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Legionella pneumophila Replication Vacuole Formation Involves Rapid Recruitment of Proteins of the Early Secretory System

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***Legionella pneumophila* vacuole biogenesis was analyzed by using a cell-free system. We show that calnexin, Sec22b, and Rab1 are recruited to the vacuole very shortly after bacterial uptake, and we have identified Rab1 as a potential host factor involved in the endoplasmic reticulum recruitment process.**

Legionella pneumophila, the causative agent of Legionnaires' disease, is an intracellular gram-negative pathogen (4). In environmental reservoirs, the bacterium is found associated with freshwater amoeba, while human disease is associated with inhalation of contaminated aerosols followed by multiplication within alveolar macrophages (16, 25). The Dot/Icm complex, a type IV secretion apparatus, is essential for intracellular growth and for translocation of bacterial effector molecules into host cells, which promote uptake and allow the establishment of the replication vacuole (2, 18, 24). Two translocated proteins that are substrates of the Dot/Icm complex have been reported thus far: RalF, a guanidine nucleotide exchange factor that activates Arf family members on the *L. pneumophila* vacuole (15); and LidA, a protein of unknown function (3).

Following uptake into target cells, the bacterium is internalized into a compartment that escapes the endocytic pathway (8, 9). Fifteen minutes after internalization, the vacuolar membrane lacks plasma membrane or endocytic markers and is surrounded by mitochondria and small vesicles (7). Based on the thickness of their membranes these vesicles are thought to be derived from the endoplasmic reticulum (ER) (22). In addition, *L. pneumophila* vacuoles intercept vesicular traffic from ER exit sites and vesicle budding from the ER appears to be required for establishment of the replication vacuole (11). Six hours after internalization, ER surrounds the vacuole and the bacterium starts to multiply (21, 22). As replication proceeds, greater than 50% of the *L. pneumophila* vacuoles acquire markers of endocytic organelles (20) and the host cell is eventually lysed.

The kinetics of ER recruitment and the nature of the vesicles recruited to the *L. pneumophila* vacuole are still unclear. ER markers have been detected around the vacuoles 6 h after infection by immunofluorescence and electron microscopy analysis (21, 22). However, it has not been shown that ER markers are associated with the vacuolar membrane at early stages of vacuolar biogenesis. In this study, we analyzed preparations of isolated *L. pneumophila* vacuoles to determine how soon after bacterial contact with host cells that ER-derived components are associated with these vacuoles and to identify the host factor(s) involved in this process.

A sucrose gradient-based procedure was used to isolate the *L. pneumophila* vacuoles. A total of 10^8 differentiated U937 cells were incubated for 10 min or 1 h with either wild-type *L. pneumophila* or with the avirulent *dot/icm* mutant (*dotA*) (1), both expressing green fluorescent protein (GFP) (19), at a multiplicity of infection (MOI) of 5 to maximize the yield of vacuoles. As *L. pneumophila* has maximum potential for promoting intracellular growth after entering the postexponential phase, we used bacteria grown to this density for all the infections described in this study. The infected cells were suspended in 2 ml of homogenization buffer (20 mM HEPES–KOH [pH 7.2], 250 mM sucrose, 0.5 mM EGTA) and lysed in a Dounce homogenizer, and the unbroken cells and nuclei were pelleted by centrifugation (3 min, 1,500 rpm). The postnuclear supernatant (PNS) was loaded onto a discontinuous sucrose gradient (Beckman centrifuge tubes, 14 by 89 mm) consisting of 6 ml of 14% (wt/wt) sucrose layered on top of 2 ml of 50% (wt/wt) sucrose in HEPES buffer (20 mM HEPES–KOH [pH 7.2], 0.5 mM EGTA) and subjected to centrifugation at 600 × *g* for 90 min at 4°C. The *L. pneumophila* vacuoles were identified in the 14 to 50% interface fraction of the sucrose gradient based on the presence of the GFP marker (Fig. 1A). The vacuoles were allowed to bind poly-L-lysine-coated coverslips prior to fixation (12) and probing with the indicated antibody. Immunofluorescence analysis of *L. pneumophila* vacuoles was the same throughout the study. The vacuolar membrane integrity was verified by immunostaining of the vacuoles containing bacteria by using a polyclonal anti-*L. pneumophila* antibody (Fig. 1B). In the absence of vacuolar membrane permeabilization, less than 20% of the *L. pneumophila* vacuoles stained positively for the bacteria. In contrast, pretreatment with a permeabilization reagent (−20°C methanol for 5 s) resulted in staining of 100% of the vacuoles (Fig. 1B). We also tested if the lysosomal marker LAMP1 was recruited to the *L. pneumophila* vacuole (anti-human CD107a; BD Pharmingen). Only 15% of vacuoles harboring bacteria with an intact Dot/Icm system were positive for LAMP1, whereas 45% of the vacuoles harboring a *dotA* mutant strain showed positive staining (data not shown). As the isolation procedure left the vacuoles largely intact and since the lack of LAMP1 recruitment to the vacuole is similar to what is observed in infected bone marrow macro-

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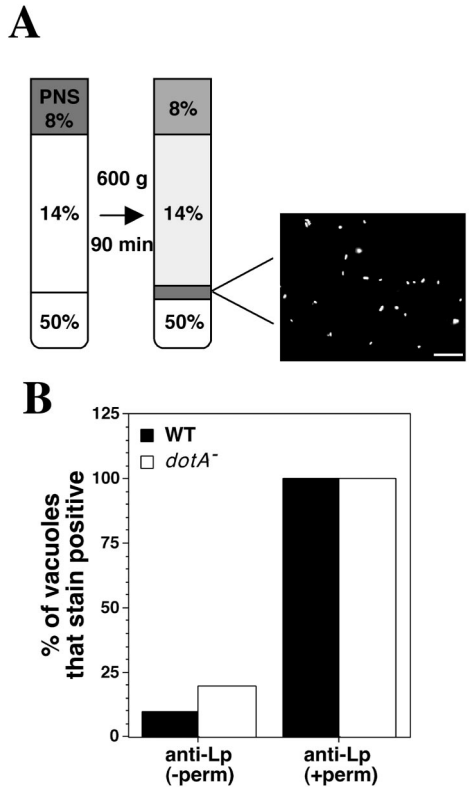

FIG. 1. Isolation and integrity of *L. pneumophila* vacuoles. (A) Schematic representation of the procedure used to isolate *L. pneumophila* vacuoles. The PNS from infected macrophages was layered on top of a discontinuous sucrose gradient. After centrifugation, the vacuoles were concentrated at the 14 to 50% interface. Bar, 5 μm. (B) Efficiency of staining *L. pneumophila* in the absence (−perm) or presence (+perm) of permeabilization of the vacuolar membranes. Displayed are the mean and standard error of three samples for each condition. Anti-Lp, anti-*L. pneumophila* antibodies. Solid bars, wild type; open bars, *dotA* mutant.

phages, we next analyzed the recruitment of proteins associated with the early secretory system to nonpermeabilized *L. pneumophila* vacuoles.

Antibodies directed against the Golgi-associated proteins p115 (G. Watters) and GM130 (BD Bioscience) failed to reveal recruitment of Golgi material at any time point after infection (data not shown). In contrast, calnexin (rabbit polyclonal antibody from Stressgen), a membrane ER protein, was detected on the surface of 77% of the vacuoles isolated from macrophages infected with wild-type *L. pneumophila* as early as 10 min after infection (Fig. 2A). The punctate pattern of the staining was consistent with the previous hypothesis that vesicles, recruited to the *L. pneumophila* vacuoles 15 min after infection, are likely to be ER derived (22) (Fig. 2B). The number of calnexin-positive vacuoles increased to 92% 1 h after infection (Fig. 2A), and the pattern of staining changed significantly from the earlier time, as the *L. pneumophila* vacuoles were completely surrounded by a thick layer of calnexin-containing material (compare Fig. 2B to C). In contrast, less than 15% of the vacuoles isolated from macrophages infected with the *dotA* mutant showed a positive staining at either time point (Fig. 2), indicating that ER recruitment was dependent on an intact Dot/Icm secretion system. This lack of coating by ER material may explain why vacuoles harboring a *dotA* mutant were twice as likely to be damaged during isolation as Dot/Icm-intact *L. pneumophila* (Fig. 1B). ER material surrounding the vacuole may provide significant protection against mechanical or osmotic lysis.

The rapid accumulation of calnexin about the vacuoles is in apparent contradiction with previous reports indicating that calnexin was only associated with intracellular *L. pneumophila* 6 h after internalization (11). This discrepancy could be due to the fact that the previous study used murine bone marrow-derived macrophages instead of human macrophages, but we favor the hypothesis that the small amount of calnexin recruited during the earliest times after uptake can only be detected when the vacuoles are isolated from other cellular material. Our results also indicate that ER recruitment continues throughout the first hour of infection, as ER accumulates around the *L. pneumophila* vacuole during this period of time.

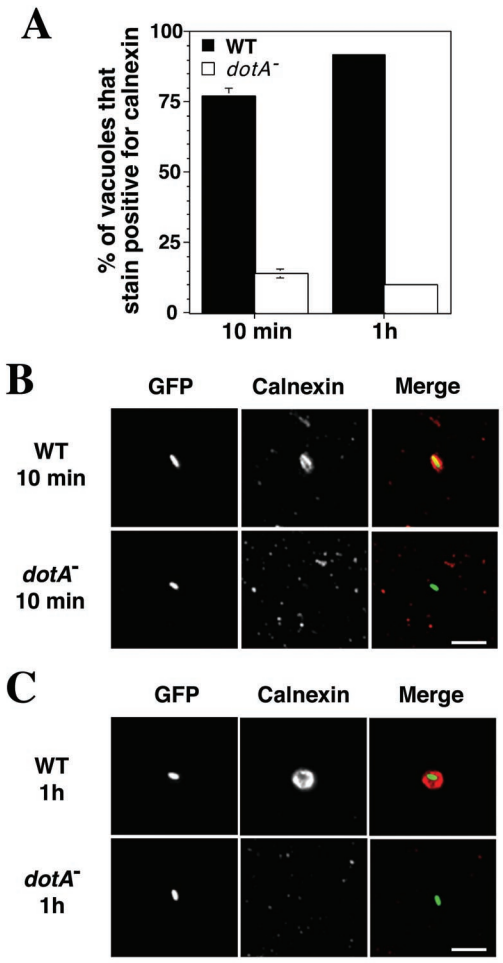

FIG. 2. ER recruitment to *L. pneumophila* vacuoles occurs within 10 min of infection in a Dot/Icm-dependent fashion. (A) Quantification of *L. pneumophila* vacuoles associated with calnexin 10 min or 1 h postinfection. Solid bars, wild type (WT); open bars, *dotA* mutant. (B and C) Enhanced recruitment of calnexin after 1 h of infection. Vacuoles were isolated 10 min (B) or 1 h (C) after incubation with either wild-type or *dotA* mutant bacteria and probed with anticalnexin antibody. Displayed are *L. pneumophila* (GFP), anticalnexin probing (calnexin), and the two images merged. Bars, 5 μm.

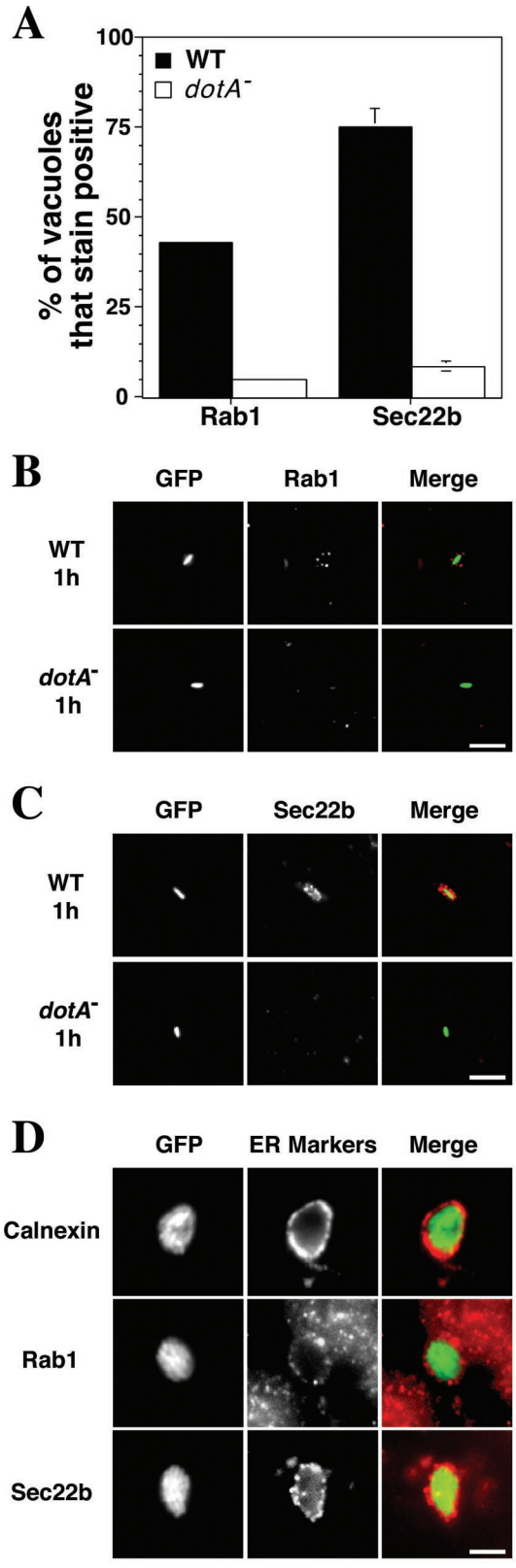

FIG. 3. Rab1 and Sec22b are recruited to the *L. pneumophila* vacuole in a Dot/Icm-dependent fashion. (A) Quantification of *L. pneumophila* vacuoles associated with Rab1 or Sec22b 1 h postinfection. Solid bars, wild type (WT); open bars, *dotA* mutant. (B and C) Punctate pattern of association of Rab1 and Sec22b with *L. pneumophila* vacuoles. Vacuoles were isolated 1 h after incubation with either wild-type or *dotA* mutant bacteria and probed with either anti-Rab1 (B) or anti-Sec22b (C). GFP, *L. pneumophila*-GFP. Merge, *L. pneumophila* displayed in green and either Rab1 or Sec22b displayed in red. Scale bar, 5 μm. (D) Calnexin, Rab1, and Sec22b are associated with vacuoles containing replicating *L. pneumophila*. Cell lysates from macrophages incubated 14 h with wild-type *L. pneumophila* were probed with either anticalnexin or anti-Rab1 or anti-Sec22b antibody in the absence of permeabilization. Merge, *L. pneumophila* displayed in green and either calnexin, Rab1, or Sec22b displayed in red. Bars, 5 μm.

Moreover, this process is dependent on the presence of an intact Dot/Icm system, indicating that the effector molecule(s) secreted by this apparatus is responsible for the proper recruitment of ER-derived material.

We believe that the recruitment of ER-derived material to the *L. pneumophila* vacuole differs from the observed ER-mediated phagocytosis previously seen with latex beads (5), since the process described here is Dot/Icm dependent (Fig. 2). The absence of calnexin recruitment to the *dotA* mutant vacuoles could be due to rapid interaction of the vacuoles with the endocytic pathway, or it could be that there was very little recruitment of ER. However, calnexin was reported to be easily detected on the surface of latex bead phagosomes as early as 15 min after uptake (5), strongly suggesting that the *dotA* mutant vacuoles never interact significantly with ER-derived material. ER-mediated phagocytosis is also proposed to play a role in presentation of antigens by major histocompatibility complex class I molecules (10). However, in the case of wild-type *L. pneumophila*, the bacteria are not degraded in the vacuole, suggesting that the ER-derived compartments harboring latex beads are very different in nature from vacuoles bearing *L. pneumophila*.

Recruitment of ER-associated material to the *L. pneumophila* vacuole may involve hijacking of host proteins that regulate vesicular traffic between the ER and the Golgi apparatus. The Rabs and the SNAREs are the largest families of proteins known to be involved in membrane trafficking (6, 17). They respectively regulate the traffic of vesicles and participate in their docking and fusion to their target membranes. Two Rab proteins, Rab1a/b and Rab2 (13, 23), and at least one SNARE complex, composed of the v-SNARE Sec22b and three t-SNAREs (syntaxin 5, membrin, and rBet1), have been shown to regulate the ER-to-Golgi traffic (26). Therefore, we investigated the recruitment of these proteins to the *L. pneumophila* vacuoles.

One hour after infection, 43% of the vacuoles isolated from macrophages infected with wild-type *L. pneumophila* were positive for the small GTPase Rab1 (rabbit polyclonal antibody from Calbiochem) (Fig. 3A). In contrast, no staining for Rab2 could be detected (data not shown) (the mouse monoclonal anti-Rab2 antibody was a kind gift from E. J. Tisdale, Wayne State University School of Medicine). At the same time point, 75% of phagosomes harboring a wild-type *L. pneumophila* strain were also positive for the v-SNARE protein Sec22b (Fig. 3A) (the rabbit polyclonal anti-Sec22b was a kind gift from J. Hay, University of Michigan). Moreover, the recruitment of both Rab1 and Sec22b to the *L. pneumophila* vacuole was dependent on the presence of the Dot/Icm complex, since less than 10% of the vacuoles isolated from macrophages infected

with the *dotA* mutant showed positive staining for either protein (Fig. 3A). The pattern of both Rab1 and Sec22b staining was punctate even 1 h after infection (Fig. 3B and C), but the staining pattern of Rab1 was significantly less densely packed than that observed with calnexin or Sec22b. The vacuoles were also analyzed 14 h after the initiation of the infection. For this purpose, the cells were infected and lysed as described for the PNS preparation but unbroken cells and nuclei were not removed. The cell lysates were allowed to directly bind coverslips and analyzed as described for isolated vacuoles. As shown in Fig. 3D, wild-type *L. pneumophila* vacuoles in which the bacteria were undergoing extensive replication were still labeled with calnexin, Rab1, and Sec22b, suggesting that these markers are associated with *L. pneumophila* vacuole throughout the replication cycle of the bacteria.

As the two Rab1 effectors p115 and GM130 are absent from the vacuole, other factors may be involved in the recruitment of ER-derived material. ER-derived vesicles dock and fuse with the Golgi apparatus by formation of a complex between Sec22b and three t-SNAREs (syntaxin 5, membrin, and rBet1) present on the Golgi target membrane (26). We performed immunofluorescence analysis using antibodies directed against these t-SNARES (the rabbit polyclonal anti-syntaxin 5, anti-membrin, and anti-rBet1 antibodies were kind gifts from J. Hay, University of Michigan), but none was found associated with the surface of the vacuole (data not shown). Therefore, if Sec22b plays a role in the formation of the *L. pneumophila* replication vacuole, it may bind to unidentified t-SNARE or t-SNARE-like proteins encoded by the host or translocated by the bacterium across the vacuolar membrane.

Rab proteins are known to cycle between a GTP-bound active form and a GDP-bound inactive form, the latter of which acts as a dominant-negative inhibitor when overexpressed in mammalian cells such as COS1 cells (African green monkey kidney cells, transformed by an origin-defective mutant of simian virus 40). To test for growth in COS1 cells, postexponential-phase *L. pneumophila* strains were introduced onto monolayers of 10^5 cells in 24-well dishes at an MOI of 0.05 by centrifugation for 5 min at 1,000 rpm. A low MOI was used to maximize the efficiency of initiation of intracellular growth (3). After incubation for 2 h at 37°C in the presence of 5% CO_2, the monolayers were washed three times to remove any extracellular bacteria and intracellular growth of *L. pneumophila* was quantified (1). As shown in Fig. 4A, *L. pneumophila* replication within COS1 cells was found to be similar to that observed in macrophage-like cell types, with intracellular growth dependent on a functional Dot/Icm secretion apparatus. This result indicated that COS1 cells are suitable to test the effect of overexpression of Rab1 isoforms on the intracellular multiplication of *L. pneumophila*. For this purpose, 3×10^4 COS1 cells were seeded onto glass coverslips in 24-well dishes and were transfected the next day with 1 μg of plasmid DNA, encoding GFP fusions to Rab1a derivatives having either the wild-type or dominant inhibitory (Rab1aS25N) sequences, using the calcium phosphate transfection method according to the manufacturer's instructions (Invitrogen). Twenty-four hours after transfection, the bacteria were incubated with the cells, as described for the intracellular growth assay, except that the MOI was 5. After incubation for 2 h at 37°C in the presence of 5% CO_2, the monolayers were

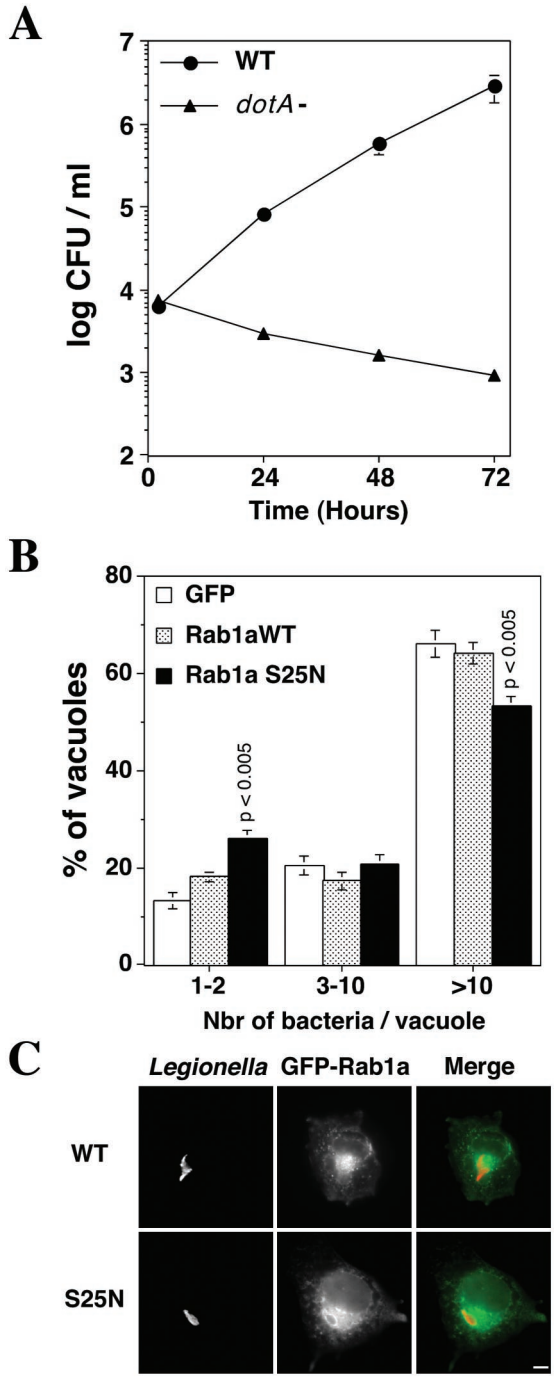

FIG. 4. Overexpression of the GDP-bound form of Rab1a reduces *L. pneumophila* replication in COS1 cells. (A) The number of viable bacteria was determined every 24 h postinfection by counting CFU. (B) Effect of overexpression of Rab1a derivatives on *L. pneumophila* intracellular multiplication. COS1 cells were transiently transfected with constructs allowing expression of GFP, the wild-type form of GFP-Rab1a (Rab1aWT), or the GDP-bound form (Rab1aS25N). The infection was allowed to proceed for 14 h, and the number of bacteria per phagosome was scored. (C) Accumulation of Rab1a around the *L. pneumophila* vacuole in COS1 cells 14 h postinfection. Displayed are *L. pneumophila* (left panels), GFP-Rab1a derivatives (middle panels), and the two images merged (right panels). Bar, 10 μm.

washed to synchronize the infection and were further incubated for 14 h at 37°C in 5% CO_2 prior to fixation. The samples were stained for extracellular and total bacteria and processed for immunofluorescence microscopy. The efficiency of replication vacuole formation was determined by visual inspection by counting the number of bacteria per vacuole, and the data were quantified by counting 100 phagosomes per coverslip. The average and standard error were calculated from three coverslips, and the *P* value was calculated by using the unpaired Student's *t* test (two samples assuming unequal variances). As shown in Fig. 4B, *L. pneumophila* growth was not affected by overexpression of either GFP or wild-type GFP-Rab1a. However, overexpression of the GDP-bound form of GFP-Rab1a (Rab1aS25N) led to a significant ($P < 0.005$) reduction of large replication vacuole formation. In comparison to the results from analysis of isolated vacuoles from U937 cells (see above), it was difficult to detect recruitment of the wild-type form of GFP-Rab1a to the *L. pneumophila* vacuole when the protein was overexpressed in intact COS1 cells, although the vacuoles were found in an area of the cell that was rich in Rab1. In contrast, the GDP-bound form of GFP-Rab1a (Rab1aS25N) strongly accumulated and labeled the *L. pneumophila* vacuole (Fig. 4C).

Our results indicate that Rab1 is required for the optimal intracellular growth of *L. pneumophila* in COS1 cells. Since Rab1 has an essential role in the docking of ER-derived vesicles with their target membrane, it is possible that Rab1 provides a similar role for *L. pneumophila*. Rab1 may direct the association of ER-derived vesicles with the *L. pneumophila* vacuole, thus assisting the formation of an environment permissive for growth. It has been previously reported that the GDP-bound form of Rab1 localizes to the cytoplasm of the cell as well as to punctate structures that are likely to be fragments of ER, ERGIC, and Golgi membranes (14). It is possible that when the inactive form of the Rab1 is overexpressed, ER-derived material rich in GDP-bound Rab1 is recruited to the *L. pneumophila* vacuole. The irreversible association of inactive Rab1 to the vacuole may interfere with cycles of recruitment of ER and, consequently, with the ability of *L. pneumophila* to multiply.

Functional redundancy of both bacterial proteins and host function may explain why we were unable to obtain a total block of *L. pneumophila* growth by overexpression of an inactive form of Rab1a. It is believed that the bacterium uses several strategies to hijack elements of the host cell secretory machinery to establish its replication vacuole. The translocated effector substrates of the Dot/Icm system are particularly subject to functional redundancy (3, 15; Z.-Q. Luo and R. Isberg, personal communication). The same argument can be made for the host factors involved in the infection process. It is possible that, in order to establish the replication vacuole, *L. pneumophila* targets several host factors of redundant function and that inactivation of more than one of them would be necessary to observe a dramatic effect on the intracellular replication of the bacteria. Our results demonstrate the power of using cell-free systems to analyze the maturation of the *L. pneumophila* replication vacuole. We have shown that the association of ER with the *L. pneumophila* vacuole is a characteristic of nascent vacuoles rather than a unique property of the replication vacuole and have identified some potential host targets of the effectors of the type IV secretion system. Future studies will emphasize the identification of the bacterial factor(s) that interacts with Rab1 and/or Sec22b in order to ensure proper delivery of ER membrane to the *L. pneumophila* vacuole.

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