Crystal structure of *Shigella flexneri* effector OspI

Many pathogenic gram-negative bacteria such as *Shigella flexneri* deliver virulence factors, called effectors, into host cells via the type 3 secretion system (T3SS) [1]. During bacterial entry into host cells and subsequent intracellular multiplication, the host cells can sense bacterial entry as damage-associated molecular patterns (DAMPs) and bacterial components as various pathogen-associated molecular patterns (PAMPs) by means of cytoplasmic pattern recognition receptors (PRRs), whereby the host cells invoke the alarm signals to activate the innate immune system [2]. To counteract this host defense, some effectors mimic or hijack the host signaling pathway, whereas other effectors interfere with the host's innate immune system. To better understand these mechanisms, we searched for additional *Shigella flexneri* effectors that modulate acute inflammatory responses to bacterial invasion, and we found that OspI plays a pivotal role. In this study, we show that OspI dampens acute inflammatory responses during bacterial invasion by suppressing the tumor-necrosis factor (TNF)-receptor-associated factor 6 (TRAF6)-mediated signalling pathway. We determined the crystal structure of OspI at 2.0 Å resolution. X-ray diffraction data sets for OspI were collected at beamline **BL44XU** [3].

The overall structure of OspI has an α/β fold, with four β-strands (β1-β4), seven α-helices (α1-α7), and a 3_{10} helix. It is organized around a central antiparallel β-sheet, with α-helices packing on both sides of the sheet (Fig. 1). A search of known structures in the Protein Data Bank reveals that OspI shares structural homology with the cysteine protease family and is most closely related to AvrPphB [4] with an r.m.s.d. value of 3.3 Å. AvrPphB is a member of a superfamily of related enzymes containing papain-like cysteine proteases, acetyl transferases, deamidases, and transglutaminases. Although there is considerable divergence across this superfamily in the overall fold, a core anti-parallel β-sheet and an N-terminal helix, which packs against the β-strands, are always present. A potential catalytic triad (Cys62, His145, and Asp160) of OspI was identified through comparison with the active site of AvrPphB. Superimposition of His145 and Asp160 of OspI onto His212 and Asp227 of AvrPphB showed an excellent fit (Fig. 2). Cys62 of OspI, however, existed in three discrete conformations in the crystal structure, and the S_γ position was located on the opposite side of the active site in AvrPphB. This result suggests that the conformational changes at Cys62 are required in the catalytic action. The fractional occupancy of each conformer was estimated

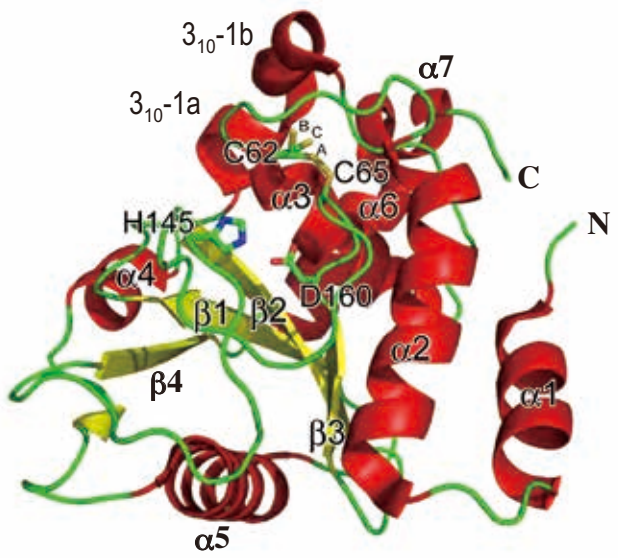

Fig. 1. Overall structure of *S. flexneri* OspI. The secondary structure elements are colored as follows: α-helices, red; β-strands, yellow; and loops, green. The active site residues are shown as stick models.

to be 0.55 (conformation A), 0.35 (conformation B), and 0.1 (conformation C). The highest occupancy site of Cys62 appeared to form a disulphide bond with Cys65 at 2.05 Å (Fig. 2). In this study, we showed that the overexpression of OspI resulted in the strong inhibition of nuclear factor-kappa B (NF-κB) activation with *Shigella* infection. OspI also selectively targeted a diacylglycerol (DAG)-dependent NF-κB signaling pathway. To characterize the active site triad (Cys-His-Asp) identified as the putative OspI catalytic center, we substituted Cys62, His145, and Asp160 with Ser (for Cys62), Ala (for His145), and Ala (for Asp160), and the resulting OspI mutants were investigated for their abilities to suppress the NF-κB activation. The elevated levels of IκBα phosphorylation and *IL-8* induction by *Δosp* infection were cancelled when infected with *ΔospI* mutant complemented by introducing each of the plasmids encoding the *ospI* (*C62S*), *ospI* (*H145A*), or *ospI* (*D160A*) gene. Consistent with this, the NF-κB reporter assay showed that OspI (C62S), OspI (H145A), and OspI (D160A) lost the ability to suppress the NF-κB activity stimulated by *Shigella*. These results supported that Cys62, His145, and Asp160 residues in OspI form the catalytic triad for suppressing NF-κB signaling.

We next investigated which of the steps in

the DAG- CARDs (CARD9, 10, 11, and 14)-Bcl10-Malt1 (CBM) complex -TRAF6-IKKβ-NF-κB pathway becomes the target for OspI. On the basis of the results in the series of experiments, we concluded that OspI interferes with TRAF6 activation in *Shigella* infection. TRAF6 is an E3 ubiquitin ligase cooperating with Ub-activating E1 and Ub-conjugating E2, such as Ubc13 and ubiquitin E2 variant (UEV1A), and to enable of self-ubiquitination, TRAF6 functions with heterodimer E2, which consists of Ubc13 and Uev1A, to synthesize K63-linked polyubiquitin chains on target proteins and TRAF6 itself [5]. This K63-linked poly-ubiquitination leads to the activation of the downstream signaling pathway. We investigated the effect of OspI on the electrophoretic mobility in a native PAGE upon incubation of OspI with each of the putative targets (TRAF6, Ubc13, Uev1A, or Ub) and found that only Ubc13 underwent mobility shift in the OspI dose-dependent manner. Hence we investigated how OspI post-translationally modified Ubc13, using LC-MS/MS. The results showed that the two overlapped tryptic digested peptides of Ubc13 underwent deamidation at Gln100 to Glu100 with OspI. To confirm deamidation of Ubc13 with OspI, we created Ubc13 (Q100E) and found it to have the same mobility shift as that of Ubc13 with Gln100 modified with OspI, but not OspI C62A, H145A, or D160A. On the basis of these results together with the capability of OspI to interact with Ubc13 *in vitro*, we concluded that OspI targets Ubc13 and causes deamination at Ubc13 Gln100, which resulted in dampening of the TRAF6-NF-κB pathway. Here, we identify OspI as a new class of T3SS effector able to selectively deamidate Ubc13, an E2 ubiquitin ligase involved in TRAF6 polyubiqitination, whereby *Shigella* can block the acute NF-κB-mediated inflammatory response at the early stage of invasion of epithelial cells (Fig. 3).

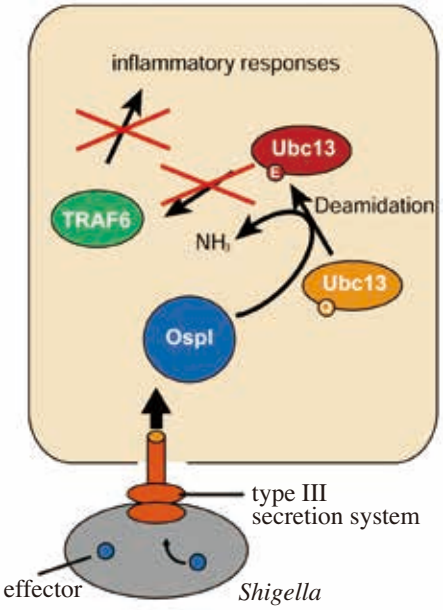

Fig. 3. *Shigella* inhibits acute inflammatory responses at the initial stage of infection. OspI acts as a glutamine deamidase and selectively deamidates Gln100 to Glu100 in Ubc13.

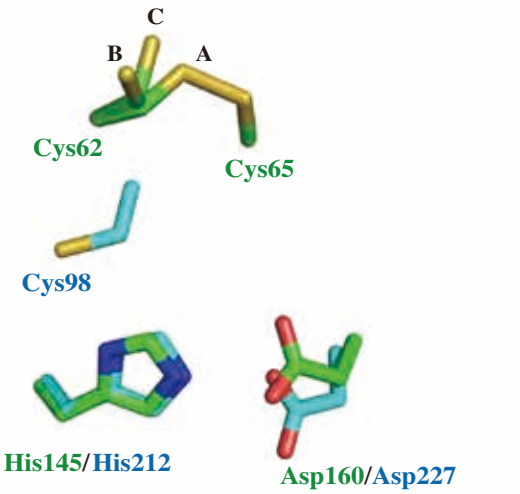

Fig. 2. Alignments of the catalytic cores of OspI with *P. syringae* AvrPphB. All atoms of histidine and the main chain atoms of aspartic acid are shown as reference. *S. flexneri* OspI (PDB ID 3B21: green) and AvrPphB (PDB ID 1UKF: cyan) are shown. C62 in OspI is represented in three alternative conformations with the three conformers labeled A, B, and C.

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