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Exploiting the ubiquitin and phosphoinositide pathways by the *Legionella pneumophila* effector, SidC

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Abstract

Intracellular bacterial pathogens use secreted effector proteins to alter host cellular processes, with the goal of subverting host defenses and allowing the infection to progress. One such pathogen, *Legionella pneumophia*, secretes ~300 such proteins into its host to alter a number of pathways including intracellular trafficking, phosphoinositide metabolism, and cell signaling. The *Legionella* effector SidC was previously found to bind to PI(4)P and was responsible for the enrichment of ER proteins as well as ubiquitinated species to the *Legionella*-containing vacuoles. Through our recent work, we have discovered that SidC contains a unique N-terminal E3 ubiquitin ligase domain and a C-terminal novel PI(4)P-binding domain. Our results demonstrate that SidC serves to link two distinct cellular pathways, ubiquitin and phosphoinositide. However, how the ubiquitin ligase activity regulates host membrane trafficking events remain to be investigated.

Ubiquitination is a eukaryotic posttranslational modification important for a plethora of cellular pathways, including protein homeostasis, cell signaling, and membrane trafficking [1, 2]. This process is carried out through the action of three classes of enzymes: E1 activating enzymes, E2 conjugating enzymes, and E3 ubiquitin ligases [3]. Ubiquitin is activated and linked to the catalytic cysteine of E1 through a thioester linkage with the C-terminal glycine of ubiquitin, and is then transferred to an E2 conjugating enzyme. The subsequent ubiquitination of a cellular substrate is mediated by the action of an E3 ubiquitin ligase, which catalyzes the formation of an isopeptide bond between the C-terminal glycine of ubiquitin and the ε-amino group of a substrate lysine. There are two main classes of E3 ubiquitin ligases: The RING (Really Interesting New Gene) family indirectly facilitates the transfer, while the HECT (Homologous to E6AP C-Terminus) family utilizes a catalytic cysteine to form an E3-ubiquitin intermediate before ubiquitinating a substrate [4, 5]. While ubiquitination is well-established as a strictly eukaryotic process, many intracellular pathogens have adopted Bacterially-encoded E3 ubiquitin Ligases (BELs), likely through horizontal gene transfer [6, 7]. Although the disruption of the host ubiquitination pathway is thought to play a crucial role in these infections, little is known about how disturbances in this pathway lead to a successful infection [8].

Phosphoinositides (PIs) are an essential class of membrane lipids found on the cytosolic surface of membranes. PIs can be reversibly phosphorylated at the 3,4, and/or 5 positions of

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the inositol ring to generate seven distinct PI species. Each of these lipids has a unique distribution within the cell and contributes to the membrane identity of cellular organelles. Besides this “zip-code”-like function, PIs also play important roles in an ever-growing number of cellular processes, including membrane trafficking, signal transduction, and cell migration [9, 10]. Accumulating data have shown that PIs are a target of intracellular bacterial pathogens, which use a variety of mechanisms to subvert host PI metabolism [11–13]. Many intracellular pathogens secrete effector proteins that directly modify the PI levels in the host, alter the activity of host PI-metabolizing enzymes, or bind to PIs for their proper intracellular targeting and function [14–16].

Intracellular pathogens have evolved many ways of performing similar tasks: uptake by host cells, evading host defenses to allow for intracellular replication, and introduction into the cytosol followed by egress from the host. For example, different species use a variety of mechanisms to enable uptake by host cells [13]. *Shigella flexneri* and *Salmonella enterica* both use type III secretion systems (T3SS) to secrete PI phosphatases into the host cytosol, altering the membrane content and inducing the phagocytosis of the bacteria. *Yersinia* spp. and *Mycobacterium tuberculosis* utilize receptor-mediated endocytosis to invade their respective hosts. Once inside, infectious bacteria also have differing replication environment preferences. *Salmonella*, *Legionella*, and *Mycobacterium* each develop unique intracellular niches to avoid detection and replicate, while *Brucella* species replicate in the cytoplasm [17–20]. The pathogenic life cycle concludes with the induction of host apoptotic pathways and lysis of the host cell, leading to the death of the host, with each pathogen using its own mechanism to do so [21–23]. There is still much to be learned about the mechanisms of bacterial pathogenesis in each species, particularly the functions of the bacterial effector proteins.

A prime example of the exploitation of both the host PI and ubiquitin systems is *Legionella pneumophila*, a gram-negative intracellular pathogen whose natural host is freshwater amoeba. *Legionella* is an opportunistic pathogen of humans, causing a severe form of pneumonia termed Legionnaires’ disease [24, 25]. *Legionella* uses an intracellular multiplication/defective organelle trafficking (Dot/Icm) type IV secretion system to secrete approximately 300 effector proteins into the host cytosol to hijack many host cellular processes, leading to the formation of a replicative niche for the bacteria, termed the *Legionella*-containing vacuole (LCV) [26, 27].

Currently, only approximately 10% of the *Legionella* effector proteins have a described function, leaving much to be learned about these proteins and the infection itself. Difficulty in studying *Legionella* effectors arises from the high level of functional redundancy observed in these proteins. Indeed, entire families of secreted effectors can be deleted without the emergence of a phenotype in a *Legionella* infection. Of the effectors with known functions, many act to disrupt host pathways. For example, SidM/DrrA has been shown to be a PI(4)P-binding guanine nucleotide-exchange factor (GEF) for the recruitment of the host GTPase Rab1 to the LCV, while LepB acts as a Rab1 GTPase activating protein (GAP), serving to regulate the activity of SidM/DrrA [28–30]. There are also recent reports of effectors proposed to regulate other *Legionella* effectors, termed “metaeffectors.” SidJ was shown to reduce the toxicity of the SidE family of effectors in yeast [31, 32]. In addition, the

U-box containing E3 ubiquitin ligase LubX directly targets SidH for degradation [33]. The complexity of the action and regulation of so many effectors is just beginning to be understood. While the majority of the effector proteins remain to be characterized, one of these effectors, SidC (and its paralog SdcA), has been recently found to have multiple functions that serve to connect both the ubiquitin and PI pathways.

SidC was shown to associate with the LCV though a C-terminal PI(4)P-binding region and was suggested to play a role in the enrichment of both ER-derived vesicles and polyubiquitinated species on the LCV [12, 34–36]. We and others determined the structure of the N-terminal domain of SidC, which contained a novel fold with an unknown function [36–38]. With careful sequence and structural analysis, we found a cluster of residues on the surface of the protein that was conserved across all SidC homologs. We observed that this cluster contained three highly conserved residues (C46, H444, and D446) arranged in a manner reminiscent of the classical Cys-His-Asp catalytic triad commonly found in cysteine proteases and deubiquitinases. However, after extensive trials we failed to detect any activity related to these hydrolytic enzymes. Surprisingly, a significant change in the ubiquitination pattern of the total cell lysate was detected in an experiment in which SidC was co-expressed with HA tagged ubiquitin in HEK293T cells. This observation led us to hypothesize that SidC may function as an E3 ubiquitin ligase, which was verified by *in vitro* ubiquitination assays. In addition, we found that SidC preferentially catalyzed K11 and K33-linked polyubiquitin chains. Thus, we named this novel N-terminal domain the SNL domain (SidC N-terminal ubiquitin Ligase).

Further structural studies led us to reveal the nearly full-length structure of SidC [39]. We found that the C-terminal PI(4)P-binding P4C domain assumed a four antiparallel α-helical bundle structure. A positively charged pocket formed at one end of this bundle was confirmed as the specific PI(4)P binding site. Intriguingly, the P4C domain masked the ubiquitin ligase active site though a hydrophobic interaction with a patch of residues near the catalytic cysteine. This “closed” conformation not only rendered SidC a lower ubiquitin ligase activity compared to the isolated SNL domain, but also a lower affinity for PI(4)P binding compared to the P4C domain alone. We found that SidC presented an increased ligase activity upon PI(4)P binding, presumably due to the “opening” of the catalytic site induced by PI(4)P. This regulatory mechanism seemed to be conserved in all SidC orthologs except that of *Legionella longbeachae*. In that species, the leucine residue that mediated the interaction between the SNL and P4C domains was altered to a glutamic acid. This substitution likely disrupted the hydrophobic interaction and gave *L. longbeachae* SidC a preference for the “open” conformation. In agreement with this observation, *L. longbeachae* SidC was shown to have ~3 fold increase in affinity for PI(4)P binding when compared to *L. pneumophila* SidC [40].

Our results indicate the discovery of another *Legionella* effector protein that merges two independent cellular pathways, by uniting a unique ubiquitin E3 ligase activity and the recognition of a specific PI in a single peptide. It remains to be seen if more bacterial effectors function to link multiple pathways together in a similar manner. These findings allow us to propose a model of SidC action. After *Legionella* infection, SidC is secreted in the closed state. Upon binding to PI(4)P on the LCV via the P4C domain, the ubiquitin

ligase active site becomes accessible, leading to an increase in E3 ubiquitin ligase activity. In this model, the precise molecular mechanism for ubiquitin ligation remains to be explored. More importantly, the specific substrates that are ubiquitinated by SidC are still unknown. Future experiments are certainly needed to address how host ER vesicle trafficking is rerouted by the ubiquitin ligase activity of SidC.

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