Structural basis of substrate recognition by a bacterial deubiquitinase important for dynamics of phagosome ubiquitination

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Manipulation of the host's ubiquitin network is emerging as an important strategy for counteracting and repurposing the post-translational modification machineries of the host by pathogens. Ubiquitin E3 ligases encoded by infectious agents are well known, as are a variety of viral deubiquitinases (DUBs). Bacterial DUBs have been discovered, but little is known about the structure and mechanism underlying their ubiquitin recognition. In this report, we found that members of the *Legionella pneumophila* SidE effector family harbor a DUB module important for ubiquitin dynamics on the bacterial phagosome. Structural analysis of this domain alone and in complex with ubiquitin vinyl methyl ester (Ub-VME) reveals unique molecular contacts used in ubiquitin recognition. Instead of relying on the Ile44 patch of ubiquitin, as commonly used in eukaryotic counterparts, the SdeA_{DUB} module engages Gln40 of ubiquitin. The architecture of the active-site cleft presents an open arrangement with conformational plasticity, permitting deubiquitination of three of the most abundant polyubiquitin chains, with a distinct preference for Lys63 linkages. We have shown that this preference enables efficient removal of Lys63 linkages from the phagosomal surface. Remarkably, the structure reveals by far the most parsimonious use of molecular contacts to achieve deubiquitination, with less than 1,000 Å² of accessible surface area buried upon complex formation with ubiquitin. This type of molecular recognition appears to enable dual specificity toward ubiquitin and the ubiquitin-like modifier NEDD8.

type IV secretion | ubiquitination | *Legionella* | phagosome | deubiquitinase

Ubiquitin, a small, 76-aa protein modifier, is involved in a wide array of eukaryotic cellular processes. The functionality of ubiquitin depends on the precise timing of the conjugation/deconjugation of the C terminus of ubiquitin to the ε-amino group of a lysine residue of a target protein. At the heart of this process are ligases (responsible for the covalent attachment of ubiquitin) and deubiquitinases (DUBs), which function to cleave isopeptide bonds between ubiquitin and substrates or within polyubiquitin chains (1). Even though many eukaryotic DUBs have already been characterized, little is known of these enzymes in prokaryotes (1–3).

Given the essential role of ubiquitination in eukaryotic cells, it is not surprising that infectious agents have evolved numerous elegant strategies to exploit host signaling mediated by ubiquitination. Many bacterial pathogens use virulence factors to hijack the host ubiquitin pathway to establish successful infections (4). Even though E3 ubiquitin ligases of bacterial or viral origin have been relatively well characterized, bacterial DUBs have not, despite their importance in the life cycles and pathogenicity of several microbial species, including *Salmonella typhimurium* (SseL), *Chlamydia trachomatis* (ChlDub1 and ChlDub2), and ElaD (*Escherichia coli*) (5–7). These bacterial DUBs belong to a larger group of peptidases called the CE clan (the MEROPS database), which comprises eukaryotic, bacterial, and viral representatives (7, 8). Although quite different from eukaryotic DUBs (<10% identity), bacterial DUBs are phylogenetically related to mammalian desumoylating enzymes (SEN1, 2, and 3) and the deneddylase Den1 (NEDP1 or SENP8). In light

of a large divergence from their eukaryotic counterparts, the overall structure of these proteases, as well as how they function and recognize ubiquitin to act as DUBs, remains to be structurally analyzed.

Modulation of host ubiquitination pathways has emerged as an important theme in the pathogenesis of the opportunistic pathogen *Legionella pneumophila* responsible for Legionnaires' disease (4, 9). Ubiquitinated species are enriched on the *L. pneumophila*-containing vacuole (LCV), and interference with such association disturbs bacterial intracellular replication (10). Among the approximately 300 effectors injected into host cells by *L. pneumophila* via the Dot/Icm type IV secretion system (11), eight proteins appear to possess F-box or U-box domains typical of some E3 ligases (12–16). This ligase activity has been demonstrated for LegU1, LegAU13/AnkB, and LubX (14, 17). A recent study revealed that SidC and SdcA are E3 ligases that catalyze the ligation reaction with a unique mechanism, and are required for efficient enrichment of ubiquitinated species on the bacterial phagosome (18).

Because balanced regulation of host cell processes is critical for the virulence of *L. pneumophila* (19), we initiated experiments to identify *L. pneumophila* proteins with DUB activity. Our efforts revealed that members of the SidE family contain a DUB domain, which catalyzes the reaction with a Cys-His-Asp (CHD) catalytic triad showing a preference for Lys63-linked polyubiquitin chains. Structural analysis of the DUB domain and its complex with the mechanism-based inhibitor, ubiquitin vinyl methyl ester (Ub-VME),

Significance

Ubiquitination and deubiquitination have emerged in recent years as novel targets for the design of therapeutic agents. To our knowledge, the structure of the deubiquitinase (DUB) domain of SdeA represents the first prokaryotic DUB determined and will thus potentially serve as a model for other bacterial deubiquitinating enzymes for use in structure-guided drug design. *Legionella pneumophila* ubiquitin E3 ligases play important roles in the biogenesis of the phagosome permissive for bacterial replication. The discovery of effectors with DUB activity highlights the importance of modulation of host processes in a regulated and balanced manner.

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Fig. 2. The SdeA DUB module is important for regulating the dynamics of ubiquitin association with the bacterial phagosome. (A) Mouse bone marrow-derived macrophages were infected with the indicated *L. pneumophila* strains (LP03 is the Dot/Icm-deficient strain; Δ4 refers to a strain lacking all SidE family members) for 1 h, and fixed cells were differentially stained for extra- and intracellular bacteria. Ubiquitinated species were labeled with the FK2 antibody. Images were acquired with a fluorescence microscope. (Scale bar: 5 μ m.) (B) Quantification of the enrichment of ubiquitinated species associated with the LCVs. Samples in triplicate were inspected for the enrichment of ubiquitin signals with the bacterial phagosomes. At least 150 vacuoles were scored for each sample.

to complement was caused by loss of enzymatic activity in the DUB module. Together, these results reveal that the DUB activity of SdeA is important for the dynamics of ubiquitin association with the bacterial phagosome.

SdeA_{Dub} Cleaves Lys11/48/63 Chains with a Preference for Lys63. To determine the chain type preference of SdeA_{Dub}, we used a gel-based diubiquitin cleavage assay. These results revealed that, even though SdeA_{Dub} cleaves all chain types examined (Fig. 3A and Fig. S2E), there was a clear preference for Lys63-linked chains, yielding a binding constant (from the K_m) of ~ 200 μ M for each DUB domain and ~ 100 μ M for the full-length SdeA construct (Fig. 3B and Fig. S2H). We were unable to obtain kinetic parameters for Lys11 and Lys48-linked diubiquitin chains as a result of the low cleavage efficiency. Interestingly, high concentrations of Lys48-linked diubiquitin appeared to inhibit SdeA_{Dub} activity (Fig. S2F and G). In line with the biochemical observations, the SdeA DUB module was able to efficiently remove Lys63-linked polyubiquitin chains from the phagosomal surface (Fig. S4C and D).

Structure of the SdeA DUB Module. Structures of SdeA_{Dub} were determined for two constructs: 1–163 SeMet (apo), 1–193 Cys118-Ala (apo), and 1–193-Ub-VME at 2.85 Å, 2.02 Å, and 2.53 Å, respectively, with each solution containing two molecules in the asymmetric unit (even though biophysical analysis suggests this protein is a monomer in solution; see Fig. S7F and G and Table S1). The overall fold of SdeA_{Dub} can be described as an arrangement of periodic secondary structures forming two distinct lobes, the α -lobe and the β -lobe (Fig. 4A and Fig. S5A), which pack closely to generate a compact globular fold, connected by loops L2 and L10 (Fig. 4A and Fig. S5A). The β -lobe is composed of a twisted, mixed, six-stranded β -sheet (β 1– β 6; Fig. S5A), which, when viewed from one edge, appears to form a partially wound β -barrel (Fig. S5B). The concave surface of this partial β -barrel allows a curved α -helix (α 3) to make tight contact. An additional, relatively short, helix (α 2) seems to cap one end of the partial barrel. The outer surface of the barrel packs against two α -helices (α 1 and α 4) of the α -lobe (Fig. S5A). The rest of the helices in the α -lobe (α 5, α 6, and α 7) arrange themselves by packing against at least one other helix within this lobe.

The active site is located at the interface between the two lobes, with Cys118 placed in the first turn of α 4 facing the catalytic histidine, His64, with the catalytic aspartic acid, Asp80, within short hydrogen-bonding distance (Fig. 4B). This CHD catalytic triad is complemented by a suitably located Asn residue

(Asn114) in loop L2 that we propose stabilizes the oxy-anion species (Fig. 4B). The catalytic residues line an open catalytic cleft (except for Glu9, which seems to function as a flap) with a predominantly negative electrostatic character complementing the distinctly electropositive C-terminal tail of ubiquitin and some UbLs. Interestingly, inspection of the structures of the three constructs reported herein reveals that the active-site residues have the propensity to assume different conformations, indicated by different orientations of the Cys and His residues, possibly reflecting plasticity in the catalytic residues, which, in addition to the open nature of the cleft, may confer some advantage in accommodating substrates with varying structure of their polyubiquitin chain.

SdeA_{Dub} Binds Ubiquitin Through Three Distinct Patches. Sequence alignments and modeling with other ULps revealed little about the nature of ubiquitin binding. To visualize the mode of ubiquitin recognition, we prepared a covalent complex of SdeA_{Dub} with ubiquitin by reacting the DUB module with Ub-VME. The structure of the complex reveals the formation of a covalent bond between Cys118 and the VME group of the suicide inhibitor. Inspection of the Ub-VME bound crystal structure reveals three distinct ubiquitin binding sites: the active-site cleft (site-1), a site centered around Tyr33 (site-2) referred to as the Y-site, and a

Fig. 3. Comparison of cleavage efficiency of diubiquitin substrates. (A) Relative efficiency of Lys11, Lys48, and Lys63 diubiquitin cleavage by SdeA_{Dub}. Each assay was done in triplicate, with error bars representing SDs. (B) Kinetic analysis of SdeA (blue) and SdeA_{Dub} (green). Fitting the plots to the Michaelis-Menten equation yields K_m values of ~ 75 μ M and ~ 200 μ M for the full length and DUB domain, respectively.

Fig. 4. The structure of the SdeA DUB module and in complex with Ub-VME. (A) Cartoon representation of SdeA_{Dub} identifying α -helices (orange), β -sheets (yellow), and loops (black). The loops L2 and L10, which connect the α - and β -lobes, are shown in blue. (B) Ubiquitin (green) binding to SdeA_{Dub} (surface rendering) is illustrated with the catalytic residues shown as blue sticks. The C-terminal tail of ubiquitin binds to the active site (site 1). (Insets) Superimposition of the catalytic residues of SdeA_{Dub} observed in three structures reported here (*Top Right*), flexibility of Glu9 observed in two distinct conformations in the apo and Ub-VME bound structures (*Top Left*), and arrangement of the C-terminal tail within the active site (*Bottom*).

site that engages the $\beta 1$ – $\beta 2$ loop of ubiquitin (site-3; Figs. 4*B* and 5). The active-site cleft engages the tetrapeptide portion of the C-terminal tail of ubiquitin (Leu73-Gly-VME, with Gly-VME corresponding to Gly76) with contacts that involve mainly interbackbone hydrogen bonding. Hydrogen-bonding contacts with tail residues stabilize the tail in an extended conformation, resulting in an arrangement in which the tail residues form the central strand of a three-stranded β -sheet that includes one arm of loop L6 and residues Glu9–Gln12 of the enzyme (Fig. 4*B*). Engagement of the tail is facilitated by charge and shape complementarity, with the α -carbon of Gly-VME fitting snugly into a narrow groove at the end of the active-site cleft adjacent to Cys118. This matching of shape ensures proper placement of the scissile isopeptide bond adjacent to Cys118 and ensures selectivity for the terminal di-Gly motif in ubiquitin and certain Ubls. Arg72, a key determinant of ubiquitin/Ubl specificity, is unengaged, explaining the reactivity of the DUB toward ubiquitin-VS and NEDD8-VS (Fig. 1*E*) (26, 27).

Most DUB-ubiquitin cocrystal structures show active-site tail binding as observed here, albeit with more extensive interactions. Tyr33 in the Y-site makes three contacts with ubiquitin, the most prominent of which appears to be Gln40, as well as making van der Waals contacts with Ile36 and Leu73. The hydroxyl group is engaged in a tight hydrogen-bonding interaction with the side-chain carbonyl group of Gln40, whose side-chain amide proton serves as a hydrogen bond donor to Asp61 from the catalytic cleft (Fig. 5C). Another commonly observed interaction between DUBs and ubiquitin involves the $\beta 1$ - $\beta 2$ loop of ubiquitin engaged at a distinct site that allows the dipeptide turn to fit in snugly. In SdeA_{Dub}, the

β 1– β 2 loop of ubiquitin interacts primarily through van der Waals contacts, with only one hydrogen bond between the backbone carbonyl of Leu8 of ubiquitin and the hydroxyl group of Ser29 of SdeA (Fig. 5D). It appears that most of these interactions can occur even if the turn segment has substitutions such as those found in ISG15, possibly explaining the observed reactivity with ISG15-VS and NEDD8-VS (Fig. 1E and Fig. S6). Surprisingly, the ubiquitin Ile44 patch is not engaged in binding (Fig. 5A), quite distinct from eukaryotic DUBs, which seem to rely on the Ile44 patch for affinity, and also from the Ulp family member Den1's recognition of NEDD8 (1, 27, 28).

Structural Similarity and Differences from Den1. Structural similarity analysis using the DALI server (29) identified Den1 (NEDP1 or SENP8) apo and in complex with NEDD8 as high-scoring hits [Protein Data Bank (PDB) ID codes 2BKR, 2BKQ, and 1XT9] (27, 28). Den1 is a member of the Ulp family of Cys proteases, whose members share a characteristic core fold comprised of a five-stranded mixed β -sheet packed against a helix (27, 28, 30). Den1 and SdeA_{Dub} are structurally similar, with most of the β -sheets (excluding $\beta 6$) and $\alpha 4$ (bearing the catalytic cysteine) adopting a similar fold (Fig. S7A). Even though the core Ulp domains were consistent, differences exist, particularly in regions used for binding to ubiquitin or Ubls (Fig. S7B). A simple S-shaped loop at the beginning of SdeA_{Dub} is replaced in Den1 by a helix followed by a β -hairpin and is used in NEDD8 binding. Notably, $\beta 2$ of SdeA, which forms a hairpin turn with $\beta 1$ to create the Tyr33 binding loop, is replaced with $\alpha 3$ in Den1, which mediates NEDD8 interaction via the Ile44 hydrophobic patch.

The structures of the Den1-NEDD8 and SdeA_{Dub}-Ubiquitin complexes also exhibit differences and similarities. The most notable similarity is the relative orientation of the C-terminal tail with respect to the adjacent arm of loop L6, which appears to be a characteristic element in the Ulp family. Loop L6 in our structures is rigid, forming a β -hairpin with a bulge (Fig. S7D). In Den1, the

Fig. 5. Molecular recognition of ubiquitin by SdeA DUB module. (A) The C-terminal tail of ubiquitin (green) is secured in the catalytic cleft, predominantly stabilized by backbone interactions (dotted lines; SdeA_{Dub} residues in orange). Glu6 and Glu9 participating in hydrogen bonding and electrostatic interactions with Arg74 of ubiquitin. (B) Gln12 hydrogen bonding with the backbone nitrogen of Leu71 of ubiquitin. (C) Tyr33 and Asp61 form hydrogen bonds with Gln40 of ubiquitin. (D) Backbone atoms of the β 1– β 2 loop of ubiquitin forms hydrogen bonds with the backbone and side chain of Ser29 of SdeA_{Dub}.

corresponding loop is quite dynamic, undergoing a significant conformational change upon binding to NEDD8. SdeA_{Dub} buries ~900 Å² [according to the proteins, interfaces, structures and assemblies (PISA) server] of surface area upon binding ubiquitin, which is substantially less than Den1 (burying nearly 2,000 Å² when bound to NEDD8) as a result of greater recognition of NEDD8 by Den1 by structural elements absent in SdeA_{Dub} (31). The most prominent of these substitutions is an N-terminal β -hairpin in Den1 that nestles against the loop segment of NEDD8 (Lys48-Asn41; Fig. S7E). This hairpin is absent in SdeA_{Dub} and would mediate the corresponding interaction with Lys48 of ubiquitin, making it exo-specific for this chain type (that is, capable of cleaving ubiquitin chains only from the most distal end). Loss of this element in the bacterial DUB may allow it to rapidly cleave from anywhere in the chain. The other prominent difference is the absence of helix-4 (Den1 numbering) in SdeA_{Dub}, which plays an important role in engaging the Ile44 patch. To probe the importance of the observed contacts in solution, we generated alanine point mutants and tested their activity against Lys-63-linked diubiquitin as well as a Nedd8 fusion construct (His-MBP-NEDD8-Ub). From these experiments, we find that some variations within the catalytic cleft are tolerated, whereas mutating Y33 and D61 (Y-site) resulted in a complete loss of activity. Moreover, results observed in experiments with the NEDD8 fusion construct mirrored those of Lys63-linked diubiquitin, suggesting a similar mode of recognition (Fig. S8).

The selectivity of SdeA_{Dub} for Lys63-linked chains may rely on chain topology. Because Lys63-linked chains adopt an extended conformation (whereas Lys48- and Lys11-linked chains do not), they may be strung more easily through the catalytic site. Based on this observation, we hypothesize that the preference observed for Lys63-linked chains is a result of conformational differences between the chains themselves rather than structural restrictions imposed by SdeA_{Dub}. However, it is possible that selective binding to the amino acids that flank Lys63 contributes to the preference for this chain type; however, this remains to be determined in future studies.

Discussion

Phagosomes harboring virulent *L. pneumophila* (i.e., LCVs) are decorated with ubiquitinated proteins whose function is incompletely understood but is thought to be important for the biogenesis and maintenance of the vacuole (9, 18). Earlier studies have identified Dot/Icm effectors with ubiquitin E3 ligase activity with important roles in the biogenesis of LCVs (18, 32). Our discovery of DUB activity associated with members of the SidE family of effectors points to the importance of spatiotemporal regulation of ubiquitination on the LCV surface. It adds another layer of complexity to the regulation of host processes by effectors of opposite biochemical activities exemplified by the reversible AMPylation and phosphorylation of the Rab1 small GTPase (33, 34). The activity of the SidE family of effectors appears to be regulated by SidJ (35, 36), a gene within the gene cluster (37). All members of this family contain the N-terminal DUB module, which appears to redundantly regulate the extent of ubiquitination of the vacuolar surface, but their natural substrates remain to be determined (24). Clearly, DUB activity is not critical for the role of SidEs in intracellular bacterial replication under laboratory infection conditions; such roles likely are conferred by the activity of other domains of these large proteins (36).

Our structural analysis establishes a core Ulp fold and a canonical CHD catalytic triad in a bacterial CE clan of cysteine protease with DUB activity. The structure of the SdeA_{Dub}-Ub-VME complex reveals the molecular contacts used by the DUB for ubiquitin recognition, providing, to our knowledge, the first structure of a Ulp-fold protease bound to ubiquitin. Bacterial CE peptidases are predicted to share structural similarity with eukaryotic proteases such as Ulp1 (yeast) and Den1 (mammals), which exhibit

exclusive specificity toward SUMO1 and NEDD8, respectively (27, 28). SdeA_{Dub} is unable to recognize SUMO because of mismatches between the C-terminal tail of the Ubl and the DUB, but is capable of deneddylating activity. Den1 binds to NEDD8 by using an extensive interface. SdeA_{Dub}, in contrast, presents a remarkably simple interface, burying only half the accessible surface area as a result of fewer contacts with the C-terminal tail and no contacts with the Lys48 loop or Ile44 patch. The latter is a feature widely used by structurally characterized eukaryotic DUBs (27, 28). Instead, SdeA_{Dub} binds to ubiquitin in an orientation that is twisted relative to the way NEDD8 docks on Den1, an orientation that brings Gln40 of ubiquitin to the forefront and permits a bifurcated engagement with Tyr33 and Asp61 of SdeA_{Dub} (Fig. S7C). This difference in binding interface between Den1 and SdeA_{Dub} may reflect their substrate preferences; Den1 releases NEDD8 from its precursor whereas SdeA_{Dub} most likely acts on polyubiquitin chains. Although a smaller interface may result in a higher K_m , as observed here, this would not be a significant impediment because the enzyme is likely to encounter a relatively high local concentration of polyubiquitin chains on the surface of the LCV (10).

The origin of ubiquitin is believed to predate that of NEDD8 and other Ubl modifiers (7, 38). This has given rise to the speculation that the evolutionary antecedents of the CE clan of peptidases with ubiquitin/Ubl hydrolyzing activity might have originated in eukaryotes, with primarily DUB activity having been acquired by pathogenic bacteria through horizontal gene transfer (7). The known eukaryotic members of the CE clan might have evolved to recognize SUMO (Ulp1) or NEDD8 (Den1) exclusively at the expense of DUB activity. The dual specificity toward ubiquitin and NEDD8 by SdeA_{Dub} is quite remarkable considering that the more widely understood eukaryotic CE peptidases have stringent specificity toward only one type of Ubl modifier. Moreover, NEDD8 and ubiquitin have respective E1 enzymes that discriminate the two based on the single amino acid at position 72 (26, 39). Interestingly, NEDD8 binding to SdeA_{Dub} appears to use a similar interface as ubiquitin, as the point mutants of Y33 and D61 abolished deneddylase activity. A binding interface with NEDD8 of the nature seen in the ubiquitin-bound structure reported here can be a solution to a problem of molecular recognition that demands both DUB and deneddylase activity. We have shown that the SdeA DUB removes Lys63-linked chains from LCVs, which could be used to antagonize the host's attempt to recruit autophagy machinery, reminiscent of the proposed function of SseL (5, 40).

The suppression of SdeA toxicity by SidJ (Fig. S3D) likely reflects an intricate interplay between the effectors of *L. pneumophila* to achieve spatiotemporal control of host cell processes, underscored by a previous observation that SidJ can remove SdeA from the phagosome after a certain time postinfection (35–37). The contribution of the catalytic activity of the N-terminal DUB module to SidJ-mediated suppression of SdeA's toxicity (caused by its central domain) adds another layer of complexity to the interplay between these two effectors (Fig. S3D). It also suggests that the DUB activity of SidE DUBs may be used for multiple functions, conferring a specific advantage to the organism in effectively establishing infection inside the host cell.

Methods

HEK293 cells were transfected with pCDNA3 HA-Ubiquitin or pCMV 4XFlag-NEDD8 by Lipofectamine 2000 (Life Technologies). Cells were collected 24 h after transfection and lysed in lysis buffer [0.2% Nonidet P-40, 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 15% (vol/vol) glycerol, and Protease Inhibitor Mixture Set III (EMD Millipore)]. The total cell lysates were then subjected to immunoprecipitation by HA agarose or Flag affinity gel (Sigma-Aldrich) for 4 h, and the beads were washed with lysis buffer three times and resuspended in cleavage buffer containing 50 mM Tris, pH 7.5, 100 mM NaCl, and 1 mM DTT. Samples were divided into aliquots and left untreated or treated with 100 μ g of SdeA_{Dub} or SdeA_{Dub}Cys118Ala for 1 h at room temperature. The beads were then washed three times with the cleavage buffer and boiled for 5 min at 100 °C in 1 $\times$ SDS/PAGE buffer. The proteins were then separated by

SDS/PAGE, and polyubiquitinated proteins or polyneddylated proteins were detected by HA and Flag antibodies, respectively. Additional descriptions of materials and methods used in the study are provided in *SI Methods*.

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