Structural Basis for the Recognition of Ubc13 by the Shigella flexneri Effector OspI

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Abstract

Ubc13 is a ubiquitin-conjugating enzyme that plays a key role in the nuclear factor-κB signal transduction pathway in human diseases. The Shigella flexneri effector OspI affects inflammatory responses by catalyzing the deamidation of a specific glutamine residue at position 100 in Ubc13 during infection. This modification prevents the activation of the TNF (tumor necrosis factor) receptor-associated factor 6, leading to modulation of the diacylglycerol–CBM (CARD–Bcl10–Malt1) complex–TNF receptor-associated factor 6–nuclear factor-κB signaling pathway. To elucidate the structural basis of OspI function, we determined the crystal structures of the catalytically inert OspI C62A mutant and its complex with Ubc13 at resolutions of 3.0 and 2.96 Å, respectively. The structure of the OspI–Ubc13 complex revealed that the interacting surfaces between OspI and Ubc13 are a hydrophobic surface and a complementary charged surface. Furthermore, we predict that the complementary charged surface of OspI plays a key role in substrate specificity determination.

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Legend: A catalytic triad in OspI selectively deamidates Gln100 of Ubc13 to Glu, and this modification suppresses downstream inflammatory signaling during bacterial infection. The structure of the OspI–Ubc13 complex provides the mechanism of substrate recognition.
Introduction

Numerous Gram-negative pathogenic bacteria deliver virulence factors, called effectors, into host cells through the type III secretion system. To establish infection, some effectors mimic or hijack the host signaling pathway, whereas others interfere with the host’s innate immune system.1

Ubiquitination is one of the most prevalent post-translational modifications. Protein ubiquitination is catalyzed by a sophisticated cascade system consisting of ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes. Among these enzymes, E3 enzymes are responsible for the selection of target proteins. E3 enzymes are a diverse family of proteins [RING (really interesting new gene) or HECT (homologous to E6-associated protein C terminus type)] and multiple protein complexes.2

TNF (tumor necrosis factor) receptor-associated factor 6 (TRAF6) is a RING-type E3 ligase. TRAF6 regulates the nuclear factor-κB activation signaling pathway to induce immune and inflammatory responses and confer protection against apoptosis. TRAF6 functions with a heterodimer E2 that consists of Ubc13 and a ubiquitin E2 variant (Uev1A) to synthesize K63-linked polyubiquitin chains on target proteins and TRAF6 itself.3 This K63-linked polyubiquitination leads to the activation of the downstream signaling pathway. Unlike K48-linked polyubiquitin chains that are hallmarks of proteosomal degradation, K63-linked polyubiquitin is nondegradative and has been shown to function as a signaling moiety in DNA damage repair processes and innate immunity pathways.2,5

OspI, a Shigella flexneri effector, is a glutamine deamidase that selectively deamidates the glutamine residue at position 100 in Ubc13 to a glutamic acid residue. This modification inhibits the E2 ubiquitin-conjugating activity, which is required for TRAF6 activation, and leads to the suppression of TRAF6-dependent inflammatory signaling during bacterial infection. We recently determined the OspI crystal structure, which has a cysteine protease-like fold and a conserved active-site catalytic triad (Cys62, His145, and Asp160).6 Mutation of the catalytic triad caused OspI to lose its Ubc13 deamidation activity. To better understand the mechanistic details underlying OspI-mediated TRAF6 inhibition, we determined the crystal structures of the OspI C62A–Ubc13 complex and the OspI C62A mutant.

Results and Discussion

Interaction between OspI and Ubc13

The structure of the OspI–Ubc13 complex revealed that OspI and Ubc13 are bound by interactions of 310–1 and loops a2–d3, 310–1–d4, β1–β2, and β3–β4 of OspI with H1, H2, and loops S3–S4 and 310–1–H2 of Ubc13 (Fig. 2a and Fig. S2). A total of 970 Å² of the accessible surface area is buried at the interface between OspI and Ubc13. Ubc13 is bound to OspI by its S3–S4 and 310–1–H2 loops against the concave surface of OspI, and Ubc13 extends its Gln100 to the active site of OspI. The residues involved in intermolecular formation are Asp59, Gly60, Ser63, Ile87, Thr92, Phe95, Asp103, Glu141, Glu142, Ala143, Thr144, Tyr170, and Asp184 of OspI and Gly3, Leu4, Arg6, Arg7, Arg14, Met64, Ser96, Pro97, Ala98, Leu99, Gin100, Thr103, and Leu106 of Ubc13. Gly60, Glu141, Glu142, Thr144, and Asn184 of OspI form a potential salt bridge and hydrogen bond with Ala98 and Arg6 of Ubc13, respectively (Fig. S2). The interacting surfaces of these two proteins are a hydrophobic surface and a complementary charged surface (Fig. 2b). To investigate the roles of residues involved in complex formation, we introduced point mutations into OspI and Ubc13. We

Overall structure of the OspI–Ubc13 complex

To define how OspI engages its substrate and catalyzes the deamidation of Ubc13, we attempted to crystallize OspI–Ubc13 complexes. To comparatively assess the binding of OspI to Ubc13, we generated catalytic cysteine-inactive mutants (C62A, C62S and C62S) and performed in vitro pull-down analysis. As shown in Fig. 1a, the OspI C62A mutant showed increased binding to Ubc13 compared with wild-type OspI. Therefore, we used an OspI C62A mutant for crystallization with Ubc13. We obtained crystals of OspI C62A in complex with Ubc13 and determined the structure by the molecular replacement method (Fig. 1b and Table 1). The refined structure model contains residues 21–212 of OspI C62A, residues 1–152 of Ubc13, and four iodine atoms.

The structure of OspI has an α/β fold, with four β-strands (β1–β4), seven α-helices (α1–α7), and a 310 helix (310–1). The electron densities of the N-terminal domain (residues 1–21) of OspI are not visible, suggesting that this region is flexible. Ubc13 adopts a canonical E2 fold, consisting of a four-stranded antiparallel β-sheet (S1–S4), four α-helices (H1–H4), and a 310 helix (310–1). The folds of the OspI C62A and Ubc13 in the complex were almost identical with those of each free form (Fig. 1c). The root-mean-square deviation (r.m.s.d.) of the Cα atoms between free and Ubc13-bound OspI was 1.2 Å for 191 residues, whereas that of the Cα atoms between free and Ubc13-bound OspI was 1.7 Å for 148 residues. However, the H1 helix of Ubc13 had a different conformation when Ubc13 was bound to OspI. The N-terminal end of the H1 helix had shifted about 1.8 Å closer to OspI (Fig. S1).
mutated selected residues of the OspI C62A–Ubc13 interface to Ala (R6A, R14A, M64A, and L99A) in Ubc13 and conducted a glutathione S-transferase (GST) pull-down experiment to monitor complex formation (Fig. 2c, left). The R6A and L99A mutants showed very weak binding to OspI. Mutation M64A also reduced binding. However, the R14A mutant had no effect. This result suggested that R6, M64, and L99 of Ubc13 participate in OspI binding. We investigated substrate deamidation with a series of Ubc13 mutants using native PAGE (Fig. S3). Consistent with their very weak binding, the R6A and L99A mutants were not deamidated by OspI. Although the M64A mutant showed reduced binding to OspI, the reduced binding was sufficient for deamidation. The R14A mutation did not affect binding to OspI and was deamidated by OspI. Next, we created a series of OspI mutants to determine the residues important for OspI–Ubc13 complex formation. We performed an in vitro pull-down assay with OspI mutants and Ubc13 (Fig. 2c, right). We found that OspI I87A, F95A, and Q142A mutants showed a decreased ability to bind to Ubc13 compared with the C62A mutant. In addition, we investigated the substrate deamidation activity with a series of OspI mutants using native PAGE. As shown in Fig. 2d, OspI mutants I87A, F95A, and Y170A have relatively low deamidation activity. These results suggest that the hydrophobic interactions are crucial for OspI–Ubc13 binding.
Catalytic site in OspI

We found that the active site of OspI showed significant conformational changes in the structure of the complex.6,19 (Fig. 3a). In the active-site Cys62 of wild-type OspI, the S" position was distantly positioned compared with that of the cysteine protease family. However, marked structural changes were found in the complex in the α2–α3 loop compared with wild-type OspI; Ala62 is shifted by 2.7 Å, and this acts to extend the α3 helix, which displaces Cys62 toward the active-site triad.

We next examined whether the conformational change in the active-site loop can occur in the uncomplexed OspI C62A mutant. The OspI C62A mutant protein was crystallized, and its structure was solved at a resolution of 3.0 Å (Fig. S4). The OspI C62A crystal structure comprises two molecules in the asymmetric unit. The overall structures of these two proteins are similar (r.m.s.d. of 0.3 Å for 192 Cα atoms of the two domains).

Table 1. Crystallographic data collection and refinement statistics

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<th>OspI C62A–Ubc13 complex</th>
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<td>b</td>
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<td>Rmerge (%)</td>
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| **Structure refinement** |                   |                  |
| Resolution range (Å)    | 33.14–2.96         | 41.63–3.00       |
| No. of reflections      | 12,150             | 7750             |
| Rcryst (%)              | 24.5               | 24.8             |
| Rmerge (%)              | 28.6               | 26.9             |
| r.m.s.d. bond lengths (Å) | 0.009              | 0.003            |
| r.m.s.d. bond angles (°) | 1.381              | 0.404            |
| No. of protein atoms    | 2699               | 2980             |
| Others (iodine)         | 4                  | 0                |
| Average B-factors (Å²)  | Protein: 72.2      | 12.9             |
|                        | Iodine: 98.5       | —                |
| Ramachandran plot (%)   | Most favored region | 77.0             |
|                        | Additional allowed  | 21.0             |
|                        | Generously allowed  | 2.0              |

Values in parentheses are for the highest-resolution shell.

Ospl and its mutants were cloned into the pGEX6P-1 vector and expressed in Escherichia coli BL21. Ospl was purified in a stepwise process using glutathione–Sepharose 4B, anion-exchange chromatography, and gel-filtration chromatography. The GST moiety was proteolytically removed by PreScission protease. For the Ospl–Ubc13 complex, we used the Ospl C62A mutant and His–Ubc13. Ubc13 was cloned into the pET28 vector and expressed in E. coli BL21. Ubc13 was purified by Ni-affinity chromatography, and the His tag was cleaved from Ubc13 using thrombin. The Ospl C62A mutant and Ubc13 were combined and purified by gel-filtration chromatography.

Fractions containing the Ospl C62A–Ubc13 complex were then concentrated to 51 mg/ml and used for crystallization. Ospl C62A was concentrated to 40 mg/ml by ultrafiltration in 25 mM Tris–HCl (pH 7.5) and 1 mM DTT. Crystallization of the Ospl C62A mutant was performed using the sitting-drop vapor-diffusion method at 293 K in drops containing a mixture of 1 μl of protein solution and 1 μl of reservoir solution, which consisted of 0.2 M magnesium acetate, 0.1 M Tris–HCl (pH 6.5), and 25% (w/v) polyethylene glycol 8000. The Ospl C62A–Ubc13 crystals were prepared using the hanging-drop vapor-diffusion method at 293 K in drops containing a mixture of 1 μl of protein solution and 1 μl of reservoir solution, which consisted of 0.4 M potassium iodide, 0.1 M 4-morpholineethanesulfonic acid (pH 6.5), 4 mM DTT, and 15% polyethylene glycol 8000. X-ray diffraction data sets for Ospl C62A and the Ospl C62A–Ubc13 complex were collected at 100 K on BL44XU (SPring-8). Data processing and reduction were conducted with HKL2000.7 The structure of the Ospl C62A mutant was determined by molecular replacement in MOLREP8,9 using as a search model the wild-type Ospl (PDB ID 3B21) structure lacking residues 59–77. The structure of the Ospl C62A–Ubc13 complex was determined using the molecular replacement technique PHENIX10 and the wild-type Ospl and Ubc1311 (PDB ID 1JBB). The models were subsequently improved through alternate cycles of manual rebuilding using Coot12 and refinement with the program REFMAC513 and CNS.14,15 There were no residues in the disallowed regions of the Ramachandran plot drawn by program PROCHECK.16 Structure figures were generated using PyMOL17 and CCP4MG.18
atoms). We will focus on chain A in the discussion below. The overall structure of OspI C62A corresponds well with that of the wild type (Fig. S4). The active-site structure of the C62A mutant is similar to that of the Ospl C62A–Ubc13 complex. The side chain of Ala62 corresponding to Cys62 of Ospl is

Fig. 2 (legend on next page)
turned out toward His145 (Fig. 3a). We previously reported that the crystal structure of Ospl is the closest to that of AvrPphB, a papain-like cysteine protease.\(^\text{19}\) In the superimposed structures, the catalytic His and main-chain atoms of Cys (Ala for Ospl C62A) and aspartic acids align well in Ospl C62A and AvrPphB (Fig. 3a). At the active site of AvrPphB, Asn93 functions to form an oxyanion hole. In Ospl, a Glu162 residue is located at a position corresponding to that of Asn93 in AvrPphB (Fig. 3a). In addition, Glu100 of Ubc13 in the complex protrudes into the active-site cleft. The distance between Glu100 of Ubc13 and Ala62 of Ospl in the complex suggests that substrate residue Glu100 can occupy an appropriate position in wild-type Ospl for deamidation (Fig. S5). Recent studies have identified several bacterial toxins and type III secreted effectors that act as glutamine deamidases.\(^\text{20,21}\) Among these deamidases, Cif family proteins deamidate Glu40 of ubiquitin and NEDD8. The crystal structures of Cif family proteins complexed with their substrates have been reported.\(^\text{22,23}\) Although the overall structures of Ospl and Cifs [Protein Data Bank (PDB) IDs 4F8C, 4HCN, and 4HCP] and their active sites are similar (r.m.s.d. range, 4.3–4.4 Å), the glutamine substrates of Cifs are located about 3.0 Å closer to the active site than that of Ospl (Fig. S6a). The structures of the interfaces between deamidases and their substrates have different shape complementarity and surface charge distribution (Fig. S6b).

The interaction surface on Ubc13

Ubc13 forms a heterodimer with its E2 variant, Uev1a, and this heterodimer interacts with specific ubiquitin ligases such as TRAF6 and CHIP (carboxy terminus of Hsp70-interacting protein). The structures of Ubc13–Uev1a,\(^\text{11,24}\) Ubc13–TRAF6,\(^\text{25}\) and Ubc13–CHIP\(^\text{26}\) have been previously determined. The structure of the Ubc13–Uev1a complex showed that the S2, S3, and S4 strands and loops S2–S3 and S4–310-1 of Ubc13 interact with Uev1a. We generated a model of the Ubc13–Uev1a–Osp1 complex by superimposing Ubc13 from the Ubc13–Uev1a and Ubc13–Osp1 structures. This model showed that Uev1a is located at one end of Ubc13, whereas Ospl is located at the other end (Fig. 3b). Previous studies have indicated that the binding region of TRAF6 and CHIP in Ubc13 covers the H1 helix and S3–S4 and 310-1–H2 loops.\(^\text{25,26}\) TRAF6, CHIP, and Osp1 recognize Ubc13 through similar interfaces (Fig. 3b and Fig. S7). The binding of TRAF6 and CHIP to Ubc13 mainly involves hydrophobic interactions. The interacting surfaces between Ospl and Ubc13 are a hydrophobic surface and a complementary charged surface. These proteins have different overall structures but present a very similar hydrophobic binding surface (Figs. 2b and 3d).

In the crystal structure of Ubc13 complexed with the deubiquitinating enzyme OTUB1 (OTU-domain-containing ubiquitin aldehyde-binding protein f), it has been recently reported that OTUB1 suppresses K63-linked polyubiquitination by binding Ubc13.\(^\text{27,28}\) OTUB1 interacts with Ubc13 through the H1 helix and S3–S4 and 310-1–H2 loops, which are the same regions involved in Ospl binding (Fig. S7). Furthermore, OTUB1 and Ubc13 interactions are mainly hydrophobic, as are Ospl–Ubc13 interactions. Although Ospl and OTUB1 interact with Ubc13 similarly, Ospl inhibits Ubc13 through a different mechanism. OTUB1 inhibits Ubc13–E3 interaction by occupying the Ubc13 interface with E3s.\(^\text{27,28}\) However, the wild-type Ospl–Ubc13 complex is not stable. The deamidation of Glu100 may decrease the affinity of Ospl and TRAF6 for Ubc13.

Next, we compared the structures of free Ubc13 and Ospl-bound Ubc13 with the known structures of three complexes (Ubc13–TRAF6, Ubc13–CHIP, and Ubc13–OTUB1). The overall structure of Ubc13 is almost identical among these complexes (r.m.s.d. range for 146–150 C atoms, 1.4–1.7 Å) (Fig. S8). Their overlapping binding sites in Ubc13 suggest that a convergence exists in E2 recognition. TRAF6, CHIP, and OTUB1 interact with not only Ubc13 but also Ubc5.\(^\text{29,31}\) However, Ospl does not specifically bind with Ubc5 (Fig. 3c). The complementary charged interactions between Ospl and Ubc13 may play a key role in specificity determination for Ubc13 (Figs. 2b and 3d and Fig. S9). The Shigella effector protein IpaH family is a ubiquitin ligase. The IpaH family was shown to have ubiquitin ligase activity in the presence of Ubc5 in vitro.\(^\text{32}\) This substrate selectivity between these effectors may be an advantage at different stages of infection.

In summary, we have solved the crystal structures of the Ospl C62A mutant and the Ospl C62A–Ubc13

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**Fig. 2.** Interactions between Ospl and Ubc13. (a) Stereo view of the Ospl–Ubc13 interface showing amino acids of Ospl (blue) and Ubc13 (orange). Broken lines indicate hydrogen bonds. (b) Surface potential representation of Ospl and Ubc13. The complementary surface patches (A and B) responsible for complex formation are depicted by a circle. Red, blue, and white represent acidic, basic, and neutral, respectively. (c) Characterization of the crucial residues in the Ospl–Ubc13 interface. A pull-down assay was performed as described in Fig. 1a using Ubc13 mutants with GST–Ospl C62A (left). A pull-down assay using GST–Ospl mutants with wild-type Ubc13 (right). GST–Ospl and its mutants were purified as described in Fig. 1a. GST-bound proteins were pulled down from lysates of bacteria expressing Ubc13–S tag. The bound proteins were immunoblotted with anti-S tag and anti-GST antibodies. (d) Deamidation activities of mutant Ospl proteins. GST–Ospl and its mutants were expressed in BL21; purified stepwise using affinity chromatography, anion-exchange chromatography, and gel-filtration chromatography; and dialyzed in reaction buffer. Ubc13 was incubated with purified GST–Ospl or its mutants at 30 °C for 10 min in the reaction buffer (20 mM Tris–HCl at pH 7.5, 100 mM NaCl, and 0.1 mM DTT). The reaction samples were separated by native PAGE and stained with Coomassie Brilliant Blue.
Fig. 3. Structural features of the activity of OspI. (a) Stereo view of comparison of the catalytic sites among AvrPphB (green), OspI WT (purple), OspI C62A (yellow), and Ubc13-bound OspI C62A (cyan). All atoms of His145 and Asp160 were superimposed on the corresponding residues of the counterpart proteins by LSQKAB in the CCP4 program suite. (b) Comparison of the Uev1a, TRAF6, CHIP, OTUB1, and OspI binding sites on Ubc13. Ubc13 is shown as a ribbon model, and Uev1a, TRAF6, CHIP, and OTUB1 are shown as Cα trace representations and are colored orange (Ubc13), blue (OspI), violet (Uev1a; PDB code 2C2V), dark green (CHIP; PDB code 2C2V), purple (TRAF6; PDB code 3HCT), gray (OTUB1; PDB code 4DHZ), and light yellow (OTUB1; PDB code 3VON). (c) UbcH5a binding activity of OspI. A pull-down assay was performed as described in Fig. 1a using UbcH5a with GST–OspI C62A. (d) Surface potential representation of the Ubc13 binding site of TRAF6, CHIP, and OTUB1. The complementary surface patches responsible for complex formation are depicted by a circle.
At the basis of the inhibition of TRAF6-dependent inflammatory signaling in response to bacterial infection, the interaction between OspI and Ubc13. Although the mutational data provided further insight into the complex. The refined structure in conjunction with mutational data provided further insight into the interaction between OspI and Ubc13. Although the mechanisms through which OspI regulates TRAF6 activity remain unclear, our results have important consequences for understanding the molecular basis of the inhibition of TRAF6-dependent inflammatory signaling in response to bacterial infection.

**Acknowledgements**

Atomic coordinates and structure factors of OspI C62A and OspI C62A–Ubc13 complex have been deposited in the Protein Data Bank with accession numbers 3W30 and 3W31, respectively.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2013.02.037

**References**


