APPLICATIONS OF CONFOCAL MICROSCOPY IN CELL MICROBIOLOGY.
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The term "cellular microbiology" was coined in 1996 (Cossart et al. 1996) to describe an emerging scientific discipline that bridged the disciplines of cell biology and microbiology. Our laboratory is focused in the study of cell biology of bacterial toxins from diahrrogenic *Escherichia coli*. One of these bacteria is enteroaggregative *E. coli* (EAEC), which has been associated with persistent infant diarrhea, especially in developing countries. We have shown that a 104 kDa EAEC protein, termed Pet (plasmid-encoded toxin), is required for EAEC-induced damage to human intestinal mucosa. Pet is a member of the autotransporter class of secreted proteins and together with Tsh, EspP, EspC, Pic, SigA, Hbp, Sat and SepA proteins comprises the serine protease autotransporter of *Enterobacteriaceae* (SPATE) subfamily. The defining feature of autotransporters is their self-contained secretion system. Pet causes raises in Isc (short-circuit current) and decreases in electrical resistance of rat jejunum mounted in the Ussing chamber, an effect that is accompanied by mucosal damage, increased mucus release, exfoliation of cells and development of crypt abscesses. 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. We have also shown that Pet cytotoxicity and enterotoxicity depend on Pet's serine protease activity, since both effects are inhibited by phenylmethylsulfonyl fluoride (PMSF) and are not induced by Pet S260I, which is mutated in the catalytic serine and thereby lacks *in vitro* protease activity. Recently, we have also shown that Pet enters the eukaryotic cell (Fig. 1) and that trafficking through the vesicular system appears to be required for the induction of cytopathic effects. Moreover, the Pet serine protease motif is the main requisite for the cytopathic effects, because the internalization assays have shown that Pet and mutant S260I are found inside epithelial cells, but that only native Pet produces cytopathic effects. All these data suggest an intracellular target for Pet. Moreover, we have found that Pet produces degradation of erythroid spectrin (Henderson et al. 2004).

Spectrin degradation by Pet depends also upon the serine-protease motif and generates a 120 kDa breakdown product after 12 h of incubation. Fodrin is a ubiquitous cytoskeletal protein involved with linking integral membrane proteins to cortical actin filaments, as well as organizing receptor domains and possibly the control of vesicle traffic at the plasma membrane. This linkage appears to be posttranslationally regulated, and several mechanisms that might control this process have been identified. Two events appear to be important for this regulation: binding of calmodulin to the α subunit of fodrin and the proteolysis of fodrin by calcium-dependent neutral proteases.

Since Pet is delivered into the intestine during the EAEC-epithelial cell interaction and fodrin is a non-erythroid spectrin that is found in epithelial cells, we have investigated further the interaction between Pet and fodrin. We have also shown that Pet is found in cellular vacuoles and then localized perinuclearly. To understand the internalization mechanism of Pet into the epithelial cells and the interaction with its intracellular target, we performed cellular microbiology strategies by using confocal microscopy.

Pet binding at the cell surface and saturation was achieved at a concentration of about 100 nM. Pet is internalized via clathrin-mediated endocytosis since monodansyl cadaverine and sucrose inhibited internalization of Pet, but not filipin, which is known to interfere with protein entry via caveolae. Pet is subsequently transferred to an endosomal compartment. Finally, Pet undergoes a retrograde transport, since confocal microscopy revels a co-localization between Pet and Golgi apparatus and then Pet and endoplasmic reticulum. Such transport does not involve KDEL (or RDEL) retrieval motif. Moreover, Brefeldin A, a drug that disrupts the Golgi apparatus, inhibits the effects of Pet on epithelial cells. We have found an intracellular target, α-fodrin (αII spectrin), for

Pet. Pet binds and cleaves (between M^{1198} and V^{1199}) epithelial fodrin *in vitro* and *in vivo*, causing fodrin redistribution within the cells to form intracellular aggregates as membrane blebs.

All these data indicated that Pet internalization into the cell involves a series of steps: (i) binding, via receptor to cell surface; (ii) uptake into the cell by clathrin-dependent endocytosis; (iii) entry of the toxin into early endosomes; (iv) transfer, by vesicular transport, of Pet from endosomes to the Golgi apparatus; (v) retrograde vesicular transport through the Golgi complex to reach the endoplasmic reticulum; (vi) translocation to the cytosol (vii) and (viii) interaction with fodrin to cleave and damage the cell cytoskeleton. Cytoskeleton contraction due to α-fodrin cleavage by Pet may explain our previous observations and from many other investigators who showed cell damage by EAEC. The enterotoxic effects produced by Pet could be due to the disruption of the membrane skeleton because α-fodrin was found to form a macromolecular complex with epithelial sodium channels, and epithelial channels mediate entry of Na from the luminal fluid into cells during the first stage of electrogenic transepithelial Na transport across Na-reabsorbing epithelia, this explains the diarrheal pathogenesis caused by EAEC. The damage of epithelial cells by EAEC strains in intestinal necropsy of Mexican children, *in vitro* organ culture model, in T84 cultured cells, or directly by Pet in HEp-2 and HT29 cultured cells, *in vitro* organ culture, or intestinal preparation mounted in Ussing chambers are due to cytoskeletal disruption and cell detachment produced by α-fodrin disarrangement. Interestingly, both enterotoxic and cytotoxic effects depended upon the serine protease motif, the active site used for α-fodrin degradation. Furthermore, Pet internalization is needed to produce the cytotoxic and cytoskeletal damage, since α-fodrin is an intracellular protein.

Thus, this mechanism appears to be a new system of cellular damage identified in bacterial toxin, which includes the internalization of the protease to allow finally specific α-fodrin degradation to destroy the cell.

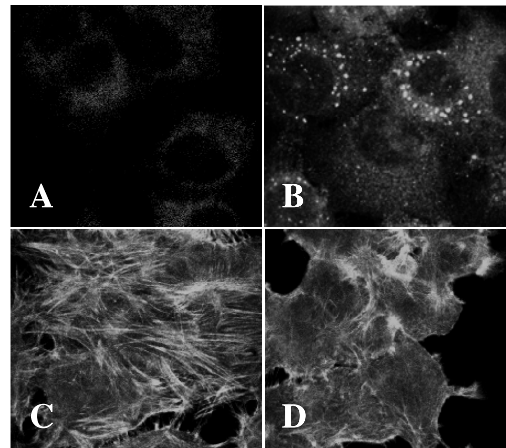

Fig. 1. Detection of Pet within epithelial cells by confocal microscopy. HEp-2 cells were treated with Pet for 3 h. After treatment the cells were fixed and processed for FAS and immunostaining. Untreated HEp-2 cells stained with anti-Pet (A) or FITC phalloidin (C); HEp-2 cells treated with Pet and stained with anti-Pet (B) or FITC phalloidin (D). Note that Pet is internalized (B) and once inside the cells Pet is able to produce cytoskeleton damage (D).

References

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