Anthrax Protective Antigen: Efficiency of Translocation Is Independent of the Number of Ligands Bound to the Prepore†

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Received January 28, 2004; Revised Manuscript Received March 12, 2004

ABSTRACT: Heptameric anthrax protective antigen (termed prepore), which assembles at the mammalian cell surface, competitively binds edema factor (EF) and/or lethal factor (LF). It then transports them to an acidic intracellular compartment and mediates their translocation across the membrane to the cytosol. Steric constraints limit to three the number of molecules of EF and/or LF that can bind simultaneously to prepore. To determine whether the number of ligand molecules bound per heptamer affects the efficiency of translocation, we measured the low-pH-triggered translocation of the radiolabeled protective antigen (PA_{63})-binding domain of LF (^{35}S-LF_N) across the plasma membrane of CHO-K1 cells as a function of the degree of saturation of the prepore. The fraction translocated remained constant at ~0.4 as ^{35}S-LF_N was varied from nil through saturating concentrations. The same constant value was observed when we held ^{35}S-LF_N at a saturating concentration and varied the number of functional ligand sites per prepore by changing the ratio of wild-type PA to a ligand-binding mutant. Thus, prepore containing only a single ligand-binding site is capable of translocating its cargo as efficiently as one containing multiple binding sites. The results as a whole imply that heptamers with one, two, or three ligands bound translocate their ligands with the same efficiency, indicating that each ligand molecule is translocated independently from the others.

The toxin produced by *Bacillus anthracis* consists of three monomeric proteins, which assemble at the mammalian cell surface to form toxic complexes. Two of the proteins, lethal factor (LF)[1] and edema factor (EF), are intracellular effectors, which enzymatically modify cytosolic target molecules. EF is a calmodulin-dependent adenylate cyclase (*1*), and LF is a zinc-dependent protease that cleaves certain mitogen-activated protein kinase kinases (*2*, *3*). The third protein, protective antigen (PA), is a receptor-binding and pore-forming entity that delivers EF and LF to the cytosol (*4*).

Assembly of the toxic complexes begins when PA (83 kDa) binds to a cell-surface receptor (ATR/TEM8 or CMG-2) (*5*, *6*) and is cleaved into two fragments by a member of the furin class of proteases. The smaller fragment, PA_{20} (20 kDa), dissociates and diffuses into the surrounding medium, leaving the complementary 63-kDa fragment (PA_{63}) bound to the receptor. Dissociation of PA_{20} allows PA_{63} to oligomerize into a ring-shaped heptamer, which we term the prepore. The prepore can competitively bind up to three molecules of EF or LF (*7*). The resulting complexes are internalized by receptor-mediated endocytosis (*8*) and trafficked to the endosome. The acidic environment of the endosome induces the prepore to undergo a conformational change that enables it to form a membrane-spanning pore and translocate EF or LF into the cytosol (*9*).

The process of translocation has been described in a broad outline, but much remains to be learned about its mechanism. Among the questions to be answered is how the efficiency of translocation is related to the number of ligand molecules bound per heptamer. We have addressed this question using two different approaches. The results of both are similar and indicate that the efficiency remains constant, regardless of the number of ligands bound per heptamer. Hence, each bound ligand molecule translocates independently from the others.

MATERIALS AND METHODS

Preparation of Proteins. PA mutants were constructed using Quickchange mutagenesis (Stratagene) according to the protocol of the manufacturer and cloned into pET22-b(+) (Novagen) expression vector. The LF_N construct used is from pET15-b, which adds a hexahistidine tag to the N terminus of LF_N. Wild-type PA, PA mutant, and LF_N were purified from *Escherichia coli* as described previously (*10*). The protein concentration was determined using Bio-Rad protein assay reagent (Bio-Rad).

Activation of PA and Heptamer Formation. PA was activated by treatment with trypsin at a molar ratio of 1:1000 at room temperature for 30 min followed by the addition of a 10-fold molar excess of soybean trypsin inhibitor to stop cleavage. PA heptamer was formed either by incubating trypsin-nicked PA (nPA) with a 4-fold molar excess LF_N in solution at room temperature for 1 h, or by loading nPA onto a Mono-Q HR 5/5 column (Amersham) and eluting with a NaCl gradient.

Cell-Surface Binding and Translocation Assay. PA binding to the cell surface and translocation of ^{35}S-LF_N were assayed as described (*11*). Briefly, CHO-K1 cells seeded in a 24-

† This work was supported by NIH Grant AI22021.

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[1] Abbreviations: PA, protective antigen; $[PA_{63}]_7$, heptameric form of PA; EF, edema factor; LF, lethal factor; LF_N, N-terminal 263 residues of LF; PA_n *n*-kDa fragment of PA; nPA, proteolytically activated PA; DTA, the A moiety of diphtheria toxin; CHO, Chinese hamster ovary.

10.1021/bi049794a CCC: $27.50
Published on Web 04/23/2004

well plate at a concentration of 2.5×10^5 cells per well were chilled on ice for 30 min before removing the medium and washing with a phosphate-buffered saline (PBS) buffer. Cells were incubated with 2.4×10^{-8} M nPA in Ham's F-12 medium buffered with 20 mM HEPES at pH 8.0. After an incubation of 2 h on ice, the cells were washed with PBS twice and then incubated with ^{35}S-labeled LF_N (produced by an in vitro transcription/translation reaction using the TNT-coupled reticulocyte lysate system, Promega) for another 2 h on ice. For the translocation assay, the cells were chased with a pH 5.0 buffer at 37 °C for 1 min, treated with 2 mg/mL Pronase at 37 °C for 8 min, and then lysed. For the binding assay, Pronase was omitted and the cells were lysed directly. After lysis, the radioactive content was determined by scintillation counting. The fraction of translocation was defined as dpm protected from Pronase/dpm bound to the cells.

Inhibition of Protein Synthesis. Protein synthesis inhibition by LF_N-DTA was used to measure the ability of PA to deliver a ligand to the cytosol and was done basically according to Milne et al. (*12*). CHO-K1 cells were plated in 96-well plates at a density of 5×10^4 cells per well at 16−18 h prior to the start of the experiment. PA at concentrations of 1×10^{-7} to 1×10^{-12} M and LF_N-DTA of 3×10^{-9} M were added, and the cells were incubated at 37 °C for 4 h to allow PA binding, heptamer formation, LF_N-DTA binding, and internalization. The medium was then removed and replaced with leucine-free Ham's F-12 medium supplemented with [^{3}H]-leucine (1 μCi/mL, Perkin-Elmer). After further incubation at 37 °C for 1 h, the cells were washed with ice-cold PBS and treated with 10% trichloroacetic acid. Protein synthesis was measured by incorporation of radioactivity into acid-insoluble material and expressed as a percentage of incorporation by unintoxicated cells.

Fluorescence Labeling and Resonance Energy Transfer. To label cysteine mutants of PA with a fluorescence probe, we incubated the protein with 0.1 M dithiothreitol (DTT) to reduce cysteine residues, removed the excess DTT by gel filtration, and then labeled the protein with a 10-fold molar excess of Alexa Fluorophore C_5 maleimide (Molecular Probe) at 4 °C overnight. PA-K563C was labeled with Alexa Fluor 488 C_5 maleimide to serve as an energy transfer donor, and PA-K563C/R178A mutant was labeled with Alexa Fluor 546 C_5 maleimide to serve as an energy transfer acceptor. The labeling efficiency was approximately 1 Alexa Fluorophore per PA monomer. PA heteroheptamer used in the resonance energy transfer experiments was formed either in solution in the presence of LF_N or by chromatography on a Mono-Q column. When PA heteroheptamer was formed in solution, the Fluorophore-labeled donor and acceptor were first nicked by trypsin and then mixed at a ratio of 1:10 (the total concentration was 1 μM) in the presence of 0.57 μM LF_N (the molar ratio of PA_{63} heptamer to LF_N was 1:4). After incubation for 30 min at room temperature, the emission spectrum was monitored with excitation at 488 nm. For the heptamer formed on the column, we mixed the PA-donor with the PA-acceptor at a ratio of 1:10, nicked it with trypsin, loaded the protein onto a Mono-Q column, pooled the heptamer fraction, and then recorded the emission spectrum.

Biotin Labeling and Streptavidin Affinity Chromatography. The heptamer formed on the column by mixing nicked PA-K563C and PA-R178A at a molar ratio of 1:10 was first reduced and labeled with a 10-fold molar excess EZ-link of Biotin-HPDP (PIERCE) at room temperature for 90 min and then passed through a PD-10 column (Amersham) to remove the excess amount of Biotin-HPDP. Biotin-labeled $[PA_{63}]_7$ was loaded onto a streptavidin column. We washed the column and then eluted the bound protein with 100 mM DTT. The eluted fraction was desalted to remove excess DTT and labeled with 20-fold excess of *N*-[ethyl-1,2-^{3}H]-maleimide (Perkin-Elmer) at room temperature for 90 min. Labeling efficiency was measured by scintillation counting.

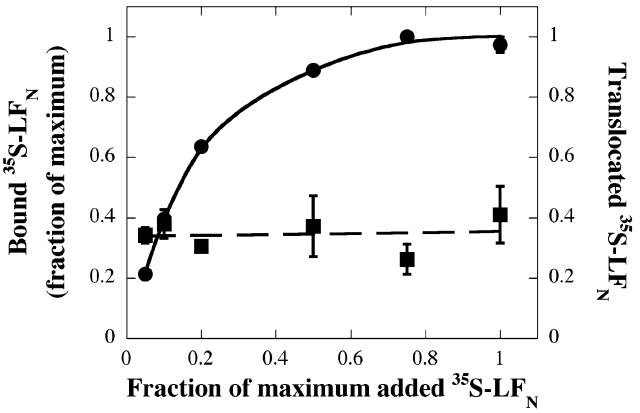

FIGURE 1: Binding and translocation of ^{35}S-labeled LF_N under nonsaturating conditions. CHO-K1 cells at a concentration of 2.5×10^5 cells per well were incubated with 2.4×10^{-8} M of nPA for 2 h on ice. The cells were then washed and incubated with various amount of ^{35}S-LF_N. After an additional incubation of 2 h on ice, the cells were treated with a pH 5.0 buffer at 37 °C for 1 min to trigger translocation. Unprotected LF_N remaining on the cell surface was then digested with Pronase. ^{35}S-LF_N translocated into cells was measured by scintillation counting (■). To measure total bound ^{35}S-LF_N, Pronase was omitted and the cells were lysed directly (●). The fraction translocated was defined as the amount of protected ^{35}S-LF_N divided by the amount bound. The amount of LF_N was normalized to the maximum ^{35}S-LF_N added. Error bars represent the standard error of the mean.

RESULTS

Varying the Degree of Saturation of Wild-Type $[PA_{63}]_7$. We performed an experiment with wild-type PA to determine whether the efficiency of translocation varied with the degree of saturation of the prepore with ligand. For this measurement, we used the cell-surface translocation assay described by Wesche et al. (*11*). CHO-K1 cells were incubated on ice for 2 h with trypsin-activated PA, to allow the protein to bind to the receptors under conditions where endocytosis is minimized. The cells were then washed and incubated with ^{35}S-LF_N for an additional 2 h. This was followed by treatment with a pH 5.0 buffer for 1 min at 37 °C and then with Pronase to digest any ligand remaining exposed at the cell surface. Finally the cells were lysed, and the Pronase-protected ligand was quantified. The total bound ligand was measured in samples in which the Pronase digestion step was omitted. As shown in Figure 1, the fraction of bound ^{35}S-LF_N molecules translocated remained approximately 0.4 as the concentration of the labeled protein was increased to saturating levels.

Varying the Number of Ligand Binding Sites per Heptamer. A mutation of any of the several residues of domain 1′ to alanine strongly inhibits the ligand binding activity of the prepore (*13*). By allowing a binding-defective PA mutant

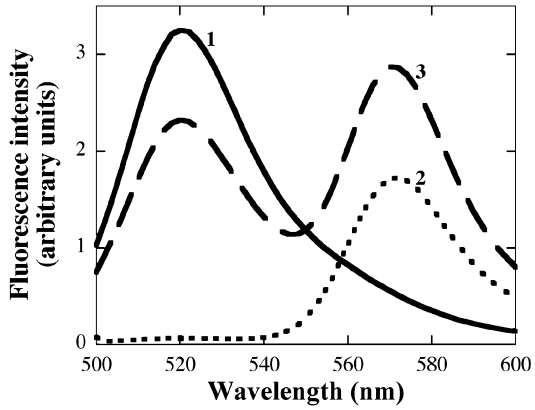

FIGURE 2: Fluorescence resonance energy transfer of heteroheptamer. Fluorophore-labeled donor (PA-K563C labeled with Alexa Fluor 488) and acceptor (PA-K563C/R178A labeled with Alexa Fluor 546) were nicked by trypsin and then mixed at a ratio of 1:10 in the presence of 0.57 μM LF_N (the molar ratio of heptameric PA to LF_N was 1:4). After incubation for 30 min at room temperature, the emission spectra were monitored with an excitation wavelength set at 488 nm. (1) Donor alone; (2) acceptor alone; (3) donor mixed with acceptor at a ratio of 1:10 in the presence of LF_N.

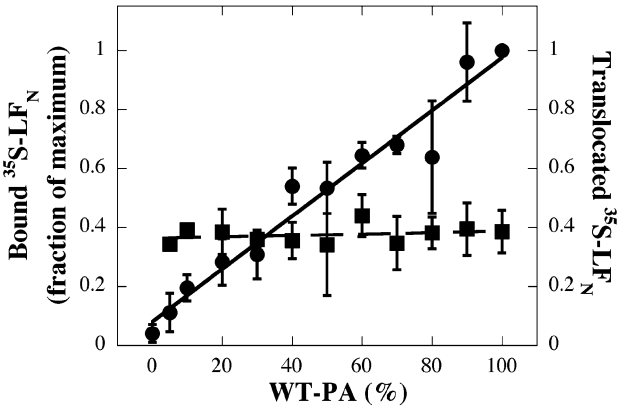

FIGURE 3: Effects of the ratio of wild-type PA to PA-R178A on binding and translocation of ^{35}S-LF_N. Wild-type PA and PA-R178A mutant were mixed at different ratios and nicked with trypsin (the total concentration of nicked PA remained constant at 2.4×10^{-8} M) before being added to the cell. ^{35}S-LF_N was used at a saturating concentration. The binding data are displayed as a fraction of 100% wild-type PA binding (●). The fraction of translocation was defined as the amount of protected ^{35}S-LF_N divided by the amount bound (■) at different percentages of wild-type PA.

to coöligomerize with wild-type PA, it should be possible to vary the average number of ligand binding sites per heptamer. We initially examined PA-R178A as a ligand-binding mutant and showed with two approaches that it coöligomerized with wild-type PA.

In the first approach, we isolated a heteroheptamer of wild-type and mutant proteins and demonstrated the presence of the wild-type component at the expected level. Unlabeled PA-R178A was mixed in a 10:1 ratio with PA-K563C that had a biotinyl group attached via a disulfide linkage to the Cys residue, and the mixture was treated with trypsin to activate both proteins. Derivatization of Cys at position 563 is efficient and without significant effect on ligand binding or translocation (data not shown). The PA_{63} fraction, in the heptameric prepore form, was then isolated by ion-exchange chromatography and applied to a streptavidin column. The biotin-labeled fraction bound to the column, and the fraction of the heptamer lacking biotin passed through and was discarded. After washing, the column was treated with 100 mM DTT to reduce the biotin−*SS*−protein linkage and release the bound protein. After removal of DTT by size-exclusion chromatography, the eluted, reduced heptamer was labeled with radiolabeled *N*-ethyl-maleimide. The incorporated label was measured and found to correspond to 0.8 Cys residues per heptamer, which is consistent within experimental error with a heteroheptamer containing a single PA-K563C subunit. This result supports stochastic coöligomerization.

The second approach involved the measurement of fluorescence resonance energy transfer (FRET) between two forms of PA, one containing the R178A mutation. The R178A/K563C double mutant was labeled with Alexa Fluor 488, as an energy donor, and the K563C single mutant was labeled with Alexa Fluor 546, as an energy acceptor. When we mixed the donor- and acceptor-labeled proteins at a ratio of 1:10, activated them with trypsin, and added a 4-fold excess of LF_N to promote oligomerization, we observed $\sim^1/_3$ decrease in the emission intensity of the donor (Figure 2). $[PA_{63}]_7$ isolated by ion-exchange chromatography after mixing and activating the two labeled proteins showed an even stronger energy transfer (65%) (data not shown). These results are consistent with Fluorophores on neighboring subunits of the heptamer being in the range of the Förster distance of 64 Å of the donor−acceptor pair used. The lower FRET efficiency in the former experiment may have resulted from incomplete oligomerization.

Having concluded that the R178A mutation did not significantly affect the ability of PA to coöligomerize with the wild-type protein, we varied the average number of binding sites per heptamer by mixing PA-R178A with wild-type PA in various ratios, holding the total concentration constant at 2.4×10^{-8} M. We incubated CHO-K1 cells with the mixtures to allow binding. The cells were then washed, incubated with saturating levels of ^{35}S-labeled LF_N, and finally subjected to the cell-surface translocation assay described above. As shown in Figure 3, the amount of ^{35}S-LF_N bound to cells increased in proportion to the fraction of wild-type PA. Consistent with the results of the experiment involving only wild-type PA, the fraction ~0.4 of bound LF_N was translocated, regardless of the ratio of the ligand-binding deficient mutant PA to the wild-type PA. The same results were obtained when we examined two other ligand-binding mutants of PA, containing either the R200A or K214A mutation (*13*).

A low level of binding activity could still be detected in PA containing the R178A by the highly sensitive LF_N-DTA assay. We therefore prepared double mutants in which various single-point mutations that were known to affect ligand binding were combined in a pair-wise fashion. Of the five double mutants tested, three (R178A/I210A, R178A/R214A, and I207A/K214A) showed no residual activity in the LF_N-DTA assay at the highest concentration tested (Table 1). One of them, PA-I207A/K214A, was examined and found to give the same constant translocation efficiency as the three single mutants examined earlier when tested as a function of the ratio of the ligand-binding mutant to wild-type PA.

Table 1: Effect of Various Ligand-Binding Mutations on the Ability of PA to Mediate Toxicity[a]

	wild type	R178A	R178A/P205A	R178A/I207A	R178A/I210A	R178A/R214A	I207A/K214A
$C_{1/2}$ (M)[b]	5×10^{-10}	2×10^{-8}	2.5×10^{-8}	7×10^{-8}	$>10^{-7}$	$>10^{-7}$	$>10^{-7}$

[a] PA-dependent toxicity of LF_N-DTA was assayed by incubating CHO-K1 cells with increasing concentrations of PA for 4 h and measuring the protein synthesis by incorporation of radioactive leucine. [b] $C_{1/2}$ = concentration of PA required for 50% inhibition of protein synthesis as compared to the unintoxicated cells.

DISCUSSION

What factors determine how much cargo (LF and EF) is transported into the mammalian cell cytosol by PA? Many variables impinge upon this question, including: the amount of cargo bound to the cell; the fraction of the complex endocytosed; the fraction of complex that forms a functional pore; the properties of the cargo; and the physical parameters of the toxic complexes. There has been no systematic effort to quantify any of these variables, and we still understand the overall process primarily in qualitative terms.

The amount of cargo that binds to a cell is limited in the first place by the amount of PA at the cell surface, which is in turn dependent on the number and affinity of the cellular receptors, ATR/TEM8 and CMG2. Receptor-bound PA must be proteolytically activated and oligomerize to bind its cargo. The PA_{63} prepore binds EF and LF strongly ($K_D \sim 1$ nM, *10*) but can accommodate only a maximum of three molecules of LF and/or EF. This surprising maximum is well-documented by measurements of the stoichiometry of complexes in solution (*7*) and is consistent with the map of the EF/LF binding site on oligomeric PA_{63} (*13*). The stoichiometry is apparently dictated by steric constraints. The molecular "footprint" of LF_N or EF_N as determined by mutagenic mapping of the site on domain 1′ of PA_{63} precludes simultaneous occupancy of adjacent sites on the prepore. Hence, only alternate sites and thus a maximum of three sites per heptamer can be occupied at one time.

Initial steps have been taken to define factors that govern the process of endocytosis and intracellular trafficking of the toxic complexes. Beauregard and co-workers have shown that internalization of PA is dependent on its proteolytic activation and oligomerization (*14*). Abrami et al. reported that clustering of the anthrax toxin receptor with heptameric PA or an antibody sandwich causes its association with cholesterol- and glycosphingolipid-rich microdomains in the plasma membrane and that endocytosis of the complexes is clathrin-dependent (*15*).

Existing data do not permit accurate estimates of the fraction of toxic complexes that make a functional pore. With $[PA_{63}]_7$ bound to the cell surface in the absence of the ligand, lowering the pH of the medium results in permeabilization of the plasma membrane, as determined by leakage of $^{86}Rb^+$ (*16*). Under similar conditions, a large fraction of PA_{63} converts from a sodium dodecyl sulfate (SDS)-dissociable to a SDS-resistant state, characteristic of the pore, suggesting that conversion to the pore is efficient.

From initial studies involving acidification of liganded PA_{63} at the cell surface, it is clear that properties of the cargo influence its translocation. LF_N entered the protease-protected state most efficiently (at ~40%) (*11*). Full-length EF or LF translocated at 15−20% efficiency, and LF_N fused to various heterologous proteins varied in efficiency from 0 to 20%. There is evidence that ligand unfolding is required for its translocation (*11*), and thus the energetics of unfolding under acidic conditions (and refolding at the neutral pH of the cytosol) may play a role in determining translocation efficiency.

Many physical parameters of the toxic complexes might influence translocation, but the number of ligand molecules bound per prepore is one that is immediately apparent. From recent studies of dominant-negative mutants of PA, conversion of the PA_{63} prepore to the pore is known to be a highly cooperative process (*17*), and this raises the possibility that either positive or negative cooperativity may exist with respect to the number of ligands bound to the perpore. To investigate this possibility, we measured the efficiency of translocation of LF_N across the plasma membrane as a function of the average number of LF_N molecules bound per heptamer. We assumed in choosing this assay system that the efficiency of translocation across the plasma membrane accurately reflects the efficiency across the endosomal membrane. LF_N was chosen because of its high efficiency and thus its good signal-to-noise ratio. We observed a constant ~40% efficiency when we varied either (i) the average number of ligand molecules bound per wild-type $[PA_{63}]_7$ or (ii) the average number of ligand binding sites per $[PA_{63}]_7$ under the conditions of LF_N saturation. The latter approach is dependent on coöligomerization of wild-type PA with ligand-binding mutants, and this was verified with PA-R178A by the two methods.

The results indicate that a ligand molecule bound to $[PA_{63}]_7$ is translocated equally well regardless of the presence or absence of others bound to the same heptamer. No positive or negative cooperativity is evident, within the limits of accuracy of the experiments. Thus, the rate-limiting event in translocation is not influenced positively or negatively by neighboring ligands. This constancy of translocation efficiency suggests that, if unfolded ligands translocate via the lumen of the pore, there must be no "crowding effect". Thus, either the pore (estimated to be only about 15 Å in diameter) can accommodate three molecules simultaneously, or alternatively up to three molecules may be translocated sequentially without loss of efficiency. The hypothesis that translocation is via the aqueous lumen of the pore remains unproven, and it remains conceivable that unfolded ligand molecules cross the membrane via the pore/lipid interface. In such an instance, a crowding effect would seem less likely because of the absence of physical constraints at that interface. Whatever the actual pathway, our results support the notion that the pore imposes little or no constraint on membrane traversal by multiple copies of the ligand bound to $[PA_{63}]_7$.

ACKNOWLEDGMENT

We thank Dr. Borden Lacy for helpful discussions. One of the authors (R.J.C.) holds equity in PharmAthene, Inc.

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BI049794A