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IpaD is localized at the tip of the *Shigella flexneri* type III secretion apparatus

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Abstract

Type III secretion (T3S) systems are used by numerous Gram-negative pathogenic bacteria to inject virulence proteins into animal and plant host cells. The core of the T3S apparatus, known as the needle complex, is composed of a basal body transversing both bacterial membranes and a needle protruding above the bacterial surface. In *Shigella flexneri*, IpaD is required to inhibit the activity of the T3S apparatus prior to contact of bacteria with host and has been proposed to assist translocation of bacterial proteins into host cells. We investigated the localization of IpaD by electron microscopy analysis of cross-linked bacteria and mildly purified needle complexes. This analysis revealed the presence of a distinct density at the needle tip. A combination of single particle analysis, immuno-labeling and biochemical analysis, demonstrated that IpaD forms part of the structure at the needle tip. Anti-IpaD antibodies were shown to block entry of bacteria into epithelial cells.

Keywords: IpaD; Needle complex; Type III secretion system; *Shigella flexneri*; Electron microscopy

1. Introduction

Many Gram-negative pathogenic bacteria use a type three secretion (T3S) system to interact with cells of their host. Each T3S system consists of a secretion apparatus (T3SA) that spans the bacterial envelope and extends on the bacterial surface, translocators that transit through the T3SA and insert into the membrane of the host cell where they form a pore, effectors that transit through the T3SA and the translocator pore to reach the cell cytoplasm, specific chaperones that associate with translocators and effectors in the bacterial cytoplasm and transcriptional regulators. Approximately 15 proteins are required for assembly of the T3SA.

Bacteria belonging to the *Shigella* genus are the causative agents of bacillary dysentery in humans [1]. Genes required for entry of bacteria into epithelial cells and inducing apoptosis in macrophages are clustered in a 30-kb region, designated the entry region, of a 220-kb virulence plasmid. The entry region contains 20 *mxi* and *spa* genes encoding components of the T3SA, the *ipaA*, *B*, *C* and *D*, *ipgB1*, *ipgD* and *icsB* genes encoding proteins that transit through the T3SA, the *ipgA*, *ipgC*, *ipgE* and *spa15* genes encoding chaperones, and the *virB* and *mxiE* genes encoding transcriptional regulators [2].

The T3SA, which is weakly active in bacteria growing in broth, is activated upon contact of bacteria with epithelial cells [3]. Inactivation of *ipaB*, *ipaC* or *ipaD*, as well as most *mxi* and *spa* genes, abolishes the ability of bacteria to enter epithelial cells, induce apoptosis in macrophages and express contact hemolytic activity. IpaB and IpaC contain hydrophobic segments and remained associated with the membrane of lysed erythrocytes, suggesting that these two proteins are components of the *S. flexneri* translocator. In addition, effector functions have been proposed for IpaB and IpaC [4–8]. Inactivation of *ipaB* and *ipaD*, but not *ipaC*, leads to a deregulated, i.e. constitutively active, T3SA, suggesting that IpaB and IpaD play a role in maintaining the T3SA inactive in

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the absence of inducers [9,10]. A small proportion of IpaD is associated with the bacterial envelope [9,11]. Picking and collaborators [12] reported that the role of IpaD in the control of the T3SA activity can be separated from its role in entry of bacteria into epithelial cells.

To get further insights on the structure of the needle complex, we performed an immuno-electron microscopic analysis on bacteria treated with the cross-linking agent BS^3, both on entire bacteria and on the mildly purified needle complex (NC). We present evidence that IpaD is a component of the NC localized at the tip of the needle and that antibodies raised against IpaD have an inhibitory effect on entry of *S. flexneri* into epithelial cells.

2. Materials and methods

2.1. Bacterial strains and growth media

Strains used in this study are the wild-type *S. flexneri* 5 strain M90T-Sm [13], its *ipaD* derivative SF622 [14]. Bacteria were grown in tryptic casein soy broth (TSB) (Sigma) at 37 °C.

2.2. Purification of NC

NCs were purified as described [15]. Bacteria in the exponential phase of growth in 1 l of TSB at 37 °C were collected by centrifugation, resuspended in 25 ml of phosphate-buffered saline and incubated in the presence of 1 mM Bis (Sulfosuccinimidyl)suberate (BS^3) for 30 min at 37 °C. The mixture was supplemented with 100 mM Tris–HCl and incubated for 15 min at 37 °C. BS^3-treated cultures were harvested and resuspended in an ice-cold lysis buffer (0.5 M sucrose, 20 mM Tris–HCl [pH 7.5], 2 mM EDTA, 0.5 mg/ml lysozyme) supplemented with 1 mM phenylmethylsulfonyl fluoride and incubated for 45 min at 4 °C and for 15 min at 37 °C. Resulting spheroplasts were incubated with 0.01% Triton X-100 for 30 min and treated with 4 mM $MgCl_2$ and 80 μg/ml DNAse (Sigma) for 20 min at 30 °C. Debris were removed by centrifugation (20,000 × *g* for 20 min at 4 °C) and the membrane fraction was pelleted by centrifugation (110,000 × *g* for 30 min at 4 °C) and resuspended in TET buffer (20 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.01% Triton X-100). Immunoblotting analysis was performed with antibodies raised against MxiJ, MxiN and IpaD as described [16].

2.3. Electron microscopy and image analysis

Whole cells and samples of purified NCs were negatively stained with 2% uranyl acetate on glow discharged carbon-coated copper grids. Electron microscopy was performed on a Philips CM120FEG equipped with a field emission gun operated at 120 kV. Images were recorded with a 4000 SP 4 K slow-scan CCD camera at 80,000× magnification at a pixel size (after binning the images) of 3.75 Å at the specimen level, with "GRACE" software for semi-automated specimen selection and data acquisition [17]. Single particle analysis including multi-reference and non-reference procedures, multivariate statistical analysis and classification was performed as described [15]. For immuno-labeling, purified NCs were incubated with affinity purified IpaD polyclonal antibodies (pAbs) at a final concentration of 0.132 ng/μl) for 1 h at 20 °C. Samples were stained with 2% uranyl acetate and observed as above.

2.4. Invasion assay

Two ml of cultures of wild-type or *mxiD* strains in the exponential phase of growth ($OD_{600\ nm}$ of 0.4) were washed twice with 2 ml EBSS before incubation. They were incubated in the presence of serum from rabbits immunized against IpaD (anti-IpaD dilution 1/2000:0.132 ng/μl to 1/50: 15.6 ng/μl) or anti-IpaB (1/50) (same concentration) antibodies for 1 h at 37 °C and bacteria were centrifuged on plates containing 2×10^5 HeLa cells for 10 min at 2000 × *g*. After 1 h incubation at 37 °C, cells were washed three times with 2 ml EBSS and incubated during 1 h with 2 ml MEM milieu containing 50 μg/ml gentamycin. After three washes with 2 ml EBSS, plates were incubated with a solution of deoxycholate 0.5% for 15 min at 20 °C and cell lysates were diluted and plated on agar plates for colony counting.

3. Results

3.1. A distinctive structure at the tip of the T3SA needle

Protein purification procedures tend to select for most stable complexes that might not contain weakly associated subunits. We recently showed that a Triton-X100 detergent concentration as low as 0.01% was sufficient to induce the release of NCs from the membrane [15]. To detect potentially labile subunits attached to the needle, we performed a cross-linking step with BS^3 on bacteria prior to any purification. Electron microscopy analysis indicated that, following BS^3 treatment, most bacteria exhibited needle appendages with an additional density at the extremity of the tip (Fig. 1).

NCs were purified from BS^3-treated bacteria after detergent solubilization of membranes as described [18]. Preparations contained a sufficient number of NCs with the additional densities at the needle tip to perform a structural analysis. To

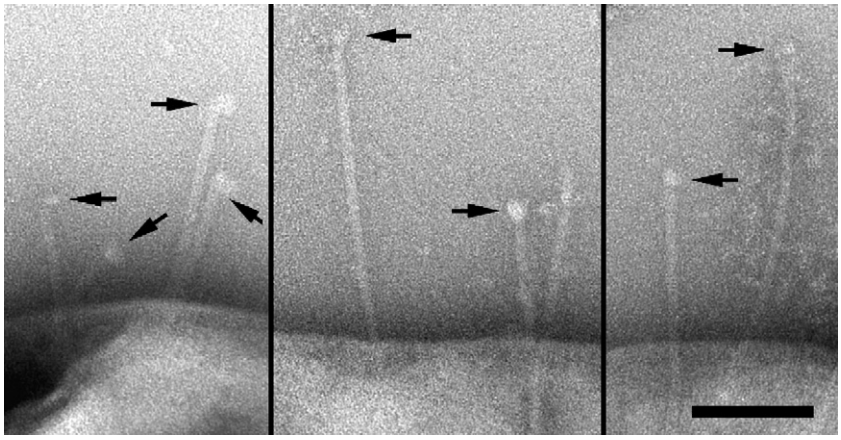

Fig. 1. Electron micrographs of negatively stained bacteria treated with BS^3. Arrows indicate distinct densities at the tip of needles protruding from the bacterial surface. The bar represents 100 nm.