 and 3 can also function as PA receptors, to explore the roles of the TEM8 cytosolic tail in PA binding, proteolytic processing, oligomerization, and internalization, and to experimentally define the transmembrane region. The three reported TEM8 variants share the same amino-terminal extracellular part but differ in length and sequence in their putative cytosolic regions. TEM8 variant 1 is the longest and extracellular part but differ in length and sequence in their three reported TEM8 variants share the same amino-terminal and to experimentally define the transmembrane region. The potential effects of PA that follow from its binding to TEM8. The clearance of PA63 (Fig. 4, —Bafilomycin a lanes) required about 2 h, indicating that the endocytic process is a slow and rate-limiting step in toxin internalization.

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