Small GTPases 3:1, 28–33; January/February/March 2012; © 2012 Landes Bioscience

The taming of a Rab GTPase by *Legionella pneumophila*

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Keywords: Legionella, Rab1, GEF, GAP, AMPylation, de-AMPylation, phosphocholination

Submitted: 09/07/11

Revised: 11/04/11

Accepted: 11/08/11

http://dx.doi.org/10.4161/sgtp.18704

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Commentary to: Neunuebel MR, Chen Y, Gaspar AH, Backlund PS, Jr., Yergey A, Machner MP. De-AMPylation of the small GTPase Rab1 by the pathogen *Legionella pneumophila*. Science 2011; 333:453–6; PMID:21680813; http://dx.doi.org/10.1126/science.1207193

Small GTPases of the Rab family represent an attractive target for microbial pathogens due to their role in controlling many aspects of intracellular cargo transport. *Legionella pneumophila* is an intravacuolar pathogen that survives inside host cells by manipulating protein trafficking pathways through a number of effector proteins secreted by the bacterium. These act as functional mimics of host proteins that modulate the activity of switch proteins such as guanosine triphosphatases (GTPases). *L. pneumophila* exploits the ER (endoplasmic reticulum)-to-Golgi vesicle transport pathway by modifying activity of Rab1, the GTPase regulating this pathway. This pathogen recruits Rab1 to the vacuole in which it resides, where effector proteins located on the surface of the vacuole regulate the activity status of Rab1 by mimicking the function of a guanine dissociation inhibitor (GDI) displacement factor, guanine exchange factor (GEF), or a GTPase-activating protein (GAP). In addition to these non-covalent modifications that alter the nucleotide binding state of Rab1, the bacterium also uses covalent modifications such as adenylylation (AMPylation) to control the dynamic of Rab1 on the *Legionella*-containing vacuole. Remarkably, AMPylation of Rab1 by SidM can be reversed by the *L. pneumophila* effector protein SidD, which exhibits de-AMPylation activity, demonstrating that *L. pneumophila* and related pathogens may utilize covalent modifications in order to transiently alter the activity of host proteins.

L. pneumophila Manipulates Host Rab GTPases

Intracellular bacterial pathogens have evolved specialized mechanisms to invade and survive within eukaryotic host cells. To efficiently replicate inside the hostile environment of a phagocyte without risking detection by the innate immune system, bacterial pathogens restructure their phagosome by exploiting various host pathways.[1] Several pathogens have the ability to manipulate host signaling cascades by delivering effector proteins into the host cell that specifically target and modify the activity of key regulatory proteins.[2] Guanosine triphosphatases (GTPases) turn entire signaling cascades on or off by simply switching between an active GTP-bound form and an inactive GDP-bound conformation. Not surprisingly, they have become the target of many pathogens that selectively exploit their activity in order to establish conditions supportive for infection and disease development.

Rab proteins are small GTPases that localize to distinct intracellular membranes in eukaryotic cells and act as molecular switches, thereby mediating intracellular processes such as vesicle trafficking between organelles.[3] The hydrophobic geranylgeranyl (prenyl) groups present at two C-terminal cystein residues allow Rab GTPases to reversibly associate with membranes. Rab GTPases alternate between two conformational states: GTP-bound (active form) and GDP-bound (inactive form). Guanine nucleotide exchange factors (GEFs) convert GDP-Rab into GTP-Rab, causing a conformational change that

prompts interaction of Rabs with multiple downstream effector proteins that are directly implicated in membrane transport, vesicle tethering and fusion.[4] Upon completion of these events, GTP-bound Rab proteins are inactivated by GTPase activating proteins (GAPs), which stimulate the intrinsic GTP hydrolysis activity of Rabs to return them to their GDP-bound inactive conformation. Subsequently, the prenylated GDP-Rab is recognized and extracted from the membrane by a GDP dissociation inhibitor (GDI). The Rab cycle is reinitiated when the Rab-GDI complex is targeted toward specific membranes by interaction with a membrane-bound protein that exhibits GDI displacement factor (GDF) activity.[3]

Following phagocytosis by the host cell, the bacterium *Legionella pneumophila* forms a specialized replication vacuole (*Legionella*-containing vacuole, LCV) that avoids the fate of lysosomal fusion other microbes face.[5] Proteome studies confirmed the presence of several small GTPases such as Rab1, Rab7, Rab8, Rab14 and Arf1 around LCVs, suggesting that *L. pneumophila* initiates intimate contact with vesicles from various transport routes.[6] Rab1 has been the recent focus of several studies that have looked at its recruitment and manipulation on the LCV. In this article we will provide an overview of the increasingly complex mechanism of Rab1 exploitation by *L. pneumophila*.

Recruitment of Rab1 to the LCV and its Noncovalent Modification

During infection, *L. pneumophila* ensures its intracellular survival by manipulating several host cell functions through a large number of effector proteins (more than 300) that are translocated into the host cytosol via a type IV secretion system (T4SS) called Dot/Icm.[5] Among these are proteins that are molecular mimics of host cell GEF and GAP proteins capable of activating or inhibiting small GTPases.[7,8] Rab1, a key regulator of ER to the Golgi and intra-Golgi vesicle transport, is recruited to LCVs shortly after bacterial uptake, consistent with the idea that *L. pneumophila* exploits this transport route to remodel the originally plasma membrane-derived vacuole into an ER-like compartment.[5] Disturbance of secretory vesicle budding and trafficking by overproduction of constitutively inactive variants of the GTPases Sar1, Arf1 and Rab1 attenuates *L. pneumophila* intracellular replication and results in enhanced lysosomal fusion of LCVs, further underscoring the importance of host vesicle flow for a successful establishment of a replication vacuole by *L. pneumophila*.[9-12]

Recruitment and activation of Rab1. Rab1 recruitment to the LCV requires the activity of the effector protein SidM (also known as DrrA).[13] SidM comprises three functionally distinct domains: a C-terminal phosphatidylinositol-4-phosphate (PI4P) binding domain, a central domain with Rab1 GEF and GDF activity, and an N-terminal domain with adenylyl transferase (AMPylation) activity.[14] Once translocated through the Dot/Icm T4SS into host cells, localization of SidM to the LCV surface is mediated by its high affinity binding to PI4P, which is present on the vacuolar membrane.[15] The central GEF/GDF domain of SidM catalyzes Rab1 recruitment to the LCV by displacing the GTPase from Rab1-GDI and activating it through GDP/GTP exchange.[7] Active Rab1 is believed to promote interaction of LCVs with secretory transport vesicles by mediating binding of its cellular ligands positioned on the opposing membranes, thereby contributing to vacuolar transformation into an ER-like compartment. Once the activity of Rab1 is no longer required by *L. pneumophila* the GTPase is removed from the LCV in a process that involves its inactivation by GAP proteins, including *L. pneumophila* LepB, as described in detail in the following section.

Inactivation and removal of Rab1 from the LCV. Mammalian cells harbor more than 65 different Rab proteins whose controlled inactivation depends on the activity of upstream regulators called GAPs. These proteins display specificity toward their cognate partner Rab and stimulate the intrinsic GTP hydrolysis activity of Rabs thereby rendering them inactive.[16] Intracellular *L. pneumophila* accumulate Rab1 on their LCV early during infection, after which it is gradually removed from the vacuolar membrane in a process that involves inactivation by the bacterial GAP LepB followed by GDI-mediated membrane extraction. LepB was originally discovered as a type IV effector involved in exocytosis of *L. pneumophila* from protozoan host cells by an unknown mechanism that avoids host cell lysis.[17] Later studies revealed LepB has Rab1-GAP activity, which may be unrelated to its function in non-lytic release from host cells.[8] In the absence of LepB, Rab1 removal from the LCV is delayed, consistent with its important role in this process.[18] The lack of a more severe effect on Rab1 removal in the absence of LepB suggests that additional GAP proteins such as TBC1D20, the mammalian Rab1 GAP, may contribute to Rab1 inactivation and removal from the LCV. Alternatively, its intrinsic GTPase activity may cause Rab1 to slowly convert itself into the GDP-bound inactive form susceptible to membrane extraction by GDI.

Until recently, it was thought that *L. pneumophila* regulates the Rab1 cycle only by mimicking the activity of host cell GEF and GAP proteins to control early activation and later inactivation of Rab1, respectively. It is now apparent that *L. pneumophila* also covalently modifies Rab1, which enables the pathogen to refine its control over this key trafficking protein.

Covalent Modification of Rab1 by Reversible Attachment of Adenosine Monophosphate (AMP)

AMPylation of Rab1. Besides transiently modifying the nucleotide binding state of Rab1, the effector protein SidM post-translationally modifies Rab1 by the covalent addition of AMP to tyrosine77 of Rab1, a process known as AMPylation (or adenylylation).[19] SidM preferentially AMPylates the GTP-bound form of Rab1 suggesting that activation of Rab1 through nucleotide exchange precedes AMPylation. One of the striking consequences of AMPylation is its ability to render Rab1 resistant to inactivation by the Rab1-GAPs LepB and TBC1D20.[19] *L. pneumophila* could benefit from transiently disabling inactivation of AMPylated active Rab1 by GAPs by potentially extending the time of Rab1 retention on the LCV, which could facilitate interception of secretory vesicles.

Another consequence of the covalent attachment of AMP within the conserved switch II region of Rab1 is that it seems to sterically hinder Rab1 binding to downstream effector proteins such as MICAL-3.[19] The ectopic production of SidM in mammalian cells results in Golgi fragmentation and subsequent cell death, most likely because AMPylation disrupts the ability of Rab1 to mediate vesicle transport within the secretory pathway, an essential trafficking route in eukaryotic cells.[18,19] The apparent adverse effects of AMPylation on Rab1 function raise the question of why *L. pneumophila* would force active Rab1 to remain on the LCV, but at the same time disrupt its interaction with host cell downstream effectors which would promote interaction of LCVs with ER-derived vesicles? It is possible that AMPylation limits interaction of Rab1 with a subset of cellular ligands, while promoting interaction with others. Alternatively, *L. pneumophila* could produce its own mimics of host cell Rab1 ligands that may be able to interact with Rab1 even in its AMPylated state thereby promoting vesicle recruitment to the LCV. In support of this notion, it was found that although AMPylated Rab1 cannot interact with its eukaryotic downstream effectors it can still interact with LidA,[19] a *L. pneumophila* effector protein that collaborates with SidM to enhance recruitment of Rab1 to the LCV.[13] Though the exact role of LidA in Rab1-controlled vesicle hijacking is yet to be determined, it was found to play an important role for *L. pneumophila* intracellular replication.[13]

De-AMPylation of Rab1. Cellular Rab1 is only transiently present on the LCV in the early phase of infection by *L. pneumophila*.[8,18] The discovery of Rab1 AMPylation and its inhibitory effect on Rab1 inactivation posed the important question of how this GTPase is eventually allowed to be inactivated by GAPs and removed from LCVs. The surprising answer was that *L. pneumophila*, in addition to its AMPylase SidM, also encodes a Rab1 de-AMPylase, the effector protein SidD.[18,20] Prior to our study, the only example of a protein harboring de-AMPylase activity was the bacterial glutamine synthetase adenylyl transferase (GS-ATase) described in the 60s.[21] The GS-ATase is present in many bacteria and it acts as a regulatory factor for nitrogen metabolism. It controls the activity of glutamine synthetase through its ability to both AMPylate and de-AMPylate this enzyme, thereby keeping it in the inactive or active form, respectively.[22] The *L. pneumophila* de-AMPylase SidD catalyzes removal of the covalently bound AMP (or GMP) from Rab1, which allows deactivation of Rab1 by LepB and TBC1D20.[18,20] The important role of SidD in triggering Rab1 inactivation is reflected in vivo where SidD proved to be a critical determinant for the timely removal of Rab1 from LCVs. Rab1 showed extended colocalization with the LCV in mouse bone marrow-derived macrophages challenged with an *L. pneumophila* mutant lacking SidD compared with wild-type. The antagonistic effects of SidD and SidM were also verified in studies where SidD reversed the cytotoxicity caused by overproduction of SidM within transiently transfected tissue culture cells.[18] Thus, AMPylation and de-AMPylation are important determinants in controlling the activity of Rab1 and are used by *L. pneumophila* to fine-tune Rab1 dynamics on the LCV membrane.

The presence of antagonistic activities among different effector proteins requires *L. pneumophila* to exert temporal and spatial control over their function. In the case of Rab1, the chronological order of events taking place during its recruitment and manipulation at the LCV is controlled by the sequence in which these various effector proteins involved at each step are translocated into host cells (**Table 1**). SidM levels are high immediately after infection, consistent with its involvement in promoting Rab1 recruitment and activation on the LCV. SidM level declines after two hours, concurrent with an increase in the level of SidD and LepB, both of which are required for removal of Rab1 from the LCV by catalyzing its de-AMPylation (SidD) and subsequent GTP hydrolysis (LepB).[18] Disturbance in the timing of effector translocation significantly affects Rab1 dynamics on the LCV. For example, host cell infection with an *L. pneumophila* variant constitutively producing plasmid-encoded SidD leads to premature translocation of SidD into host cells and significantly diminishes Rab1 accumulation on LCVs compared with a wild-type strain. Thus, de-AMPylation primes Rab1 for premature inactivation and subsequent extraction from LCVs.[18] Early translocation of LepB, on the other hand, had no detectable effect on Rab1 accumulation on LCVs, indicating that the blockage of GTP hydrolysis caused by AMPylation is maintained even in the presence of elevated levels of LepB. Thus, *L. pneumophila* employs AMPylation and de-AMPylation to control timing of the effect that other regulatory factors such as GAPs have on Rab1 activity.

Our recent discovery of SidD's activity combined with earlier findings provide a more complete model of the Rab1 cycle on the LCV (**Fig. 1 and Table 1**). SidM initiates the Rab1 cycle by recruiting Rab1 to the LCVs surface early during infection. SidM mediates release of Rab1 from the cytosolic chaperone RabGDI and activation through GDP/GTP exchange. SidM then AMPylates Rab1 to transiently lock it in the active conformation by preventing its premature inactivation by bacterial LepB or the host cell GAP TBC1D20. Once formation of the replication vacuole is completed, *L. pneumophila* translocates

Table 1. *Legionella pneumophila* effectors that control Rab1 recruitment to the LCV and its activity status

Translocation post-infection	Effectors	Mode of action	Effect on recruitment to the LCV and activity of Rab1
Early	SidM	GDF, GEF AMPylase	Recruits to LCV and activates[13] Blocks inactivation by GAPs[19]
↓	LidA	Unknown	Enhances recruitment to the LCV[13]
↓	SidD	De-AMPylase	Triggers inactivation by GAPs[18]
Late	LepB	GAP	Inactivates[8]
Unknown	AnkX	Phosphocholinase	Unknown[23]

GDF, guanine displacement factor; GEF, guanine exchange factor; GAP, GTPase activating protein; LCV, Legionella-containing vacuole.

the de-AMPylase SidD as well as the GAP LepB into host cells. SidD catalyzes removal of AMP from Rab1-GTP, thereby enabling Rab1 inactivation by LepB and subsequent membrane extraction of Rab1-GDP by host cell RabGDI (**Fig. 1**).

(De-)AMPylases in other genomes. The existence of both AMPylase and de-AMPylase activity within *L. pneumophila* effector proteins strongly suggests that other microbes have developed similar tools to manipulate GTPases and other host factors. Identifying novel de-AMPylases will be challenging because SidD does not possess significant homology to genes in any other genome. Its predicted secondary structure, however, is likely to be similar to protein phosphatases, with two conserved aspartate residues at position 92 and 110, which were recently shown to be crucial for its catalytic activity.[20] This is not surprising given that both a de-AMPylase and a protein phosphatase catalyze hydrolysis of a phosphoester bond. SidD most likely evolved from a protein phosphatase that was acquired through gene duplication or horizontal gene transfer and that was repurposed into a protein de-AMPylase. A more detailed study of SidD will be necessary to better understand its molecular function and to potentially determine a unique signature of residues within SidD that would help identify protein de-AMPylases in other pathogenic organisms. However, in the absence of such unique structural features, a reasonable place to look for de-AMPylases would be the genomic region proximal to genes encoding proteins with Fic domains, which in other pathogenic models have been associated with AMPylation of small GTPases.[24] When it comes to bacterial genomes it is not uncommon to find that genes encoding proteins of related functions are located adjacent to each other on the genome and are co-inherited, as is the case for *sidD* and *sidM*. By refining their own genetic make up to efficiently manipulate signaling pathways of the host cell, *L. pneumophila* and related pathogens may have increased their chance of survival in a broad array of hosts. Hence, we expect to identify additional examples of effector proteins in *L. pneumophila* and other pathogenic microbes that have been similarly fine-tuned for a function inside the host cell.

Despite identification of a growing numbers of effector proteins that *L. pneumophila* invests in manipulating the activity of Rab1, the presence of this GTPase on the LCV does not seem to be essential for successful survival and replication inside the host cell. Disturbance in the control of Rab1 by deleting or over-expressing SidM or SidD does not affect replication and survival inside the host cell.[13,18] This is in accordance with the emerging consensus that *L. pneumophila* effectors displays a high degree of functional redundancy during infection which makes it challenging to detect replication phenotypes when individual host cell pathways are disrupted during growth in

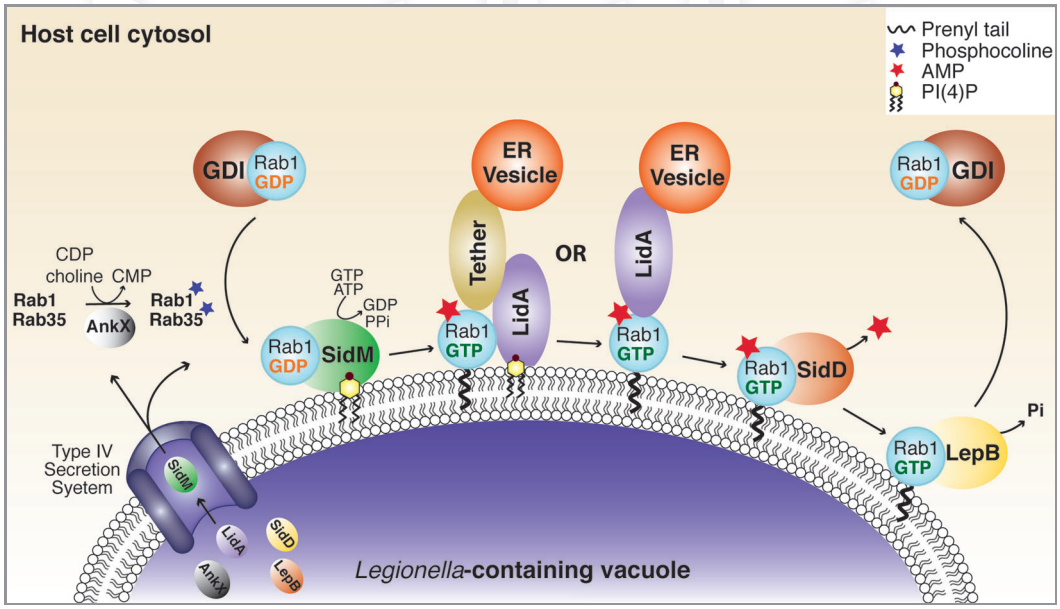

Figure 1. Manipulation of the Rab1 activity cycle by bacterial effector proteins on the *Legionella*-containing vacuole (LCV). *L. pneumophila* sequentially translocates effector proteins into the host cell cytosol via a type IV secretion system (Dot/Icm). Early during infection, SidM attaches to the LCV via PI(4)P binding to recruit Rab1 to the LCV by displacing it from the Rab1-GDI complex and it activates Rab1 by exchanging GDP for GTP. SidM then covalently modifies GTP-bound Rab1 by adding an AMP moiety at tyrosine 77 to lock Rab1 in an active conformation, as GAPs are unable to access AMPylated Rab1; interaction with LidA is not disrupted by AMPylation. We favor a model in which the role of AMPylation is to prolong the presence of Rab1 on the LCV to allow its interaction with downstream effector proteins in the presence or absence of LidA leading to attraction of ER vesicles to the LCV. Later during infection, SidD is translocated into the host cell to remove AMP from modified Rab1, which makes it available to inactivation through LepB-stimulated GTP hydrolysis. Inactive GDP-bound Rab1 is subject to GDI-mediated removal from the LCV membrane. Following recruitment of Rab1 by SidM to the LCV, Rab1 can be phosphocolinated by AnkX, but the role of this new covalent modification has yet to be determined. (ATP/AMP, adenosine tri-/monophosphate; CMP/CDP, cytidine mono-/diphosphate; GDP/GTP, guanosine di-/triphosphate; PI(4)P, Phosphatidylinositol 4-phosphate; PPi, pyrophosphate; Pi, phosphate).

laboratory cell lines. Legionella likely targets multiple vesicle trafficking routes simultaneously to direct membrane material to the LCV.

Covalent Modification of Rab1 by Attachment of Phosphocholine

Fic domains are well conserved and widely distributed within bacterial proteins, but a few examples can be found even in humans.[24] However, only a handful of these bioninformatically identified Fic proteins have been experimentally validated, Fic domains were generally thought to be associated with AMPylation. Subsequent to our recent study identifying the Rab1 de-AMPylase, a new study uncovered that Fic domains can catalyze another covalent modification known as phosphocholination. The ankyrin repeat containing protein LegA8 (AnkX) from *L. pneumophila* uses cytidine diphosphate choline (CDP-choline) as a substrate for phosphocholine transfer to Rab1 as well as Rab35, a GTPase involved in endosomal trafficking.[23] The serine 79 in Rab1A (Ser76 in Rab1B) phosphocholinated by AnkX directly precedes the tyrosine residue that is AMPylated by SidM (Tyr80 in Rab1A, Tyr77 in Rab1B). Given the proximity of those residues it is not surprising that AMPylation of Tyr77 and phosphocholination of Ser76 in Rab1 appear to be mutually exclusive, most likely because modification at one residue would sterically impede covalent modification of the other. Consistent with this, only one of the two forms of modification were detected at one time within the same Rab1 molecule isolated from *L. pneumophila* infected cells.[23]

The biological relevance of Rab phosphocholination during *L. pneumophila* infection is unclear. AnkX was predicted to interfere with microtubule-dependent delivery of the lysosomal compartment to LCVs. Phosphocholination of Rab35 interferes with binding of its cognate GEF, connecdenn,[23] and the morphology d of endosomes caused by ectopic production of AnkX in eukaryotic cells seem to be similar to those caused by disturbance of Rab35 levels.[23] Binding of Rab35 to its respective downstream effector proteins needs to be tested to obtain a more clear perspective as to the role of Rab35 phosphocholination. It is also unclear if phosphocholination of Rab GTPases during *L. pneumophila* infection can be reversed in a way similar to the SidD-mediated reversal of Rab1 AMPylation.

The discovery of AnkX as a Fic protein with phosphocholine transferase activity hints at the fact that the Fic domains may be capable of a wider range of enzymatic activities than previously anticipated. Consistent with this, the conserved histidine residue of the Fic motif is essential for both AMPylation and phosphocholination activity, suggesting that proteins with these different activities have a similar catalytic core.[23]

Conclusions

As molecular switches, Rab GTPases play pivotal roles in eukaryotic pathways and are thus not surprisingly a common target for bacterial pathogens. The fact that *L. pneumophila* dedicates multiple effector proteins to manipulate Rab1 by non-covalent and covalent modifications underscores the importance of Rab-regulated vesicle transport pathways for the survival of the pathogen inside the host cell. Modifications mediated by *L. pneumophila* effector proteins upon infection do not seem to perturb the general homeostasis of the host cell most likely because they are largely confined to a small fraction of the target host proteins present on the LCV. Even though it is challenging to bioinformatically identify novel effectors that target and modify host cell proteins because of the lack of significant homology, it is likely that the repertoire of *L. pneumophila* effectors that target Rab1 during infection will continue to expand. Moreover, there may be additional post-translational modifications employed by *L. pneumophila* to hijack the activity of Rab proteins and other host factors. Given the abundance of Fic domain-containing proteins among bacterial species and their functional adaptability it will be interesting to see if other microbial pathogens make use of enzyme pairs that transiently control localization and activity of host target factors such as small GTPases through reversible post-translational modifications such as AMPylation and de-AMPylation.

Acknowledgments

This work was supported by the Intramural Research Program of the NIH.

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