J. Microbiol. Biotechnol. (2017), 27(3), 610–615
https://doi.org/10.4014/jmb.1611.11066

jmb

Host Cell Nuclear Localization of *Shigella flexneri* Effector OspF Is Facilitated by SUMOylation

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Received: November 24, 2016
Revised: December 15, 2016
Accepted: December 19, 2016

First published online
December 20, 2016

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pISSN 1017-7825, eISSN 1738-8872

When *Shigella* infect host cells, various effecter molecules are delivered into the cytoplasm of the host cell through the type III secretion system (TTSS) to facilitate their invasion process and control the host immune responses. Among these effectors, the *S. flexneri* effector OspF dephosphorylates mitogen-activated protein kinases and translocates itself to the nucleus, thus preventing histone H3 modification to regulate expression of proinflammatory cytokines. Despite the critical role of OspF, the mechanism by which it localizes in the nucleus has remained to be elucidated. In the present study, we identified a potential small ubiquitin-related modifier (SUMO) modification site within OspF and we demonstrated that *Shigella* TTSS effector OspF is conjugated with SUMO in the host cell and this modification mediates the nuclear translocation of OspF. Our results show a bacterial virulence factor can exploit host post-translational machinery to execute its intracellular trafficking.

Keywords: *Shigella flexneri*, effector, OspF, SUMO, nuclear localization

Introduction

Shigella spp. are gram-negative mucosal pathogenic bacteria that infect colonic epithelial cells and cause infection in the large intestine in humans [1]. The bacteria are highly infectious since a small number of microorganisms may cause severe inflammatory colitis, called bacillary dysentery or shigellosis [2]. *Shigella* infection is associated with the infection and death of macrophages, followed by invasion and dissemination within the host epithelium conferring the modulation of host innate immune response [3, 4].

The pathogenicity of *Shigella* is attributed to a subset of effector proteins called virulence factors, which are delivered through the type three secretion system (T3SS) in the host cell cytoplasm [5, 6]. Intracellular effector proteins exert their function by interfering with eukaryotic enzymes, such as protein kinases, phosphatases, and E3 ubiquitin-ligases, and subvert the host cell immune response to promote bacterial survival and further colonization within the host epithelium [7].

To evade the host innate immune response, *Shigella* delivers more than 10 effector proteins. They can be divided into two groups based on the cellular compartment of their act. The first group of virulence proteins remain in the cytoplasm after injection into the host cell, where they modulate the host immune response by interfering with mitogen-activated protein kinases (MAPK) and NF-κB signaling pathways. The second group of virulence factors targets the nucleus of the host cell to suppress the expression of genes stimulated by transcription factors for innate immune responses [2, 3, 8].

OspF, along with other Osp proteins such as OspZ,

OspC1, OspB, translocate from the host cytoplasm to the nucleus [9, 10]. OspF confers phosphothreonine lyase activity and removes phosphate groups from MAPKs, such as phospho-ERK, phospho-p38, and phospho-JNK [11–13]. Moreover, nuclear-localized OspF recruits retinoblastoma protein to down-regulate histone modification, resulting in the suppression of proinflammatory cytokines such as IL-8 and CCL-20 [10, 11]. Nevertheless, the mechanism of nuclear localization of OspF has not been illuminated.

OspF is 27 kDa, and molecules smaller than 40 kDa can translocate to the nucleus by simple diffusion. However, considering the function of OspF in the nucleus and the fact that OspF is found in the nucleus within minutes of infection, it can be assumed that there is an active transport of OspF from the cytoplasm to the nucleus [11].

Protein transport to the nucleus is facilitated by conventional nuclear localization sequences (NLS), or post-translational modifications by small ubiquitin-related modifier (SUMO) [14–16]. No previously known NLS was found within the amino acid sequence of OspF. In this study, we identified two putative SUMO modification consensus sequences (ΨKXE/D) within OspF and demonstrated that one of these SUMOylation sites was indeed a target for SUMO modification. Furthermore, we showed that SUMO modification of OspF mediates its nuclear localization and it is a prerequisite for OspF to exert its nuclear activities, especially histone dephosphorylation.

Materials and Methods

Bacterial Strains and Cell Cultures

The bacterial strains used in this study were *S. flexneri* M90T (an invasive *Shigella flexneri* 5a), BS176 (a non-invasive derivative of M90T), Δ*ospF* (an *ospF* gene-disrupted mutant strain of M90T), and Δ*ospF*+pF (an OspF *trans*-complemented Δ*ospF* strain) [11]. Δ*ospF*+pF K19R and K158R (each strain was constructed by site-directed mutagenesis) were derivatives of Δ*ospF*+pF, on which SUMOylation target sites, the 19th and 158th amino acid lysine residues [14], were substituted with arginine.

Antibodies

Anti-*myc* antibody (Sigma-Aldrich, USA), anti-SUMO-1, SUMO-2/3 antibodies (Santa Cruz, USA), anti-histone H3A antibody (Abcam, USA), anti-histone H3 phosphorylated at Ser10 and acetylated at Lys 14 (Upstate, USA), and anti-histone H3 phosphorylated at Ser10 (Santa Cruz, USA) antibodies were used for immunoprecipitation and western blot analysis according to the manufacturer's manual.

In Vivo SUMOylation Assay

In vivo SUMOylation of OspF was performed as previously described with slight modifications [17]. Briefly, HEK293T cells were transfected with pRK*myc*5-OspF, pcDNA-*His*$_6$-SUMO-1/2/3, and p*FLAG*-Ubc9 in 100 mm plates. After 48 h of transfection, cells were harvested in 50 μl of SDS sample buffer (0.5% SDS, 30% glycerol, and 150 mM Tris-Cl (pH 7.8)) and incubated for 5 min. One milliliter of lysis buffer (50 mM Tris-Cl (pH 7.8), 120 mM NaCl, 0.5% NP-40) containing protease inhibitor cocktail (Roche, Germany) and 2 mM NEM was added and further incubated for 10 min. This lysate was sonicated for 30 sec and then centrifuged for 15 min at 10°C. The supernatant was incubated with 50 μl of Ni-NTA resin (Clonetech, USA) for 16 h at 4°C. Co-precipitated OspF was detected by immunoblot assay using myc antibody (Sigma-Aldrich, USA).

Bacterial Infections

HeLa cells (1×10^7 cells in 60 mm plate) were infected with each *S. flexneri* strain at a multiplicity of infection of 100 in an antibiotics-free medium. After 1 h incubation, bacteria outside the cells were washed with gentamicin [18]. Cells were further incubated for the indicated duration and harvested for the immunoblot analyses.

Cytoplasmic and Nuclear Fraction Separation

Cells were lysed with CLB (10 mM HEPES (pH 7.8), 10 mM NaCl, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$) and homogenized by using a dounce homogenizer (Wheaton, USA). The lysate was centrifuged at 7,500 ×*g* for 5 min. The pellet (nuclei and cell debris) and supernatant (cytoplasm and membrane protein) were analyzed separately by immunoblot assay.

Results

Lys19 Is the Target Site for SUMOylation of OspF

In order to elucidate the mechanism of OspF nuclear trafficking, we searched the database for any entity within the OspF sequence that may trigger its cytoplasmic-nuclear translocation. No previously recognized canonical NLS was found within OspF. However, we identified two potential SUMO consensus sequences (ΨKXE/D) on OspF (VKSE 18~21 and VKSD 157~160). To investigate the possible SUMOylation on these SUMO consensus sequences, the lysine residues of these two SUMO consensus sites were converted to arginine individually or simultaneously. Two single-mutation clones (OspF-K19R and OspF-K158R) and a double-mutation clone (OspF-K19, 158R) of *ospF* were constructed.

HEK-293T cells were co-transfected with plasmids expressing *myc*-tagged wild-type or mutant OspF, *FLAG*-tagged SUMO conjugating enzyme Ubc9, and His-tagged SUMO-1 (SUMO-2-*His6* and SUMO-3-*His6*, when necessary).

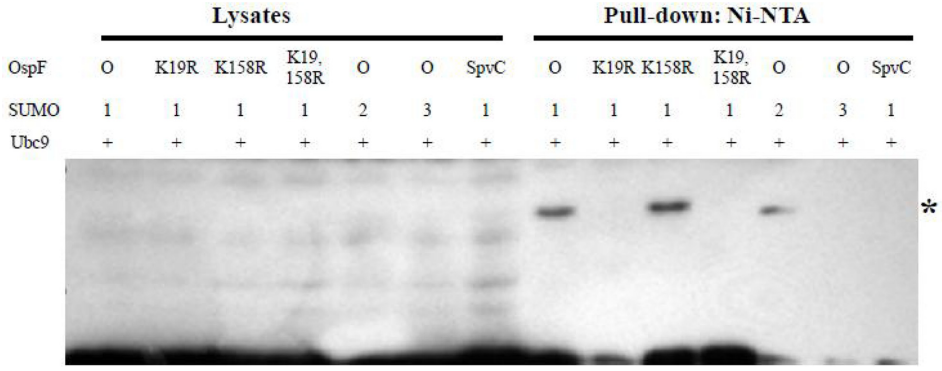

Fig. 1. K19 of *Shigella* type three secretion system effector OspF is a SUMOylation site in host cells.
HEK-293T cells were transiently transfected with SUMO-His, *FLAG*-Ubc9, and *myc*-OspF. The lysates of transfected cells were applied for pull-down by Ni-NTA. OspF was detected by western blot assay with anti-*myc* antibody. The asterisk indicates SUMOylated OspF.

SUMOylated proteins were immnuoprecipitated with Ni-NTA agarose beads and SUMOyaltion of OspF was analyzed by immunoblot assay with anti-myc antibody. As shown in Fig. 1, the wild-type OspF and OspF-K158R mutant were co-precipitated with SUMO-1, whereas OspF-K19R and the OspF-K19, 158R double mutant were not precipitated by SUMO. These results indicate that the major SUMO-binding site is located on lysine 19 of OspF.

Salmonella protein SpvC is known to have the same function as OspF in the cytoplasm to modulate host immune responses, but it does not translocate to the host nucleus [19]. We have recognized that the *Salmonella* SpvC protein does not contain any SUMO consensus sequence, and investigated if SpvC is SUMOylated at the same condition as OspF [20]. As expected, *Salmonella* SpvC was not SUMOylated under the same condition, indicating the site-specific SUMOylation of OspF (Fig. 1).

SUMOylated OspF Translocates to the Nucleus

Translocation of injected bacterial effector OspF to the nucleus has been previously reported [9]. However, the mechanism by which OspF moves in the nucleus has remained unidentified. In order to verify whether SUMOlyation is necessary for OspF translocation to the host cell nucleus, cell lysates of HEK-293T cells transfected with *myc*-tagged wild-type or mutant OspF were separated into cytoplasmic and nuclear fractions. Each fraction was analyzed for the presence of OspF by immunoblotting with anti-myc antibody. Wild-type OspF was detected in both fractions, but the signal intensity was greater in the nuclear fraction (Fig. 2). The SUMO-binding deficient mutant OspF-K19R was mainly detected in the cytoplasm although some were detected in the nucleus (Fig. 2). This result demonstrates that SUMOylated OspF translocates to the nucleus, whereas non-SUMOylated OspF remains mostly in the cytoplasm.

To verify our results and to confirm the SUMO-dependent nuclear localization of OspF, a semiconfluent monolayer of HeLa cells co-transfected with SUMO-1 together with wild-type or SUMOylation site-deficient OspF were analyzed by fluorescence microscopy. After 24 h of transfection, SUMO-1 was ubiquitously expressed in the cells. In correlation with the results on Fig. 3, the myc signal for wild-type OspF was primarily localized within the nucleus and with lesser amount in the cytoplasm, whereas the OspF-K19R mutant was mainly present in the cytoplasm (Fig. 3). These findings indicate that SUMOylation of OspF is essential for its efficient translocation to the host cell nucleus.

SUMOylation-Deficient OspF Remains in the Cytoplasm and thus Does Not Contribute to Chromatin Remodeling

OspF mediates post-translational modifications on amino acid terminal tails of histone; particularly, OspF impairs

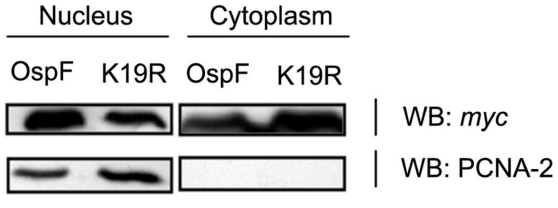

Fig. 2. SUMOylated OspF tends to translocate to the host nuclear.
HeLa cells transfected with OspF-*myc* were separated into the cytoplasm and nuclear fractions. Each fraction was immunoblotted with anti-myc antibody. Anti-PCNA-2 antibody was used as the control for the nucleus fraction.

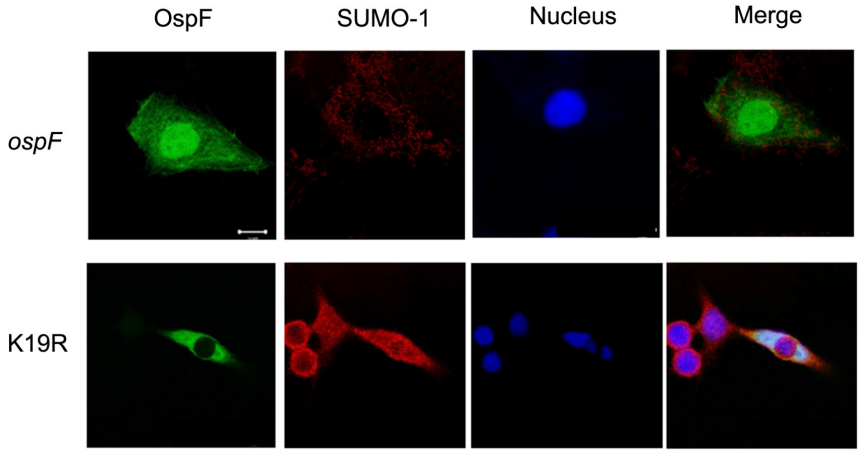

Fig. 3. Nuclear localization of SUMOylated OspF.
HeLa cells were transfected with pRK*myc*5-OspF and pRK*myc*5-OspF K19R. Co-localized OspF (green), nucleus (blue), and SUMO-1 (red) were captured using confocal microscopy. The scale bar indicates 10 μm.

the phosphorylation and acetylation of histone H3 [11]. These modifications have been correlated with transcriptional activity, as they may alter chromosome compaction and accessibility of regulatory proteins and transcription factors to the their target binding sites. To study the effect of SUMOylation on OspF functions in remodeling the host chromatin, we compared the modification level of histone H3 by SUMOylation-site mutated OspF with that of wild-type OspF (Fig. 4).

HeLa cells infected with M90T showed relatively low levels of histone H3 modification when we used antibody to histone H3 with phosphorylated Ser10 and acetylated Lys14, and antibody to histone H3 with phosphorylated Ser10. The Δ*ospF* mutant, due to the absence of OspF, maintained high levels of phosphorylation and acetylation of histone H3, as shown in Fig. 4. [11]. As a result of overexpressed OspF, HeLa cells infected with Δ*ospF*/pF *Shigella* showed reduced level of histone H3 modification, similar to the cells infected with M90T. However, the Δ*ospF*/pF K19R mutant showed a high level of modification of histone H3A (Fig. 4). These results indicate the requirement of SUMOylation for active nuclear localization of OspF.

Discussion

Post-translational modification of eukaryotic proteins by ubiquitin-like protein, SUMO, regulates the localization and functions of proteins in various cellular processes. Some pathogenic bacteria can interfere with this post-translational modification. For example, *Listeria monocytogenes*

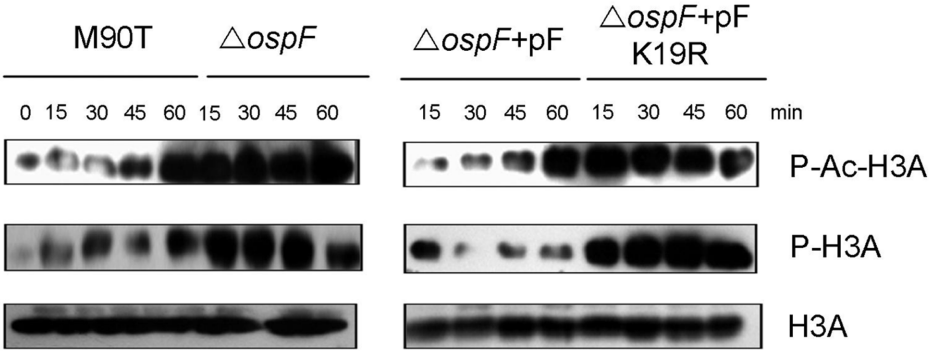

Fig. 4. SUMOylation-defective OspF does not show histone modification functions.
SUMOylation-deficient mutant OspF was not translocated in the nucleus. To detect OspF localization in the nucleus, histone modification was measured. Immunoblot assay of cell lysates by using anti-histone H3A (H3A), anti-histone H3 phosphorylated at Ser 10 (p-H3A), and anti-histone H3 phosphorylated at Ser 10 and acetylated at Lys 14 (p-Ac-H3A) antibodies.

virulence factor LLO decreases the level of SUMOylated proteins by triggering the degradation of Ubc9 [21]. *Yersinia pestis* YopJ, a cysteine protease, separates SUMO from SUMO-conjugation proteins to inhibit multiple signaling pathways in host cells [22]. *Xanthomonas campestris* TTSS effector XopD is a cysteine protease, and hydrolyzes SUMO from SUMO-conjugated proteins in plants [23]. However, no bacterial effector proteins have been shown to be SUMOylated.

Although these effector molecules alter the SUMOylation level of host proteins in order to promote their own survival and dissemination, no bacterial protein has been shown to be SUMOylated in the host cell. In the present study, we found that once injected into the host cytoplasm through TTSS, the *Shigella* effector OspF undergoes SUMO modification, and this SUMO modification is essential for the active cytoplasmic-nuclear translocation of OspF.

OspF localizes in the host cells and irreversibly dephosphorylates phosphor-Erk [10, 11]. Earlier in vitro experiments showed that OspF possesses phosphothreonine lyase activity and removes phosphate groups from MAPKs. To exert phosphothreonine lyase activity, the concentration of OspF should exceed that of MEK kinase to out-compete the activity of endogenous MEK kinase [10]. In addition, OspF modulates and controls gene expression by suppressing chromatin remodeling, and thus OspF can suppress the expression of proinflammatory cytokines, such as IL-8 and CCL-20.

This study identifies that *Shigella* OspF, a bacterial effector molecule, undergoes SUMO modification in the host cell. We have shown that the nuclear localization of *Shigella* effector OspF is facilitated by SUMOylation. The SUMOylation-deficient OspF did not localize in the nucleus and displayed an aberrant histone modification. We are examining if the SUMO modification only assures OspF translocation without interfering with its functional activity. The phosphorylation of MAPKs and production of proinflammatory cytokines and modulation of host immune responses by OspF with a defective SUMOylation site are under investigation.

Acknowledgments

This work was supported by a research fund of Hanyang University (HY-2012-P).

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