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Ubiquitin Chains Modified by the Bacterial Ligase SdeA Are Protected from Deubiquitinase Hydrolysis

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Abstract

The SidE family of *Legionella pneumophila* effectors is a unique group of ubiquitin-modifying enzymes. Along with catalyzing NAD⁺-dependent ubiquitination of certain host proteins independent of the canonical E1/E2/E3 pathway, they have also been shown to produce phosphoribosylated free ubiquitin. This modified ubiquitin product is incompatible with conventional E1/E2/E3 ubiquitination processes, with the potential to lock down various cellular functions that are dependent on ubiquitin signaling. Here, we show that in addition to free ubiquitin, Lys63-, Lys48-, Lys11-, and Met1-linked diubiquitin chains are also modified by SdeA in a similar fashion. Both the proximal and distal ubiquitin moieties are targeted in the phosphoribosylation reaction. Furthermore, this renders the ubiquitin chains unable to be processed by a variety of deubiquitinating enzymes. These observations broaden the scope of SdeA's modulatory functions during *Legionella* infection.

Ubiquitin (Ub) signaling regulates many key processes in eukaryotic cells.¹ This occurs by post-translationally modifying a substrate protein with the conserved 76-residue protein ubiquitin. Traditionally, ubiquitination has been known to occur via a coordinated three-step mechanism, requiring the E1 (ubiquitin-activating), E2 (ubiquitin-conjugating), and E3 (ubiquitin-ligating) enzymes along with the cofactor ATP, ultimately resulting in an isopeptide bond between the C-terminus of Ub and an acceptor lysine residue of the

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Supporting Information

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Notes

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substrate protein. Often, polyubiquitin chains are generated on substrate proteins via successive addition of Ub groups to the first one.¹ Ubiquitin possesses seven Lys residues that, along with the N-terminal amino group of Met1, serve as points through which Ub groups in polyubiquitin chains may be connected. The simplest of these polyubiquitin chains are homotypic polymers that are produced by linking through the same Lys residue; accordingly, the homotypic chains are named after the linking Lys, such as Lys63- or Lys48-linked chains. In addition, complex chain types involving mixed linkages and branching generate an immense diversity of polyubiquitin chains serving different signaling functions. For example, polyubiquitin chains linked via Lys48 of ubiquitin (K48) are known to be a signal for proteasomal degradation, while K63-linked chains are implicated in lysosomal degradation, cellular trafficking, and DNA repair.¹ Importantly, ubiquitination is a reversible process. The hydrolysis of Ub isopeptide linkages is achieved by a group of enzymes collectively known as deubiquitinases, or deubiquitinases (DUBs).^{1–3} Several categories of human DUBs have been identified to date; these include the USP, UCH, OTU, MJD, and MINDY⁴ families, cleaving ubiquitin linkages through a cysteine protease mechanism, and the JAMM family, utilizing a zinc metalloprotease mechanism.^{5,6} Precise processing of ubiquitin chains by DUBs serves an important regulatory function in eukaryotes and is crucial for normal cellular function.

While prokaryotes do not possess a ubiquitin system of their own, remarkably there are several examples in nature of pathogens that are able to control host ubiquitin signaling for their benefit,^{7,8} through both ubiquitin ligation and deubiquitination. The infectious bacterial organism *Legionella pneumophila*, responsible for Legionnaire's disease,⁹ is one such pathogen. It exerts its control by injecting hundreds of effector proteins via the Dot/Icm type IV secretion system into the cytoplasm of the host cell during infection.¹⁰ Among these effectors is the SidE family, which includes four proteins, the paralogs SdeA, SdeB, SdeC, and SidE. This family is required for optimal *Legionella* replication within the host. SdeA was recently observed to possess two noteworthy functions. First, it was able to catalyze deubiquitination¹¹ through its N-terminal domain (the SdeA^{DUB} domain, spanning the first 200-residue segment). The second function, and pertaining to the focus of this study, is that it ubiquitinated certain human substrate proteins in a manner completely different from the canonical E1/E2/E3 cascade,¹² with NAD⁺ instead of ATP as a necessary cofactor and ADP-ribosylated Arg42 of Ub (Ub-ADPR) as the activated Ub intermediate. The Ub-ADPR intermediate is produced by a mono-ADP ribosyl transferase domain present within the segment of residues ~500–1000 of the effector (SdeA^{mART}). A further study¹³ showed that this intermediate is subsequently attacked by a substrate Ser residue, resulting in ubiquitination of the Ser residue through a phosphor-ribosyl (PR) linkage between the target and Arg42 of Ub. This ubiquitination step is catalyzed by a phosphotransferase domain (dubbed the PDE domain)¹³ harbored within the segment of residues ~200–500 of the protein. These studies showed ubiquitination taking place in any fashion besides E1/E2/E3 and through a linker besides the isopeptide bond for the first time. The Ub-ADPR intermediate is also subject to hydrolysis, with water cleaving the bond between the phosphate groups, in a reaction that is also catalyzed by the same PDE domain (Figure 1). The hydrolysis results in the generation of free phosphoribosylated ubiquitin (Ub-PR) (Figure 1). Both outcomes are accompanied by the release of AMP as a byproduct. Ub-PR

was shown not to participate in any of the three steps of the canonical ubiquitination pathway.¹³ However, the ADP-ribosylating and PDE activity of SdeA against ubiquitin chains as well as their effects on DUBs has remained unexplored. An understanding of these effects is of great interest due to the fact that polyubiquitinated proteins are known to associate with the LCV (*Legionella*-containing vacuole) during infection.¹⁴

In this study, we demonstrate that Ub chains of different linkage types are modified by SdeA. We also show that both Ub monomers of diubiquitin (diUb) chains undergo modification, suggesting that SdeA is nonselective in its ADP-ribosylating activity as well as its PDE activity. These modified ubiquitin chains were then tested against several different DUBs. We report that ADP ribosylation as well as phosphoribosylation of diUb by SdeA leads to a significant protection against DUBs that engage Arg42 of Ub during hydrolysis. We explored the basis for this effect through selective modification of the proximal versus the distal ubiquitin species and structural modeling. These data suggest that SdeA may prevent the processing of existing ubiquitin chains by host DUBs.

In a recent study,¹⁵ Kotewicz et al. demonstrated that K63-linked tetra-Ub chains were ADP-ribosylated by SdeA. However, which ubiquitin monomers underwent modification remained unknown. To investigate this, we tested the activity of SdeA *in vitro* against a diUb substrate. K63-, K48-, K11-, and M1-linked diUb were incubated with two constructs of SdeA: the ubiquitinating construct SdeA^{181–1000} and SdeA^{519–1100}, which contains the mART domain but lacks the PDE domain. The reaction mixture was analyzed using top-down liquid chromatography and mass spectrometry (LC–MS) (Figure 2A and Figure S1) to determine whether one or both ubiquitin moieties were modified. The masses of the reaction products indicated that a doubly modified diUb species was generated (Table S1). Therefore, SdeA does not distinguish between free Ub and Ub that is part of a polyubiquitin chain. In addition, within the time scale of our reaction, no preference for either the proximal (C-terminus-engaged) or distal (Lys-engaged) moiety was observed, due to an absence of peaks for singly ADP-ribosylated or phosphoribosylated diUb species.

There appeared to be no preference for chain type, with K11-, K48-, K63-, and M1-linked chains all serving as viable substrates, being converted to their modified form within the time of our observation. We also found that SdeA is able to transfer diUb to Rab1, a previously reported substrate, as well as to itself (self-modification, a common feature of ubiquitin ligases) (Figure 2B).

It has also been suggested that modified Ub chains are resistant to hydrolysis from the DUB domain of SdeA.¹⁵ To better understand this process and determine whether this effect could also be extended to other DUBs, the activities of the DUB domain of SdeA, the related bacterial DUB ChlaDUB1,¹⁶ and the recently described *L. pneumophila* DUB SidJ¹⁷ along with that of the human DUBs USP2, USP7, OTULin, AMSH, and UCHL1 and the yeast DUB MIY1 were tested against unmodified, ADP-ribosylated, and phosphoribosylated ubiquitin substrates. K63-linked diUb was used as the substrate for USP2, AMSH, SdeA, and ChlaDUB1; K48-linked diUb was used for USP7, and M1-linked diUb was used for OTULin. Ubiquitin with a C-terminal tryptophan (Ub-W) was used as a substrate for UCHL1 because of its preference for cleaving small peptides from the C-terminus of Ub rather than

diUb linkages.^{18,19} Substrates were incubated with SdeA in the presence or absence of NAD⁺ for 1 h before the addition of DUBs (Figure 3A).

The occurrence of ADP ribosylation or phosphoribosylation protected the substrate from DUB hydrolysis in all cases except for that of AMSH and UCHL1 (Figure 3B, Figure S2, and Table S2). Their activity may have been unhindered because of a lack of steric clashes between the –ADPR/–PR group of ubiquitin and the enzyme. Unexpectedly, for several of the DUBs examined, the presence of SdeA^{181–1000} in the absence of NAD⁺ caused an apparent increase in DUB activity compared to trials in which SdeA^{519–1100} or no SdeA was included. It was expected that SdeA would compete with the DUB for diUb binding and therefore cause an inhibitory effect, but the opposite was observed. This may be due to the longer SdeA construct binding to the diUb substrate and holding it in a conformation that is more accessible for the DUB to act. This acceleration of activity was observed in the cases of SdeA^{DUB}, ChlaDUB1, and USP7, with this effect being most prominent in the case of ChlaDUB1. It is known that SdeA^{181–1000} will ubiquitinate itself upon being incubated with Ub and NAD⁺.¹² An upward shift in the mass of SdeA, indicative of autoubiquitination, continued to be observed when diUb instead of Ub was included. The addition of NAD⁺ alone caused little to no inhibition of DUBs (Figure S3A). Decreasing the NAD⁺ concentration to 100 μ M resulted in a similar level of Rab1 ubiquitination as well as similar protective effects from DUB activity (Figure S3B,C).

According to structural data, R42 of M1-linked diUb is located at the DUB–substrate interface, whereas the R118 residue (proximal acceptor) is farther from the enzyme being exposed to solvent²⁰ (Figure 4A). Therefore, we expected the addition of an –ADPR or –PR group to R42 (distal acceptor) rather than R118 to cause a disruption to DUB binding and a corresponding loss of activity. To further investigate the basis for protection of diUb from hydrolysis, we generated two mutants of M1-linked diUb, with each mutant having either R42 or R118 changed to a lysine residue. Consistent with our hypothesis, the R118K mutant behaved like wild-type (WT) diUb in that the DUB activity of OTUlin was lost after modification with SdeA. The R42K mutant, in contrast, was cleaved by OTUlin with or without modification at a comparable level (Figure 4B). We noticed that the R42K mutant was a poorer substrate for OTUlin than the wild type or R118K mutant was, highlighting the importance of the distal R42 for binding (Figure S4). To compensate for the loss of activity upon Arg42 to Lys mutation, the reaction time was increased in the assay involving the R42K mutant. These data provide an example of the structural basis for the protection of Ub chains from DUB hydrolysis. Via the addition of steric bulk in the form of –ADPR or –PR, the interface between the enzyme and Ub chain is disrupted. Selective ADP ribosylation and phosphoribosylation by SdeA of certain Ub moieties of chains may serve as a useful and simple enzymatic tool in future studies for probing proximal versus distal binding of uncharacterized ubiquitin-interacting enzymes such as DUBs.

The role of SdeA during *Legionella* infection has been the subject of intense study. A growing list of functions have been described, including ubiquitination of ER-associated Rab proteins,¹² ADP ribosylation, and phosphoribosylation of free ubiquitin possibly leading to subsequent loss of activity of E1/E2/E3 machinery,¹³ and remodeling of host ER tubules.¹⁵ The results presented here offer new insights into the role of SdeA in controlling

the host's ubiquitin system. Our data suggest that along with free Ub, Ub that is part of a chain is not spared from modification by SdeA, with all Ub monomers serving as a target. SdeA-catalyzed modification of polyubiquitin chains can be extensive and most likely protects the chain from hydrolysis by most host DUBs as well as the DUB module of SdeA itself. The LCV surface is decorated with polyubiquitinated proteins during infection, a modification that seems to serve an important role in the life cycle of the bacterium.^{14,21} In such a scenario, protection of polyubiquitin chains from host DUBs might prove advantageous to *L. pneumophila*. At least one other example of Ub chain modification has been observed, phosphorylation at Ser65 by PINK1.²² Interestingly, this also impairs hydrolysis by DUBs. Future studies will elucidate the cellular effects of SdeA's activity, including the protection of Ub chains, as well as the structural basis of SdeA's Ub binding ability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Proposed two-step mechanism for SdeA-catalyzed ubiquitination of the substrate Rab33b (A) as well as generation of Ub-PR (B) upon hydrolysis of the Ub-ADPR intermediate. Reaction byproducts and leaving groups are colored red.

Figure 2.

Reactions of diubiquitin with SdeA. (A) MS of diUb incubated with SdeA constructs and NAD⁺ (see Figure S1 for LC–MS data of other diubiquitin chain types and Table S1 for MS data analysis). (B) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of diUb and SdeA^{181–1000} incubated with the substrate Rab1 in the presence or absence of NAD⁺.

Figure 3.

DUB cleavage assays of modified vs unmodified diUb species. (A) Overall scheme of assays and list of DUBs used. (B) SDS-PAGE gels of reactions between DUB and diUb that had been pretreated with SdeA constructs in the presence or absence of NAD⁺. For UCHL1, Ub-W was used as a substrate and reactions were analyzed by LC-MS. See Figure S2 for complete LC-MS results and Table S2 for detailed analysis of MS data.

Figure 4. Mutational analysis. (A) Crystal structure of OTUlin bound to M1 diUb,¹⁸ retrieved from the Protein Data Bank, with R42 and R118 of diUb colored red. The inset focuses on the two OTUlin residues that interact with R42. (B) SDS-PAGE of cleavage assays with indicated mutant M1 diUb substrates.