INVOLVEMENT OF ATP-DEPENDENT PSEUDOMONAS EXOTOXIN TRANSLOCATION FROM A LATE RECYCLING COMPARTMENT IN THE LYMPHOCYTE INTOXICATION PROCEDURE

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Pseudomonas exotoxin (PE) is a cytotoxin which, after endocytosis, is delivered to the cytosol where it inactivates protein synthesis. Using diaminobenzidine cytochemistry we found over 94% of internalized PE in transferrin (Tf) -positive endosomes of lymphocytes. When PE translocation was examined in a cell-free assay using purified endocytic vesicles more than 40% of endosomal ^{125}I-PE was transported after 2 h at 37°C, while a toxin inactivated by a point mutation in its translocation domain was not translocated.

Sorting endosomes did not allow cell-free PE translocation, whereas active PE transmembrane transport was observed after > 10 min endocytosis when PE and fluorescent-Tf were localized, by confocal immunofluorescence microscopy within a rab5-positive, rab4- and rab7-negative recycling compartment in the pericentriolar region of the cell. Accordingly, when PE delivery to this structure was inhibited using a 20°C endocytosis temperature, subsequent translocation from purified endosomes was impaired. Translocation was also inhibited when endosomes were obtained from cells labeled with PE in the presence of brefeldin A, which caused fusion of translocation-competent recycling endosomes with translocation-incompetent sorting elements. No PE processing was observed in lymphocyte endosomes, the full sized toxin was translocated and recovered in an enzymatically active form.

ATP hydrolysis was found to provide directly the energy required for PE translocation. Inhibitors of endosome acidification (weak bases, protonophores or bafilomycin A1) when added to the assay did not significantly affect ^{125}I-PE translocation, demonstrating that this transport is independent of the endosome-cytosol pH gradient. Nevertheless, when ^{125}I-PE endocytosis was performed in the presence of one of these molecules, translocation from endosomes was strongly inhibited, indicating that exposure to acidic pH is a prerequisite for PE-membrane traversal.

When applied during endocytosis, treatments that protect cells against PE intoxication (low temperatures, inhibitors of endosome acidification and brefeldin-A) impaired ^{125}I-PE translocation from purified endosomes.

We conclude that PE translocation from a late receptor-recycling compartment is implicated in the lymphocyte intoxication procedure (Mol. Biol. Cell, Februrary 1998).

THE PORE-FORMING TOXIN AEROLYSIN INTERACTS WITH CHOLESTEROL RICH MICRODOMAINS OF HOST CELL PLASMA MEMBRANE

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Aerolysin is one of the major virulence factors produced by the human pathogen *Aeromonas hydrophila*. The toxin is secreted as an inactive precursor, proaerolysin. In vitro studies have shown that activation occurs through proteolytic removal of a C-terminal peptide. The toxin then acquires the capacity to perforate biological membranes. We have studied the interactions of proaerolysin with cultured mammalian cells. We have established that proaerolysin binds to BHK and CHO cells via GPI-anchored receptors. The protoxin is subsequently processed to the active form by host cell proteases (see below) and can thereby form channels in the plasma membrane allowing selective transport of small ions. Presumably due to its effect on the plasma membrane, the toxin also leads to dramatic vacuolation of the endoplasmic reticulum without however affecting other intracellular compartments. The aim of the present work was to examine the early steps of the interaction with the plasma membrane, i.e.locate the toxin precisely on the plasma membrane and identify the host proteases that activate the protoxin. We have first shown biochemically that the protoxin is associated with detergent insoluble microdomains, or cholesterol-glycolipids rafts. By double labeling immunofluorescence, we then demonstrated that the toxin clusters in caveolae as well as in non-caveolar microdomains also containing clustered alkaline phosphatase.

We have then identified the proprotein convertase furin as being the main endoprotease involved in the processing of proaerolysin in CHO cells by using a furin inhibitor as well as CHO cells deficient in this enzyme. Proaerolysin could also be activated by purified furin in vitro as well as PC4, PC5 and PC6, other members of this family of proprotein convertase. Proteolytic cleavage appeared not to occur in the plasma membrane microdomains. Indeed treatment of cells with cholesterol depleting drugs as beta-methylcyclodextrin, which disrupts cholesterol-glycolipids rafts, led to a significant increase in the kinetics of protoxin cleavage. However since association of the toxin with microdomains lead to clustering, we speculate that this local increase in this toxin concentration will promote oligomerization and thereby channel formation. This however remains to be demonstrated.

STUDY OF SHIGA TOXIN B-FRAGMENT RETROGRADE TRANSPORT: DEVELOPMENT OF INTRACELLULAR TARGETING TOOLS WITH POTENTIAL APPLICATIONS IN CANCER THERAPY

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We have previously shown that in HeLa cells, Shiga toxin B-fragment is transported from the plasma membrane via endosomes and the Golgi apparatus to the ER (Johannes et al. (1997), J. Biol. Chem., 272, 19554-19561). We have now analyzed B-fragment transport in cells of the hematopoietic lineage, i.e. in macrophages and dendritic cells. In these cells, which expressed the toxin receptor Gb_3, the protein was internalized into early endosomes via a phagocytosis-independent mechanism. A significant fraction of internalized molecules was then degraded in late endosomes or lysosomes. No protein seemed to be transported into the biosynthetic/secretory pathway, as judged from morphological studies and from the absence of glycosylation and sulfation on modification site carrying B-fragments. Surprisingly, however, we found in cell fractionation and immunofluorescence experiments that about 10% of internalized B-fragment was transported to the nucleus. In the nucleus, the protein was localized in nucleoli, and we identified the nucleolar protein B23 as a B-fragment interacting partner. This finding is of potential importance for the understanding of Shiga toxin function since the toxin's target, i.e. ribosomal rRNA, is transcribed in nucleoli and pre-ribosomes are assembled here. We are currently studying the mechanism of B-fragment transport into the nucleus of these cells.

In the light of its capacity to cross membranes and considering the fact that the B-fragment receptor is specifically found on cells of the immune system (this study and C. Lingwood, personal communications), we then tested whether the B-fragment could be used to target immunogenic peptides into the MHC class I restricted antigen presentation pathway, a prerequisite to induce cytotoxic T lymphocytes which have been shown to represent an important component of the protective and therapeutic immune response to viral infections and tumors. We show that a B-fragment coupled to an immunogenic peptide (Mage 1) could be presented by peripheral blood mononuclear, B lymphoblastoid and dendritic cells in an MHC class I restricted manner, and that presentation depended on intracellular processing of the protein. The ability of Shiga toxin B-fragment to target dendritic cells and B cells and to direct antigen into the class I restricted pathway makes it an attractive non-living and non-toxic vaccine vector.

SHIGELLA IPA PROTEINS: BIOCHEMISTRY AND INTERACTIONS WITH HOST CELLS

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Shigella flexneri is the etiological agent of shigellosis, also called bacillary dysentery. This Gram-negative bacterium causes disease by invading the colonic mucosa. Entry into epithelial cells is triggered by host cell contact-dependent secretion of a group of proteins termed Ipa proteins (invasion protein antigen). The secretion of these proteins leads to the rapid formation of a macropinocytic-like ruffle which engulfs the bacterium and allows it to gain access to the cell interior.

Our goal is to identify the biochemical interactions between Ipa proteins and their mechanism of translocation to host cells and their cellular targets. To this aim we have developed a method to induce the bacteria to secrete Ipa proteins in concentrated form. The secreted proteins are soluble as judged by sucrose density gradient analysis. Gel filtration chromatography allowed us to determine the "native" molecular weight of these proteins and their possible intermolecular associations.

We are now investigating the interaction of these proteins with host cells. We have found that the secreted protein suprenatants lead to neither actin rearrangments nor trypan blue permeability in HeLa cells. Unlike for *Yersinia*, we also see no induction of trypan blue permeability even after incubation of wild-type bacteria with host cells for several hours in the presence of cytochalasin D. We are thus investigating in parallel using radio-labelled bacteria or secretion supernatants the potential association/translocation of Ipa proteins with/into cells. Preliminary data indicate that IpaB and IpaC, which are expected from their null mutants phenotypes and hydrophobicity to interact with cell membranes, are indeed present in the microsomal fraction of cells exposed to bacteria under conditions which prevent bacterial entry. IpaB and IpaC seem to associate much less efficiently, if at all, with the membranes of cells exposed to soluble Ipa proteins instead of bacteria.

Our future aims are to study the translocation of Ipa proteins into host cells using *in vitro* techniques developed for studying the post-translational translocation/association of proteins into/with the endoplasmic reticulum and to gain insight into the secretion mechansim on the bacterial side by reconstituting it using a permeabilised cell system.