Structure-Function Analysis of the Enteroaggregative Escherichia coli Plasmid-encoded Toxin Autotransporter Using Scanning Linker Mutagenesis*

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The plasmid-encoded toxin (Pet) from enteroaggregative Escherichia coli is the predominant protein of a large family of bacterial autotransporter toxins. To further elucidate the structure-function relationships of this toxin, we employed transposon-based scanning linker mutagenesis. A subset of insertions throughout the Pet mature toxin (passenger) domain reduced secretion to the extracellular space. Many of these mutants were undetectable, but secretion of a subset of mutants with insertions in the N-terminal half of the toxin could be restored to wild-type levels if cultured in the presence of 0.1% Triton X-100. Secretion of two mutants with insertions at the extreme C terminus was partially restored when co-expressed with a minimal clone of EspP, a related autotransporter protein. Several well secreted mutants with insertions in the N-terminal third of the molecule reduced protease activity over 20-fold, suggesting that the protease domain is located within this N-terminal region of Pet. We have also identified two insertional mutants in the middle of the passenger domain that were proteolytic but no longer cytotoxic; these mutants displayed decreased binding and internalization upon incubation with HEp-2 cells. Our data suggest the existence of separate functional domains mediating Pet proteolysis, secretion, and cell interaction.

Enterotoxigenic Escherichia coli is an emerging diarrheal pathogen in both developing (3–5) and industrialized countries (6–8). Enterotoxigenic E. coli strains are characterized by their distinctive aggregating patterns of adherence to HEp-2 cells (6,7), and histopathology from human autopsies (7) and animal models reveal substantial damage to the mucosal epithelia (8). The action of secreted cytotoxins has been hypothesized to be the cause of the observed pathology (9,10).

The plasmid-encoded toxin (Pet) is the predominant protein in culture supernatants of enterotoxigenic E. coli prototype strain 042 and has been extensively studied by our laboratory (9,11,12). Previous studies have identified this 104-kDa protein to be a member of the serine protease autotransporters of enterobacteria (11,12). Autotransporters are a unique family of proteins, whose ability to translocate through the outer membrane is conferred by a dedicated C-terminal domain (13). An N-terminal signal sequence directs secretion of the protein precursor into the periplasm, presumably via the Sec apparatus; once in the periplasm, the signal sequence is cleaved, and the C terminus is presumed to form an outer membrane pore, perhaps through oligomerization (14). Through this channel the passenger domain is exported to the extracellular milieu. For Pet and many of the autotransporter proteins, the translocated protein is cleaved, resulting in the release of the mature passenger domain into the culture supernatant (11).

Our lab has previously shown that Pet contributes to the damage caused by enterotoxigenic E. coli strain 042 in vitro organ cultures (9), whereas concentrated culture supernatants containing the Pet protein elicited enterotoxic effects in Ussing chambers mounted with rat jejunal tissue (11,15). Moreover, Pet was shown to induce cytotoxic effects on HEp-2 cells grown in monolayers, including loss of focal contacts, dissolution of actin microfilaments, contraction and darkening of the cytoplasm, and eventual rounding and detachment from the glass substratum (12). The cytotoxic effects were shown to be dependent on the secreted protease activity of Pet; co-incubation with serine protease inhibitor or mutation of the predicted catalytic serine abolish both proteolytic and toxic effects (12,15).

Although the complete mechanism of action has yet to be elucidated, the available data suggest that Pet is internalized into epithelial cells (16). Immunofluorescence and confocal images have revealed entry of Pet into HEp-2 cells, whereas brefeldin A, which inhibits vesicular trafficking, abolishes Pet cytotoxic effects (15,16). In addition, Villaseca et al. (17) have shown that Pet cleaves eukaryotic spectrin in vitro and causes redistribution of spectrin in intoxicated cells; it is therefore hypothesized that proteolysis of spectrin is the fundamental mechanism of action.

As a prototypical serine protease autotransporter of enterobacteria protein, elucidation of structure-function relationships will prove enlightening to research on Pet and other members of this family (13,14,18,19). Accordingly, we have applied the technique of scanning linker mutagenesis (20,21) to identify regions of the Pet molecule involved in various aspects of activity. This effort was facilitated by the recent introduction of transposon-based methods for the introduction of linker insertions. Our data suggest that distinct functions can be correlated with various regions of the secreted toxin.

EXPERIMENTAL PROCEDURES

Strains and Plasmids Used—The strains and plasmids used in these studies are listed in Table I.

Construction of Permissive Linker Mutants—The minimal pet clone
pCEFNI (11) was randomly mutagenized in vitro with a unique Tn5-derived transposon EZ::TN (NotIKAN-3) (nicknamed PEZ) using the EZ::TN in-frame linker insertion kit (Epicenter Technologies, Madison, WI) according to the manufacturer’s instructions. The mutagenized plasmids were transformed into competent E. coli DH5α. Kanamycin- and ampicillin-resistant colonies were screened by PCR for insertions in the passenger region of pet. Primers used for this reaction were the following: forward, 5’-ATACTAAATATAAAATGCGGCC-3’, and reverse, 5’-CCCATAGGTTGTGGAGTGTTG-3’. Plasmid DNA from transformants was extracted and digested with NotI to excise the kanamycin resistance gene. The restricted plasmid fragments were separated by agarose gel electrophoresis, religated using a Fast-Link DNA ligation kit (Epicenter Technologies), and then were transformed into E. coli HB101. The site of transposon insertion was determined by sequence analysis using a primer designed from the transposon (5’-ACTTCAACAAAGCTCTCATCAACC-3’). In a similar protocol, the pCEFNI plasmid was mutagenized with the Tn7-derived GPS-LS transposon (New England Biolabs, Inc., Beverly, MA) according to the manufacturer’s instructions. Mutagenized plasmids were transformed into E. coli DH5α, and the colonies growing on agar plates containing both kanamycin and ampicillin were selected for screening by PCR for insertions in the passenger region, as above. Plasmid DNA was extracted and digested with PmeI to remove the kanamycin resistance gene. The restricted plasmid fragments were separated by agarose gel electrophoresis, religated, and transformed into E. coli HB101 as above. The site of insertion was determined by sequence analysis using a primer designed from the transposon (5’-ACTTCAACAAAGCTCTCATCAACC-3’). DNA sequencing was performed at Davis Sequencing, LLC (Davis, CA) or at the Biopolymer Sciences (Department of Microbiology and Immunology, University of Maryland School of Medicine). The S260A mutant was constructed by QuickChange mutagenesis of pCEFNI and is described elsewhere (15).

**Toxin Preparation**—Toxin was prepared as previously described (12). Briefly, overnight LB cultures of E. coli HB101 expressing the various Pet protein mutants were centrifuged at 6,000 × g for 10 min. The culture supernatants were passed through a 0.22-μm filter to remove residual bacteria and concentrated to 30–kDa cut-off filter devices (Millipore, Bedford, MA). Pet and mutant Pet proteins were visualized by Coomassie Blue staining, and protein concentrations were calculated by densitometry on a PhosphorImager Storm 840 (Amer sham Biosciences) after SYPRO Orange (Bio-Rad) staining. The images were analyzed with ImageQuant 5.0 software (Amer sham Biosciences).

When indicated, Triton X-100 (Bio-Rad) was added to LB medium to a final concentration of 0.1%. The protein was then prepared as described above; prior to visualization on SDS-PAGE, the detergent was removed with a PAGE Prep protein clean-up and enrichment kit (Pierce) according to the manufacturer’s instructions.

**Immunologic Methods**—Rabbit antiserum specific for the Pet protein was derived previously (11). Prior to use, antiserum was absorbed with E. coli proteins conjugated to agarose (Sigma) according to the manufacturer’s instructions. For immunoblot detection, the proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride (Millipore, Bedford, MA) or nitrocellulose (Schleicher & Schuell) and detected with rabbit antiserum at dilutions of 1:8,000. Pet protein was visualized with goat anti-rabbit antibodies conjugated with horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD) using an ECL Plus Western blotting detection system (Amer sham Biosciences) or, for more sensitive detection, SuperSignal West Femto (Pierce), according to the manufacturer’s instructions.

**Limited Trypsin Digestion**—2 μg of protein was digested with 0.7 μg of trypsin (Sigma). The reaction was stopped at 2 and 10 min by boiling, and the products were separated using 12% SDS-PAGE. The digestion products were visualized using the immuno blot techniques described above.

**Bacterial Cell Fractionation**—Overnight bacterial cultures (100 μl) were subcultured into 10 ml of LB containing the appropriate antibiotics, grown at 37 °C to an A600 of 0.7. The cells were collected by centrifugation and resuspended in 1 ml of 0.2 M Tris, 20% sucrose, and 0.5 mM EDTA, pH 7.8. 25 μl of 0.5 mg/ml lysozyme was added and incubated at room temperature for 10 min. The cells were examined under light microscopy to document spheroplasting. The cells were centrifuged again, and the supernatant containing the periplasmic proteins was precipitated with trichloroacetic acid and analyzed with SDS-PAGE and immunoblottings techniques as described (11). The outer membrane fractions were prepared by Sarkosyl insolubility as described (22). The outer membrane and the periplasmic fractions were separated using 12% SDS-PAGE. The digestion products were visualized with the immunoblot techniques described above.

**Protease Assay**—Concentrated supernatants containing 5 μg of Pet or Pet mutant proteins were added to 1 mM solution of Ala-Ala-Pro-Ala oligopeptide (Calbiochem, La Jolla, CA) conjugated with p-nitroanilide, and proteolysis of the oligopeptide was determined as described previously (12). The reactions were carried out in triplicate at 37 °C for 5 h, and the absorbance readings at 405 nm were measured. The absorbance value obtained for Pet was set to 100%, and the values for mutants were reported as the mean percent of Pet activity.

**Cell Intoxication and Binding Studies**—HEp-2 cells were maintained and intoxicated as previously described (12, 15). To calculate equivalent activity units, the reciprocal of the in vitro proteolytic activity was multiplied by 10 (i.e. for a mutant with 50% of the proteolytic activity of native Pet (20 μg) was used). A maximum of 60 μg was used for any one mutant. To quantitate cell binding, 10 μg of toxigen was applied to cultured HEp-2 cells as described above. The cells were incubated for 2 h at 4 °C, then washed twice with PBS, and washed a third time with PBS at 150 or 200 μl of PBS or a solution of 50 mM glycerol and 100 mM NaCl (pH 2) to disrupt receptor-ligand interactions (24). The third wash was analyzed by enzyme-linked immunosorbent assay using anti-Pet serum as described (12). In addition, HEp-2 cells and toxin were visualized after the third wash using confocal immunofluorescence techniques as described (11). Immunofluorescence images were acquired using a standard confocal microscope.

**RESULTS**

**Generation of Linker Insertions**—To characterize Pet structure-function relationships, the pet clone pCEFNI clone was subjected to permissive linker mutagenesis with two different transposon-based systems, each designed to generate in-frame oligopeptide insertions. The Tn5-derived EZ::TN (NotI/kan-3) (PEZ) transposon generates 57-bp insertions, encoding 19 amino acids, after restriction digestion of the target plasmid...
Pet Structure Function Studies

Mutants are listed in order from N to C termini. PEZ mutants (designated P-) contain an insertion of 19 amino acids. GPS mutants (designated G-) contain an insertion of 5 amino acids. Mutants designated a and b represent independent insertions at the same amino acid.

### Mutant location of insertion

| Mutant location of insertion | Well secreted | Secreted with Triton (Class Ia) | Poorly or nonsecreted (Class Ib) | Proteolytic<sup>a</sup> (adjusted for protease activity) | Cytopathic (adjusted for protease activity) |
|-----------------------------|--------------|----------------------------------|----------------------------------|------------------------------------------------------|---------------------------------------------|
| P-M55                       |              |                                  | NA                               | NA                                                   | NA                                          |
| P-W61                       |              |                                  | NA                               | NA                                                   | NA                                          |
| P-G70                       |              |                                  | NA                               | NA                                                   | NA                                          |
| P-N71                       |              |                                  | NA                               | NA                                                   | NA                                          |
| P-1114                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-1183                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-Y186                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-V188                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-F209                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-221                       |              |                                  | NA                               | NA                                                   | NA                                          |
| G-S260                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-N286                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-Q290                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-E304                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-N317                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-D362                      |              |                                  | NA                               | NA                                                   | NA                                          |
| P-E361                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-E362                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-A452                      |              |                                  | NA                               | NA                                                   | NA                                          |
| P-E463                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-T465                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-J466                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-S500                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-T505                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-L515                      |              |                                  | NA                               | NA                                                   | NA                                          |
| P-G527                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-H537                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-D540                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-R547                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-D551a                     |              |                                  | NA                               | NA                                                   | NA                                          |
| G-D551b                     |              |                                  | NA                               | NA                                                   | NA                                          |
| G-D553                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-S560                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-T573                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-T573                      |              |                                  | NA                               | NA                                                   | NA                                          |
| P-A576                      |              |                                  | NA                               | NA                                                   | NA                                          |
| P-F589                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-J657                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-N665                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-G667                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-S715                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-D731                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-D760                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-M777                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-G790                      |              |                                  | NA                               | NA                                                   | NA                                          |
| P-N792                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-K796                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-A818                      |              |                                  | NA                               | NA                                                   | NA                                          |
| P-G837                      |              |                                  | NA                               | NA                                                   | NA                                          |
| P-S841                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-O843                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-G844                      |              |                                  | NA                               | NA                                                   | NA                                          |
| P-S845                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-S851                      |              |                                  | NA                               | NA                                                   | NA                                          |
| P-L859                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-T863                      |              |                                  | NA                               | NA                                                   | NA                                          |
| P-L963                      |              |                                  | NA                               | NA                                                   | NA                                          |
| P-T908                      |              |                                  | NA                               | NA                                                   | NA                                          |
| P-T908                      |              |                                  | NA                               | NA                                                   | NA                                          |
| P-G910                      |              |                                  | NA                               | NA                                                   | NA                                          |
| P-A911                      |              |                                  | NA                               | NA                                                   | NA                                          |
| G-B915                      |              |                                  | NA                               | NA                                                   | NA                                          |
| P-E921                      |              |                                  | NA                               | NA                                                   | NA                                          |
| P-G922                      |              |                                  | NA                               | NA                                                   | NA                                          |

<sup>a</sup>: >20% proteolytic activity of Pet from Fig. 3.

The table shows that PEZ insertion was not random, with most insertions occurring at the Pet C terminus. The pet clone was therefore also mutagenized with the Tn7-derived GPS-LS transposon to yield 51 permissive pentapeptide linker insertions; these mutants were well distributed throughout Pet. The mutants are designated G for the GPS-LS method or P for PEZ method, followed by the amino acid immediately after which the linker is inserted. Locations and phenotypes of all insertions are listed in Table II and are illustrated in Fig. 1.

Effect on Secretion—Many linker insertions in the passenger domain resulted in >90% diminution in abundance of the mature toxin species in the supernatant fraction, as determined by densitometry (Table II). These mutants were designated Class I. With the exception of P-N1018, all Class I mutants produced the expected 30-kDa β domain in the outer membrane (not shown). Although P-N1018 did not exhibit the 30-kDa β domain in the outer membrane fraction, it did yield an unprocessed 130-kDa Pet preprotein in the outer membrane fraction, indicating that the mutant protein was properly translocated to and inserted into the outer membrane but that the mature toxin was not cleaved from the β domain.

To determine the fate of the passenger domain, Class I mutants were grown in the presence of 0.1% Triton X-100, which removes noncovalently bound bacterial surface proteins. The 104-kDa Pet passenger species was observed in culture supernatants of 16 Class I mutants when cultured in LB supplemented with 0.1% Triton X-100 (Table II and Fig. 2). These 16 mutants are herein designated as Class Ia. Incubation of whole cell pellets of Class Ia mutants with trypsin released fragments that were reactive with anti-Pet antibodies on immunoblots (data not shown), corroborating their presence on the cell surface. Interestingly, all 16 Class Ia mutants mapped to the N-terminal half of the passenger domain (upstream of residue 505). In contrast, all but one Class I mutant whose secretion was not detected in the presence of Triton X-100 mapped downstream of residue 450; these 29 mutants are designated Class Ib mutants.

Rescue of Junction Region Mutations—With the exception of
P-N1018, Class Ib mutants yielded a 30-kDa β domain species in the outer membrane but no detectable passenger species. Western immunoblots of whole cell pellets did not reveal passenger species in Class Ib mutants (not shown), suggesting that the passenger domain is degraded at some point in the secretion process.

We noted that 13 of the 29 Class Ib mutants carried insertions in the C-terminal 100 amino acids of the passenger domain. In homologous proteins, this region has previously been designated the autotransporter junction region and has been suggested to play a role in secretion (25–28). Ohnishi et al. (26) have shown that nonsecreted deletion mutants in the junction region of SSP, an autotransporter from Serratia marcescens, could be rescued for secretion when the junction region is supplied in trans. We therefore tested the ability of the junction region to rescue secretion of three Class Ib mutants. For these experiments, we employed the junction region of the Pet homolog EspP, provided in clone pEPL (Fig. 3A).
pEPL, Class Ib mutants P-T1003 and P-F1013 were detected in culture supernatants. No change in secretion was detected for mutant Class Ib mutant P-A576 (Fig. 3B).

Effect of Linker Insertions on Protease Function—The effects of insertion mutations on proteolytic activity were analyzed for normally secreted mutants and for Class Ia mutants released by Triton X-100. These assays employed an oligopeptide previously shown to be cleaved efficiently by Pet (12). No effect of 0.1% Triton X-100 on protease function was observed on native Pet (Fig. 4, third panel). The most significant decrease (≈90% diminution) was observed for insertions in the N-terminal third of the passenger domain (Fig. 4, first panel). Interestingly,
despite the sensitivity of the N-terminal third with regard to protease activity, four insertions within the N-terminal third (G-N174, G-Y186, G-V188, and G-F209) had little or no effect. These insertions suggest that the region between residues 174 and 209 (but not including residue 183) is permissive to insertion. More broadly, these data also suggest that the N-terminal third of the passenger domain is essential to protease function.

We hypothesized that nonproteolytic mutants in the N terminus were improperly folded. We therefore compared the partial trypsin digestion profiles of nonproteolytic N-terminal mutants P-W61, P-N71, G-T166a, G-T166b, and G-167 with those of native Pet. All five of these nonproteolytic mutants yielded digestion patterns dramatically different from native Pet (Fig. 5), suggesting that misfolding of the N-terminal domain accounted for the loss of protease activity.

**Cytopathic Activity**—Pet and its linker mutants were tested for their capacity to elicit cytotoxic effects on HEp-2 cells. Even when the toxin dose was normalized for proteolytic activity (Fig. 4), G-D553, G-S560, G-I657, G-G667, G-Q843, and P-G922 were unable to intoxicate HEp-2 cells. These were designated Class II mutants.

Mutants G-D553, G-I657, and G-Q843 demonstrated nearly wild type levels of protease activity but decreased cytotoxic effects. These mutants were therefore analyzed for ability to bind to and be internalized by epithelial cells. Epithelial cells were incubated with Pet, G-D553, G-I657, or G-Q843 at 4 °C, a temperature that permits binding but not internalization. The cells were washed twice with cold PBS to remove excess protein and washed a third time with PBS or acidic glycine solution to remove receptor-bound protein from the cell surface. The third wash was assayed by enzyme-linked immunosorbent assay to quantitate toxin removed. G-Q843 and G-D553 displayed cell binding ability similar to native Pet (p < 0.05 acid versus PBS by a one-tailed Fisher test). In contrast, no significant acid-dissociable binding was observed for G-I657. Toxin binding studies were confirmed by immunofluorescence. The cells treated with native Pet at 4 °C exhibited a diffuse green fluorescence, whereas after the acid wash, green fluorescence was clearly diminished (Fig. 7). Such an effect could also be appreciated with G-Q843 and G-D553-treated cells but not with cells treated with G-I657; for G-I657, the intensity of green fluorescence was similar after PBS or acid washes. These data confirm that G-I657 has a decreased ability to bind to HEp-2 cells.

To investigate the ability of G-D553, G-I657, and G-Q843 to be internalized by epithelial cells, HEp-2 cells were treated with Pet or its mutants and incubated at 37 °C to permit toxin uptake. The cells were then washed with acidic glycine solution to remove surface-bound protein, and Pet was visualized using confocal microscopy. By 2 h of incubation, the majority of cells incubated with Pet exhibited a uniform green fluorescence resistant to acid wash, indicating toxin internalization. Similar observations were made for the G-Q843 mutant. However, for the G-I657 and G-D553 mutants, green fluorescence was faint (Fig. 8), suggesting that these two mutants were defective for cell entry.

G-Q843, G-I657, and G-D553 were assayed for their abilities to cleave spectrin, because the cleavage of spectrin is hypo-
esized to be the primary mechanism through which Pet acts to induce cytopathic effects. All three mutants were able to cleave spectrin in vitro over a similar time period (Fig. 9), suggesting that the inability to elicit cytopathic effects was not due to an inability to cleave the putative target molecule.

**DISCUSSION**

Scanning linker mutagenesis is a powerful approach to study protein structure-function relationships (20). We employed a recently developed, highly efficient linker mutagenesis method to characterize structure-function relationships of Pet, a prototypical autotransporter protein. Secretion of autotransporters has been examined using a variety of reporter and deletion constructs but rarely with the full-length protein (28–30). These previous studies have generally implicated the extreme C terminus of the passenger domain (i.e. the last 250 amino acids) in secretion. Our method has permitted us to examine the effects of mutations throughout the Pet passenger domain on secretion as well as toxin function.

Because many of the linker insertion Class I mutants were not detected in the culture supernatants, different compartments of the bacterial cell were examined. Outer membrane preparations revealed the 30-kDa /H9252 domain in all Class I mutants, with the exception of P-N1018, indicating that Class I mutants were competent for insertion into the outer membrane. P-N1018 (containing an insertion at the predicted cleavage site Asn1018-Asn1019) could be detected only by immunoblot as an uncleaved 130-kDa species in the outer membrane fraction. The insertion of 19 additional amino acids near this location may have resulted in a decreased efficiency of processing by the responsible protease (which remains unknown).

Many linker insertions located in the N-terminal half of the passenger domain (Class Ia) were observed to be secreted at levels lower than native Pet but were shown to remain associated with the cell surface, dissociable upon treatment with a low concentration of Triton X-100. These data suggest that Class Ia mutations in the N-terminal region permit secretion but result in an aberrantly folded protein whose unnaturally exposed residues mediate noncovalent association with the surface of the bacterial cell.

Another set of insertion mutations (Class Ib), located predominately at the C terminus of the passenger, abolished Pet secretion. Our data suggest that these passengers were degraded at some point in the secretion process. However, secretion of P-T1003 and P-F1013 was restored when the C-terminal region was provided in trans. Recent studies with the Bordetella pertussis autotransporter BrkA have identified a C-terminal region that aids in folding and secretion of the mature protein. Moreover, when this region is expressed in trans, it is capable of rescuing mutants in the corresponding region (25, 27). Our mutants lie just downstream of the region implicated by Oliver et al. (25), but our data are consistent with this report. Although the mechanism of autotransporter secretion

![Fig. 7. Binding of GPS linker mutants to epithelial cells.](image1)

![Fig. 8. Internalization of noncytopathic mutants.](image2)
has not been completely elucidated, a recently suggested model predicts the formation of an oligomeric outer membrane channel comprising multiple autotransporter β domains, through which the passenger domain translocates (14). Such a model would be consistent with our findings concerning Class Ib mutants, in that linker insertions in the C terminus of the passenger may have prevented the oligomerization of the β domains and the interactions of the multiple junction regions, thereby abolishing pore formation. The junction region, which is strongly predicted to be α-helical in nature (25), may help to stabilize the oligomeric channel. Presumably, passenger species that are not translocated are degraded by periplasmic proteases.

Our data also permit inferences regarding Pet functional regions. Fink et al. (31) previously characterized the protease domain of the Hap autotransporter and suggested by homology that the catalytic triad of Pet lies in the N-terminal region of the passenger domain. This hypothesis is consistent with the presence of the catalytic serine residue at position 260 (15) and our observation that linker insertion mutants in the N-terminal third of Pet abolish proteolytic activity. We also note that a region surrounding amino acid residue 200 may be permissive to insertion; we are currently investigating the utility of this observation in antigen delivery. Of note, three nonproteolytic mutants were mapped to the C terminus of the Pet passenger domain (P-L879, P-T964, and P-G1009a; Fig. 1). The curious existence of these mutations in an area in which many mutants did not affect proteolysis will be examined in future studies. Such mutations could be explained by a folded structure in which these sites contribute to the protease active site.

Although the full mechanism of Pet-induced cytopathology remains to be elucidated, the current hypothesis is that Pet must bind to the HEp-2 cell surface, be internalized, and cleave the target eukaryotic protein spectrin. However, regions of the Pet molecule that orchestrate these first two events have yet to be identified. In these studies, several linker insertions with decreased ability to intoxicate cells mapped between amino acids Asp553 and Gln843. Four of these mutants, G-D553, G-I657, G-G667, and G-Q843, retained close to wild type levels of protease activity. Because these mutants were able to cleave erythroid spectrin, we suspected that an earlier step in the intoxication process may be defective. Compared with the wild type Pet protein, G-I657 displayed less acid-dissociable binding, based on both the amount of protein released after an acid wash of intoxicated cells and visualization with immunofluorescence. G-G667, harboring an insertion only 10 amino acids away from that of G-I657, appears to behave similarly (not shown). These data suggest that this region may be specifically required for toxin binding to the cell surface. Unlike G-I657, G-D553 displayed acid-dissociable binding to the cell surface but reduced cellular entry. Thus, residue Asp553 could be located within an internalization region, although this hypothesis requires further verification. An alternative interpretation of our data would be that the central domain may mediate a toxin effect independent of protease activity, and this possibility is being investigated. A schematic representation of Pet functional domains is shown in Fig. 10.

Our data shed additional light on structure-function relationships of Pet and related autotransporters and suggest a multi-domain modular structure for this growing family of proteins. The data also permit the identification of multiple regions permissive to insertion of foreign epitopes, which will facilitate antigen display and delivery efforts.

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