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Involvement of Domain 3 in Oligomerization by the Protective Antigen Moiety of Anthrax Toxin

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Protective antigen (PA), a component of anthrax toxin, binds receptors on mammalian cells and is activated by a cell surface protease. The resulting active fragment, PA_{63}, forms ring-shaped heptamers, binds the enzymic moieties of the toxin, and translocates them to the cytosol. Of the four crystallographic domains of PA, domain 1 has been implicated in binding the enzymic moieties; domain 2 is involved in membrane insertion and oligomerization; and domain 4 binds receptor. To determine the function of domain 3, we developed a screen that allowed us to isolate random mutations that cause defects in the activity of PA. We identified several mutations in domain 3 that affect monomer-monomer interactions in the PA_{63} heptamer, indicating that this may be the primary function of this domain.

Anthrax toxin, a major virulence factor of *Bacillus anthracis*, consists of three proteins: two enzymic proteins (edema factor [EF] and lethal factor [LF]) plus a third (protective antigen [PA]), which serves as the vehicle for delivery of the enzymic moieties to the cytosol of mammalian cells (10). EF is a calmodulin-dependent adenylate cyclase (9), whereas LF is a zinc-dependent protease that cleaves mitogen-activated protein kinase kinases 1, 2, and 3 (4, 17, 20). The combination of PA and EF causes edema as a gross manifestation of its activity, but its primary function is probably to protect the invading bacteria from destruction by professional phagocytes. The combination of PA and LF selectively affects macrophages, causing, at high concentrations, their death and the death of animals (5).

PA binds to an unidentified receptor on the surface of mammalian cells, where it is activated by furin or a furin-like protease (7, 16). Cleavage of PA leads to the release of an amino-terminal fragment (PA_{20}) and to heptamerization of the remainder of the protein (PA_{63}) through monomer-monomer interactions on the cell surface (15). PA_{20} has no further role in the intoxication process. PA_{63} binds EF and LF, and these complexes are internalized by endocytosis (5, 6). The low pH environment of the endosome causes the PA_{63} heptamer to insert into the membrane, apparently by forming a 14-stranded transmembrane β-barrel (1, 12, 18). Conversion of the heptameric prepore to a pore is accompanied by translocation of the enzymic moieties from an endosomal to a cytosolic environment (8).

PA is a 735-amino-acid protein organized into four domains, as defined by X-ray crystallography (18). Furin cleavage within domain 1 (amino acids 1 to 258) leads to the release of PA_{20}, which in turn exposes a surface on PA_{63} that may be involved in binding EF and LF. Domain 2 (amino acids 259 to 487) contains an unstructured loop (D2L2), which inserts into membranes to form a cation-selective channel (2, 14), and the domain also contributes to a binding interface between PA_{63} monomers. Domain 3 (amino acids 488 to 595) has no reported function, but its location suggests that it could participate in any of various functions, including oligomerization, binding the receptor, and/or binding the enzymic moieties. Domain 4 (amino acids 596 to 735) has been shown to bind the anthrax toxin receptor (11, 19).

In order to explore the role of domain 3 in the intoxication process, we have combined random mutagenesis of this domain with a novel screen for mutations that affect the overall delivery function of PA. We found mutations in distinct regions, including within a loop of domain 3 (amino acids 510 to 518), that impair heptamerization of PA_{63}. These oligomerization-deficient mutants are also defective in their ability to bind LF_N (the amino-terminal PA-binding domain of LF), suggesting that oligomerization of PA_{63} is required for LF or EF to bind PA_{63}.

MATERIALS AND METHODS

Plasmid construction. The plasmid pET22b-PA contains the entire PA gene, except for the portion that encodes the signal sequence (1). QuikChange mutagenesis was performed according to the instructions of the manufacturer (Stratagene) to introduce site-directed mutations. Oligonucleotides were obtained from Integrated DNA Technologies. The oligonucleotide, GAGTGAAG TGGTACCGCAAATTCA, and its complement were used to introduce a *Kpn*I site into the vector pET22b-PA to make pET22b-PA_K. The oligonucleotide, CATAGCAGTTGGGATCGATGAGTCAGTAG, and its complement were used to introduce a *Cla*I site into the vector pET22b-PA_K to make pET22b-PA_{KC}.

Mutations in pET22b-PA were introduced by GTGAAGTGTTACCGGCAA TTCAAGAAACAAC, CCGCAAATTCAAGCAACAACTGCACG, GGTTA ATCCTAGTGCTCCATTAGAAACG, CCTAGTGATCCAGCAGAAACGA CTAAACC, GAAACGACTAAACCGGCTATGACATTAAAAG, CTAAACC GGATATGATATTAAAAGAAGCCC, GCAAAAATGAATATTTTAATAG CAGATAAACGTTTTC, and their complements to make pET22b-PA483QA, pET22b-PA486EA, pET22b-PA512DA, pET22b-PA514LA, pET22b-PA520DA, pET22b-PA522TI, and pET22b-PA592RA, respectively.

Mutagenesis. A 100-μl PCR mixture containing 1× *Taq* DNA polymerase buffer, 1 ng of pET22b-PA_{KC} DNA/μl, 2.5 ng of GAGTGAAGTGGTACCGC AAATTCA DNA/μl, 2.5 ng of CTACTGACTCATCGATCCCAACTGCTATG DNA/μl, 100 μM deoxynucleoside triphosphate, 1μM 6-(2-deoxy-β-D-ribofuranosyl)-3,4-dihydro-8H-pyrimido-[4,5-c][1,2]oxazin-7-one triphosphate (dPTP) (Amersham Life Science), and 0.05 U of *Taq* DNA polymerase (Stratagene)/μl was used to amplify a DNA fragment encoding domain 3 of PA. The

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mixture was initially incubated at 95°C for 45 s. A cycle consisting of a 45-s incubation at 95°C, a 45-s incubation at 55°C, and a 40-s incubation at 72°C was then repeated 30 times. Five microliters of this first reaction mixture was used in a second reaction mixture that lacked pET22b-PA_{KC} DNA and dPTP (incubation temperatures and times were the same as described above). The product from the second reaction was purified using a QIAquick PCR purification kit (Qiagen), cut with *Cla*I and *Kpn*I, and again purified using a QIAquick PCR purification kit. This fragment was ligated into pET22b-PA_{KC}, which had been grown in the *Escherichia coli* strain SCS-110 (Stratagene), cut with *Cla*I and *Kpn*I, and purified from an agarose gel using a QIAquick gel extraction kit (Qiagen).

Preparation of PA and LF_N-DTA lysates. pET22b-PA_{KC} vectors containing mutations introduced into PA (see above) were transformed into BL21 (DE3) cells (Stratagene) and spread on 1.5% agarose plates containing Luria broth (LB) and 50 μg of ampicillin/ml. The resulting colonies were used to inoculate 100 μl of LB containing 50 μg of ampicillin/ml in 96-well plates. The cultures were grown overnight, and then 100 μl of 50% glycerol was added to each well. Fifty microliters was then transferred into 100 μl of LB containing 50 μg of ampicillin/ml in a second 96-well plate. Cells were grown at 30°C with shaking. When the cells reached log phase, 20 μl of bacteriophage T7 lysate (supernatant of a T7 infection of log-phase XL1-blue *E. coli* cells, which was then diluted 10-fold with LB) was added. When the cells had lysed (approximately 3 h after infection), plates were centrifuged at 1,500 × *g* for 10 min to pellet the cellular debris.

LF_N-DTA lysates were prepared by infecting a 30°C log-phase culture of BL21(DE3) cells containing the vector pET15b-LF_N-DTA (13). When the cells had lysed, the culture was centrifuged to separate the cellular debris from the supernatant, which contained LF_N-DTA.

Screen for PA mutants. CHO-K1 cells (ATCC CCL-61) were grown in Ham's F-12 medium supplemented with 10% calf serum, 500 U of penicillin G/ml, and 500 U of streptomycin sulfate/ml. Twenty microliters of PA lysate and 10 or 2.5 μl of LF_N-DTA lysate were added to confluent CHO-K1 cells in 100 μl of medium per well of a 96-well plate and incubated overnight. CHO-K1 cells were scored as dead if they were rounded.

Preparation of proteins. PA, PA_{63}, LF_N, and LF_N-DTA were purified from *E. coli* as described previously (12, 21). Protein concentrations were determined using Bradford protein assay reagent (Bio-Rad).

Translocation assay. Translocation assays were performed essentially as described previously (21). Briefly, CHO-K1 cells were incubated on ice with trypsin-nicked PA for 1 h. The cells were washed twice with phosphate-buffered saline (PBS) and then incubated on ice with ^{35}S-labeled LF_N for 1 h. The cells were washed twice with PBS and exposed to MES-gluconate buffer (140 mM NaCl, 5 mM sodium gluconate, 20 mM 4-morpholineethanesulfonic acid) at pH 4.8 for 2 min. The cells were either lysed with lysis buffer (0.1 M NaCl, 20 mM NaH_2PO_4, 10 mM EDTA, 1% Triton X-100) or treated with 2 mg of pronase/ml at 37°C for 10 min. Cells that had been treated with pronase were centrifuged, resuspended in medium containing Mini Complete protease inhibitor (Boehringer Mannheim), centrifuged, and resuspended in lysis buffer. Radioactive content was determined by scintillation counting.

Rubidium release assay. Confluent CHO-K1 cells in 24-well plates were exposed overnight to medium containing ^{86}Rb at 1 μCi/ml. Cells were incubated at 4°C for 30 min, washed with cold PBS, and exposed to medium containing 2×10^{-8} M trypsin-nicked PA for 1 h. The cells were washed twice with PBS and exposed to 500 μl of MES-gluconate buffer at pH 4.8. After 30 min, 100 μl of buffer was removed. Radioactive content of the supernatant was determined by gamma radiation counting.

Protein synthesis inhibition assay. The protein synthesis inhibition assay was performed essentially as described previously (13). Various amounts of PA or mutant PA were incubated with CHO-K1 cells for 4 h at 37°C in the presence of LF_N-DTA. The cells were washed with PBS and then incubated with leucine-deficient medium supplemented with 1 μCi of [^{3}H]leucine/ml. The cells were incubated for 1 h at 37°C and then washed twice with cold PBS. The cells were incubated for 10 min at room temperature, first with 10% trichloroacetic acid and then with 0.2 M KOH. An equal volume of 0.1 M HCl was added, and the amount of tritiated protein was determined by scintillation counting.

Native gel electrophoresis. Trypsin-nicked PA, PA_{63}, and LF_N were mixed, as indicated, in 20 mM Tris-HCl (pH 8.0)–150 mM NaCl (in a volume of 8 μl) and incubated for approximately 10 min at room temperature. Two microliters of loading dye (bromophenol blue in 50% glycerol) was added to the mixtures, which were then loaded onto 4 to 20% acrylamide, Tris-glycine gels (BioWhittaker Molecular Applications). 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

Preparation of mutant PA lysates. We used the nucleotide analog dPTP in PCRs to introduce mutations in the DNA encoding domain 3 of PA. This procedure causes A-to-G, T-to-C, G-to-A, and C-to-T transitions (22). A concentration of dPTP was used that resulted in an error rate of approximately one mutation per PCR product (data not shown). At this level of mutagenesis by dPTP, we estimated that only 2% of the mutations obtained would be nonsense mutations. To facilitate cloning of mutagenized domain 3 DNA, we introduced into the PA gene a *Kpn*I site (which mutated amino acid 481 from Leu to Val) and a *Cla*I site (which mutated amino acid 607 from Ala to Ile). A population of constructs containing mutagenized domain 3 DNA was transformed into BL21(DE3) cells. Colonies from this transformation were used to inoculate LB-ampicillin in 96-well plates, and after overnight growth, the cells were used to inoculate a second 96-well plate. When the cells reached log phase, they were infected with bacteriophage T7, thereby lysing them and releasing PA from the periplasm into the medium. Lysates were centrifuged to separate the cellular debris from the supernatant.

Screen for defective PA mutants. CHO-K1 cells in 96-well plates were incubated overnight with 20 μl of (mutant) PA lysate and 10 μl of LF_N-DTA lysate (LF_N-DTA consists of the amino-terminal, PA-binding domain of LF fused to the catalytic domain of diphtheria toxin, which inhibits protein synthesis by catalyzing the ADP ribosylation of elongation factor-2). The cells were observed under a microscope and scored as dead if they had rounded. Killing of the CHO-K1 cells depended on the presence of both PA lysate and LF_N-DTA lysate (data not shown).

Of the 93 lysates screened, 45 killed CHO-K1 cells and 48 did not. Western blotting revealed that 10 of the 48 lysates contained full-length PA, and we sequenced seven of the 10 corresponding clones (Table 1 and Fig. 1). Three did not have PCR-induced mutations (these clones expressed reduced levels of PA for reasons that are unclear), three contained single missense mutations, and one had two missense mutations.

In a second screen, we modified our protocol to distinguish between lysates that contained full-length PA and those that contained only truncated PA. To do this, PA lysate was used in combination with two amounts of LF_N-DTA lysate, 10 μl (which was not limiting in the cell killing assay) and 2.5 μl (the lowest amount of LF_N-DTA that killed cells with wild-type PA). Lysate containing wild-type PA, or mutant PA with wild-type activity, would kill CHO-K1 cells with either 10 or 2.5 μl of LF_N-DTA lysate. We reasoned that lysates containing only truncated PA would not kill with either amount of LF_N-DTA lysate, whereas lysates containing full-length, partially defective PA might kill CHO-K1 cells with 10 μl but not with 2.5 μl of LF_N-DTA lysate. In the second screen, lysates from 192 clones were characterized. Of these lysates, 126 killed CHO-K1 cells with both amounts of LF_N-DTA lysate, 47 did not kill with either amount, and 19 killed with 10 μl but not with 2.5 μl of LF_N-DTA lysate. Western blotting revealed that all of the 19 PA lysates that killed with 10 μl but not with 2.5 μl of LF_N-DTA lysate contained full-length PA. These clones were sequenced (Table 1 and Fig. 1). One did not have PCR-induced mutations, 14 had single missense mutations, three con-

TABLE 1. PA mutants isolated as being defective at killing CHO-K1 cells with LF_N-DTA

Group[a]	Mutation	Activity with the following amt (μl) of LF_N-DTA[b]:	
		10	2.5
1	514LF	−	ND
	514LS	+	+/−
	515ER	−	ND
	515EA, 553ND	+	−
	516TA, 521MT, 547TM	+	+/−
2	483QH	+	−
	485QH	+	+/−
	486EG	+	−
3	520DG	+	−
	522TI	+	−
	525EG	−	ND
	529IT, 546DG	−	ND
4	592RK	+	+/−
	597HR, 496KE	+	−
	602ND	+	−
	602NS, 545KE	+/−	−
5	491IT	−	ND
	492IT	+	+/−
	494NS	+	−
	506AV	+	−
	542YC	+	−
	554FL	+	−
	554FS	+/−	−

[a] Mutants in group 1 contain mutations in the amino acid loop 510 to 518, which forms part of the monomer-monomer interface. Mutants in group 2 contain mutations in domain 2 (amino acids 259 to 487). Group 3 mutations are in domain 3 (amino acids 488 to 595) but do not form part of the interface. Group 4 mutations, in domains 3 and 4 (amino acids 596 to 735), are close to each other in a region where the two domains abut. Group 5 mutations are in buried amino acids.

[b] Plus signs indicate that the PA lysate caused rounding of almost all of the CHO-K1 cells. A minus sign indicates that the lysate did not change the morphology of the cells. A plus-or-minus sign indicates that an intermediate level of cell rounding was observed. The level of rounding was not determined (ND) with 2.5 μl of LF_N-DTA in the first screen. One mutant, 514LF, was obtained in a preliminary screen.

tained double missense mutations, and one had three missense mutations.

Because the mutants isolated in the screen contained mutations at amino acids 481 and 607 to facilitate cloning, we used site-directed mutagenesis to introduce some of the mutations into a wild-type PA background. Except for two mutations, we changed the amino acids to Ala instead of to the amino acids that were isolated in the screen so as to determine whether the wild-type amino acid had a positive contribution to function, rather than if the mutated one had a negative effect on PA function. Amino acids 522 and 529 were changed to the amino acids that were isolated in the screen because, based on the structure, we predicted mutations to Ala would be silent.

The mutations that we made in the wild-type background were 483QA, 486EA, 514LA, 520DA, 522TI, 529IT, and 592RA. We also mutated amino acid 512D to A because of its proximity to amino acids 514 to 516 and because the structure suggested it might be involved in a hydrogen bond with the adjacent monomer. Each mutant, except for PA529IT, expressed well and was purified.

Native gel electrophoresis of PA mutants and LF_N. Electron microscopy has revealed that, in solution, LF_N can convert trypsin-nicked PA into ring-shaped heptamers (data not shown). The heptamers can be detected as a low mobility species when the mixture is electrophoresed on a nondenaturing polyacrylamide gel (Fig. 2A, compare lane 3 to lanes 1 and 2). Heptamers were formed when LF_N was mixed with nicked PA486EA, PA522TI, and PA592RA (Fig. 2A, lanes 7, 15, and 17). No low-mobility species was observed, however, when LF_N was mixed with nicked PA483QA, PA512DA, or PA514LA (Fig. 2A, lanes 5, 9, and 11). A reduced amount of the low mobility band was observed with PA520DA (Fig. 2A, lane 13).

To assess whether preformed mutant heptamers bind LF_N, we first purified them by loading trypsin-nicked mutant PA onto a MonoQ column and eluting PA_{63} heptamers with approximately 0.3 M NaCl. We then electrophoresed wild-type and each mutant heptamer (except for PA512DA, which did

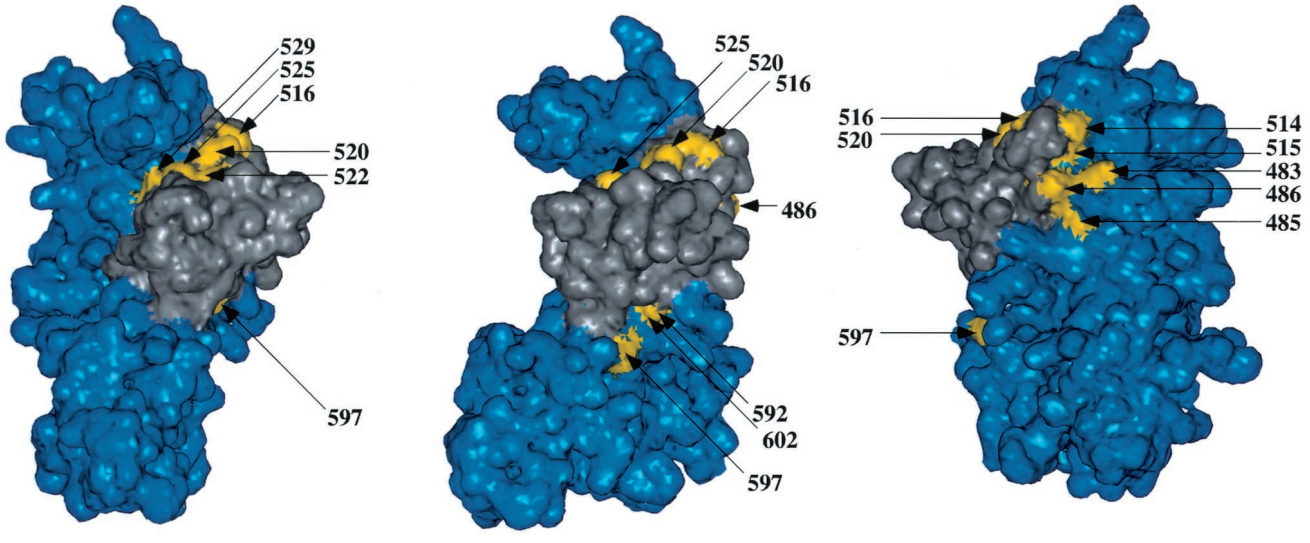

FIG. 1. Structure of PA_{63} with domain 3 mutations. Domain 3 is colored grey. Mutations in the yellow-colored amino acids were isolated in the screen.

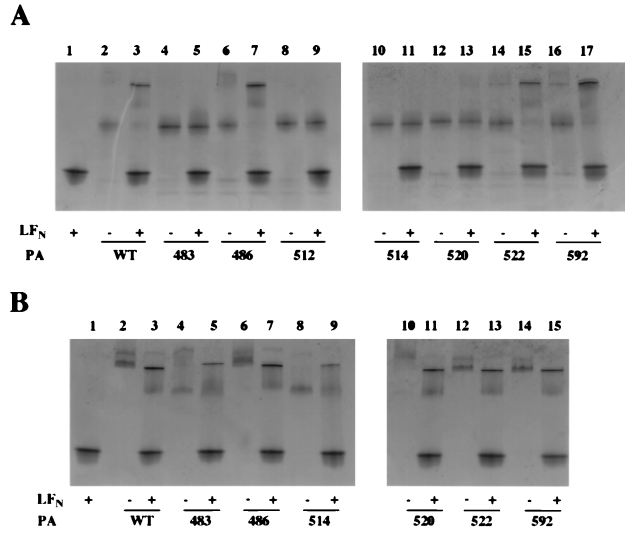

FIG. 2. Native gel electrophoresis of PA mutants. Trypsin-nicked PA (A) or heptameric PA_{63} (B) was incubated in the presence (+) or absence (−) of LF_N and electrophoresed on 4 to 20% acrylamide Tris-glycine gels. The gels were stained with Coomassie blue.

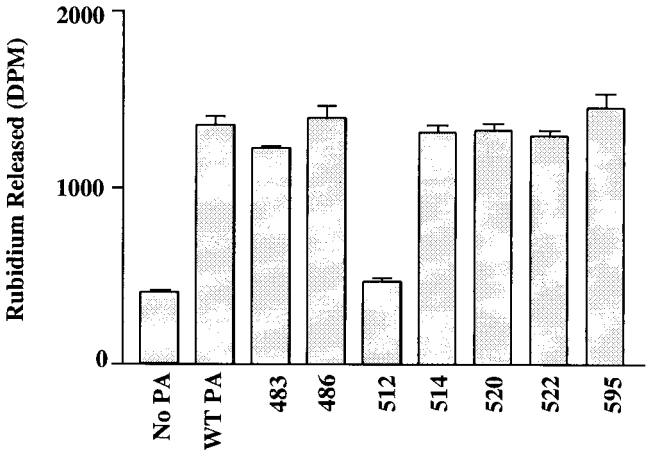

FIG. 3. Release of ^{86}Rb from CHO-K1 cells by PA and PA mutants. Trypsin-nicked wild-type or mutant PA was incubated with CHO-K1 cells at 4°C. The cells were washed twice with PBS and then exposed to buffer at pH 4.8 to induce channel formation. After 30 min, buffer was removed and its radioactive content was determined by gamma radiation counting. Error bars, standard deviations.

not form heptamers on the MonoQ column) on native gels in the presence or absence of LF_N (Fig. 2B). Wild-type PA_{63} ran as diffuse bands on the native gel (Fig. 2B, lane 2). When LF_N was mixed with PA_{63}, a sharper band of higher mobility was formed. This was observed when LF_N was mixed with each of the PA_{63} mutants tested (lanes 5, 7, 9, 11, 13, and 15). Thus, LF_N could interact with the oligomeric PA_{63} mutants 483QA, 514LA, and 520DA (the nicked forms of which did not stably associate with LF_N) as well as with oligomeric 486EA, 522TI, and 592RA.

Rubidium release mediated by PA mutants. On the two-dimensional surface of cells, trypsin-nicked PA self-associates into oligomers which, when exposed to low-pH buffer, form cation-selective channels through the membrane. We loaded cells with ^{86}Rb and tested whether they released it when exposed to wild-type or mutant PA (Fig. 3). Mutations at amino acids 483, 486, 514, 520, 522, and 592 of PA had no significant effect on channel formation. PA512DA, which did not form oligomers on native gels or on a monoQ column, was almost completely defective at releasing ^{86}Rb.

Translocation of LF_N by PA mutants. Channel formation is thought to be necessary for translocation of the enzymic moieties of anthrax toxin, but it is unclear whether the two processes are distinct and separable. To investigate whether the mutations affected the ability of PA to translocate LF_N across a cell membrane, we first incubated trypsin-nicked PA with CHO-K1 cells at 4°C so that it would oligomerize and remain on the cell surface. LF_N labeled with ^{35}S was bound to the oligomers, and a low-pH buffer was added to induce translocation. The translocation efficiency can be determined by dividing the amount of LF_N that is protected from extracellularly added protease by the amount of LF_N bound by cells. The PA mutants all bound similar amounts of LF_N, except for PA514LA (55% compared to wild-type) and PA512DA (28%) (Fig. 4). The translocation efficiencies of wild-type PA, PA486EA, PA514LA, PA520DA, PA522TI, and PA592RA were similar (69, 77, 70, 66, 70, and 67%, respectively). The translocation efficiencies of PA483QA (32%) and PA512DA (51%) were lower than that of the wild type.

Cytotoxity of PA mutants. We assessed the activity of the site-directed PA mutants in a cytotoxicity assay using LF_N-DTA. In this assay, PA and LF_N-DTA are added to CHO-K1 cells and viability is assessed after 4 h by the cells' ability to incorporate tritiated leucine into proteins. The concentration of wild-type PA that inhibits leucine incorporation by 50% under the conditions chosen is $(10 \pm 1.0) \times 10^{-11}$ M. Similar concentrations of the PA mutants PA483QA [$(11 \pm 1) \times$

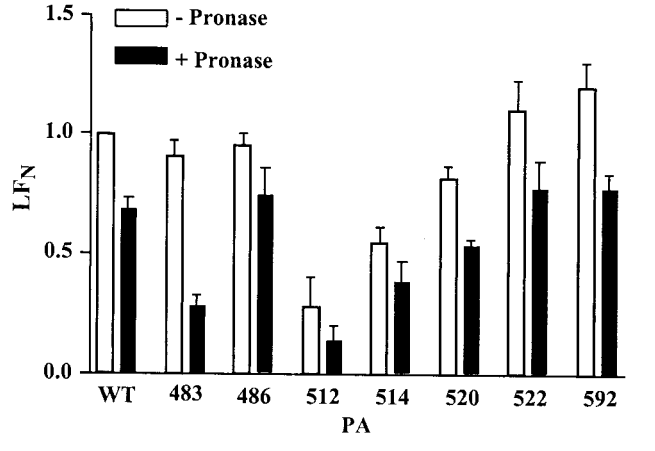

FIG. 4. Translocation at the cell surface of LF_N by wild-type and mutant PA. Trypsin-nicked wild-type or mutant PA was incubated with CHO-K1 cells at 4°C. The cells were washed twice with PBS and incubated with ^{35}S-LF_N at 4°C. The cells were exposed to buffer at pH 4.8 and either solubilized with lysis buffer, to determine the amount of bound LF_N (− Pronase), or treated with pronase, to determine the amount of translocated LF_N (+ Pronase). Radioactive content was measured by scintillation counting. Nonspecific binding of LF_N to cells (less than 10%) was subtracted from the experimental measurements to determine specific binding. Error bars, standard deviations.

10^{-11} M], PA486EA [$(9 \pm 2) \times 10^{-11}$ M], PA522TI [$(12 \pm 2) \times 10^{-11}$ M], and PA592RA [$(10 \pm 4) \times 10^{-11}$ M] were required to inhibit leucine uptake by 50%. The concentrations of PA512DA [$(38 \pm 9) \times 10^{-11}$ M], PA514LA [$(21 \pm 6) \times 10^{-11}$ M], and PA520DA [$(16 \pm 3) \times 10^{-11}$ M] required to inhibit leucine uptake were significantly higher than that of the wild type.

DISCUSSION

Of the four domains of PA, domain 3 (amino acids 488 to 595) is perhaps the least well understood. We investigated the function of this domain by isolating mutations in it that are detrimental to PA activity. To do so, we introduced random mutations into domain 3 by error-prone PCR and screened for proteins that were defective in mediating killing of CHO-K1 cells by LF_N-DTA. The source of PA protein in this assay, lysate derived from PA-expressing *E. coli* cells that had been infected with and lysed by bacteriophage T7, allowed us to screen a large number of PA mutants without having to purify them individually. We used T7 to lyse cells because it requires fewer manipulations and is more efficient than either osmotic shock or freeze-thawing protocols.

In our first screen, we exposed CHO-K1 cells to mutant PA lysates and an amount of LF_N-DTA that killed the cells with wild-type PA. The fact that 38 of the 48 defective clones isolated did not express full-length protein came as a surprise since, at the level of dPTP-induced mutagenesis we used (approximately one mutation per clone), we predicted that only 2% of the mutations would be nonsense mutations. We believe that the majority of truncated proteins we detected had mutations that destabilized the structure of domain 3, making the proteins sensitive to proteases. In the second screen, we distinguished between truncated PA and full-length PA molecules with activity defects by using two concentrations of LF_N-DTA. The mutants from both screens were sequenced (Table 1).

We classified the mutants into five groups based on the location of the mutations in the crystal structure of the PA_{63} heptamer. The mutants of the first group (Table 1) have mutations in a loop (amino acids 510 to 518) that inserts into a cleft in domain 1 of the adjacent monomer (Fig. 5). The cleft consists of amino acids 192 to 205 ($1\beta_{12}$-$1\beta_{13}$ hairpin) and amino acids 239 to 249. The $1\beta_{12}$-$1\beta_{13}$ hairpin is the region of PA that moves the most upon oligomerization (18). We made single alanine mutations at amino acids 512 and 514 in a wild-type background. Amino acid 512 was chosen after identifying the cluster of mutations in the 510-to-518 loop, because we thought it might make a hydrogen bond with an amino acid in the adjacent monomer (this is unclear because of the low resolution of the heptamer structure). PA512DA was defective in all of the assays we performed, and we could not detect the assembly of the heptameric form of this mutant using a MonoQ column (data not shown). Although the heptameric form of PA514LA bound LF_N, trypsin-nicked PA514LA did not form oligomers on native gels when mixed with LF_N. On cells, nicked PA514LA bound less LF_N than did wild-type PA. This mutant also showed a defect in the cell killing assay with LF_N-DTA. Nevertheless, PA514LA formed channels in the rubidium release assay, indicating that the sensitivity of this assay is not sufficient to detect this mild mutation. These data indicate that the loop comprised of amino acids 510 to 518, and amino acid 512 in particular, is important for the oligomerization of PA_{63}.

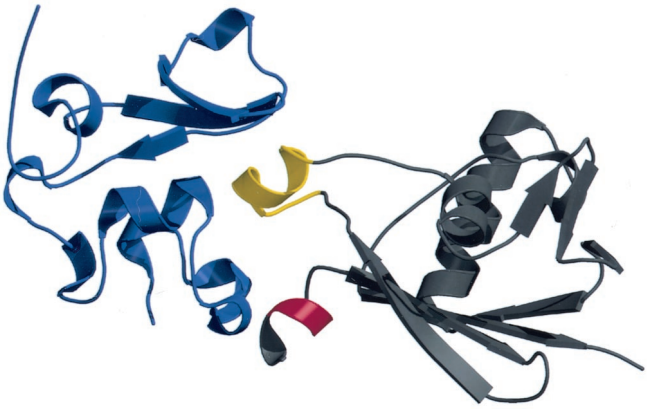

FIG. 5. Involvement of domain 3 in the oligomerization of PA_{63}. Domain 1 (amino acids 173 to 258, colored blue) and domain 3 (amino acids 488 to 595 plus amino acids 481 to 487 of domain 2, colored grey) of adjacent monomers are shown. Amino acids 483 to 486 are colored red, and amino acids 510 to 518 are colored yellow.

The second group of mutants consists of the domain 2 mutants, PA_{KC}483QH, PA_{KC}485QH, and PA_{KC}486EG. These amino acids are situated at the monomer-monomer interface of the PA_{63} heptamer (Fig. 5). We introduced single alanine mutations at positions 483 and 486 into a wild-type PA background to assess the importance of these amino acids to PA activity. A high-molecular-weight band was observed when LF_N and wild-type nicked PA were electrophoresed together on a native gel but not when LF_N and trypsin-nicked PA483QA were. Since this band results from LF_N inducing the heptamerization of PA_{63}, this experiment suggested that amino acid 483 is involved in oligomerization. Consistent with this, MonoQ chromatography of nicked PA483QA yielded lower amounts of heptamer than did the wild type (data not shown). Although oligomers presumably form on the cell surface, these oligomers must not be assembled efficiently, because they do not translocate as efficiently as wild-type heptamer. PA486EA behaved like wild-type PA in all of the assays performed, which may be explained by the fact that a Glu-to-Gly mutation was isolated in the screen. The glycine residue might have disrupted the alpha-helix, consisting of amino acids 476 to 487, whereas the alanine mutation might not have. Thus, amino acid 486 of PA probably does not have a direct role in the oligomerization process.

The mutants in the third group have mutations in a pocket on the outer face (opposite the lumen) of the heptamer. We introduced the single mutations 520DA and 522TI into a wild-type background to determine the functional defect in this group. PA522TI showed a wild-type phenotype in all of the assays performed. Trypsin-nicked PA520DA did not form oligomers when mixed with LF_N and electrophoresed on native gels; it showed a mild defect in the cell killing assay, but it appeared normal in other assays. This suggests that the mutants in this group are mildly defective in oligomerization. Although these amino acids are not directly involved in monomer-monomer interactions, mutations at these positions might affect the positioning of the nearby loop that consists of amino

acids 510 to 518. The mutant background (PA481LV 607AI) that was used in the screen may have enhanced the effect of these mutations.

The fourth group of mutants consists of PA_{KC}592RK, PA_{KC}597HR 496KE, PA_{KC}602ND, and PA_{KC}545KE 602NS. The arginine at position 592, which is in domain 3, may form a hydrogen bond with the asparagine residue at position 602, in domain 4. Amino acid 597 is also in domain 4, close in space to these amino acids. We made an arginine-to-alanine mutation at amino acid 592 and found that it did not affect activity in any of the assays we performed. The original PA_{KC}592RK mutant that we isolated was only mildly defective at killing CHO-K1 cells with the lower concentration of LF_N-DTA. The mild defect may have been a result of a lower concentration of PA in the lysate, or the mutation may have rendered the protein less stable. In fact, it has been suggested previously that the region consisting of amino acids 592 to 608 is important for the stability of PA (3).

A number of mutations we isolated were in buried amino acids (491IT, 494NS, 506AV, 542YC, 554FL, and 554FS). These mutations, comprising group 5, probably caused structural rearrangements in the protein, so we did not pursue them further. Even though our screen was not saturating, it is interesting that we did not isolate any single mutations between amino acids 530 and 590 other than mutations in buried amino acids. Although this region of PA is close to regions that are thought to interact with EF, LF, and the receptor, we did not obtain any mutants that were defective in either interaction. In addition, the site-directed mutants PA549EK, PA563KE, and PA568EK did not show activity defects (J. Mogridge, unpublished data).

The results of our screens suggest that domain 3 is important for the heptamerization of PA_{63}. A single mutation at amino acid 512 is sufficient to inhibit oligomerization, as determined by native gel electrophoresis and its reduced ability to form channels on cell membranes. This mutant protein must not be completely defective for oligomerization, however, because it is still able to mediate cell death by LF_N-DTA, although concentrations higher than wild-type concentrations are required.

An unexpected result from our examination of PA512DA and PA514LA was that these proteins were also defective in binding LF_N on the cell surface. Although it is conceivable that these mutations affect both oligomerization and LF_N binding, we think that this result suggests that oligomerization of PA_{63} is required for LF_N binding. This notion would also explain how LF_N can stimulate the heptamerization of PA_{63} from nicked PA, by stabilizing PA_{63} dimers and lower-order oligomers. It would also explain why LF_N binds PA_{63} heptamers formed from PA514LA but does not bind nicked PA514LA in native gels. Thus, these results suggest that EF and LF stably associate with PA_{63} only after it has oligomerized.

The screen we devised to isolate mutations in PA that affect its function can be easily adapted to isolate muations in other toxins or other proteins that act at the surface of mammalian cells and induce an observable phenotype. This screen is also ideally suited to the isolation of dominant negative toxin mutants, which may be useful as therapeutic agents.

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