Pet Toxin from Enteroaggregative *Escherichia coli* Produces Cellular Damage Associated with Fodrin Disruption

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**Pet toxin** is a serine protease from enteroaggregative *Escherichia coli* which has been described as causing enterotoxic and cytotoxic effects. In this paper we show that Pet produces spectrin and fodrin (nonerythroid spectrin) disruption. Using purified erythrocyte membranes treated with Pet toxin, we observed degradation of α- and β-spectrin chains; this effect was dose and time dependent, and a 120-kDa protein fraction was observed as a breakdown product. Spectrin degradation and production of the 120-kDa subproduct were confirmed using specific antibodies against the α- and β-spectrin chains. The same degradation effect was observed in α-fodrin from epithelial HEp-2 cells, both in purified cell membranes and in cultured cells which had been held in suspension for 36 h; these effects were confirmed using antifodrin rabbit antibodies. The spectrin and fodrin degradation caused by Pet is related to the Pet serine protease motif. Fluorescence and light microscopy of HEp-2 Pet-treated cells showed morphological alterations, which were associated with irregular distribution of fodrin in situ. Spectrin and fodrin degradation by Pet toxin were inhibited by anti-Pet antibodies and by phenylmethylsulfonyl fluoride. A site-directed Pet mutant, which had been shown to abolish the enterotoxic and cytotoxic effects of Pet, was unable to degrade spectrin in erythrocyte membranes or purified spectrin or fodrin in epithelial cell assays. This is a new system of cellular damage identified in bacterial toxins which includes the internalization of the protease, induction of some unknown intermediate signaling steps, and finally the fodrin degradation to destroy the cell.

**Enteraggregative *Escherichia coli* (EAEC)** is a group of bacteria characterized by the ability to adhere to cultured cell monolayers in a “stacked brick” adhesion phenotype (27). There is increasing evidence that EAEC is strongly associated with persistent diarrheal disease in children in India, Brazil, Mexico, Bangladesh, and other areas in the developing world (4, 9, 14, 18, 26). The participation of EAEC strains in several outbreaks of diarrhea in children and adults has also been reported in developing and developed countries such as Serbia (8), Mexico (C. Eslava, J. Villaseca, R. Morales, A. Navarro, and A. Cravioto, Abstr. 93rd Gen. Meet. Am. Soc. Microbiol. 1993, abstr. B-105, 1993), Japan (21), the United Kingdom (36), and Germany (20). In addition, the participation of EAEC as the causative agent of diarrheal disease in human immunodeficiency virus-infected adults in the developed world has also been suggested (24).

The pathogenesis of EAEC infection is not completely understood, although histopathologic alterations of intestinal epithelium from patients and animal models infected with EAEC have been reported. Formation of a thick mucous gel on the intestinal epithelium mucosa was observed in gnotobiotic piglets inoculated with EAEC (38). Hicks et al. (19), using an in vitro organ culture model, observed that EAEC strains were embedded within a mucus-containing biofilm and exfoliation of enterocytes from the mucosal surface of intestinal biopsies. Vial et al. (39), using the rabbit and rat ileal loop models inoculated with EAEC strains, observed lesions characterized by shortening of the villi, hemorrhagic necrosis of the villous tip, and a mild inflammatory response with edema and mononuclear infiltration of the submucosa. Similar histologic alterations were observed in autopsy samples of the ileum from children who died as a consequence of persistent diarrhea associated with EAEC infection (Eslava et al., Abstr. 93rd Gen. Meet. Am. Soc. Microbiol. 1993), as well as in rat jejunal preparation mounted in Ussing chambers and treated with a supernatant from EAEC (29). All these observations suggested that some of the alterations caused during EAEC infection were associated with the production of a cytotoxin.

Eslava et al. (Abstr. 93rd Gen. Meet. Am. Soc. Microbiol. 1993) identified two high-molecular-weight proteins from EAEC strains isolated from children who died as a consequence of persistent diarrhea caused by EAEC. These proteins were tested in the rat ileal loop model and were observed to cause shortening of the villi, hemorrhagic and necrotic alterations, and ulceration of the upper epithelium. The gene for one of these two high-molecular-weight proteins located on the 65-MDa EAEC virulence plasmid was cloned, and the protein was named Pet, for plasmid-encoded toxin (13). Pet sequence shows a high homology with the type IV class autotransporter-secreted proteins, including the subfamily that has been called SPATE (Tsh, EspC, and EspP from *E. coli* and ShMu and SepA from *Shigella*) (17). It has also been shown that Pet induces cytopathic effects on HEp-2 and HT29 C 1 culture cells, characterized by release of the cellular focal contact from glass substratum and rounding and detachment of cells, as well as cytoskeleton contraction and loss of actin stress fibers (30). Navarro-Garcia et al. showed with the Ussing chamber model that Pet induces enterotoxic and cytotoxic effects (29) and that these activities depend upon the serine protease motif (30). However, the specific action mechanism of Pet toxin on epithelial cells has not yet been elucidated. This study shows that
Pet toxin causes disruption of spectrin and fodrin (nonerythroid spectrin, which is distributed among the majority of cell types, including epithelial cells), proteins of the membrane skeleton that are connected with the cytoplasmic actin network. Fodrin degradation could explain the previously mentioned cellular alterations and the diarrheal pathogenesis caused by EAEC.

(Materials and Methods)

Strains and plasmids. The minimal Pet clone pCEFN1 (previously described) was constructed by cloning the pet gene of EAEC strain 042 into the BamHI/KpnI site of pSPORT1 and is expressed in E. coli HB101 (13). HB101(pCEFN1) was used to obtain Pet protein, and HB101(pSPORT1) was used as a control for cell experiments. Site-directed mutagenesis was performed to obtain the Pet serine motif mutant (Pet S260I), using the QuikChange site-directed mutagenesis kit from Stratagene exactly as described (30) and cloned in the same vector, HB101(pCEFN2). The strains were maintained on L agar or L broth containing 100 μg of ampicillin.

Protein purification. Pet protein was obtained from a culture supernatant of pet clone E. coli HB101(pCEFN1), precipitated with 75% ammonium sulfate, and further precipitated with 1.15 and 1.75 M potassium phosphate buffer, eluted from a Q-Sepharose column and then from last-protocol liquid chromatography (FPLC) Mono S HR 5/5 columns. The protein fractions were determined by the Bradford method (5), and the purified protein was analyzed by sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE) (22).

N-terminal sequence. The N-terminal sequence was determined by automated Edman degradation on a gas-phase protein sequencer (L 3000; Beckman Instruments) equipped with an online Beckman System Gold high-performance liquid chromatography (HPLC) system. The HPLC equipment included a model 126 pump and a 168-diode array detector set at 268 and 293 nm for signal and reference, respectively. The HPLC column used was the Beckman Spherogel Micro PTH (2 by 150). The standard Beckman sequencing reagents were used for the analysis.

Protein samples for sequencing were prepared by SDS-PAGE and electrophoretic transfer to nitrocellulose or polyvinylidene difluoride membranes (Millipore Co.), as described by Towbin et al. (37), as well as by direct application of desalted purified protein on Beckman protein supports.

Spectrin assay. Sheep red blood cells (SRBC; Microlab, Mexico City, Mexico) and HEp-2 cells suspended in phosphate buffer (310 mosM) were centrifuged at 1,000 × g for 10 min (three times), and the pellet was washed with the same buffer and then incubated in a phosphate buffer (20 mosM). The lysed cells were centrifuged at 20,000 × g for 40 min and the pellet obtained was washed by resuspension in hypotonic phosphate buffer followed by centrifugation at 20,000 × g for 20 min (three times) to obtain erythrocyte and HEp-2 cell membranes, which are spectrin or fodrin enriched fractions, respectively. These membranes were incubated with different Pet protein concentrations or with E. coli HB101 culture supernatants from 3 to 24 h at 37°C. Reaction mixture samples of 100 μl containing 10 ng of SRBC or 100 μg of HEp-2 cells and 0.1 to 10 μg of Pet were analyzed by SDS-6% PAGE (22). In some experiments purified spectrin (from Sigma Chemical Co., St. Louis, Mo.) was also used.

For antibody inhibition experiments, Pet protein (5 μg) was incubated for 3 h at 37°C with 10 μg of antibodies against Pet protein in 100 μl of RPMI medium (29). To analyze the participation of the serine protease motif, a reaction was performed in the presence of 2 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co.). To further confirm the role of the serine protease motif, similar concentrations of the Pet S260I protein mutant were used instead of Pet protein (30).

Pet effects on HEp-2 cells in suspension. Cultures from HEp-2 cells were detached with Puck’s solution (Gibco BRL) and washed three times with phosphate-buffered saline (PBS). HEp-2 cells held in suspension were adjusted to a final concentration of 30 μg/ml of Pet protein for 3, 6, 12, 18, 24, or 36 h. After incubation, the cells were washed three times (15 min each) by centrifugation with PBS and were lysed with SDS-PAGE Laemmli sample buffer. The HEp-2 cell proteins were separated by SDS-PAGE (22). Western immunoblot. Untreated and Pet-treated erythrocyte and HEp-2 cell membrane preparations separated by SDS-6% PAGE were transferred to nitrocellulose sheets (Schleicher & Schuell, Keene, N.H.) as described by Towbin et al. (37). Rabbit anti-alpha and anti-beta spectrin chain antibodies (Sigma Chemical Co.) were used (10−4) to analyze Pet activity on spectrin from cell membranes. The reaction was visualized using goat anti-rabbit antibodies (10−4) conjugated with alkaline phosphatase (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). To detect the α-fodrin of HEp-2 cells in suspension, rabbit antibodies against brain α-fodrin (rabbit anti-fodrin antibody 9053, kindly provided by R. Bloch) in a concentration of 10 ng/ml were used. The reaction was visualized using goat anti-rabbit antibodies (10−4) conjugated with horseradish peroxidase (Kirkegaard & Perry Laboratories) and developed using Western-light chemiluminescent reagent (Du Pont, NEN). Detection of Pet effects on spectrin in situ. HEp-2 cell suspensions were incubated during 3 h with 5 μg of Pet/ml. The cell preparations were fixed with glutaraldehyde (3% in PBS, pH 7.4) and permeabilized with Triton X-100 (0.1% in PBS, pH 7.4). The permeabilized cells were incubated with anti-alpha and anti-beta spectrin chain (Sigma Chemical Co.) rabbit antibodies or were stained with Coomasie blue (Sigma Chemical Co.) for 10 min. For immunofluorescence, the reaction was visualized using goat anti-rabbit IgG antibodies labeled with fluorescein (Kirkegaard & Perry Laboratories). The slides were observed by epifluorescence or light microscopy (Karl Zeiss).

Results

Purification of Pet protein. Pet protein was purified from the minimal clone HB101(pCEFN1); the process of purification included ammonium sulfate precipitation, and passing through a Q-Sepharose column and then an HPLC Mono S HR 5/5 column (Fig. 1). The recuperation efficiency rate of Pet protein was about 3.14%, which corresponds to 3.3 mg from 10 liters (105 mg) of overnight culture. The N-terminal amino sequence of the purified protein was determined and the sequence found (ANMDISKAWARDYLDLAQN) was the same as the previously predicted product from the pet gene of the 042 EAEC strain (13).

Effects of the Pet protein on erythrocyte membranes. In order to explore the possible effects of Pet on the cell membrane, purified erythrocyte membranes were used. After 6, 12, 18, and 24 h of incubation of 10 μg of membrane proteins with 5 μg of Pet protein in a total volume of 100 μl, Pet induced a change on the normal SDS-PAGE profile of erythrocyte membrane proteins. This was characterized by the degradation of two protein fractions of 240 and 220 kDa, molecular masses...
that corresponded to the α- and β-spectrin chains. In addition, a new protein fraction of 120 kDa was observed which corresponds to a possible main subproduct of degraded spectrin bands (Fig. 2A). A similar effect was found when a sample of 2 μg of purified erythrocyte spectrin was treated with 1 μg of Pet protein in 20 μl of reaction mixtures, showing degradation of the same protein fractions of 240 and 220 kDa and the production of a 120-kDa breakdown product (Fig. 2B).

In order to verify if the two protein fractions degraded by Pet protein correspond to α- and β-spectrin chains, a Western blot assay of Pet-treated erythrocyte membrane proteins was performed using specific antibodies against α- and β-spectrin chains. The results confirmed that the degraded 240- and 220-kDa protein fractions correspond to α- and β-spectrin chains and that the 120-kDa subproduct appeared to come from spectrin (Fig. 2C). It was also seen that the α-spectrin chain was more sensitive to Pet and that the effect was dose and time dependent. Erythrocyte membrane proteins (10 μg) treated with different doses of Pet protein (ranging from 10 ng to 5 μg of Pet in 100 μl) for 3 h of incubation showed that whereas the α- and β-spectrin bands were decreasing, the 120-kDa subproduct band was increasing. Similar results were found when the erythrocyte membrane proteins were incubated with 5 μg of Pet for different lengths of time (data not shown).

To know if the Pet effects on spectrin were specific, antibodies against Pet were used to inhibit them. These antibodies have been shown to neutralize the enterotoxic and cytotoxic activity of Pet (29, 30). Pet protein was preincubated with polyclonal anti-Pet antibodies and then incubated with erythrocyte membranes. These experiments showed that α- and
β-spectrin bands were partially degraded, with some subproducts appearing; however, the 120-kDa subproduct was not seen (Fig. 3), suggesting that this partial degradation occurred on another site.

**Role of the serine protease motif on spectrin degradation.** In order to evaluate the role of the serine protease activity of Pet on spectrin, the serine protease inhibitor PMSF was used. Pet protein, previously incubated with 2 mM PMSF, was then used in the spectrin degradation assay. PMSF inhibited the effects of Pet on spectrin (Fig. 3). In order to confirm the role of the serine protease motif on spectrin degradation by Pet, a culture supernatant partially purified from the previously described serine protease mutant, Pet S260I, was used to incubate with erythrocyte membranes. This mutant protein was unable to produce spectrin degradation (Fig. 3).

To establish the cleavage site of spectrin by Pet, the 120-kDa subproduct obtained from purified spectrin and erythrocyte spectrin degradation was analyzed to determine its N-terminal sequence. The results from the N-terminal sequence showed it to be the same as that of mature α-spectrin, which suggested that the cleavage site occurred at the C-terminal site.

**Effects of Pet on epithelial cell membranes.** To determine if Pet produces the same alteration on epithelial cells as previously seen with erythrocyte membranes, purified HEp-2 cell membranes were incubated with Pet protein for 3 h. After incubation, the SDS-PAGE protein profile showed a degradation zone around the 240- and 220-kDa protein fractions. However, a fodrin subproduct of 120 kDa was not seen, and no other subproduct was seen (Fig. 4). Similar assays, using precipitated supernatants from *E. coli* HB101, which lacks Pet protein, were unable to produce alteration in the SDS-PAGE protein profile (Fig. 4).

In order to identify Pet activity on live HEp-2 cells, cultured cells in suspension were exposed to Pet protein (10, 50, and 100 µg per ml) for 3, 6, 12, 18, 24, and 36 h and then analyzed for α- and β-fodrin degradation by Western blotting using polyclonal antibodies against brain fodrin. Using this methodology, it was seen that Pet protein caused α-fodrin degradation after 18 h of incubation (Fig. 5A) and degradation of both α-fodrin and another protein of 220 kDa (which probably corresponds to β-fodrin) at 36 h of incubation (Fig. 5B). Although the anti-brain fodrin polyclonal antibodies were unable to detect some specific subproducts, a fraction of approximately 83 kDa was increasing at the same rate as the α- and β-fodrin were degrading (Fig. 5B). The effects of Pet on HEp-2 cell fodrin also were time and dose dependent.

**Effects of Pet on HEp-2 cells in situ.** In order to detect the Pet effects on fodrin in HEp-2 cells, HEp-2 cells in suspension were treated with Pet toxin for 3 h at 37°C, stained with Coomassie blue, and observed by light microscopy. These cells showed morphological alterations characterized by damage of the cell membrane in the form of cell swelling (Fig. 6B). In contrast, the untreated cells (Fig. 6A) and those treated with Pet S260I (Fig. 6C) did not show morphological alterations and maintained their normal structure.

To determine if the morphological alterations of the HEp-2 cell membrane caused by Pet were related to fodrin disruption, the HEp-2 cells held in suspension were treated with Pet protein and visualized by immunofluorescence using anti-α- and β-spectrin antibodies. The control slides from untreated cells showed a homogeneous distribution of fluorescence, indicating that fodrin was not modified (Fig. 6D). On the other hand, the Pet-treated cell preparations showed cellular modification characterized by cellular swelling and irregular distribution of fluorescence, indicating fragmentation of fodrin (Fig. 6E) and, as a consequence, a disarrangement of the cell membrane. On the other hand, when the HEp-2 cells were treated with precipitated supernatant from the mutant, Pet S260I, the cells appeared to be normal, as seen in the control cells (Fig. 6F).

**DISCUSSION**

The cytoskeleton is a target for many intracellular microorganisms, and in some bacterial and parasite pathogens this effect is accomplished by triggering a rearrangement of the membrane skeleton (33, 34). Recently it was shown that Pet EAEC toxin elicits cytopathic effects characterized by release
of the cellular focal contact from glass substratum, as well as rounding and detachment of cells, and that these effects were associated with damage to the actin cytoskeleton (30).

The present study shows that cytoskeletal effects by Pet on epithelial cells are associated with the degradation of fodrin, an analog of spectrin. The spectrin protein accounts for 75% of the membrane skeleton protein mass in erythrocytes, and spectrin analogs (such as fodrin) are widely distributed among the majority of cell types. The spectrin-based membrane skeleton is a submembranous, spatially limited, two-dimensional lattice that binds a subset of membrane proteins (2).

The results obtained showed that one of the action mechanisms of Pet is the degradation of both α- and β-spectrin chains (from erythrocyte membranes) and α- and β-fodrin chains (from epithelial HEp-2 cell membranes). These data may explain previous observations from many other investigators who showed cell damage by EAEC. Hicks et al. (19), using the in vitro organ culture model, showed that EAEC strains induced exfoliation of enterocytes from the mucosal surface of intestinal biopsies from children. Nataro et al. (25), utilizing T84 cultured cells, observed that EAEC induced vesiculation of the microvillar membrane followed by exfoliation of cells from the monolayer. On the other hand, intestinal necropsy of Mexican children who died as a consequence of EAEC infection showed that the effector delivery may take place through a membrane fusion event or by release through exocytotic microvesicles (15). However, the morphologic effects on erythrocytes and epithelial cells as a consequence of spectrin degradation by this spectrin protease and Pet toxin are the same: cell rounding, detachment, and cell death (1, 30).

Interestingly, the most common targets for microbial pathogens, which interact with the host cells’ cytoskeleton, are in fact components of the cytoplasmic network, mostly actin, although the ability to target spectrin in the host cell has been reported for the intracellular protozoans *Plasmodium falciparum* and *Plasmodium berghei* (10).

Spectrin proteases are involved in many other mechanisms of substrate degradation on erythrocytes (spectrin) and non-erythroid cells (fodrin), such as those that occur under normal and pathophysiological conditions. Under normal conditions, there are membrane-bound proteinases that preferentially de-
grade oxidatively damaged erythrocyte membrane proteins as a secondary antioxidant defense (3). This secondary antioxidant defense mechanism for the removal of the oxidatively damaged cell membrane proteins by proteinases includes degradation of spectrin by a membrane-bound serine protease of 80 kDa (16), which produces a spectrin breakdown product of around 120 kDa, while the proteolytic activity is inhibited by the serine protease inhibitor diisopropylfluorophosphate (3). These last reports suggest that Pet toxin, the serine protease secreted by EAEC, could use the same cell pathway to degrade spectrin and fodrin from erythrocytes and epithelial cells. On the other hand, under pathophysiological conditions, calcium-activated proteases, such as the calpains, are important intermediaries connecting [intracellular Ca\(^{2+}\)] with cell death (11) through degradation of the preferred calpain substrate \(\alpha\)-spectrin (6).

Cleavage \(\alpha\)-fodrin (nonerythroid spectrin) has been detected during apoptosis in a variety of cell lines of murine and human origin and is inhibited under conditions where apoptosis is inhibited. Interestingly, in cell cultures that have undergone extensive apoptosis, fodrin is cleaved to a single detectable fragment of 120 kDa. However, in cultures containing fewer apoptotic cells, a large fragment of 150 kDa was observed (23), suggesting that the 120-kDa fragment is a further breakdown product of the 150-kDa fodrin fragment. In addition, the formation of these apoptotic nuclei in JURKAT T cells, after Fas antigen ligation, was blocked by the serine protease inhibitors TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone) and DCI and by the interleukin 1β-converting enzyme (ICE) inhibitor, VAD-FMK; but chromatin degradation and morphological changes were inhibited only by TPCK (7, 35). Nath et al. (28) have found that in cell necrosis (e.g., maitotoxin-treated neuroblastoma SH-SYSY cells), the \(\alpha\)-fodrin breakdown product of 150 kDa was produced by cellular calpains, whereas in neuronal cells undergoing apoptosis an additional breakdown product of 120 kDa was observed. The formation of the 120-kDa fragment was insensitive to calpain inhibitors but was completely blocked by ICE-like protease inhibitors. Furthermore, the authors propose that calpain and ICE can each cleave \(\alpha\)-fodrin at two sites; one is VY \(\downarrow\) GMMP for a 150-kDa fragment, which is located within a sequence in repeat 11 and just N terminal of the calmodulin-binding domain, whereas ICE cleavage for a 120-kDa fragment must be C
terminal to the PEST sequence located between repeats 12 and 13 (28). Interestingly, Pet toxin produced the 120-kDa breakdown product, and its N-terminal sequence was the same as the mature α-spectrin, indicating that the cleavage site must be C-terminal and similar to the ICE cleavage site.

In summary, many autotransporter proteins have been implicated as important or putative virulence factors in many gram-negative pathogens (17); however, none of them have been as well characterized as Pet protein from enteropathogenic E. coli, which is part of the SPATE (serine protease autotransporters of Enterobacteriaceae) subfamily. Pet toxin caused enterotoxic and cytotoxic activity involving its serine protease motif. Cytoskeleton contraction and loss of actin stress fibers were also observed, suggesting that one or more components of this cellular structure were the Pet target (30). This study showed that Pet toxin produces damage to the epithelial cells through a novel mechanism of the bacterial toxin involving internalization of the serine protease and α-fodrin degradation. Such alterations of the membrane skeleton could explain previous observations in Pet-intoxicated intestinal segments, HEP-2 and HT29 C1 cells, which showed an induction of a net secretory state, a cytoskeleton contraction, and a loss of actin stress fibers (29, 30). The proteolytic demolition of spectrin within these cells may induce a disaggregation of the membrane skeleton and of its connections with the cytoplasmic actin network, leading to membrane alteration and finally to cell death. The α-fodrin degradation by Pet toxin may occur by following the normal or pathophysiological pathway shown above, and the death of enterocytes may occur due to apoptosis, as suggested by the production of the 120-kDa spectrin breakdown product.

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