Entry of anthrax edema factor (EF) and lethal factor (LF) into the cytosol of eukaryotic cells depends on their ability to translocate across the endosomal membrane in the presence of anthrax protective antigen (PA). Here we report attributes of the N-terminal domains of EF and LF (EF_N and LF_N, respectively) that are critical for their initial interaction with PA. We found that deletion of the first 36 residues of LF_N had no effect on its binding to PA or its ability to be translocated. To map the binding site for PA, we used the three-dimensional structure of LF and sequence similarity between EF and LF to select positions for mutagenesis. We identified seven sites in LF_N (Asp-182, Asp-187, Leu-188, Tyr-223, His-229, Leu-235, and Tyr-236) where mutation to Ala produced significant binding defects, with H229A and Y236A almost completely eliminating binding. Homologous mutants of EF_N displayed nearly identical defects. Cytotoxicity assays confirmed that the LF_N mutations impact intoxication. The seven mutation-sensitive amino acids contain a common domain for binding PA. Indeed, it was shown that the N-terminal domain of LF (LF_N) was sufficient for binding PA and could act as a carrier for delivery of heterologous proteins across membranes in the presence of PA. The three-dimensional crystal structure of LF shows that LF_N forms a distinct structural domain and explains how the binding function can be separated from the rest of the molecule.

In the current study we have identified attributes of LF and EF that are crucial for their binding to PA. First, we showed by deletion mutagenesis that the first 36 residues of LF_N are dispensable for binding to PA and translocation. Next we identified seven positions in LF_N (Asp-182, Asp-187, Leu-188, Tyr-223, His-229, Leu-235, and Tyr-236) and the corresponding positions in EF_N where mutation to Ala significantly impairs binding to PA and toxin action. These residues are located in three different α-helices that interact to form a surface-exposed patch on one face of the LF structure. These patches on EF and LF are proposed to represent the PA63 recognition sites of these proteins.

**EXPERIMENTAL PROCEDURES**

Reagents and Chemicals—Oligonucleotides were synthesized by Integrated DNA Technologies. Supplies for cell culture media were from Invitrogen. Sigma supplied all chemicals unless noted otherwise.

Preparation of LF_N Constructs—The plasmid pET15b-LF contains the entire LF gene, except for the portion that encodes the signal sequence. The oligonucleotide 5′-GAGGAACATATGCCGGCGGT CATGGTGATG-3′ was used to introduce an NdeI site and serve as a forward primer in the amplification of LF_N(1-250). The forward primers for LF_N(250-286), LF_N(286-323), LF_N(323-363), LF_N(363-406), and LF_N(406-436) were constructed to permit the appropriate truncation and also introduce an NdeI site. In all constructs, 5′-CTAGGATCTTACCGTTGATCTTTAAGTTCTTCC-3′ was used to introduce a BamHI site and act as the reverse primer. Sequences were amplified from a pET15b-LF template by PCR. The PCR products and pET15b (Novagen) were gel-purified and digested with NdeI and BamHI. The digested fragments and nicked vector were gel-purified again, ligated, and transformed into Escherichia coli DH5α. Transformants were screened by digestion and verified by sequencing.
Preparation of LF N and Mutants—Mutations were made using the QuikChange method of site-directed mutagenesis using a protocol supplied by the manufacturer (Stratagene). All mutants were made in pET15b-LFN(28–263). DNA for each mutant was fully sequenced and then used to create 35S-LFN protein by in vitro transcription/translation using the TNT-coupled Reticulocyte Lysate System (Promega).

Preparation of EF N and Mutants—The plasmid pET15b-EF N(1–254) was prepared similarly to the LFN constructs above. Oligonucleotides 5'-GGAGAACATGGAAGAACATTACGAG-3' and 5'-CTAGGATCCAAACCTTTCTCTCAACTTTC-3' were used as forward and reverse primers, respectively, to PCR-amplify EF N(1–254) from a pET15b-EF template containing the entire EF gene. The oligonucleotides also introduced NdeI and BamHI sites to facilitate the ligation back into a pET15 vector. Mutants of this construct and their corresponding 35S-labeled proteins were as described above for LFN.

Preparation of LF N-DTA Mutants—Mutations in LF N-DTA were made using the same primers and protocol used for constructing the LFN mutants. Each mutant was transformed into E. coli BL21(DE3) (Novagen) and purified using the protocol described previously for wild-type LF N-DTA (11).

Preparation of PA and LF N Proteins—PA, LF N, and LF N(Y236A) were purified from E. coli as described previously (11, 17). Protein concentrations were determined using the Bradford protein assay reagent (Bio-Rad). A 0.2 mg/ml solution of PA was activated using a trypsin/PA ratio of 1:2000 (w/w). The mixture was incubated at room temperature for 30 min and quenched with a 10 mM excess of soybean trypsin inhibitor. Wild-type LF N(28–263) and a Y236A mutant were incubated with trypsin at trypsin:LFN ratios of 1:10, 1:100, 1:1000, and 1:10,000 (w/w) for 60 min. Trypsinization was quenched with soybean trypsin inhibitor, and digestion profiles of wild-type and mutant LF N were compared by SDS-PAGE to assess protein stability.

Cell Culture—CHO-K1 cells (ATCC CCL-61) were grown in Ham's F-12 medium supplemented with 10% calf serum, 500 units/ml penicillin G, and 500 units/ml streptomycin sulfate. Cells were maintained as monolayers and grown in a humidified atmosphere of 5% CO2.

Cell Surface Binding and Translocation Assay—The protocol for measuring PA-mediated binding and translocation of 35S-labeled LF N was previously described (11). Briefly, CHO-K1 cells (2 × 106 cells/well) were washed once with ice with trypsin-nicked PA for 1 h. The cells were washed with PBS and then incubated on ice with 35S-labeled EF N or LF N for 1 h. Cells were washed once with PBS and then treated with a low pH buffer at 37°C for 1 min. The cells were exposed to Pronase or a no-Pronase control for 8 min at 37°C and then protease inhibitors were added. Cells were treated with lysis buffer, and the radioactive content was determined by scintillation counting. To test the level of binding at pH 7, the cells were washed three times with PBS after the incubation with EF N. Reduced binding was observed in D182A, D187A, L188A, Y223A, and L235A (Fig. 3 and Table I). These residues were conserved with respect to the structure revealed only one significant cluster of conserved surface-exposed amino acids. This cluster (colored red in Fig. 2D) was formed by the intersection of two α-helices (1α6 and 1α10) and a tyrosine residue (Tyr-223) projecting from a third buried helix (1α9). We hypothesized that this cluster represented the PA-binding site for LF N.

Defining the PA-binding Site Using 35S-LFN Mutants and a Cell Surface Binding Assay—The conserved residues of the cluster shown in Fig. 2A were individually mutated to alanine in the LF N(28–263) construct, and the mutant proteins were 35S-labeled using in vitro incorporation of [35S]Met at the five methionine sites of the construct. Each 35S-LFN mutant was visualized on a 4–20% SDS gradient gel using a PhosphorImager and/or film (data not shown). The concentration for each mutant was measured by scintillation counting and normalized such that each 35S-LFN sample was applied at 10–15 μM. Samples were tested for their ability to bind cells in the presence and absence of PA. The difference in binding was then compared with that for LF N(28–263) and displayed as a fraction of wild-type binding.

Mutations at Glu-142, Ser-183, Asp-184, Glu-196, and Val-232 showed 79–100% of the binding observed with wild-type LF N(28–263) (Fig. 3), and those residues are shown in green in Fig. 2B. Reduced binding was observed in D182A, D187A, L188A, Y223A, and L235A (Fig. 3 and Table I). These residues are...
are shown in purple in Fig. 2B to signify a significant although not complete binding defect. The H229A and Y236A mutants showed almost undetectable levels of binding (Fig. 3 and Table I) and are shown in red in Fig. 2B. Additional mutations were made in some of the non-conserved residues surrounding the cluster (Y108A, K110A, E139A, T141A, N179A, S181A, Q186A, Q228A, D231A, Q234A, E239A, D245A, and E249A). All showed high (>65% of wild-type) levels of binding (Fig. 3) and are shown in green in Fig. 2B. One mutation (Y125A) was made on the opposite side of the molecule and bound at 74% of that seen for wild-type (Fig. 3). Finally, the His-229 position was mutated to both glutamine and asparagine to further characterize the role the nitrogen atoms of this residue might play in binding. As with H229A, binding for both mutants, H229N (0 ± 1%) and H229Q (9 ± 5%), was severely impaired.

The Mutations in LF_N That Affect Binding Also Show Defects in Cytotoxicity—Mutations that showed a significant defect in ability to bind PA were further characterized by a cytotoxicity assay using LF_N-DTA. LF_N-DTA is a protein fusion between LF_N and the catalytic domain of diphtheria toxin, which inhibits protein synthesis by catalyzing the ADP-ribosylation of elongation factor-2. CHO-K1 cells were incubated with PA and LF_N-DTA and then checked for ability to incorporate tritiated leucine into proteins. With a chosen set of conditions, including a 10^{-11} M concentration of PA, the concentration of wild-type LF_N-DTA required to inhibit leucine incorporation by 50% (EC_{50}) was 8 ± 2 pm (Fig. 4). The EC_{50} value for each of the seven LF_N-DTA mutants (D182A, D187A, L188A, V223A, H229A, L235A, and Y236A) was higher than that of wild-type LF_N-DTA, and all except that of D187A differed by at least a factor of 10 (Fig. 4 and Table I).

Mutations in LF_N and LF_N-DTA Do Not Affect Structural Stability—To ensure structural stability, we expressed and purified the LF_N mutant with the most dramatic PA-binding defect (Y236A) to homogeneity and compared its trypsin digestion profile to that of wild-type LF_N. The mutant expressed at wild-type levels (100 mg/liter) and eluted as a discrete symmetric peak from an anion exchange column. Incubation of the mutant and wild-type proteins with trypsin for 1 h at room temperature resulted in only slight digestion with a 1:100 w/w trypsin:LF_N ratio, and a 1:10 (w/w) ratio was required for full digestion. At both ratios the digestion product profiles on SDS-PAGE were identical between mutant and wild-type (data not shown). Each of the seven mutant proteins with a PA-binding defect was expressed and purified in the LF_N-DTA construct. The mutations had no effect on expression yield, and in all cases the proteins eluted as a single symmetric peak off an anion exchange column.

Homologous Mutations in EF_N Show Similar Defects in Binding PA on Cells—Mutations were made in EF_N at positions corresponding to the seven LF_N positions where mutation caused a defect in binding PA. A cell surface binding assay was performed, and binding was calculated as a percentage of that observed for 35S-EF_N. Each mutant (D169A, D174A, L175A, Y214A, H220A, L226A, and Y227A) displayed reduced binding relative to wild-type EF_N (Fig. 5 and Table I).

DISCUSSION

We have used the recently solved crystal structure of LF to guide new experiments probing the relationship between structure and function in ATx intoxication (16). The structure reveals that the bulk of LF_N residues 40–263, forms a compact structural domain, whereas the α-helix containing residues 28–39 (1α1) projects out, away from the rest of the molecule (Fig. 2A). The first 27 residues of the structure were not visible, indicating either lack of structure or significant motion. Constructs that take advantage of these structural properties (LF_N-(1–263) and LF_N-(28–263)) showed enhanced E. coli expression as compared with LF_N-(1–255), with no effect on cell surface binding and translocation. In fact, LF_N with a 27-, 32-, or 36-residue N-terminal truncation showed wild-type levels of binding and translocation, whereas a 39- or 42-residue truncation showed a ~50% reduction in binding but wild-type translocation efficiency.

The data suggest that the alterations in function observed by us and others with N-terminal deletions are due to a defect in
binding rather than in a later step in the process. It should be noted that the method of in vitro transcription/translation does not allow for removal of the N-terminal hexahistidine tag and thrombin cleavage site which adds an additional 20 residues to the N terminus of each protein. Our data indicate that the first 36 residues are not specifically required for binding and translocation, but we do not address whether a flexible "tail" of any sequence is required for function. If a flexible N terminus is required, it is unable to fully compensate after 39 residues have been deleted. Starting at residue 37, the side chains of the N terminus begin to interact with the main body of the LF N structure. Therefore, these N-terminal residues are likely to affect binding indirectly by providing structural support to the rest of the molecule. Other researchers have reported that the LF mutants Y148A, Y149A, I151A, and K153A are unable to lyse macrophages (21), but structural information now indicates that these residues are part of 1α4, the central helix of the LF N fold with no to relatively low surface exposure. Mutation at these sites is likely to affect binding indirectly by altering structural stability.

To identify the PA-binding sites of EF and LF, we assumed that the binding site would consist only of surface-exposed residues and that the site would be similar, if not identical, between EF and LF. We identified only one significant conserved cluster of surface-exposed residues on the LF structure and hypothesized that this cluster would represent the PA-binding site for LF. The results using alanine scanning and the cell surface binding assay support our hypothesis, because mutation of most of the conserved residues within the cluster caused a decreased ability to bind PA, whereas none of the mutations in non-conserved residues affected binding (Fig. 2). Mutation of both conserved and non-conserved residues surrounding the cluster did not affect binding but served to delimit the boundary of the binding site (green in Fig. 2B). Finally, the results indicate the relative importance of residues within the cluster to binding. In this assay, D187A and L235A showed a ~50% reduction in binding of PA, whereas the D182A, L188A, Y223A, H229A, and Y236A mutants bound at only ~25–35% of that seen with wild-type (Table I). The H229A and Y236A mutations had the most dramatic effects on binding and indicate that these residues are critical in the binding interaction between LF and PA.

To test the role of these seven residues by another method, we made the same single alanine mutations in an LF N-DTA construct. Being able to perform a protein synthesis inhibition assay with LF N-DTA had several advantages. Using purified protein eliminated the possibility of contaminants affecting the results and also allowed us to accurately measure concentrations. Most importantly, the assay serves as a measure of more than binding, as cytotoxicity depends on multiple factors. In addition to LF N-DTA needing to bind PA, the process requires proper endocytosis and trafficking, pH-induced structural changes in PA resulting in pore formation, and the proper unfolding and refolding of LF N-DTA to allow for its efficient translocation and catalysis.

The results from the protein synthesis inhibition assay reinforce the idea that the seven residues identified in the binding assay (Asp-182, Asp-187, Leu-188, Tyr-223, His-229, Leu-235, and Y236) form a PA-binding site. The EC_{50} value for each of the seven LF N-DTA mutants was higher than that for wild-type LF N-DTA, and the relative cytotoxicity profile generally
correlated well with the profile of observed binding differences (Table I). For example, the Y236A mutation, as in the binding assay, showed the most dramatic defect, requiring a concentration 100 times that of wild type to achieve 50% inhibition. Slight profile differences included the H229A mutation, which had a drastic defect in the cell binding assay but a more modest defect when incorporated into LF₅-DTA. In an effort to characterize further the role of His-229 in binding PA, we constructed two additional mutants, H229N and H229Q. These mutations are more similar to histidine than alanine in terms of steric bulk and can be used to probe the role, if any, of the two nitrogen atoms of histidine (22). In this case, however, both ³⁵S-LF₅ H229N and ³⁵S-LF₅ H229Q nearly ablated binding, supporting the idea that the dramatic reduction in cell surface binding observed for H229A is significant. Furthermore, although it does not prove that the histidine nitrogens are not involved, this result suggests that it could be unique properties of the imidazole ring and/or the titratable charge that forms the basis of this binding interaction.

Using the argument of sequence conservation to identify a binding site implies that the homologous seven mutations in EF will also show defects in binding. This was confirmed using the cell surface binding assay for each of the seven corresponding EF₅ mutants (Table I). The fact that the profiles mirror those observed with the LF₅-(28–263) mutations is notable and may indicate that the profile differences between the binding and cytotoxicity assays indicate roles for some of these residues later in the intoxication process.

The seven residues identified in this report form a patch on one face of the LF structure. Although distant in primary sequence, the patch is close enough to the N terminus of the molecule to suggest that the structural integrity of the N terminus (starting after residue 36) would affect the properties of the binding site. As visualized in the uncomplexed LF structure, the patch has a net negative charge (largely due to Asp-182 and Asp-187) and contains both a pocket (composed of residues His-229, Tyr-223, and Leu-188) and a protruding loop (containing Leu-235 and Tyr-236). Although the charge and contour properties of the patch do not suggest an obvious binding site on the surface of (PA₉)₅, we hope to use these mutations to guide the search for the LF/EF-binding site on (PA₉)₅. A model of these molecules in complex will provide the framework for addressing the next steps in the intoxication pathway of anthrax toxin.

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Mapping the Anthrax Protective Antigen Binding Site on the Lethal and Edema Factors

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