RESEARCH ARTICLE

AMPylation Is Critical for Rab1 Localization to Vacuoles Containing *Legionella pneumophila*

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ABSTRACT ***Legionella pneumophila*** **is an intracellular pathogen that resides within a membrane-bound compartment that is derived from vesicles exiting the endoplasmic reticulum (ER). To create this compartment, these bacteria use a type IV secretion system to deliver effector proteins that subvert host cell functions. Several *Legionella* effector proteins modulate the function of the host protein Rab1, which is a GTPase that is recruited to the *Legionella*-containing vacuole (LCV). Here, we examined which of the Rab1-directed enzymatic activities displayed by *Legionella* effectors are important for localizing the Rab1 protein to the LCV membrane. The guanine nucleotide exchange factor (GEF) domain in the effector protein DrrA (SidM) was essential for Rab1 recruitment to the LCV and Rab1 AMPylation by the nucleotidyltransferase domain in DrrA was important for Rab1 retention. *Legionella* organisms producing mutant DrrA proteins that were severely attenuated for GEF activity *in vitro* retained the ability to localize Rab1 to the LCV. Rab1 localization to the LCV mediated by these GEF-defective mutants required AMPylation. Importantly, we found that efficient localization of Rab1 to the LCV occurred when Rab1 GEF activity and Rab1 AMPylation activity were provided by separate proteins. Rab1 phosphocholination (PCylation) by the effector protein AnkX, however, was unable to substitute for Rab1 AMPylation. Lastly, the defect in Rab1 localization to the LCV in AMPylation-deficient strains of *Legionella* was partially suppressed if the GTPase-activating protein (GAP) LepB was eliminated. Thus, our data indicate that AMPylation of Rab1 is an effective strategy to maintain this GTPase on the LCV membrane.**

IMPORTANCE **Activities that enable the intracellular pathogen *Legionella pneumophila* to subvert the function of the host protein Rab1 were investigated. Our data show that a posttranslational modification called AMPylation is critical for maintaining a pool of Rab1 on the LCV membrane. AMPylation of Rab1 led to the accumulation of GTP-bound Rab1 on the LCV membrane by protecting the protein from inactivation by GAPs. Importantly, PCylation of Rab1 by the *Legionella* effector protein AnkX was neither necessary nor sufficient to maintain Rab1 on the LCV, indicating that AMPylation and PCylation represent functionally distinct activities. We conclude that modification of Rab1 by AMPylation is an effective strategy to spatially and temporally regulate the function of this GTPase on a membrane-bound organelle.**

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Legionella pneumophila is a Gram-negative bacterium capable of replicating inside eukaryotic host cells. Protozoa living in freshwater and soil environments are the natural hosts for *Legionella* (1), but *Legionella* also has the ability to replicate inside human alveolar macrophages. Human infections are often caused by inhalation of aerosolized water contaminated by *Legionella* and sometimes result in a severe pneumonia known as Legionnaires' disease (2). After uptake, *Legionella* must manipulate the host cell in which it resides to persist and survive during the course of the infection. This is accomplished through the activity of over 280 bacterial proteins that are translocated into the host cytoplasm by a type IV secretion system called Dot/Icm (3, 4). Bacterial proteins translocated into host cells are known as effectors (5), and collectively these effector proteins function to prevent fusion of the *Legionella*-containing vacuole (LCV) with lysosomes and promote vacuole remodeling by vesicles derived from the endoplasmic reticulum (6–11).

A hallmark of *Legionella* manipulation of host signaling events is the localization of endoplasmic reticulum (ER) proteins at the vacuole membrane (11–13). Vesicles exiting the ER are actively recruited to the LCV to create a specialized compartment that supports bacterial replication (11). *Legionella* regulates vacuole maturation by co-opting small GTPases involved in membrane transport. The functions of Rab1 (12, 13), ARF1 (11, 14), and Sar1 (11) are important for LCV biogenesis. Rab1 has a conserved role in eukaryotic cells in promoting the tethering and fusion of vesicles exiting the ER with the Golgi (15). *Legionella* subverts Rab1 function to promote vesicle fusion with the LCV (12, 13, 16–20). Thus, localization of Rab1 to the LCV membrane is one mechanism to stimulate the recruitment and fusion of ER-derived vesicles.

Rab1 function is modulated by multiple *Legionella* effectors (Fig. 1) (21). Some of these effector proteins mimic the biochemical activities of eukaryotic proteins. The effector DrrA (SidM) is a

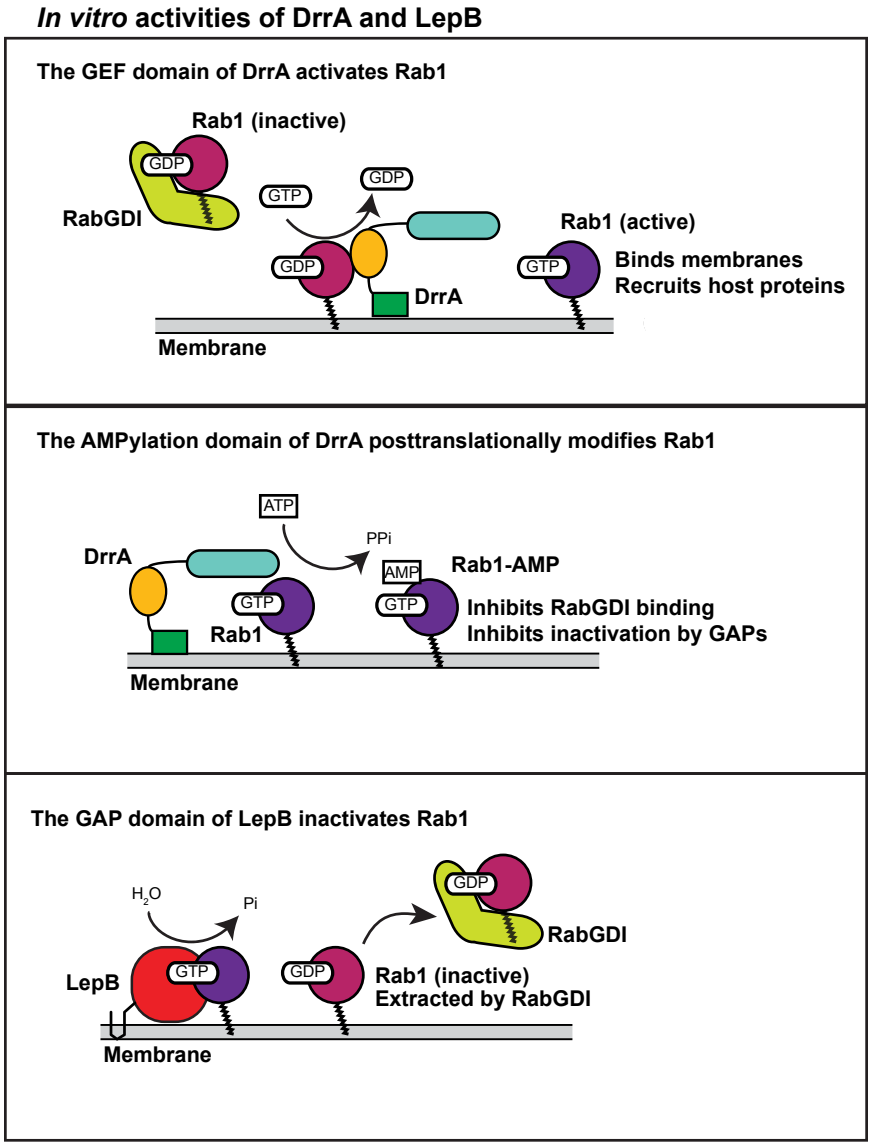

FIG 1 *In vitro* activities displayed by DrrA and LepB. The DrrA protein localizes to membranes and has the ability to activate soluble Rab1 protein bound to the chaperone protein RabGDI by catalyzing the exchange of GDP for GTP through a central GEF domain. Activated Rab1 is associated with membranes. The AMPylation domain in DrrA posttranslationally modifies Rab1, which interferes with Rab1 inactivation by GAP proteins and RabGDI binding. LepB is a GAP that localizes to membranes and inactivates Rab1 by stimulating GTP hydrolysis, which promotes RabGDI-mediated membrane extraction.

potent Rab1-specific guanine nucleotide exchange factor (GEF) that activates Rab1 by catalyzing the replacement of GDP for GTP (16, 17). Conversely, the effector LepB deactivates Rab1 by functioning as a GTPase-activating protein (GAP) (18). DrrA is detected on the LCV membrane within 30 min of infection and cycles off after several hours, whereas the protein LepB begins to appear on the LCV membrane after several hours and remains enriched on the vacuole during bacterial replication (18, 22). The dynamics by which DrrA and LepB appear on the LCV membrane coincide with the cycling of Rab1 on the membrane, suggesting that the GEF and GAP activities of these proteins mediate the temporal association of Rab1 on the early LCV.

Legionella effectors have been identified that posttranslationally modify Rab1. The amino-terminal region of DrrA contains a nucleotidyltransferase domain that mediates the covalent attachment of an adenosine monophosphate (AMP) moiety onto Rab1b Tyr-77 (Rab1a Tyr-80) through a process called AMPylation (23). The effector AnkX contains a FIC motif that catalyzes the covalent attachment of a phosphocholine moiety onto Ser-76 in Rab1b (Ser-79 in Rab1a) through a process termed phosphocholination (PCylation) (24). *Legionella* also translocates the effectors SidD and Lem3, having cognate Rab1-demodifying activities that reverse the process of AMPylation and PCylation of Rab1, respectively (22, 25).

It is predicted that Rab1 is recruited by DrrA and retained on the LCV membrane until GTP hydrolysis is stimulated by a GAP protein, which would make Rab1 susceptible to extraction from the membrane by Rab GDP dissociation inhibitor (GDI) (26). Both PCylation and AMPylation of Rab1 have been shown *in vitro* to prevent deactivation by GAP proteins (23, 27, 28), which

TABLE 1 Alleles of *drrA* and *ankX* used in this study

Allele	Mutation(s)	Phenotype	Rab1 GEF activity (%)	Reference
drrA	Wild-type allele	WT	100	16, 17
drrA1	N451A, R453A, A454E	GEF deficient	<1	31
drrA2	W410D	GEF deficient	<1	31
drrA3	N451A, R453A, D480A, S483A	GEF deficient	<1	29, 31
drrA4	G431D	GEF deficient	<1	31
drrA5	A435D	GEF deficient	<1	31, 32
drrA6	N451A, R453A	GEF deficient	~5–33	29, 31
drrA7	W410D, N451A, R453A, A454E	GEF deficient	<1	31
drrA8	W410D, M444A, D445A	GEF deficient	<1	31
drrA110	D110A, D112A	AMPylation deficient	100	23
*drrA3*_110	D110A, D112A, N451A, R453A, D480A, S483A	AMPylation deficient, GEF deficient	<1	This study
*drrA8*_110	D110A, D112A, W410D, M444A, D445A	AMPylation deficient, GEF deficient	<1	This study
$drrA^{\Delta GEF}$	Δ340-500	GEF deficient	0	This study
$drrA110^{\Delta GEF}$	D110A, D112A, Δ340-500	AMPylation deficient, GEF deficient	0	This study
ankX	Wild-type allele	WT	NA	24
ankX229	H229A	PCylation deficient	NA	24

should prolong membrane retention. Analysis of *Legionella* strains overproducing SidD or deficient in LepB further suggests that these effectors modulate Rab1 dynamics on the LCV by de-AMPylating Rab1 or inactivating Rab1, respectively (22, 25). Thus, biochemical and *in vivo* studies suggest that the efficiency by which *Legionella* effectors activate, modify, and deactivate Rab1 should impact the temporal association of Rab1 on the LCV membrane.

To further understand the *in vivo* role of *Legionella* effectors that modulate Rab1 activities, we set out to use *Legionella* strains having mutations that disrupt Rab1-specific activities to investigate how these biochemical functions affect the temporal dynamics of Rab1 localization to the LCV during infection. These studies indicate that Rab1 AMPylation by DrrA, but not PCylation by AnkX, is important for retaining Rab1 on the LCV membrane by blocking the process of rapid Rab1 deactivation by GAP proteins.

RESULTS

Analysis of Rab1 recruitment to the LCV by GEF-deficient DrrA proteins. DrrA contains an AMPylation domain spanning residues 1 to 339 (23), a central GEF domain spanning residues 340 to 533 (29), and a membrane-targeting domain spanning residues 534 to 647 (30) (see Fig. S1 in the supplemental material). To test the role of the GEF domain in Rab1 localization to the LCV, we made GEF-deficient DrrA proteins either having the central GEF domain deleted ($DrrA^{\Delta GEF}$) or containing site-directed mutations in the GEF domain that were shown previously to disrupt catalytic activity *in vitro* (29, 31, 32). To investigate whether GEF-deficient DrrA proteins were capable of recruiting Rab1 to the LCV during *Legionella* infection, the mutant DrrA proteins described in Table 1 were produced from a plasmid in a Δ*drrA* Δ*ankX* strain of *Legionella* (24), and Rab1 localization to the LCV was assessed.

Immunoblot analysis and immunofluorescence localization studies showed that the mutant DrrA proteins were produced by *Legionella* (see Fig. S2 in the supplemental material) and were associated with the LCV (see Fig. S3 in the supplemental material) at levels that were similar to those in wild-type DrrA. Localization of endogenous Rab1b to the LCV was measured by immunofluorescence microscopy after infection of RAW macrophages for 1 h (Fig. 2A). Rab1 was detected on the majority of vacuoles containing *Legionella* producing the wild-type DrrA protein and was not detected on vacuoles containing *Legionella* having vector alone, which indicated that localization of Rab1 required DrrA (Fig. 2A and B). Rab1 localization was not detected on vacuoles containing *Legionella* producing the $DrrA^{\Delta GEF}$ protein, which indicated that GEF activity was required for Rab1 localization to vacuoles (Fig. 2B). Unexpectedly, when the catalytically deficient DrrA proteins with site-directed mutations that decrease GEF activity were analyzed (29, 31), we found that localization of Rab1 to vacuoles was attenuated but still within a detectable range (Table 1; Fig. 2A and B). *Legionella* Δ*drrA* Δ*ankX* strains overproducing these GEF-deficient DrrA proteins from a plasmid exhibited Rab1 localization deficiencies ranging from 2-fold to 10-fold compared to isogenic strains overproducing wild-type DrrA. These data are in contrast to the >100-fold deficiency in nucleotide exchange activity determined for most of these mutant DrrA proteins (Table 1). Thus, mutant DrrA proteins that have extremely low Rab1 GEF activity *in vitro* retain the ability to recruit Rab1 to the LCV. Taken together, these data indicate that the DrrA GEF domain is necessary, but not sufficient, for the dynamic processes that mediate Rab1 localization to the vacuole.

AMPylation is important for Rab1 localization to the LCV. We next addressed whether the AMPylation activity displayed by DrrA was important for Rab1 localization to the LCV. For these studies, the aspartic acid residues D110 and D112 in DrrA were changed to alanine, which abolishes AMPylation activity (23). AMPylation activity was abolished in the DrrA protein having a wild-type GEF domain and in GEF-deficient mutants. Plasmids encoding AMPylation-deficient DrrA proteins were introduced into the *Legionella* Δ*drrA* Δ*ankX* strain, and similar levels of DrrA production (see Fig. S2 in the supplemental material) and DrrA localization to the LCV (see Fig. S3 in the supplemental material) were confirmed. Elimination of AMPylation activity in a DrrA

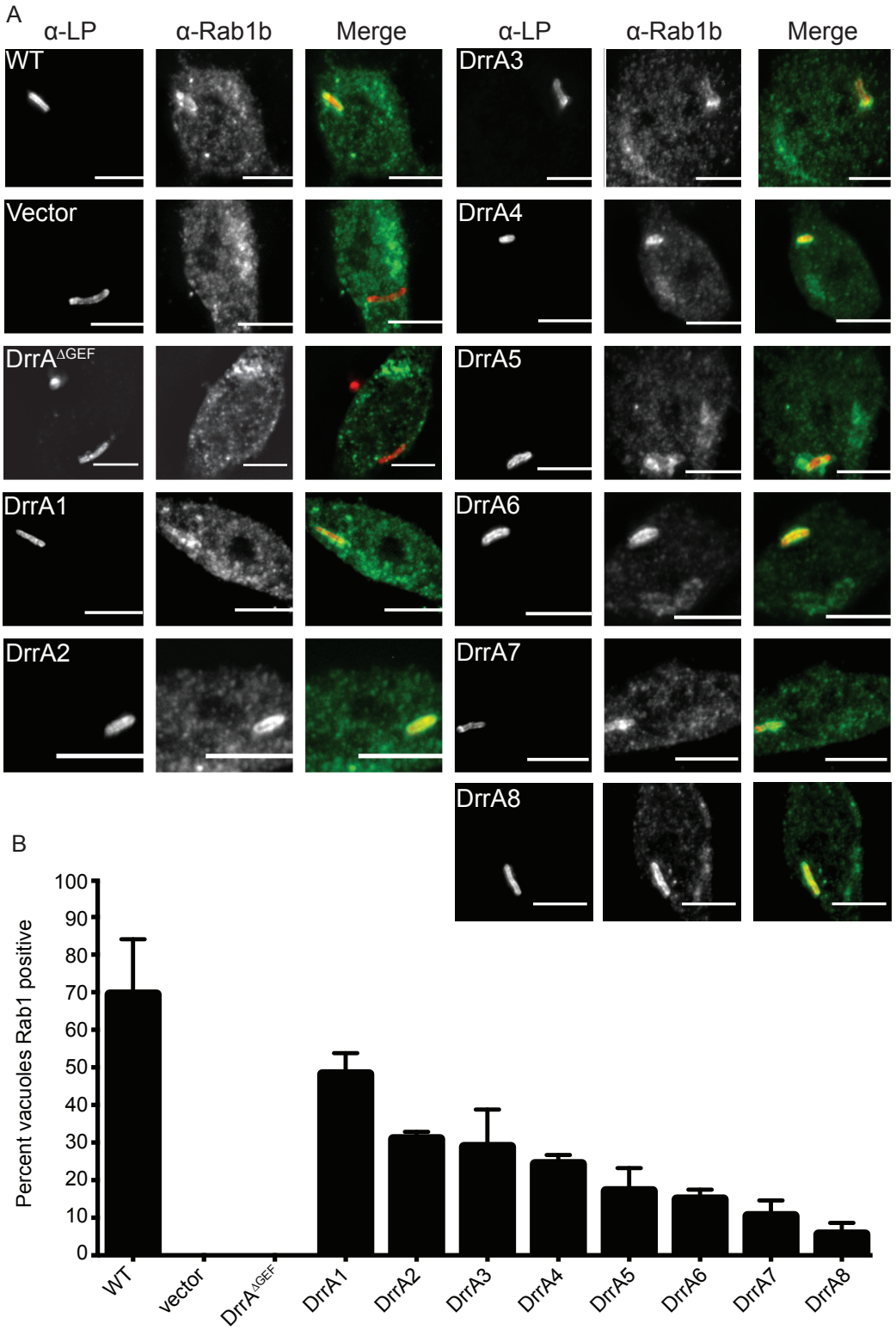

FIG 2 Analysis of Rab1 recruitment to the LCV by GEF-deficient DrrA proteins. RAW cells were infected with *Legionella* Δ*drrA* Δ*ankX* strains with plasmids encoding wild-type (WT) DrrA, the indicated GEF-deficient mutants, or empty vector. Cells were fixed 1 h after infection and labeled using an anti-Rab1b antibody. (A) Representative single-channel and merged immunofluorescence micrographs show Rab1b localization (anti-Rab1b, green) to vacuoles containing the indicated *Legionella* strains (anti-LP, red). Scale bar = 5 μm. (B) Average percentage of Rab1b-positive vacuoles containing *Legionella* producing the indicated DrrA protein. At least 150 vacuoles were scored for each experimental condition, and data were acquired from three independent replicates. Data are averages ± standard errors of the means (SEM).

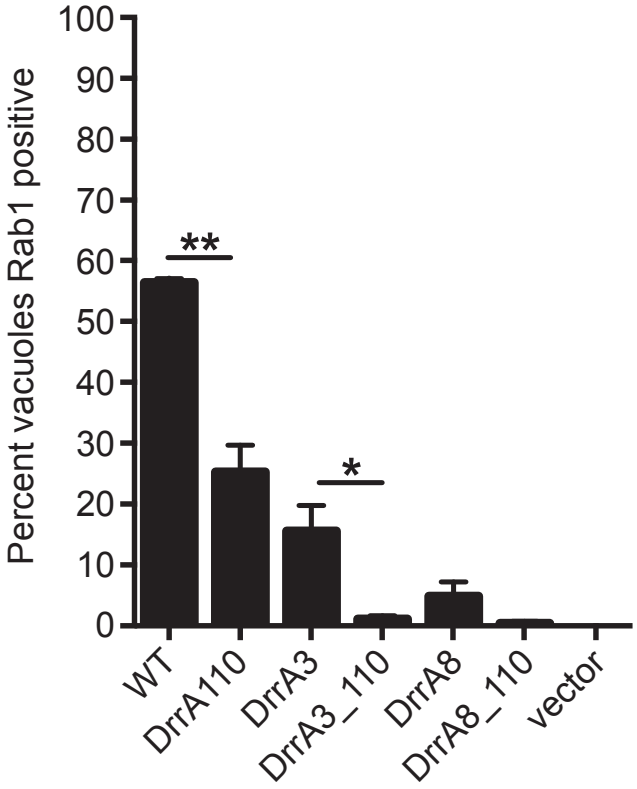

FIG 3 AMPylation is important for Rab1 localization to the LCV. RAW cells were infected with *Legionella* Δ*drrA* Δ*ankX* strains with plasmids encoding the indicated DrrA proteins. Cells were fixed 1 h after infection and labeled using an anti-Rab1b antibody. Data are the percentages of Rab1b-positive vacuoles containing *Legionella* organisms producing the indicated DrrA protein. AMPylation-sufficient controls were compared to isogenic strains producing the corresponding AMPylation-deficient DrrA protein. At least 150 vacuoles were scored for each experimental condition, and data were acquired from three independent replicates. Data are averages ± SEM. *, $P < 0.05$, and **, $P < 0.005$, compared to the AMPylation-sufficient control.

protein with a wild-type GEF domain (DrrA110) resulted in a significant decrease in the localization of Rab1 to the LCV at 1 h (Fig. 3). Rab1 localization was not detected on vacuoles containing *Legionella* producing DrrA3_110 or DrrA8_110, which are AMPylation-deficient proteins with defective GEF domains (Fig. 3). Thus, Rab1 AMPylation by DrrA is critical for localization of this GTPase to the LCV, especially under conditions where Rab1 GEF activity is severely attenuated.

AMPylation functions can be provided in *trans* to retain Rab1 on the LCV. The AMPylation and GEF functions of DrrA represent distinct biochemical activities that can be measured independently in an *in vitro* system; however, it is unknown whether these functions can be provided on separate proteins during infection. This question was addressed using the *Legionella drrA110* Δ*ankX* strain, which lacks the AnkX protein and has the chromosomal allele *drrA110* encoding the AMPylation-deficient DrrA110 protein in place of the wild-type *drrA* allele. Thus, this strain produced the DrrA110 protein at normal physiological levels from the endogenous promoter. Compared to the control strain containing the wild-type *drrA* allele, a significant defect was observed in Rab1 localization to vacuoles containing *Legionella* carrying the chromosomal *drrA110* allele and having an empty vector in *trans* (Fig. 4A and B). When the GEF-deficient DrrA8 protein having a functional AMPylation domain was produced in *trans* to the chromosomally encoded DrrA110 protein, the percentage of vacuoles that scored positive increased to levels that were similar to those in the control strain producing a wild-type DrrA protein from a chromosomal allele and were significantly higher than when the DrrA8 protein was produced in the *Legionella* Δ*drrA* Δ*ankX* strain (Fig. 4A). Rab1 localization was not restored when the AMPylation-deficient derivative of the DrrA8 protein (DrrA8_110) was produced in the *Legionella drrA110* Δ*ankX* strain. Thus, the AMPylation activity of the DrrA8 protein and the GEF activity of the DrrA110 protein could be provided on separate proteins to synergistically enhance Rab1 localization to the LCV.

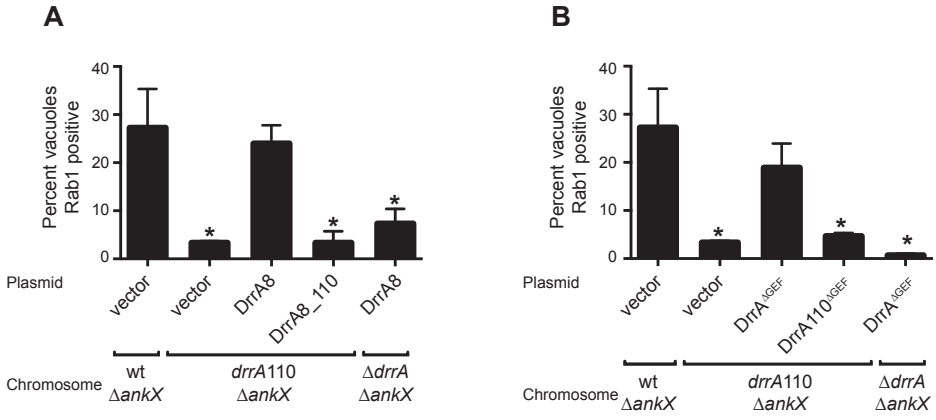

FIG 4 AMPylation functions can be provided in *trans* to retain Rab1 on the LCV. The graphs show the average percentage of Rab1b-positive vacuoles detected after RAW cells were infected for 1 h with *Legionella* organisms producing the plasmid-encoded DrrA protein indicated below each column or containing empty vector. The genotypes listed below the lines indicate the chromosomal *drrA* and *ankX* alleles present in the *Legionella* strains producing the different plasmid-encoded DrrA proteins. At least 150 vacuoles were scored for each experimental condition, and data were acquired from three independent replicates. Data are averages ± SEM. *, $P < 0.05$. (A) Rab1 localization was measured for *Legionella* organisms producing the AMPylation-deficient DrrA110 protein from a chromosomal allele and complemented with the indicated plasmid-encoded DrrA proteins with inefficient GEF domains. (B) Rab1 localization was measured for *Legionella* producing the AMPylation-deficient DrrA110 protein from a chromosomal allele and complemented with the plasmid-encoded $DrrA^{\Delta GEF}$ protein, which lacks the GEF domain. *, $P < 0.05$ compared to the control *Legionella* Δ*ankX* strain having a wild-type *drrA* allele.

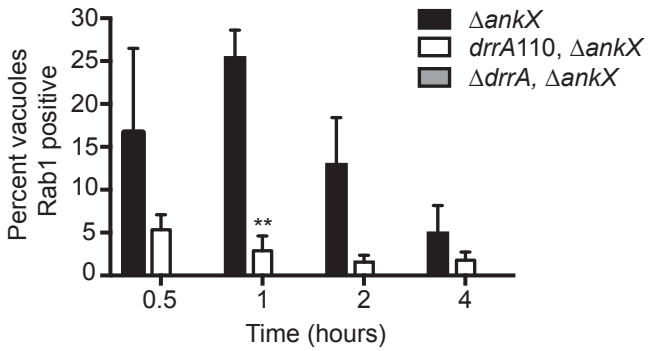

FIG 5 AMPylation promotes accumulation of Rab1 on the LCV in absence of PCylation. The graph shows the average percentage of Rab1b-positive vacuoles detected after RAW cells were infected for the times indicted on the *x* axis. *Legionella* carrying a wild-type *drrA* allele (Δ*ankX*; black bars) was compared to *Legionella drrA110*, which produces the AMPylation-deficient DrrA protein from the chromosomal allele (*drrA110* Δ*ankX*; white bars), and *Legionella* Δ*drrA*, which does not produce DrrA (Δ*drrA* Δ*ankX*; gray bars). At least 150 vacuoles were scored for each experimental condition, and data were acquired from three independent replicates. Data are averages ± SEM. **, $P < 0.005$ compared to the value for the wild-type control at the same time point.

To further test whether AMPylation in *trans* could restore Rab1 localization to the LCV, an in-frame deletion of the central GEF domain was made in both the wild-type DrrA protein and the AMPylation-deficient DrrA110 protein, which resulted in DrrA$^{\Delta GEF}$ and DrrA110$^{\Delta GEF}$, respectively. A plasmid encoding the DrrA$^{\Delta GEF}$ protein restored Rab1 localization when produced in the *Legionella drrA110* Δ*ankX* strain and a plasmid encoding the DrrA110$^{\Delta GEF}$ protein did not (Fig. 4B). Importantly, no Rab1 localization to the LCV was observed when the plasmid-encoded DrrA$^{\Delta GEF}$ protein was produced in the *Legionella* Δ*drrA* Δ*ankX* strain. Thus, AMPylation activity alone is not sufficient to localize Rab1 to the LCV; however, AMPylation functions provided in *trans* to the GEF domain of DrrA were sufficient to restore Rab1 localization to the LCV.

AMPylation promotes accumulation of Rab1 on the LCV. Time course studies were conducted to better understand how AMPylation may affect the dynamics of Rab1 localization to the LCV using the *Legionella* Δ*ankX* strain, producing the wild-type DrrA protein, and the *Legionella drrA110* Δ*ankX* strain, producing the AMPylation-deficient DrrA110 protein. Rab1 localization to the LCV was lower for *Legionella* producing the AMPylation-deficient DrrA110 protein at all stages of infection examined, with the most significant defect being at 1-h postinfection (Fig. 5). Thus, the Rab1 protein recruited by the DrrA GEF domain is not efficiently retained on the LCV in the absence of AMPylation.

AnkX-mediated PCylation is not a substitute for DrrA-mediated AMPylation of Rab1. When vacuoles containing a wild-type (WT) strain of *Legionella* encoding fully functional DrrA and AnkX proteins were compared to vacuoles containing an isogenic mutant deficient in AnkX (Δ*ankX*), there was no sig-

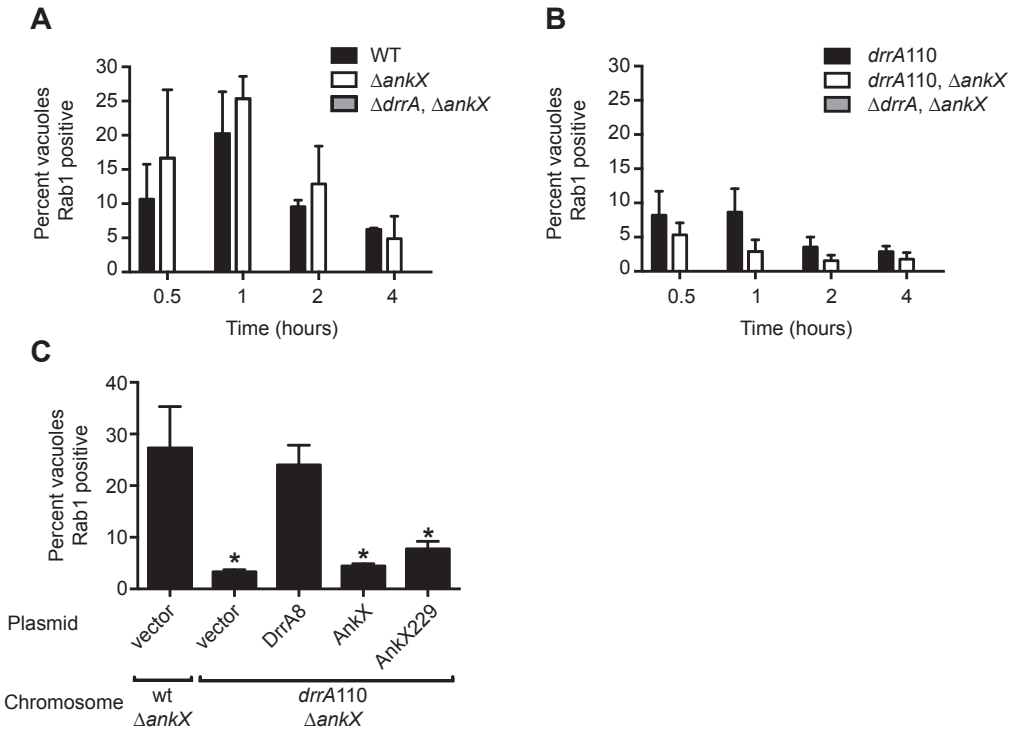

FIG 6 AnkX does not complement the Rab1 localization defect displayed by DrrA110. The graphs show the average percentage of Rab1b-positive vacuoles detected after RAW cells were infected with the indicated strains of *Legionella*. At least 150 vacuoles were scored for each experimental condition, and data were acquired from three independent replicates. Data are averages ± SEM. (A) Wild-type *Legionella* (WT; black bars) were compared to a PCylation-deficient mutant (Δ*ankX*; white bars) at the times indicated on the *x* axis. (B) AMPylation-deficient *Legionella drrA110* (black bars) was compared to a *Legionella* strain that was deficient for both AMPylation and PCylation (*drrA110* Δ*ankX*) at the times indicated on the *x* axis. (C) RAW cells were infected for 1 h with the indicated strains of *Legionella*. The genotype listed below each line indicates the chromosomal *drrA* and *ankX* alleles present in the *Legionella* strains producing the different plasmid-encoded DrrA or AnkX proteins indicated below each column. *, $P < 0.05$ compared to the control Δ*ankX* strain containing a WT *drrA* allele.

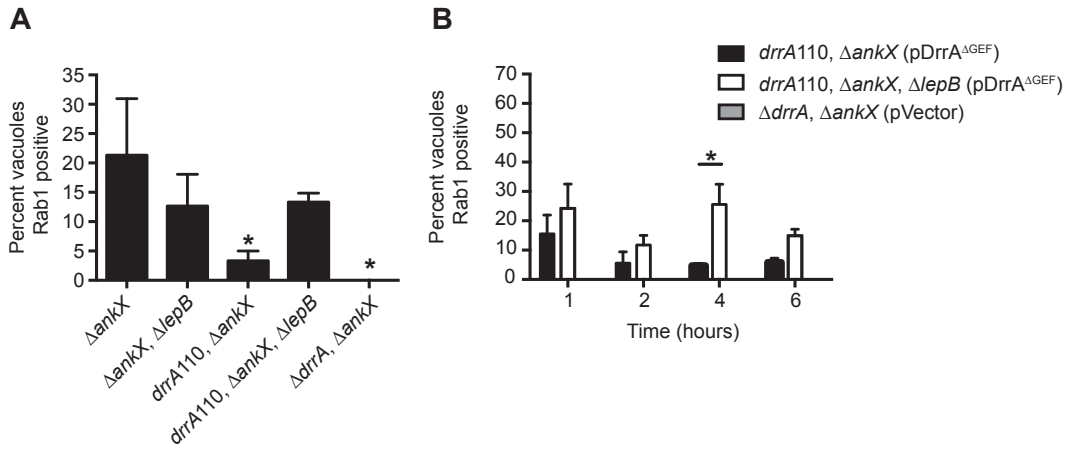

FIG 7 AMPylation protects Rab1 on the LCV from deactivation by GAPs. At least 150 vacuoles were scored for each experimental condition, and data were acquired from three independent replicates. Data are averages ± SEM. (A) Average percentage of Rab1b-positive vacuoles detected after RAW cells were infected with *Legionella* for 1 h. The genotype of the strain used is indicated below each column. *, $P < 0.05$ compared to the control strain (Δ*ankX*). (B) The graph indicates the average percentage of Rab1b-positive vacuoles detected after RAW cells were infected for the times indicted on the *x* axis. A plasmid encoding the AMPylation-sufficient DrrA$^{\Delta GEF}$ protein was used to complement isogenic *Legionella* mutants with the AMPylation deficient *drrA110* allele that either encoded LepB (*drrA110* Δ*ankX* pDrrA$^{\Delta GEF}$; black bars) or were deficient for LepB (*drrA110* Δ*ankX* Δ*lepB* pDrrA$^{\Delta GEF}$; white bars). *, $P < 0.05$ compared to the LepB-sufficient control at the same time.

nificant difference in Rab1 localization observed at any time point (Fig. 6A). Because the potential benefits of AnkX-mediated PCylation in promoting the retention of Rab1 on the LCV might not be detected when AMPylation is functional (28), the AMPylation-deficient *drrA110* allele was introduced into a wild-type strain of *Legionella* to obtain isogenic AMPylation-deficient strains that either encoded a functional AnkX protein (*drrA110*) or were AnkX deficient (*drrA110* Δ*ankX*). Time course studies indicated that Rab1 localization to the LCV was attenuated to similar levels in both strains, which suggests that AnkX-mediated AMPylation is unable to substitute for DrrA-mediated AMPylation in retaining Rab1 on the LCV (Fig. 6B). To test this further, AnkX proteins were produced from a plasmid in the AMPylation-deficient *Legionella drrA110* Δ*ankX* strain, and AnkX production was verified by immunoblot analysis (see Fig. S2 in the supplemental material). Overproduction of the wild-type AnkX protein did not restore localization of Rab1 to levels obtained when AMPylation by DrrA remained intact or when AMPylation was restored in *trans* by the DrrA8 protein, and no significant difference was observed between the Rab1 localization phenotypes in strains overproducing wild-type AnkX and that in the control strain producing the PCylation-deficient AnkX229 protein (Fig. 6C). Thus, the PCylation activity of AnkX was not a functional substitute for the AMPylation activity of DrrA.

AMPylation *in vivo* prevents Rab1 deactivation by GAPs. The percentage of vacuoles containing wild-type *Legionella* that stained positive for Rab1 peaked after roughly 1 to 2 h of infection and then began to decrease (Fig. 6B; also, see Fig. S4A in the supplemental material). When wild-type *Legionella* and a Δ*lepB* mutant were compared, there were no significant differences in the percentage of vacuoles that stained positive for Rab1 (see Fig. S4A). Similarly, when the *Legionella* Δ*ankX* mutant was compared to the isogenic *Legionella* Δ*ankX* Δ*lepB* mutant, no significant differences in Rab1 localization were observed (Fig. 7A; also, see Fig. S4B in the supplemental material). This suggests that it is difficult to detect GAP activity for LepB *in vivo* by measuring Rab1 localization to the LCV and that other Rab1-deactivating factors, such as host GAPs, may be dominant over LepB under these conditions. To determine if AMPylation protected Rab1 from deactivation by LepB, a *Legionella drrA110* Δ*ankX* mutant deficient in both AMPylation and PCylation was compared with the isogenic strain deficient in AMPylation, PCylation, and GAP activity (*drrA110* Δ*ankX* Δ*lepB*). One hour after infection, the AMPylation-deficient strain (*drrA110* Δ*ankX*) displayed a significant defect in Rab1 localization to the LCV compared to the control strain (Δ*ankX*), and elimination of LepB (*drrA110* Δ*ankX* Δ*lepB*) suppressed this defect (Fig. 7A). When the role of LepB was analyzed using strains deficient in AMPylation, the difference in the percentage of vacuoles that stained positive for Rab1 was greatest at 1 h and then began to diminish at later times (see Fig. S4C in the supplemental material), which is consistent with previous data suggesting that the deAMPylation activity displayed by SidD will reverse the benefits of AMPylation at these later times (22, 25).

In contrast to the dynamics observed for *Legionella* strains producing chromosomally encoded effectors expressed from their native promoters, when the DrrA protein was overproduced from a plasmid by a heterologous promoter, the localization of Rab1 to the LCV was more efficient, it was protracted, and it was insensitive to mutations that eliminate *lepB* (see Fig. S5 in the supplemental material). These phenotypes result from increased production and delivery of DrrA into host cells, which should increase both the GEF activity and the AMPylation activity on the LCV. To examine more specifically how increased AMPylation of Rab1 would affect dynamics in the absence of increased GEF activity, we produced the GEF-deficient DrrA$^{\Delta GEF}$ protein in the *Legionella* strain deficient in AMPylation and PCylation (*drrA110* Δ*ankX*) and in the corresponding LepB-deficient strain (*drrA110* Δ*ankX* Δ*lepB*) (Fig. 7B). There was no longer a significant LepB-dependent difference in the percentage of vacuoles that stained positive for Rab1 observed at 1 h postinfection when the AMPylation domain was overproduced, which is consistent with AMPy-

lation being important for protecting Rab1 from LepB-mediated deactivation at these early times postinfection. Overproduction of the AMPylation domain also resulted in an increase in the percentage of vacuoles that stained positive for Rab1 at later time points, but only for *Legionella* strains that were defective in LepB. These data suggest that overproduction of the AMPylation domain reduced the effectiveness of deAMPylation by SidD at these late times and enhanced retention of Rab1 on the LCV by increasing the resistance of Rab1 to GAP activity (Fig. 7B). These data support a model whereby the kinetics of AMPylation and deAMPylation control the temporal association of Rab1 with the LCV and suggest that LepB contributes to these dynamics by functioning as a Rab1 GAP.

DISCUSSION

An early event that occurs during *Legionella* infection of host cells is the recruitment of ER-derived vesicles to the LCV, which involves the subversion of Rab1 (12, 13, 16, 17, 20). Although several *Legionella* effectors have been shown to modulate Rab1 function *in vitro*, little is known about how these effectors function during infection. To better understand the *in vivo* function of these effectors, we examined how these proteins affect the dynamics of Rab1 localization to the LCV using mutant *Legionella* deficient in specific activities.

Cytosolic GDP-bound Rab proteins in association with RabGDI have the ability to bind membranes transiently (Fig. 8). If a cognate Rab GEF is present on the membrane, this would result in activation during membrane sampling and stable Rab association (29, 31, 32). If the membrane does not contain a cognate GEF, then the GDP-bound Rab protein would be rapidly extracted by RabGDI. Accordingly, the recruitment of a Rab protein to a membrane-bound organelle should correlate with the catalytic efficiency by which an associated GEF protein mediates nucleotide exchange.

Given this model, we did not expect to find that DrrA proteins with site-directed mutations that resulted in undetectable GEF activity *in vitro* would retain the ability to recruit Rab1 to the LCV when delivered by the Dot/Icm system during infection. *Legionella* producing DrrA proteins with GEF activities that were >100-fold lower than that of the wild-type DrrA protein had only modest defects in their ability to localize Rab1 to the LCV, whereas DrrA proteins with the central GEF domain deleted were unable to localize Rab1 to the LCV. Thus, although the GEF domain is essential for Rab1 recruitment to the LCV, DrrA proteins with very weak GEF activity could promote Rab1 accumulation on the vacuole.

The catalytic efficiency of a GEF should influence the amount of a Rab protein localized to a membrane; however, steady-state levels of membrane association should also be subject to the rate of RabGDI-mediated extraction, which is regulated by the activity of Rab GAPs. Because *in vitro* studies revealed that Rab1 AMPylation by DrrA renders the GTPase insensitive to interactions with RabGDI and deactivation by GAP proteins (18, 23, 24, 33), we investigated whether Rab1 localization to the LCV mediated by proteins with weak GEF activity required the AMPylation activity of DrrA. These data showed that AMPylation was critical for Rab1 localization to the LCV. Defects in AMPylation resulted in significant defects in Rab1 localization to vacuoles containing *Legionella* organisms producing DrrA proteins with wild-type or mutant GEF domains. Thus, AMPylation plays an important role in maintaining Rab1 of the LCV, even under conditions where the DrrA protein has a GEF domain that efficiently activates Rab1.

The expression of DrrA proteins with a functional AMPylation domain but a defective GEF domain in *trans* to DrrA proteins that were deficient for AMPylation but retained GEF activity restored efficient localization of Rab1 to the LCV. These *in vivo* data validate *in vitro* studies that suggested a model where AMPylation would occur in *trans* after Rab1 activation by the GEF domain of DrrA (23, 34). We found that overproduction of the DrrA$^{\Delta GEF}$ protein in a Δ*drrA* strain was not sufficient to localize Rab1 to the LCV. This indicates that DrrA does not efficiently target Rab1-GDP for AMPylation during a transient membrane-sampling event, which rules out AMPylation of Rab1-GDP on the membrane as a mechanism for Rab1 recruitment to the LCV. Importantly, these data indicate that the majority of Rab1 localized to the LCV membrane is GTP bound and AMPylated (Fig. 8).

Because *in vitro* studies showed that PCylated Rab1 is resistant to inactivation by GAP proteins and interaction with RabGDI (24, 27, 28, 33), our initial studies were conducted in *Legionella* Δ*ankX* strains to eliminate PCylation of Rab1 during infection. Unexpectedly, the Rab1 localization defects displayed by strains defective in AMPylation were not affected upon reintroduction of AnkX, which indicated that PCylation functions conferred by AnkX do not substitute for the AMPylation functions mediated by DrrA. This suggests that the pool of Rab1 targeted for PCylation by AnkX is distinct from the pool of Rab1 that is targeted for AMPylation by DrrA. Thus, it is unlikely that Rab1 on the LCV membrane is the intended target for AnkX-mediated PCylation. Indirect evidence supporting this hypothesis includes the inability to localize AnkX to the LCV membrane and data showing that AnkX displays punctate staining of peripheral structures when overproduced in mammalian cells (35). We hypothesize that AnkX and DrrA have divergent roles in modulating the function of Rab1 family members in the cell, with DrrA being a factor that controls Rab1 dynamics specifically on the LCV membrane and AnkX regulating the function of Rab proteins on other cellular organelles. Studies focusing specifically on AnkX function *in vivo* should clarify the role for this protein in modulation of Rab dynamics.

Elimination of LepB partially suppressed defects in Rab1 localization observed for vacuoles containing AMPylation-deficient strains of *Legionella*. These data are in agreement with studies examining the *in vivo* role of the deAMPylase protein SidD (22, 25, 36). *Legionella sidD* mutants display protracted Rab1 localization to the LCV membrane (22, 25). These data suggested that a defect in Rab1 deAMPylation resulting from the elimination of SidD would lead to the accumulation of a pool of AMPylated Rab1 on the LCV membrane, and this would prevent Rab1 removal stimulated by LepB-mediated GAP activity. This model was further supported by data showing a reduction in Rab1 localization to the LCV when plasmid-encoded SidD was produced from a heterologous promoter (22, 25). Taken together, these *in vivo* studies strongly suggest that AMPylation of Rab1 by DrrA protects Rab1 from deactivation by LepB and that the deAMPylation activity of SidD controls the timing of deactivation (Fig. 8).

The strong Rab1 localization defect displayed by AMPylation-deficient alleles of *drrA* was only partially suppressed by elimination of LepB, which indicates that AMPylation also plays an important role in protecting Rab1 from host GAP proteins. The ability of AMPylation to protect Rab1 from host GAPs explains

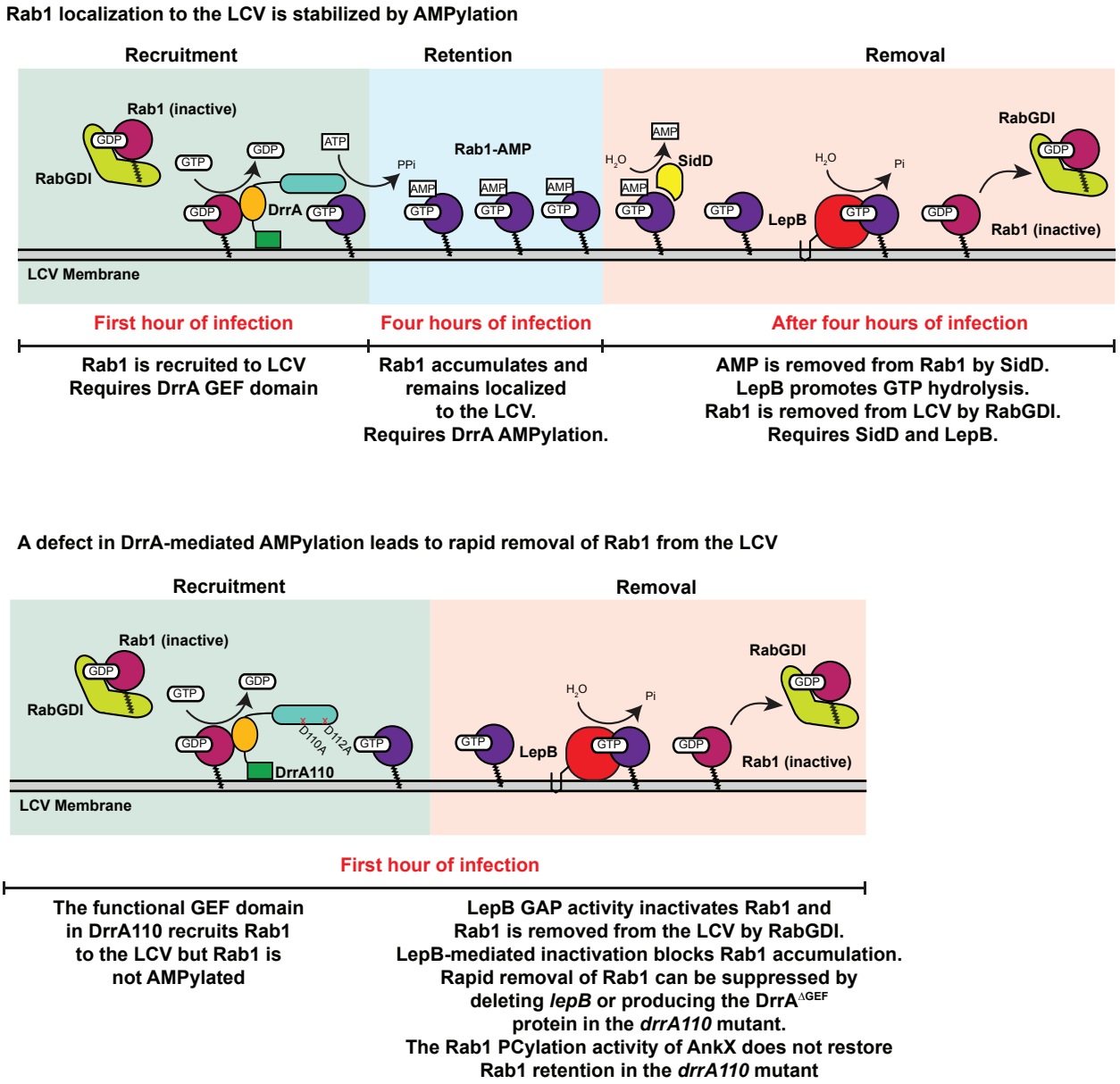

FIG 8 Rab1 dynamics on the LCV are tightly regulated by AMPylation. (Top) Rab1 localization to the LCV membrane is temporally controlled in three distinct stages. Recruitment of Rab1 is mediated within the first hour of infection by the GEF domain of DrrA. The active membrane-associated Rab1 protein is AMPylated by DrrA, which mediates the retention and accumulation of AMPylated Rab1 on the LCV membrane over the next 4 h. After 4 h, the deAMPylation activity of SidD stimulates the generation of unmodified Rab1, which is then inactivated by LepB-stimulated GTP hydrolysis. Inactive Rab1 protein is removed from the LCV by RabGDI. (Bottom) Infection of cells with *Legionella* producing the DrrA110 protein. Here, the recruitment of Rab1 by the DrrA GEF domain still occurs within the first hour of infection; however, the DrrA110 protein is unable to AMPylate Rab1. Without AMPylation, LCV-localized Rab1 is rapidly deactivated by LepB, which leads to membrane extraction of Rab1 by RabGDI. Thus, in the absence of AMPylation, there is reduced retention and accumulation of Rab1, which results in lower levels of Rab1 on the LCV during the first hour of infection and rapid removal thereafter.

why comparison of an isogenic *lepB* mutant with wild-type *Legionella* does not reveal a difference in the dynamics of Rab1 localization (see Fig. S4A in the supplemental material). The protein TBC1D20 would be a likely candidate for controlling Rab1 dynamics independent of LepB, given that TBC1D20 has Rab1 GAP activity and is localized to the ER (37). This would place TBC1D20 in a position to function as a host GAP that stimulates removal of Rab1 from the membrane after ER-derived vesicles have successfully remodeled the LCV into a replicative niche. Alternatively, one of the other *Legionella* effectors could inactivate Rab1 by a mechanism that is yet to be determined. Thus, the mechanism by which Rab1 is removed from vacuoles containing a *lepB* mutant remains to be determined.

Overall, these data suggest that AMPylation evolved as a mechanism to enhance Rab1 subversion by preventing the rapid removal of Rab1 proteins from the LCV membrane through the activity of host GAPs. Although speculative, it is possible that protozoan hosts for *Legionella* have GAP proteins on early phago-

somes that have a cell-autonomous defense function and assist in preventing subversion of Rab GTPases by pathogens. Effectors such as SidD and LepB would have then evolved as factors that enable *Legionella* to fine-tune the dynamics of Rab1 localization to the LCV. It is intriguing that PCylation and AMPylation are chemically distinct modifications that similarly affect the function of Rab1 proteins. Data from this study suggest that AnkX evolved separately to control Rab1 functions that are spatially and temporally distinct from those targeted by DrrA. Because these modifications are chemically distinct, it also provided *Legionella* with the opportunity to independently control different aspects of Rab1 function through the use of the demodifying enzymes SidD and Lem3. This suggests that *Legionella* targets Rab1 family members that reside on the LCV to promote vacuole biogenesis and also targets Rab1 proteins on other cellular organelles, perhaps as a mechanism to avoid host defense.

MATERIALS AND METHODS

Bacterial strains. Homologous recombination was used to generate isogenic strains harboring mutant alleles of *drrA* in *Legionella pneumophila* Philadelphia1 strains that were initially derived from Lp01 (7). Plasmids that produce the wild-type and mutant DrrA proteins were created by ligating alleles of *drrA* into pJB1806-M45 (38). The resulting DrrA expression plasmids were electroporated into isogenic Lp01-derived strains of *Legionella*. Details of how the constructs were prepared are found in Text S1 in the supplemental material.

Immunofluorescence. Sterile glass coverslips were placed in each well of a 24-well tissue culture plate, and 2×10^5 RAW cells were added to each well. Bacteria from a 48-h heavy patch were used to infect the RAW cells to an estimated multiplicity of infection (MOI) of 5 bacteria for each host cell in the dish. To discriminate between extracellular and intracellular bacteria, inside-out staining was performed as previously described (39) at various time points. To score Rab1 localization, cells were incubated for 1 h with mouse anti-*Legionella* and rabbit anti-Rab1b primary antibodies at dilutions of 1:1,000 and 1:250, respectively. Details of how cell culture and immunofluorescence was performed are found in Text S1 in the supplemental material.

Statistical analysis. Tests for statistical significance were performed with the unpaired *t* test using GraphPad Prism. All error bars displayed on graphs represent the standard errors of the means (SEM).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01035-13/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.
Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.
Figure S3, PDF file, 0.3 MB.
Figure S4, PDF file, 0.1 MB.
Figure S5, PDF file, 0.1 MB.
Table S1, DOCX file, 0.1 MB.

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