

Epithelial cell intoxication by Plasmid-encoded toxin (Pet) requires intracellular trafficking including clathrin-dependent endocytosis and retrograde transport

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Enteroaggregative *Escherichia coli* (EAEC) has been associated with persistent infant diarrhea, especially in developing countries [1]. The pathogenesis of EAEC infection is not completely understood, although histopathologic alterations of the intestinal epithelium from patients and animal models infected with EAEC have been reported [2,3]. Similar histological alterations were observed in autopsy samples of the ileum from children who died as a consequence of persistent diarrhea associated with EAEC infection [4]. We have shown that a 104 kDa EAEC protein, termed Pet (plasmid-encoded toxin), is required for EAEC-induced damage to human intestinal mucosa [5]. We have also found that Pet bears nucleotide homology to a class of serine protease autotransporter proteins (SPATEs) from *E. coli* and *Shigella* [6].

Pet increases Isc (short-circuit current) and decreases electrical resistance of rat jejunum mounted in the Ussing chamber, effects that are accompanied by mucosal damage, increased mucus release, exfoliation of cells, and development of crypt abscesses [7]. Pet appears to be a cytoskeleton-altering toxin, since it induces contraction of the cytoskeleton, loss of actin stress fibers, and release of focal contacts in HEp-2 and HT29/C1 cell monolayers, followed by complete cell rounding and detachment [8]. We have also shown that Pet cytotoxicity and enterotoxicity depend on Pet serine protease activity, since both effects are inhibited by PMSF and are not induced by Pet S260I, which is mutated in the catalytic serine and thereby lacks *in vitro* protease activity [8].

Recently, we found an intracellular target for Pet. Pet is able to cleave fodrin *in vivo*; Pet-treated HEp-2 cells reveal intracellular redistribution of fodrin after 2 h of incubation. After 3 h, when Pet has produced the cytoskeletal effects, almost all the fodrin is found in intracellular aggregates, which appear to be inside blebs. Fodrin degradation was confirmed in *in vitro* experiments, using a recombinant GST-fusion protein, representing repeat units 8-14 of human fetal brain fodrin (109 kDa). Pet generated two breakdown subproducts of 37 and 72 kDa. This proteolytic activity was time-dependent and was observed during the first 10 min until its complete degradation after 2 h. Sequencing of the 37 kDa fragment, which did not have the GST in its N-terminal, demonstrated that Pet cleavage site is localized within fodrin's 11th repetitive unit, in the helix C between M1198 and V1199, and inside the calmodulin binding domain. The change of these amino acids by site-directed mutagenesis prevented fodrin degradation by Pet. A mutant in the serine protease motif of Pet was also unable to generate fodrin redistribution in epithelial cells and to cleave recombinant fodrin-GST [9].

In addition, we have shown that Pet enters the eukaryotic cell and this internalization is required for the induction of cytopathic effects [10]. Moreover, the Pet serine protease motif is the main requisite for the cytopathic effects, because internalization assays have shown that Pet and mutant S260I are inside epithelial cells, but only native Pet produces cytopathic effects [10]. However, the mechanisms of Pet uptake and trafficking in epithelial cells are unknown. Here, we show that Pet toxin binds to the cell surface and is endocytosed by clathrin-coated vesicles. Besides, Pet toxin undergoes vesicular trafficking, which include endosomes, the Golgi apparatus, and the endoplasmic reticulum (ER). Finally, Pet is translocated from ER to cytosol by using the Sec61p complex to interact with fodrin, one of its intracellular targets.

Pet internalization by clathrin-coated vesicles was found to be the essential mechanism because two reagents for blocking this pathway totally inhibited Pet internalization (monodansylcadaverine and sucrose). Whereas drugs for blocking endocytosis (filipin and methyl- β -cyclodextrin), through caveolae mechanism, was unable to inhibit Pet effects on the cytoskeleton. These data suggest that Pet uptake in epithelial cells seems to be similar to the mechanism used by diphtheria toxin, *Pseudomonas* exotoxin A, or Shiga toxin.

Efficient Pet internalization into HEp-2 cells was affected by a tyrosine-kinase inhibitor, genistein, suggesting that phosphorylation events in tyrosine are involved in Pet endocytosis induction. Perhaps, genistein interferes with Pet endocytosis by altering the structural organization of clathrin-coated pits, since inhibition of tyrosine kinase activity blocks clathrin redistribution in the cellular periphery [11]. Another hypothesis is that the Pet receptor holds tyrosine kinase activity, such is the case for epidermal growth factor receptor, insulin receptor or albumin receptor, in which it has been determined that binding of the ligand to its membrane receptor stimulates its phosphorylation into tyrosine, leading to active endocytosis by either clathrin or caveolae dependent mechanisms [12].

An interesting finding that supports Pet endocytosis by clathrin-coated pits was Pet endocytosis inhibition by altering either actin or microtubules cytoskeleton, evidencing the participation of these cytoskeletal elements in the function of clathrin-coated pits and trafficking of proteins. Actin cytoskeleton is involved in vesicles formation and clathrin-dependent endocytosis [13]. On the other hand, the role of microtubules in endocytosis has been extensively studied and it is clear that late steps of endocytosis depend on an integrated

network of microtubules [14]. Additionally, microtubules also participate in early steps of endocytosis. Thus, it is clear that, in our system, Pet endocytosis depends on an intact actin and microtubules network for an efficient internalization.

Pet endocytosis was a rapid event in HEp-2 cells, since it was possible to find Pet in early endosomes, at 8 min of interaction. The rapid Pet endocytic process has also been observed in various bacterial toxins, in which, after their endocytosis through clathrin-dependent or -independent mechanisms [15], toxins are delivered to early endosomes and a small fraction is subsequently delivered to lysosomes for its degradation [16]. In the endocytic transport of Pet, we were able to observe that a small fraction reached the lysosomes for its possible degradation. However, most of Pet followed a transport to other organelles.

Furthermore, brefeldin A (BFA) is able to inhibit the cytoskeletal effects caused by Pet [10]. BFA disrupts the Golgi apparatus and causes multiple alterations in the vesicular transport. Inhibition of cell intoxication by BFA is classically associated with toxins, which undergo retrograde transport from the Golgi apparatus to the ER, such as cholera toxin, Shiga toxin and ricin [17]. In this work, we confirmed these findings by localizing, at sequential times, Pet in the Golgi apparatus and then in the ER. ER is an attractive compartment for translocation, since it contains factors that facilitate entry to the cytosol, by reducing or displaying proteins before their departure from the ER membrane. Additionally, the ER contains a protein machinery for translocation, named Sec61p, which is involved in the reverse translocation of misfolded proteins from the ER lumen to the cytosol. The Sec61p translocon complex appears to be responsible, in this essential pathway, for the intoxication by diverse toxins [18], since they have the ability to associate with the Sec61p complex, such as the A subunit of ricin, Pseudomonas exotoxin A, and cholera toxin. In this work, we show that Pet is able to colocalize with Sec61 α , the major protein in the Sec61p complex, and this colocalization occurred immediately after Pet localization in the ER. Additionally, antibodies against Sec61 α were able to precipitate Pet and vice versa, antibodies against Pet immunoprecipitate Sec61. Furthermore, unpublished preliminary data (Teter K, Burlingame K, Navarro-Garcia F) have shown that Pet was unable to intoxicate mutant CHO cells (cholera toxin resistant), which have translocation defects [19]. Finally, we were able to colocalize Pet with one of its intracellular targets, fodrin, just after its translocation from the ER to the cytosol, as published previously [9].

In summary, we have found that Pet toxin is endocytosed by clathrin-coated vesicles after its binding to an unknown receptor on the cell membrane, which is being further studied in our laboratory. Pet toxin follows vesicular trafficking, including endosomes, the Golgi apparatus, and the endoplasmic reticulum. From this latter organelle, Pet toxin is translocated by using the Sec61 translocon to the cytosol, where it interacts with fodrin by producing a proteolytic cleavage in this molecule, leading to cytoskeleton disruption as shown in a previous study [9]. Fodrin degradation correlates with cell damage caused by Pet, including actin cytoskeleton retraction, loss of the stress fibers, cell rounding with membrane blebs, and cell detachment from the substrate [9, 8].

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