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The *Shigella flexneri* OspB effector: an early immunomodulator

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ABSTRACT

Through the action of the type three secretion system (T3SS) *Shigella flexneri* delivers several effectors into host cells to promote cellular invasion, multiplication and to exploit host-cell signaling pathways to modulate the host innate immune response. Although much progress has been made in the understanding of many type III effectors, the molecular and cellular mechanism of the OspB effector is still poorly characterized. In this study we present new evidence that better elucidates the role of OspB as pro-inflammatory factor at very early stages of infection. Indeed, we demonstrate that, during the first hour of infection, OspB is required for full activation of ERK1/2 and p38 MAPKs and the cytosolic phospholipase A_2 ($cPLA_2$). Activation of $cPLA_2$ ultimately leads to the production and secretion of PMN chemoattractant metabolite(s) uncoupled with release of IL-8. Moreover, we also present evidence that OspB is required for the development of the full and promptly inflammatory reaction characteristic of *S. flexneri* wild-type infection in vivo. Based on OspB and OspF similarity (both effectors share similar transcription regulation, temporal secretion into host cells and nuclear localization) we hypothesized that OspB and OspF effectors may form a pair aimed at modulating the host cell response throughout the infection process, with opposite effects. A model is presented to illustrate how OspB activity would promote *S. flexneri* invasion and bacterial dissemination at early critical phases of infection.

Introduction

The human enteropathogenic bacterium *Shigella flexneri* invades the colonic mucosa and induces an intense inflammatory reaction that leads to tissue destruction and the dysenteric syndrome. Efficient invasion of epithelial cells of the colonic mucosa by *S. flexneri* occurs mainly at the basolateral membrane, through access to the submucosa. This access is accomplished via three distinct mechanisms: transcytosis through M cells, tight junction disruption or induction of polymorphonuclear (PMN) leucocyte transepithelial migration (Ashida et al., 2011). The latter two mechanisms maximize the available surface for bacterial entry into host cells. Following invasion, bacteria break free in the cell cytosol, replicate intra-cellularly and, using actin-based motility, disseminate from cell to cell without extracellular steps (Ashida et al., 2011).

Critical to its virulence, *S. flexneri* expresses a type III secretion system (T3SS) to inject type III effectors into the surrounding space and directly into the host cell cytoplasm (Parsot, 2009; Sasakawa, 2010; Ashida et al., 2011). Secreted effectors enable the bacteria to be internalized, to survive intra-cellularly, to spread the infection to adjacent cells and to modulate the host inflammatory and immune responses (Sasakawa, 2010). Effectors are classified into three categories depending on whether their expression is not, partially or fully controlled by the T3SS activity (Le Gall et al., 2005; Parsot, 2009). Early effectors are produced and presumably stored in the cytoplasm prior to activation of the T3SS and, therefore, are readily secreted into host cells to induce bacterial internalization (Le Gall et al., 2005; Ashida et al., 2011). Late effectors are produced only after activation of the T3SS apparatus and are likely to be involved at a later stage. In addition, middle effectors are produced both before and after T3SS activation and could be involved

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in both situations. It has been shown that the capacity of *S. flexneri* to divert host immune response for its growth and deeper dissemination success relies mainly on the control of mitogen-activated protein kinase (MAPK) signaling pathways (Ashida et al., 2011; Sperandio and Sansonetti, 2014). Consequently, the activity of key factors involved in all the three well-defined MAPK pathways, the extracellular signal-regulated protein kinases (ERK1/2), the p38 kinases, and the c-Jun NH_2-terminal kinases (JNK1/2), is subjected to attack by multiple bacterial effectors (Zurawski et al., 2006; Arbibe et al., 2007; Li et al., 2007; Zurawski et al., 2009). Although much progress has been made recently in the molecular and biochemical characterization of these T3SS effectors (Marteyn et al., 2012; Carayol and Tran Van Nhieu, 2013), a defined role for the OspB effector in *S. flexneri* pathogenesis has not been elucidated yet. Along with VirA, OspF, and OspC1, OspB belongs to the middle effectors and it was shown to be involved in the modulation of the innate immune response (Santapaola et al., 2002; Zurawski et al., 2009). More specifically, it has been suggested that OspB may play a dual role depending on the stage of infection. At an early stage, OspB appears to induce inflammation, while at later stages it appears to attenuate the inflammatory response. However, so far, there are no data available on the targets or the molecular mechanism by which OspB modulates the host inflammatory response at the very early stages of infection.

In this study, we show for the first time how OspB manipulates the host inflammatory response at the early phase of infection. We demonstrate that, during the first hour of epithelial cell infection, the absence of the OspB protein causes (i) a marked slowdown in the re-phosphorylation kinetics of ERK1/2 and p38 MAPKs and in the activity of the cytosolic phospholipase A2 (cPLA2) enzyme; and (ii) a significant reduction in the secretion of a PMN chemoattractant(s) into the culture medium, independently of the secretion of inflammatory cytokines, such as interleukin-8 (IL-8). Moreover, by using an in vivo infection model (Sereny test), we also show that lack of OspB activity is linked to a dramatic reduction of the intensity and a significant delay in the onset of inflammatory response. The data reported herein add new insights on the role of OspB in *S. flexneri* pathogenesis, extending our knowledge on the molecular mechanism utilized by this pathogen during bacterial invasion and multiplication.

Materials and methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *S. flexneri* strains were plated on Trypticase soy broth agar (TSA) plates (BBL Microbiology Systems) containing 0.01% Congo Red (CR) and grown on Luria-Bertani (LB) broth (Sambrook and Russel, 2001). When required, antibiotics were included at the following concentrations: ampicillin (Ap), 100 μg/ml; chloramphenicol (Cm), 30 μg/ml; tetracycline (Tc), 5 μg/ml.

Tissue culture

The human cell lines Caco-2 and HeLa (Santapaola et al., 2006) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and grown in the presence of 5% CO_2 at 37 °C. *S. flexneri* invasion of semi-confluent monolayers was carried out at a multiplicity of infection (MOI) of 150, using the gentamicin protection assay, as previously described (Santapaola et al., 2002).

DNA manipulations

Plasmid DNA extraction, purification of DNA fragments, construction of recombinant plasmids, restriction digestion, DNA transformation, electroporation, and electrophoresis were performed by standard methods (Sambrook and Russel, 2001). The *ospB*::3×FLAG fusion encompassing the native promoter and the entire *ospB* gene fused in frame with the 3×FLAG tag was obtained by PCR amplification using HND549 (M90T *ospB*::3×FLAG) DNA as the template and the primer pair reported in Table 1. The amplicon was *Eco*RI-digested and cloned into the corresponding sites of the pACYC184 vector (Fermentas), thus yielding plasmid pOspB. This plasmid was checked by DNA sequencing and introduced by electroporation into the *S. flexneri ospB* null mutant, strain HND201, thereby generating *ospB*/pOspB.

Serény test

The Serény test was performed as previously described (Serény, 1957). Briefly, 5.0×10^8 CFU of each strain were used to infect one guinea pig eye. Three guinea pigs were used to evaluate each strain used, and symptoms were monitored and scored over the course of four days. The degree of the inflammatory responses was rated on the basis of time of development, severity and rate of clearance of symptoms with the following scores: 0, no reaction or mild irritation; 1, mild keratoconjunctivitis or late development and/or rapid clearing; 2, keratoconjunctivitis, but not purulent; 3, fully developed keratoconjunctivitis with purulence; 4, eyes as in 3, but unusually swollen with excessive purulence. Experiments were repeated at least twice. Animal handling and experiments were performed in accordance with Italian legislation on animal

Table 1
Bacterial strains, plasmids and primers.

Strain or plasmid or primer	Description	Source
Strain		
M90T	Wild-type *S. flexneri* serotype 5a	Sansonetti et al. (1982)
HND201	M90T Δ*ospB*; susceptible	Santapaola et al. (2002)
HND53	M90T Δ*virB::aphA*-3; Km^r	Santapaola et al. (2002)
HND549	M90T *ospB*-3×FLAG; susceptible	Santapaola et al. (2002)
Plasmid		
pACYC184	Low copy number cloning vector; Cm^R	Fermentas
pOspB	pACYC184 derivative carrying the *ospB* gene fused with 3×FLAG	This study
Primer[a]		
ospBFw	5′-GGGGAATTCGGTAAGAGAAGTTCATCATAC-3′	This study
ospBRv	5′-GGGGGGAATTCCTTACTATTTATCGTCGTCATCTTT-3′	This study
ospBFw	5′-ATTTAGATGGTGTTAGACCATACTGT-3′	Nicoletti et al. (2008)
ospBRv	5′-GATAGAACATCATTGCTATCACAA-3′	Nicoletti et al. (2008)
rrsAFw	5′-CACGATTACTAGCGATTCCGACTT-3′	Nicoletti et al. (2008)
rrsARv	5′-CGTCGTAGTCCGGATTGGA-3′	Nicoletti et al. (2008)

Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance.
[a] The *Eco*RI restriction sites are underlined.