Mapping the lethal factor and edema factor binding sites on oligomeric anthrax protective antigen

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Assembly of anthrax toxin complexes at the mammalian cell surface involves competitive binding of the edema factor (EF) and lethal factor (LF) to heptameric oligomers and lower order intermediates of PA₆₃, the activated carboxyl-terminal 63-kDa fragment of protective antigen (PA). We used sequence differences between PA₆₃ and homologous PA-like proteins to delineate a region within domain 1' of PA that may represent the binding site for these ligands. Substitution of alanine for any of seven residues in or near this region (R178, K197, R200, P205, I207, I210, and K214) strongly inhibited ligand binding. Selected mutations from this set were introduced into two oligomerization-deficient PA mutants, and the mutants were used in various combinations to map the single ligand site within dimeric PA₆₃. The site was found to span the interface between two adjacent subunits, explaining the dependence of ligand binding on PA oligomerization. The locations of residues comprising the site suggest that a single ligand molecule sterically occludes two adjacent sites, consistent with the finding that the PA₆₃ heptamer binds a maximum of three ligand molecules. These results elucidate the process by which the components of anthrax toxin, and perhaps other binary bacterial toxins, assemble into toxic complexes.

B*acillus anthracis*, the causative agent of anthrax, produces a tripartite toxin comprising a receptor-binding moiety termed protective antigen (PA) and two effector moieties termed edema factor (EF) and lethal factor (LF; ref. 1). Secreted from the bacteria as nontoxic monomers, these proteins assemble on the surface of receptor-bearing eukaryotic cells to form toxic noncovalent complexes. PA binds to cells, coordinates self-assembly of the complexes, and ultimately delivers EF and LF to the cytosol. EF is a calmodulin-dependent adenylate cyclase, the activity of which protects the bacteria from phagocytic destruction (2, 3). LF is a Zn²⁺-dependent protease that cleaves members of the mitogen-activated protein kinase family and possibly other intracellular substrates (4, 5). The events leading from substrate cleavage to death of toxin-treated animals remain poorly defined.

PA binds to the extracellular von Willebrand factor type A domain of ATR, the cell-surface receptor of the anthrax toxin (6), and is cleaved into two fragments by members of the furin class of cell-associated proteases (7). The smaller 20-kDa fragment (PA₂₀) dissociates into the medium, allowing the larger, 63-kDa carboxyl-terminal receptor-bound fragment (PA₆₃) to self-associate into ring-shaped heptamers (8, 9). The heptamer binds a maximum of three molecules of EF and/or LF competitively and with high affinity ($K_d \approx 1$ nM; refs. 10 and 11). The resulting heterooligomeric complexes are endocytosed and trafficked to an acidic intracellular compartment (12). There the low pH induces conformational changes in the heptameric PA₆₃ moiety that allow it to form a membrane-spanning pore and translocate bound EF and/or LF across the membrane to the cytosol (13, 14).

The process by which the anthrax toxin proteins assemble into toxic complexes has been described only to a first level of detail. Removal of PA₂₀ eliminates a steric constraint to oligomerization and exposes a surface on PA₆₃ that has been proposed to function in EF/LF binding (9). The amino-terminal ≈ 250 -

residue domains of EF and LF termed EF_N and LF_N, respectively, are homologous and mediate binding to PA₆₃ (15, 16). In the accompanying paper we report that PA₆₃ must dimerize or oligomerize to stably bind EF or LF (17).

In the current study we mapped the binding site for EF and LF on PA₆₃. Replacement of any of seven residues of domain 1' (R178, K197, R200, P205, I207, I210, and K214) with alanine almost completely eliminated effector binding. Several of these residues are located in the 1 β_{12} –1 β_{13} hairpin, which interacts with an adjacent PA₆₃ subunit in the PA₆₃ heptamer. This result, together with the finding that ligand binding depends on PA₆₃ oligomerization, suggested that the ligand site might span the subunit–subunit interface. Combining ligand-binding mutations in PA with mutations that block oligomerization, we showed that this indeed is the case. Further, the spatial distribution of residues comprising the ligand site strongly suggests that each bound ligand sterically occludes two adjacent binding sites, consistent with the PA₆₃ heptamer's binding a maximum of three ligand molecules.

Materials and Methods

Cell Culture, Media, and Chemicals. Chinese hamster ovary (CHO) K1 cells were obtained from the American Type Culture Collection and grown as described (18). Cells were seeded at a concentration of 2.5×10^5 cells per ml into 24-well plates (Costar), 16–18 h before the experiment. All supplies for cell culture media were obtained from GIBCO/BRL. All chemicals were obtained from Sigma unless specified.

Construction of PA Mutations. QuikChange mutagenesis was performed according to manufacturer protocol (Stratagene). Site-directed mutations were introduced in the pET22b-PA plasmid (14) by using complementary oligonucleotides (Integrated DNA Technologies, Coralville, IA). The resulting constructs were transformed into *Escherichia coli* BL21 DE3 Star (Invitrogen) and expressed as described (19).

Preparation of Proteins. PA and LF_N were purified from *E. coli* as described (19, 20). Protein concentration was quantified by using the Edelhoch method (21).

Activation of PA and Oligomer Formation. PA was activated by incubation with trypsin at a final trypsin/PA ratio of 1:1,000 (wt/wt) for 30 min at room temperature. The reaction was stopped with the addition of a 10-fold molar excess of soybean trypsin inhibitor. Oligomeric PA₆₃ was prepared from activated PA (2 mg) by purification on a MonoQ HR 5/5 column

Abbreviations: PA, protective antigen; EF, edema factor; LF, lethal factor; PA_n, *n*-kDa fragment of PA; LF_N, amino-terminal domain of LF; CHO, Chinese hamster ovary.

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(Amersham Pharmacia) in 20 mM Tris·HCl (pH 8.0) with a 0–0.5 M NaCl gradient.

Cell-Surface Binding Assay. The cell-surface assay used to measure the PA-mediated binding of ^{35}S -labeled LF_N was a modification of the previously described translocation assay (20). Briefly, CHO-K1 cells were incubated on ice with 2.4×10^{-8} M trypsin-activated PA for 2 h. The cells were washed twice with cold Dulbecco's PBS and then incubated on ice with ^{35}S LF_N for 2 h. The ^{35}S LF_N was produced by *in vitro* transcription/translation using the TNT Coupled Reticulocyte lysate system (Promega) in Ham's F-12 medium buffered with 20 mM Hepes-NaOH (pH 8.2). The cells were washed twice with cold Dulbecco's PBS and then lysed with lysis buffer (0.1 M NaCl/20 mM NaH_2PO_4 /10 mM EDTA/1% Triton X-100). Radioactive content was determined by scintillation counting.

Native Gel Electrophoresis. PA₆₃ oligomer (5 μg) was incubated for 30 min at room temperature in the absence and presence of LF_N (1 μg) in 20 mM Tris·HCl (pH 8.0)/150 mM NaCl in a volume of 20 μl . Five microliters of loading dye (bromophenol blue in 50% glycerol) was added to the samples, which were then loaded onto 4–20% acrylamide, Tris-glycine gels (BioWhittaker). The running buffer was 25 mM Tris base/192 mM glycine. The gels were stained with 0.05% Coomassie blue R-250/50% methanol/10% acetic acid and then destained with 10% acetic acid/10% methanol.

Results

Mutations Within Domain 1' That Block Ligand Binding. The surface of domain 1' of PA₆₃ that is exposed after removal of PA₂₀ represents a potential site of interaction with EF and LF. Although the hydrophobic residues of this surface are likely to contribute to ligand binding (9), we did not make mutations in them because of their likely role in maintaining the structural integrity of native PA. Instead, we decided to test whether some of the polar or more peripheral residues might contribute to binding. We compared the sequence of PA with the receptor-binding components of other binary toxins including *Clostridium perfringens* Iota toxin Ib (22), *Clostridium spiroforme* Sb component (23), *Clostridium difficile* CdtB component (24), component-II of the *Clostridium botulinum* C2 toxin (25), and vegetative insecticidal protein-1 (VIP-1) from *Bacillus cereus*. Whereas these proteins are homologous with PA, their cognate enzymatic moieties do not show sequence similarity with EF and LF. Hence the recognition sites for these enzymatic moieties on their respective PA-like proteins would be expected to differ from the EF/LF site on PA₆₃. By sequence comparison, we identified a stretch of residues in domain 1' (residues 193–217) that lacked similarity with the other PA-like proteins and hypothesized that this stretch is involved in EF and LF binding.

We mutated selected residues within and proximal to this stretch to alanine. Also, E465, located in an adjacent area of domain 2, and N537 and E568 on either side of the exposed shoulder of domain 3, were mutated. The purified mutant proteins were screened for the ability to bind ^{35}S LF_N by means of a cell-surface binding assay (20). LF_N binds PA₆₃ with the same affinity (11) and stoichiometry (10) as EF and LF. Wild-type and mutant forms of PA were activated with trypsin and incubated with CHO-K1 cells. Unbound PA then was removed, radiolabeled LF_N was incubated with the cells, and PA-dependent binding of ^{35}S LF_N was determined by scintillation counting.

Alanine substitutions for any of six residues of the identified stretch (K197, R200, P205, I207, I210, and K214) caused >90% loss of LF_N binding (Fig. 1A). These residues are colored red in the surface display of monomeric PA₆₃ shown in Fig. 1B. Intermediate (25–75%) reductions of binding were seen at seven

Fig. 1. Effects of mutations on ligand binding by cell-bound PA. (A) PA-dependent cell binding of ^{35}S LF_N. Trypsin-nicked wild type (WT)-PA or PA mutant (2.4×10^{-8} M) was incubated with CHO-K1 cells for 2 h on ice. Cells were washed twice with PBS, incubated for 2 h with ^{35}S LF_N, and lysed. ^{35}S LF_N content was determined by scintillation counting. Nonspecific binding of ^{35}S LF_N to cells (less than 10%) was subtracted from the experimental measurements to determine specific binding. Bars are shaded black, medium gray, and gray to represent samples in which the mutation caused >90% loss of ligand binding, 25–75% loss of ligand binding, and little or no effect on ligand binding, respectively. The error bars represent SE of the mean. (B) Space-filling representation of monomeric PA₆₃ showing the locations of mutated residues. Domain 1' is colored blue, and domains 2 and 3 are colored gray. Domain 4 was omitted for clarity. The sevenfold axis of symmetry of heptameric PA₆₃ is indicated by a black dot. Residues where mutation caused >90% loss of ligand binding are colored red, those where an intermediate reduction (25–75%) was seen are colored orange, and those where little or no effect was seen are colored dark blue. This figure was generated with MOLSCRIPT (29) and RASTER 3D (30).

other residues of the stretch (D195, N198, K199, F202, H211, K213, and L216) and two adjacent residues (E190 and K218); these residues are colored orange in Fig. 1B. No significant effect on binding was seen at four positions within the stretch (S204, N209, E212, and T217) and at the three residues mutated outside of domain 1'; these residues are colored dark blue in Fig. 1B.

These findings provided support for the hypothesis that domain 1', and the 193–217 stretch in particular, is involved in ligand recognition.

Three residues at which a large effect was seen, K197, R200, and K214, are basic, and two of them, K197 and R200, are located at the tip of the $1\beta_{12}$ – $1\beta_{13}$ hairpin. In the heptamer, K197 and R200 are close to R178 of an adjacent subunit. We inferred that the nearby R178 might serve together with K197 and R200 as a highly basic subsite spanning the subunit–subunit interface and therefore tested the R178A mutation. It too was found to disrupt the ability of PA to bind LF_N , supporting this hypothesis of a basic intersubunit subsite.

Nature of the Functional Defect Introduced by the Domain 1' Mutations. The mutations in domain 1' that blocked LF_N binding are in solvent-accessible residues, making it likely that they directly affect ligand recognition. Nonetheless, we performed controls to ensure that the failure to bind LF_N was not an indirect consequence of inhibition of oligomerization (data not shown). (i) Similar to wild-type PA, the trypsin-activated mutant proteins formed SDS-resistant, high molecular mass oligomers on the surface of CHO-K1 cells when the cells were treated with low-pH buffer. (ii) The elution characteristics of nicked PA from the mutants on an anion-exchange column (MonoQ) were similar to those of the wild-type protein, in which the PA_{63} moiety elutes as the heptamer. In contrast, the oligomerization-deficient PA mutants identified in a screen for the function of domain 3 yielded little or no heptamer (26). (iii) Electrophoresis of material from the PA_{63} peak on non-denaturing PAGE showed a low-mobility band indicative of oligomerization. These findings provide evidence that the mutations in domain 1' do not impair the oligomerization function of PA_{63} .

PA_{63} heptamers derived from activated wild-type PA or ligand-binding mutants were assessed for the ability to bind LF_N on native gels. Three molecules of LF_N were added per heptamer, and disappearance of the LF_N band was used as an indicator of LF_N binding. None of the PA mutants tested bound LF_N except S204A and N209A, correlating well with the results of the cell-based assay (data not shown).

Defining the Ligand Site on Dimeric PA_{63} . Several pieces of evidence suggested that the EF/ LF binding site might lie at the PA_{63} subunit–subunit interface and comprise residues from two adjacent subunits. To identify residues from two monomers that form one ligand site, we made use of two complementary oligomerization-deficient forms of PA. One form contains a mutation, D512K, on the counterclockwise face of PA_{63} (viewing the domain 1' face of the heptamer). The other contains three mutations, K199E/R468A/R470D, on the clockwise face (17). Neither form alone is able to oligomerize or bind ligand. When the two forms are combined, however, they interact via their respective oligomerization-competent faces to generate a dimer, and the dimer stably binds one molecule of ligand (17). By introducing individual ligand-binding mutations into each of the oligomerization-deficient forms of PA, we were able to identify residues within the dimer that comprise the ligand site.

Single alanine mutations were made at positions R178, K197, R200, I207, I210, and K214 in each of the two oligomerization-deficient PA mutants. The mutant proteins expressed well except for those in which K197A or R200A was combined with K199E/R468A/R470D. These two PA mutants could not be obtained in sufficient amounts for testing, perhaps because mutation of two of the three basic residues in the region 197–200 destabilized the protein. After purification, the proteins were activated by trypsin, and each was combined with an equal amount of the complementary oligomerization-deficient

Fig. 2. PA-dependent cell binding of ligand to PA_{63} dimer mutants. Trypsin-nicked ligand-binding mutants and their complementary oligomerization-deficient PA mutant pair (1.2×10^{-8} M of each) were mixed and incubated with CHO-K1 cells for 2 h on ice. Cells were washed twice with PBS and incubated for 2 h with [35 S] LF_N . Cells were washed and lysed, and the bound [35 S] LF_N content was determined by scintillation counting. Nonspecific binding of [35 S] LF_N to cells (less than 10%) was subtracted from the experimental measurements to determine specific binding. Bars shaded gray represent samples in which the ligand-binding mutation was in PA–D512K, and those shaded black represent samples in which the ligand-binding mutation was in PA–K199E/R468A/R470D. The error bars represent SE of the mean. WT, wild type.

mutant (trypsin-activated). The mixtures then were incubated with CHO-K1 cells, unbound PA was removed, [35 S] LF_N was added, and PA-specific binding of the labeled ligand was quantified by scintillation counting. Mutations in R178, I207, I210, and K214 inhibited binding of LF_N to the PA_{63} dimer by $\approx 90\%$ when present in the K199E/R468A/R470D mutant but $\leq 30\%$ when in the D512K mutant. Mutation of K197 and R200 inhibited LF_N binding ≈ 75 and $\approx 90\%$, respectively, when present in the D512K mutant (Fig. 2). These findings serve as the basis for defining the ligand site of oligomeric PA_{63} as shown in Fig. 3.

Discussion

The most straightforward interpretation of our findings is that they define a minimal recognition site for EF and LF on oligomeric PA_{63} . Several pieces of evidence support this interpretation: (i) the high concentration of mutation-sensitive residues in this area; (ii) the fact that these residues become exposed only after PA_{20} is removed from the molecule (9); (iii) the consistency of our findings with the dependence of ligand binding on PA_{63} oligomerization (17); and (iv) the consistency with the stoichiometry of anthrax toxin complexes (10). Also, mutations in domains 2 and 3 and those in residues of domain 1', the side chains of which projected down toward domain 3 (E212 and T217), did not affect ligand binding, suggesting that the binding surface is confined to residues with side chains projecting from the top surface of domain 1'. Without direct structural evidence it is not possible to exclude the possibility that one or more of the mutations that disrupt binding of LF_N do so by perturbing the conformation of the site (P205 is a residue where this may be likely). However, no indications of gross misfolding were observed.

Discovery that stable binding of ligand depends on oligomerization of PA_{63} suggested that a single ligand molecule might

Fig. 3. Ligand sites on heptameric PA₆₃. (A) A ribbon model of domain 1' of two neighboring PA₆₃ subunits. Residues that participate in ligand binding are highlighted in yellow, red, and green. Residues in red form a complete ligand-binding site spanning the dimer interface. The figure was created with MOLSCRIPT (29) and RASTER 3D (30). The green spheres represent the two Ca²⁺ in domain 1'. (B) Three sequential ligand-binding sites, each spanning a subunit-subunit interface, are shown on the PA₆₃ heptamer in yellow, red, and green. The heavy black lines mark the boundaries between domain 1' of the PA₆₃ subunits. Domain 1' (blue) and domains 2 and 3 (gray) are shown, and domain 4 was omitted for clarity. The figure was created with the INSIGHT(R) II molecular modeling system.

bridge two adjacent subunits (17). Further, the fact that the heptamer simultaneously binds only three molecules of ligand indicated that a single ligand molecule might block the sites on both subunits (10). Results obtained in the current study with the oligomerization-deficient forms of PA support both concepts.

By mutagenesis of wild-type PA we identified six residues in the residue 193–217 stretch plus another outside this stretch (R178) that caused >90% loss of activity. In the following analysis we focus on these residues as likely major points of contact with ligand. P205 was excluded from the analysis for the reason indicated above. For the purpose of discussion we classify the remaining six residues into three subsites (refer to Fig. 1B). Subsite I consists of a single residue, R178, at the counterclockwise tip of domain 1'. Subsite II consists of K197 and R200, located in the 1 β_{12} –1 β_{13} loop, at the clockwise tip of the domain 1'. Subsite III consists of I210 and K214, located on the short 1 α_2 helix, plus I207, in the loop preceding the helix. Subsite III is near subsite II but slightly farther from the sevenfold axis of symmetry.

The fact that a PA₆₃ dimer, formed from two oligomerization-deficient mutants, binds only a single molecule of ligand allowed us to identify residues that comprise the ligand site. Our results provide strong evidence that the single site on the mixed PA₆₃ dimer consists minimally of subsites I and III from one PA₆₃ monomer plus subsite II from the adjacent monomer in the counterclockwise direction. Three sequential sites, each spanning a subunit-subunit interface, are colored yellow, red, and green in the heptamer in Fig. 3. As suggested in *Results*, subsite I from the clockwise subunit and subsite II from the counterclockwise subunit may function together as a single, highly basic patch. Because we were unable to test K197 or R200 in combination with K199E/R468A/R470D, we cannot exclude the possibility that these residues from the clockwise subunit also may contribute to the ligand site on the dimer.

The proposed ligand site contains basic residues at two locations: K214 at one location, and at the other, R178 of the same subunit plus K197 and R200 of its counterclockwise neighbor. These positively charged patches could function to direct and orient the binding of ligand to the oligomer. Between these basic patches are several hydrophobic residues including two that were shown to be important for binding: I207 and I210. These centrally located hydrophobic residues could participate in binding by providing favorable stability at the ligand-oligomer interface (27). The PA binding site on EF/LF contains hydrophobic residues and has a net negative charge mainly contributed by residues D182 and D187 (16). Modeling the ligand-oligomer complex will provide a framework for testing specific residue interactions in the complex.

The locations and spacings of the subsites I, II, and III provide a potential explanation of the fact that the PA₆₃ heptamer simultaneously binds a maximum of only three molecules of ligand. Residues K197 from subsite II and K214 from subsite III are located on the same PA₆₃ subunit, but belong to different ligand sites. The distance separating the C α atoms of these residues is 11Å. Considering the dimensions of LF_N (28), occupancy of subsite II could obscure occupancy of the adjacent ligand-binding site. Assuming this to be the case, only alternate sites, and thus a maximum of three of the seven sites of the heptamer, can be occupied simultaneously.

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