INTRODUCTION

Intracellular bacterial pathogens have evolved highly specialized mechanisms to enter and survive intracellularly within their eukaryotic hosts. In order to do this, bacterial pathogens need to avoid host cell degradation, obtain nutrients and biosynthetic precursors, as well as evade detection by the host immune system. To accomplish these tasks, intracellular pathogens have been shown to (i) escape the phagosome and replicate within the nutrient-rich cytosol, (ii) inhibit or delay the maturation of the phagosome to restrict fusion with lysosomes, or (iii) exit or inhibit interactions with the endocytic/lysosomal pathway completely (reviewed in references 60, 67, and 174). Pathogens that inhibit the maturation of the phagosome or exit the endocytic pathway do so by modifying the identities of their phagosomes in order to exploit host cell trafficking pathways and create an intracellular niche that favors bacterial replication. Organelle identity is determined, in part, by the composition of active Rab GTPases on the membranes of each organelle (15, 144, 170, 212). This review describes our current understanding of how selected bacterial pathogens regulate host trafficking pathways by the selective exclusion or retention of Rab GTPases on membranes of the vacuoles that they occupy in host cells during infection.

THE Rab GTPase FAMILY

The Rab family constitutes the largest member of the Ras superfamily of small guanosine triphosphatases (GTPases). There are more than 60 members of the Rab family, which are highly conserved among eukaryotic cells (138, 139). Rabs play an essential role in both endocytic and exocytic traffic in eukaryotic cells (177, 183, 212). The fact that the human genome contains nearly fivefold more Rabs than does the Saccharomyces cerevisiae genome suggests that they play a role in specialized trafficking pathways in differentiated cell types. Indeed, the expression of individual Rab family members, as well as their regulators and effectors, is unique to different tissues in mice and humans. This led Gurkan and colleagues to suggest that Rab coordinates signaling “hubs” through which membrane traffic is regulated (80).

Rab Functions

Rabs are thought to act as molecular switches, being active in their GTP-bound state and inactive in their GDP-bound state. Because Rabs do not have high intrinsic guanine nucleotide exchange or hydrolysis activities, they are regulated by other proteins, guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). In their GDP-bound state, Rabs are typically soluble and bound to guanine nucleotide dissociation inhibitor (GDI). At the acceptor membrane, the Rab-GDI complex is thought to interact with GDI displacement factor, which removes GDI and allows Rab membrane insertion (143). Next, a GEF converts Rab to its GTP-bound, active conformation, allowing it to interact with its downstream effectors. Through their effectors, Rabs control many aspects of membrane traffic, including vesicle formation,
vesicle motility along the actin/microtubule cytoskeletons, vesicle tethering, transport, and fusion (80, 177, 212).

**Rab Localization**

At steady state, Rabs appear to be concentrated to specific subcellular compartments in eukaryotic cells (for a review, see reference 212). Multiple Rabs can be present on single intracellular compartment, each occupying its own distinct “microdomain” (13, 178). Targeting of Rabs to these compartments is thought to be mediated, at least in part, by the posttranslational addition of one or two prenyl modifications of a C-terminal cysteine residue(s) (137). Rab targeting to microdomains within specific organelles is thought to occur via interactions with their effectors and/or lipids present in this compartment (142, 143). Recent studies suggested the importance of both phospholipids (specifically, phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] and PI(3,4,5)P₃) and membrane surface charge in the targeting of some Rab GTPases to the plasma membrane (91, 211). The role of phospholipids and membrane surface charge in targeting Rabs to other compartments (e.g., endosomes) remains to be determined.

**Rab Conversions**

Rabs appear to function by coordinating a continuum of membrane traffic. For example, the endosomal system involves a highly coordinated and interdependent network of Rabs that control many aspects of cargo delivery from the cell surface to lysosomes. Many studies focused on the role of Rab5 and Rab7, which are enriched on early and late endosomes, respectively, and thus play key roles in determining organelle identity. Although it is clear that cargo destined for degradation transits from the cell surface through early endosomes to eventually reach lysosomes, the specific mechanism by which this trafficking occurs is still open to debate (78). Two models have been proposed to explain endocytic trafficking. The first model proposes that cargo is delivered from one stable organelle of distinct and defined composition to a second stable organelle via vesicle-mediated trafficking mechanisms. The second model proposes that organelles are not static and instead mature along with their cargo as it is trafficked to the lysosome (150, 185). In this model, organelles are dynamic structures, and endosomes would mature along with their cargos as they are delivered to the lysosome.

Recent studies by Rink and colleagues using fast live-cell imaging provided convincing data to support the organelle tethering, transport, and fusion (80, 177, 212).

**Rabs Associated with the Phagosome**

Phagocytosis is a specialized form of endocytosis that plays a critical role in both innate and adaptive immunity as well as tissue remodeling during development (169). After their formation, phagosomes are thought to undergo a maturation sequence similar to that of endosomes, ultimately resulting in their fusion with degradative lysosomes. Like smaller endosomes, Rab conversion is thought to control the maturation sequence of phagosomes. Initial studies focused on Rab5 and Rab7, which are localized to early and late phagosomes, respectively (50). Vieira and colleagues examined the relationship between these two GTPases and phagosome maturation (204). The expression of dominant negative Rab5 inhibited Rab7 accumulation and phagosome maturation. This is consistent with the Rab conversion model for the endosomal pathway noted above (150). Rab5 is known to coordinate phosphatidylinositol signaling on early endosomes (32, 173). Therefore, Vieira and colleagues studied the effect of wortmannin (an inhibitor of phosphatidylinositol 3-kinase) on phagosome maturation. Wortmannin was found to delay the dissociation of Rab5 from phagosomes but had little effect on the acquisition of Rab7. Importantly, wortmannin effectively blocked phagosome maturation. One important conclusion from those studies is that Rab7 recruitment to phagosomes is not sufficient to promote their maturation. That study suggested that factors other than Rab5 and Rab7 contribute to the control of phagosome maturation.

To further characterize phagosome maturation, we examined the association of 48 different Rabs with model phagosomes containing a noninvasive (ΔinvA) mutant of *Salmonella enterica* serovar Typhimurium (176). These bacteria were engineered to express the *Yersinia* invasion protein, which promotes bacterial uptake by β1 integrin-mediated phagocytosis (98). This mutant traffics to lysosomes and is degraded, allowing us to determine which Rabs localize to a maturing phagosome. In total, we observed that 18 Rabs associated with phagosomes at some point during their maturation. This number reflects an arbitrary threshold value of 20% phagosomes positive for a given Rab at the time examined. Other Rabs were found to associate with phagosomes to a lesser extent, not meeting the threshold value used here, and may also contribute to phagosome maturation. Remarkably, we observed that up to 12 different Rabs are present on phagosomes at a given stage of maturation. This is consistent with the finding that endosomes are composed of membrane domains coordinated by Rabs (13, 178). These data suggest that a complex network of Rab GTPases regulates phagosome maturation. We hypothesize that this network acts through a series of Rab conversion events (similar to those discussed above) that sequentially control phagosome maturation. The details of how this Rab network is controlled remain unclear.
One of the exciting findings of our “Rab screen” of the phagosome was the identification of novel Rabs present on this compartment. These Rabs included Rab8B, Rab13, Rab23, Rab32, and Rab35, which had not been observed on phagosomes previously (see Fig. 4) (176). The expression of dominant negative mutants of Rab23 and Rab35 was found to significantly impair the fusion of model phagosomes that contained the \( \Delta\text{invA} \) mutant of \( S.\ enterica\) serovar Typhimurium with lysosomes preloaded with fluorescent dextran (176). Both Rabs are thought to play roles in endosomal recycling (56, 109). Since endosomal recycling pathways are known to contribute to phagosome maturation (37, 43), it is possible that Rab23 and Rab35 promote phagosome maturation by mediating recycling from this compartment.

**Intracellular Bacterial Pathogens and Rab Modulation on Phagosomes**

Rab GTPases are central to membrane traffic in eukaryotic cells. Therefore, it is not surprising that intracellular bacterial pathogens target Rab functions to colonize vacuolar compartments (modified phagosomes) in cells of their host. The study of Rabs in cells infected by these pathogens has provided insight into how microorganisms can manipulate host cell function to avoid destruction in lysosomes and to cause disease. Here, we focus on intracellular bacterial pathogens that manipulate/exploit Rab function to colonize cells of their host during infection.

**MYCOBACTERIUM TUBERCULOSIS**

*Mycobacterium tuberculosis* is one of the most successful pathogens on the planet, infecting nearly one-third of the human population and killing approximately 1.7 million people each year (158). During infection, these bacteria colonize the lung, predominantly in macrophages. Related mycobacterial species, including *M. avium*, *M. bovis*, and *M. marinum*, are similarly known to colonize macrophages during infection of their hosts and are often used as models of *M. tuberculosis* infection. These *Mycobacterium* species share the ability to colonize a nondegradative phagosome in macrophages (8, 41, 186). However, recently reported data demonstrated that *M. marinum*, *M. tuberculosis*, and *M. leprae* also have the ability to escape from phagosomes and grow within the cytosol of host cells (179, 196). Remarkably, *M. marinum* undergoes actin-based motility in the cytosol, allowing the spread of the organism to neighboring cells (179). Escape of these mycobacteria from the phagosome occurs after a significant delay (48 h postinfection [p.i.] for *M. tuberculosis*) (196). Therefore, a pathogen-induced block in phagosome-lysosome fusion is an essential prerequisite for escape to the cytosol.

The maturation of mycobacterium-containing phagosomes is blocked at the Rab5-positive stage (Fig. 1). Therefore, they are described as being “four-minute phagosomes,” a reflection on their positioning in the normal phagosome maturation sequence (159). Mycobacterium phagosomes are accessible to early endosomal contents. In fact, the bacterial cell wall component phosphatidylinositol mannoside stimulates the fusion of early endosomes with mycobacterial phagosomes (201). Rab5 facilitates the mycobacterial acquisition of iron within phagosomes by ensuring proper endocytic sorting (105). Despite the retention of Rab5, mycobacterial phagosomes do not recruit Rab7, even at 7 days p.i. (202). In addition, the Rab5 effector early endosomal antigen 1 (EEA1) was not recruited. Bacterial products target the formation/retention of PI3P on *M. tuberculosis* phagosomes to block phagosome maturation. EE, early endosome; RE, recycling endosome; LE, late endosome; Lys, lysosome.

**FIG. 1.** *M. tuberculosis.* *M. tuberculosis* cells replicate in a phagosome where the normal maturation process has been blocked at an early stage. The phagosome retains Rab5, Rab14, and Rab22 but does not acquire Rab7 and does not fuse with lysosomes. The phagosome receives iron by continued fusion with early/recycling endosomes and also receives cargo from the trans-Golgi network (such as immature cathepsins). Despite the presence of Rab5 on *M. tuberculosis* phagosomes, its downstream effector EEA1 was not recruited. Bacterial products target the formation/retention of PI3P on *M. tuberculosis* phagosomes to block phagosome maturation. EE, early endosome; RE, recycling endosome; LE, late endosome; Lys, lysosome.
Coxiella burnetii, an obligate intracellular bacterium, is the etiologic agent of the zoonotic disease Q fever (116). C. burnetii undergoes a biphasic developmental cycle consisting of two cell types, the non-metabolically-active, environmentally-resistant, infectious small-cell variant and the replicative and more fragile large-cell variant (36). Unlike most intracellular pathogens that actively avoid fusion with lysosomes, C. burnetii requires a low-pH environment to activate its metabolism and thus replicates within an acidified parasitophorous vacuole that shares many characteristics with lysosomes (84, 88).

In vivo, C. burnetii has a tropism for mononuclear phagocytic cells such as alveolar macrophages and Kupffer cells in the liver (106, 182). However, epithelial and fibroblast cell lines have been used most often as in vitro model systems to study the intracellular development of this bacterium. In vitro, C. burnetii undergoes a 1- to 2-day lag phase followed by 4 days of exponential growth. By 48 h p.i., C. burnetii replicates within large spacious vacuoles termed the replicative Coxiella vacuole (RCV) (36). The RCV is decorated with the lysosomal glycoproteins LAMP-1 and LAMP-2 (73, 88), LAMP-3 (73), the vacuolar H^+ ATPase (73, 88), and Rab7 (17). In addition, the RCV is accessible to fluid-phase markers (88) and is acidified (84) (Fig. 2). Furthermore, the vacuole contains lysosomal hydrolytic enzymes such as acid phosphatase (1, 28, 95), cathepsin D (73, 88), and 5'-nucleotidase (28), suggesting that the RCV is phenotypically similar to lysosomes.

Because the RCV shares many traits with lysosomes, Coxiella was initially thought to passively traffic through the default endocytic pathway to eventually fuse with lysosomes (88). However, recent data suggest that although the RCV shares many characteristics with lysosomes, the RCV is not a typical phagolysosome but is rather a bacterium-modified phagolysosome. Even though C. burnetii does not block the acquisition of LAMP-1 (96, 154), the bacterium still actively modifies the parasitophorous vacuole to regulate the formation and maintenance of the spacious RCV (96). Spacious vacuoles fail to form in cells treated with chloramphenicol (a bacterial protein synthesis inhibitor). In contrast, RCV formation is normal in cells treated with penicillin (a cell wall biosynthetic inhibitor) or nalidixic acid (a DNA synthesis inhibitor) (96). These data demonstrate that bacterial protein synthesis, but not replication, is required for the formation of the spacious RCV. In addition, unlike lysosomes, Coxiella-containing vacuoles (CVs) undergo both homo- and heterotypic fusion with a large variety of different compartments, including pinocytic vesicles containing thorium dioxide, horseradish peroxidase, or ferritin (1, 11), as well as phagosomes containing beads (198), yeast (198), zymosan (198), Mycobacterium species (45, 74), or Leishmania amazonensis (199). Finally, as described below, C. burnetii actively promotes interactions with the autophagy pathway (17, 81, 154), which is thought to delay fusion with lysosomes.
Because of the phagolysosomal characteristics of the RCV, the roles of Rab5 and Rab7 in the biogenesis of the RCV have been extensively examined. A kinetic analysis examining early times p.i. demonstrated that the early CV sequentially acquired wild-type green fluorescent protein (GFP)-Rab5 and GFP-Rab7 with kinetics similar to those observed for a typical latex bead-containing phagosome (154). The recruitment of Rab5 was observed as early as 5 min p.i., with maximal recruitment by 60 min p.i. By 60 min, the majority of CVs lacked Rab5. In contrast, a gradual increase in Rab7 recruitment was observed over time, with maximal staining by 40 min. Additionally, Rab7 recruitment was maintained over the course of infection and was present on the large spacious RCV observed at 48 and 72 h p.i. (17, 154). In cells expressing the constitutively active GTPase-defective mutant GFP-Rab5(Q79L) at 60 min p.i., there was an increase in both the number of infected cells and the size of the vacuole (154). In addition, multiple bacteria were present in each vacuole. Since the generation time of *Coxiella* is at least 10 to 12 h, the increased size of the vacuole is most likely due to the Rab5(Q79L)-mediated induction of homotypic fusion events and not an increase in bacterial replication (155, 184). In contrast, in cells expressing the dominant negative GDP-restricted Rab5(S34N) mutant, there was a decrease in the number of infected cells. Collectively, these data demonstrate that the overexpression of wild-type and constitutively active Rab5 promotes bacterial entry, while the overexpression of Rab5(S34N) inhibits entry. In contrast, at 20 min p.i., similar numbers of infected cells were observed in cells irrespective of the expression of any of the Rab7 guanine nucleotide binding mutants, demonstrating that Rab7 does not play a role in mediating entry. However, similar to what was observed in Rab5(Q79L)-expressing cells, in cells expressing the constitutively active GTPase-defective Rab7(Q67L) mutant, the CV contained multiple bacteria when examined at early times prior to bacterial replication (154).

To further define the role of Rab5 and Rab7 in the maturation of the RCV and to eliminate defects associated with impaired entry, guanine nucleotide binding mutants were introduced after *Coxiella* infection had been established. In infected Chinese hamster ovary (CHO) cells that were superinfected with Sindbis virus expressing wild-type or constitutively active GTPase-defective forms of Rab5 [Rab5(Q67L)] and Rab7 [Rab7(Q67L)], both wild-type and constitutively active Rab7 were localized to the RCV (17). These data demonstrate that Rab7 can be recruited to mature CVs by either active recruitment or fusion with Rab7-positive compartments (17). In contrast, constitutively active Rab5 but not wild-type Rab5 localized to the RCV. Therefore, unlike wild-type Rab5, the Rab5 mutant is either mistargeted to or remains associated with the RCV. Since the expression of Rab5(Q67L) is thought to promote the aberrant fusion of endosomes with phagosomal or lysosomal compartments (155), it is likely that the expression of Rab5(Q67L) induces the aberrant fusion of endosomes with the RCV, consistent with the presence of Rab5(Q67L) but not wild-type Rab5 on the RCV.

In cells expressing either GFP-Rab5(S34N) or GFP-Rab7(T22N), instead of large spacious vacuoles, small bacterium-containing vacuoles are formed (154). These data suggest that both Rab5 and Rab7 are required for the formation of large spacious RCVs. Because the expression of Rab5(S34N) inhibited RCV formation without the presence of wild-type Rab5 on the RCV, these data suggest that Rab5 is required for continual membrane flow through the endocytic pathway and not directly involved in vacuolar formation. A similar model was proposed for the Rab5(S34N)-mediated inhibition of *H. pylori* VacA-induced vacuole formation (136). On the other hand, since wild-type Rab7 is localized to the RCV, the inhibition of the maturation of the RCV resulting from the overexpression of GFP-Rab7(T22N) suggests that the large spacious vacuole is formed as a result of Rab7-mediated fusion events between the RCV and late endosomes or lysosomes (26, 57, 206).

Although *Coxiella* passively transits through the default endocytic pathway, it has recently been shown that *Coxiella* actively interacts with the autophagy pathway to delay trafficking to the lysosome (17, 81, 154). Autophagy is a normal cellular process that nonselectively degrades organelles and other cellular components in times of nutrient starvation to provide a source of carbohydrates and amino acids (107, 148). As early as 5 min p.i., the nascent CV interacts with the autophagic pathway, as demonstrated by the presence of microtubule-associated protein light chain 3 (LC3), a well-defined marker of autophagic vacuoles, on the CV membrane (154). LC3, the mammalian homologue of yeast Atg8, localizes to and is required for the formation of autophagosomes (101). At later times p.i., monodansylecadaverine (MDC) (18) and Rab24 (81, 129), which are both additional markers of autophagosomes, were localized to the RCV. The localization of MDC and Rab24 to the CV were not analyzed at times earlier than 12 h. From 12 to 48 h, in CHO cells stably expressing GFP-LC3, the construct remained strongly associated with the majority of RCVs (17). However, by 72 h p.i., GFP-LC3 was no longer associated with the RCV.

Autophagy is a constitutive process that can also be upregulated by nutrient limitation or through the use of rapamycin, which inhibits TOR, a kinase critical for autophagy (107). By 12 h p.i., both nutrient limitation and treatment with rapamycin increased the percentage of infected cells, the size of the vacuoles, and *Coxiella* replication but had no effect on bacterial entry. These data demonstrate that the induction of autophagy promotes *Coxiella* infection and the formation of the RCV at a postentry stage (81). Furthermore, the pharmacological inhibition of phosphatidylinositol 3-kinase with wortmannin (21) or 3-methyladenine (172), both inhibitors of autophagy, reversed the stimulatory effect of nutrient limitation on *Coxiella* infection. These data confirm that nutrient limitation promotes *Coxiella* infection through the induction of the autophagic pathway (81). Similar to nutrient starvation, the overexpression of both GFP-LC3 and GFP-Rab24 has also been shown to increase the number of autophagosomes (129). Consistent with a positive role for autophagy during *Coxiella* infection, cells overexpressing either GFP-LC3 or GFP-Rab24, an increase in both the number of infected cells and the size of the vacuole was observed by 12 h p.i., a time when vacuoles are normally barely visible. Finally, the induction of autophagy induces the posttranslational modification of LC3. First, immediately after synthesis, a carboxy-terminal fragment is proteolytically cleaved, yielding LC3-I (101). Subsequently, LC3-I is conjugated with phosphatidylethanolamine, generating LC3-II (102). LC3-II is the form of LC3 that is localized to the
autophagosome and is essential for autophagosomal maturation. Autophagosome maturation can also be inhibited by the overexpression of an LC3 mutant, LC3(G120A), that cannot be posttranslationally modified to LC3-II or a nonfunctional allele of Rab24 (129). The formation of the RCV is delayed in cells expressing either of these mutant proteins that are no longer targeted to autophagosomes (81). In summary, although autophagy is induced as a host defense mechanism to eliminate invading pathogens in some cases, in the case of C. burnetii, the induction of autophagy promotes Coxiella infection and the formation of the RCV, while the inhibition of autophagy inhibits Coxiella infection (17).

The data described above demonstrate that the induction of autophagy enhances Coxiella infection, but there is also evidence to suggest that infection by Coxiella induces this innate immune mechanism to promote its intracellular growth. First, in Coxiella-infected cells, both GFP-Rab24 and GFP-LC3 are present in punctate and ring-like vesicular compartments with or without bacteria, which is a pattern of redistribution seen in uninfected cells induced for autophagy (129). Second, similarly to uninfected cells grown under nutrient-limited conditions, LC3-1 is processed to LC3-II in cells that have been infected for 72 h or 96 h with Coxiella (154). The redistribution and localization of LC3 to the vacuole, processing of LC3-I to LC3-II, and formation of the spacious vacuole are all dependent on Coxiella protein synthesis (154). Finally, under nutrient-starved conditions, the acquisition of cathepsin D, a lysosomal marker, to the CV is delayed (154). Collectively, these data suggest that Coxiella actively induces and interacts with the autophagy pathway to delay fusion with lysosomes.

It is not clear why Coxiella delays fusion with lysosomes. One possible model suggests that the delay allows time for the differentiation of the small-cell variant into the large-cell variant. However, the actual timing of the differentiation of the small-cell variant into the large-cell variant is not entirely clear and may occur prior to (95) or after (36) delivery to lysosomes. Alternatively, fusion with autophagosomes may be a mechanism used by Coxiella to obtain nutrients and membrane required for the growth of the spacious vacuolar membrane or to trigger its differentiation or metabolism. Due to the obligate nature of the organism and the lack of a tractable genetic system, the identities of the Coxiella proteins that regulate the maturation of the spacious vacuole or interception of the autophagy pathway have remained elusive. However, a type IV secretion system has been identified in C. burnetii (171). Therefore, type IV effectors secreted into the host cytosol may function to delay maturation of the phagosome, intercept autophagosomes, or alter the fusogenicity of the vacuole.

HELCOBACTER PYLORI

Helicobacter pylori is a gram-negative, spiral-shaped bacterium that infects the human stomach and contributes to the development of gastritis, gastroduodenal ulcers, and stomach adenocarcinoma (54, 188). Although over half of the world’s population is infected with H. pylori, only a fraction of those infected develop disease (188). Several factors are thought to be important for the development of disease, including the presence of two virulence factors, VacA and CagA, encoded within the cag pathogenicity island in the infecting bacterium (38, 54, 122, 123), host genetic immune determinants (55, 156), as well as undefined environmental factors. Although H. pylori is generally considered to be an extracellular pathogen that colonizes the mucus layer of the gastric mucosa, there is both in vitro and in vivo data to suggest that H. pylori may also invade and reside within gastric epithelial cells (52).

The identities of the specific bacterial virulence factors and host factors that promote disease are not fully understood. However, VacA, a secreted vacuolating toxin, is considered to be an important virulence factor that is directly involved in the pathogenesis of the organism (39). In vivo, VacA is important for the colonization of the stomach (162). In vitro, many functions have been attributed to VacA (44), including the induction of apoptosis through the release of cytochrome c from mitochondria (65), the inhibition of T-cell development (22, 71), antigen presentation (121), and the induction of cellular vacuolation (9, 39, 40).

VacA-mediated vacuolation requires the presence of weak anionic bases such as ammonium chloride and the activity of the vacuolar ATPase proton pump (124). These data suggest that vacuolation results from the formation of anion-selective pores, which allow the accumulation of osmotically active, membrane-permeant, weak bases into the vacuoles, resulting in the swelling of the vacuoles. Immunofluorescence microscopic examination of VacA-induced vacuoles, together with the biochemical characterization of purified VacA-induced vacuoles, demonstrated that the vacuoles were enriched for Rab7 and LAMP-1 and were accessible to the fluid-phase marker Lucifer yellow. On the other hand, the early endocytic markers Rab5 and transferrin (Tfn) receptor, the late endosomal marker mannose-6-phosphate receptor, and the lysosomal hydrolase cathepsin D were absent from VacA-induced vacuoles (135). Collectively, these data suggest that VacA induces the accumulation of vacuoles that display characteristics of both late endosomes and lysosomes and, as such, have been described as a postendosomal hybrid of late endosome and lysosomal compartments.

To elucidate the host proteins that were required for the formation of VacA-induced vacuoles, the ability of VacA to induce Rab7-positive vacuoles in HeLa cells transiently expressing nucleotide binding mutants of Rab5, Rab7, and Rab9 was examined (136). VacA-mediated vacuole formation was greatly inhibited by the dominant negative Rab7 mutants Rab7(T22N) and Rab7(N125I) and inhibited to a lesser extent by the dominant negative Rab5 mutants Rab5(S34N) and Rab5(N133I) (136). In addition, the expression of the constitutively active GTPase-defective Rab7 mutant Rab7(Q67L) enhanced the effect of VacA. None of the Rab9 guanine nucleotide binding mutants altered the ability of VacA to induce the formation of vacuoles (136). The absence of Rab5 on the vacuole, together with the inhibitory effect of the dominant negative Rab5 mutants, suggests that continual membrane flow through the endocytic pathway is necessary for vacuole formation. Furthermore, the enrichment of Rab7 on the vacuoles and the inhibitory effect of the dominant negative Rab7 mutants suggest that Rab7 is actively required for vacuole formation. However, since the expression of the constitutively active Rab7 mutant did not mimic the phenotype of VacA-treated cells, Rab7 activation is necessary but not sufficient for VacA-induced vacuole formation, suggesting the involvement of
other factors. Several other guanine nucleotide binding proteins have also been shown to localize to VacA-induced vacuoles and to be required for VacA activity, including Rac1 (94) and dynamin (189). However, similar to Rab7, constitutively active mutants of both proteins failed to mimic the activity of VacA.

Late endosomes are also referred to as multivesicular bodies (MVBs) due to the presence of internal membrane-bound vesicles (145). In yeast, two proteins, Vps4 (10) and Fab1p (134), regulate the formation of MVBs. Similarly to VacA, the expression of a dominant negative mutant of PIKfyve, the mammalian homologue of the yeast Fab1p, induced the formation of enlarged vacuoles that colocalized with late endocytic markers (97). Furthermore, the overexpression of wild-type PIKfyve and microinjection of the PIKfyve lipid product PI(3,5)P₂ abrogated the ability of VacA to induce vacuoles (97). These data are consistent with the idea that VacA functions to inhibit PIKfyve activity and decrease the levels of PI(3,5)P₂ and that this inhibition is essential to the induction of vacuoles. The molecular mechanisms by which VacA might inhibit PIKfyve or how Rab7 is involved in this process remains to be determined.

In order to further characterize the mechanism of VacA-induced vacuole formation, early events in VacA-induced intoxication were examined. In the absence of weak anionic bases, although large vacuoles were not formed, VacA induced the redistribution and clustering of late endocytic compartments enriched for Rab7 and LAMP-1 (120). The addition of ammonium chloride to these VacA-treated cells induced the formation of large Rab7-positive vacuoles from the clustered Rab7-positive compartments (120). Finally, the expression of dominant negative Rab7 but not Rab9 inhibited the VacA-dependent redistribution of late endocytic compartments (120). Collectively, these data suggest that the Rab7-dependent redistribution and clustering of late endocytic compartments precede and are required for the formation of VacA-induced vacuoles and that VacA alters late endocytic trafficking of Rab7- and LAMP-1-positive vesicles.

*H. pylori* can also invade gastric epithelial cells (52), and recent data suggest that VacA is also required for the enhanced intracellular survival of *H. pylori* in gastric AGS epithelial cells (191). Although *H. pylori* can invade AGS cells in a VacA-independent manner, the formation of large *H. pylori*-containing vacuoles is VacA dependent. In addition, although the intracellular survival rates of vacA mutant and wild-type strains were similar when examined at 24 h p.i., at later times, there were significant differences in CFU between the wild type and the mutant. This suggests that the VacA-mediated formation of large vacuoles contributes to the enhanced survival of the organism. Both vacA and wild-type *H. pylori* strains invade AGS cells and are contained within vacuoles derived from late endocytic organelles, as demonstrated by the presence of Rab7, LAMP-1, and CD63 (191) (Fig. 3). The decreased survival of the vacA mutant was correlated with the greater retention of cathepsin D and the increased degradative capability of the vacA-derived bacterium-containing vacuoles. Moreover, the addition of purified VacA or conditioned culture supernatant derived from wild-type-infected cells rescued the intracellular survival defect of the vacA mutant and generated spacious vacuoles from VacA-negative tight vacuoles. Finally, wild-type *H. pylori*, but not vacA mutant bacteria, induced the fusion of bead-containing phagosomes with *H. pylori*-containing vacuoles (191).

Similarly to VacA-induced vacuolation, the VacA-dependent formation of large spacious bacterium-containing vacuoles was dependent on Rab7 (191). In cells expressing GFP-Rab7(F45A), a lack-of-function mutant that does not associate with endocytic organelles, GFP-Rab7(F45A), failed to localize

![FIG. 3. *H. pylori*. *H. pylori*-containing vacuoles originate from the fusion of late endocytic vesicles in a process mediated by the VacA-dependent retention of active Rab7 and the interaction of Rab7 with RILP. Rab7 retention on the bacterium-containing vacuoles inhibits further maturation of the vacuole, thus limiting the degradative capabilities of the vacuole and enhancing the intracellular survival of the organism. In the absence of VacA, spacious vacuoles fail to form, and vacA-containing vacuoles acquire cathepsin D, resulting in a decrease in the intracellular survival of the bacteria. EE, early endosome; LE, late endosome; Lys, lysosome.](http://mmb.asm.org/ Downloaded from)
to *H. pylori*-containing vacuoles, and its expression had no effect on the formation of spacious vacuoles (191). In addition, GFP–Rab-interacting lysosomal protein (RILP), a Rab7 effector that binds only to GTP-Rab7 (29), was recruited to the VacA-induced bacterial vacuoles, demonstrating that active Rab7 was present on the vacuoles. Fluorescence recovery after photobleaching demonstrated that the rate of fluorescence recovery of GFP-Rab7 on VacA-mediated bacterium-containing vacuoles is much slower than that on endosomes, which suggests that the recycling of Rab7 was slower on the bacterium-containing vacuole (191). These data suggest that the bacterial vacuole retains active Rab7 by either inhibiting the intrinsic GTPase activity of Rab7 or inactivating a Rab7-GAP.

Not only is RILP recruited to the bacterium-containing vacuole, the interaction between Rab7 and RILP is required to generate the spacious vacuole (191). First, in cells expressing GFP–Rab7(V180A), a mutant that is unable to interact with RILP but is still recruited to bacterium-containing vacuoles, the generation of large vacuoles was inhibited. Second, the expression of myc-tagged RILP(ID25A), which is defective in Rab7 binding, in AGS cells also inhibited the formation of large vacuoles and caused a redistribution of Rab7 adjacent to the nucleus. Finally, the formation of large vacuoles but not the recruitment of Rab7 was inhibited by the expression of a truncated RILP mutant, RILPC33-GFP (191). RILPC33-GFP retains the ability to bind to active Rab7 but is unable to bind to microtubule motors and, as a result, inhibits late endosome and/or lysosomal fusion. Collectively, these data suggest that VacA functions to retain active Rab7 at the bacterium-containing vacuole and that RILP/Rab7 interactions are important for the formation of the large spacious vacuole. Finally, the retention of active Rab7 alters endocytic trafficking such that the maturation of the bacterium-containing vacuole is inhibited at a late endocytic stage prior to the delivery of active lysosomal hydrolases.

**SALMONELLA ENTERICA SEROVAR TYPHIMURIUM**

*S. enterica* serovar Typhimurium typically causes gastroenteritis in humans and a systemic, typhoid-like disease in genetically susceptible mice (194). These bacteria are facultative intracellular pathogens that can infect a wide variety of cell types in their host during infection. Since they initially interact with intestinal epithelial cells during infection, many in vitro studies of *Salmonella* pathogenesis have involved polarized or nonpolarized epithelial cell lines. Murine macrophages are also studied since this cell type is the primary cellular niche occupied by the bacteria during systemic infection of mice (149, 163). Most of the research described below involves *S. enterica* serovar Typhimurium infection of these cell types. Despite differences in cellular trafficking within epithelial cells and macrophages, it is noteworthy that the key phenotypes associated with *S. enterica* serovar Typhimurium infection in vitro (growth in nondegradative vacuoles and the formation of tubular endosomal structures) (see below) are common to both cell types. This indicates that these bacteria employ a common strategy that allows them to infect both cell types. However, there is also evidence that *S. enterica* serovar Typhimurium replicates less efficiently in some cell types than in others, in part due to differences in the intracellular trafficking of these cell types (perhaps due to Rab expression) and/or differential virulence gene expression (the reader is directed to reviews in references 24, 66, 92, 108, and 192, which discuss infection of different cell types by *S. enterica* serovar Typhimurium).

*S. enterica* serovar Typhimurium invades and replicates within host cells using two different type III secretion systems (T3SS) (64). These needle-like structures deliver bacterial virulence proteins, called effectors, into the host cell. During initial contact with host cells, the first group of effectors is delivered across the plasma membrane to modulate host signal transduction pathways, including the activation of Rho family GTPases, to induce actin rearrangements that drive the ruffling of the cell surface and the uptake of the bacteria into *Salmonella*-containing vacuoles (SCVs) (166). After entry, the bacteria typically remain in SCVs, although a small population can damage this compartment and either escape (140) or become targeted by the autophagy system of the host cell (20). By modifying the SCV using type III secreted effectors and other virulence factors, the bacteria are able to block SCV fusion with lysosomes (27, 42, 70, 119, 176).

SCVs are thought to undergo a multistep maturation process that is distinct from the normal phagosome maturation pathway (Fig. 4). In the first step (0 to 15 min p.i.), the SCV interacts with early endosomes and acquires the markers Rab4, Rab5, and EEA1 (175, 181). The role of Rab5 in SCV biogenesis was explored by Baldeon and colleagues (12). The expression of constitutively active Rab5(Q79L) caused the retention of the early endosomal marker EEA1. This observation is consistent with the ability of Rab5 to promote homotypic early endosome fusion. Rab5(Q79L) expression also promoted the early acquisition of LAMP-2 (12). The accumulation of Rab5 on wild-type SCVs was found to be higher than on model phagosomes containing the Δmva/inv mutant of *S. enterica* serovar Typhimurium (176). This suggests that wild-type bacteria might be promoting fusion with early endosomes to control the maturation of SCVs and that this may be regulated by bacterial effector proteins delivered into cells by the SPI-1 T3SS (180). Indeed, the SPI-1 effector protein SopB was found to promote SCV maturation (LAMP-1 acquisition). The mechanism(s) by which SopB (and possibly other effectors) contributes to SCV maturation is not clear.

The second step (15 to 60 min p.i.) of SCV maturation involves the recruitment of lysosomal glycoproteins (Lgp) such as LAMP-1 and LAMP-2. In contrast to model phagosomes, SCVs acquire Lgp without interacting with mannose-6-phosphate-containing late endosomes or lysosomes (25, 118). Instead, SCVs are thought to acquire Lgp by interacting with a Rab7-positive/Lgp-positive/M6PR-negative/cathepsin D-negative compartment (118). The expression of dominant negative Rab7(T22N) delayed LAMP-1 acquisition by SCVs (118). Pulse-chase analysis suggested that SCVs acquire LAMP-2 derived from the cell surface rather than from the biosynthetic route (12). In support of this hypothesis, LAMP-2 acquisition on SCVs occurred normally in cells lacking a functional AP3 adaptor complex (12), and LAMP-1 acquisition by SCVs was not affected by blocking protein synthesis (118). During this stage of SCV maturation, active Rab7 recruits its effector RILP and promotes centripetal SCV movement to the microtubule organizing center (MTOC) (85). The overexpression of RILPC33, the truncated form of RILP that lacks the dynein-
recruiting domain (29, 99), impaired centripetal SCV movement during the first 2 h p.i. (85). Therefore, Rab7 promotes Lgp acquisition and contributes to the control of SCV positioning within host cells during infection.

During these first two stages of maturation, the SCV is subject to endosomal recycling events. This is evidenced by the fact that many cell surface proteins present on early SCVs are subsequently removed during later stages of SCV maturation (68). SCV interactions with various endosomal recycling factors have been observed. For example, SCVs recruit Rab11 and syntaxin 13, with a maximal association at 60 min p.i. (175). Using a dominant negative approach, we determined that Rab11 regulates the recycling of CD44 from SCVs but had no effect on major histocompatibility complex class I recycling. In contrast, syntaxin 13 regulated the recycling of major histocompatibility complex class I. In recycling. In contrast, syntaxin 13 regulated the recycling of major histocompatibility complex class I but not the recycling of CD44. We also determined that Lgp acquisition by SCVs was slowed when recycling by Rab11 and/or syntaxin 13 was impaired. These findings suggest that protein movement through the endocytic recycling system is regulated through at least two concurrent pathways and that efficient interaction with these pathways is necessary for the maturation of SCVs. Other factors involved in endosomal recycling (e.g., Arf6, Rab4, and Rab10) are also localized to SCVs and may promote the recycling of specific cargo from this compartment (175, 176).

Despite evidence for extensive interactions with the endosomal system, SCVs do not fuse with lysosomes. Our "Rab screen" of the SCV revealed that a number of Rabs enriched on model phagosomes (Rab8b, Rab13, Rab23, Rab32, and Rab35) are excluded from SCVs containing wild-type bacteria (176) (Fig. 4). This suggests that S. enterica serovar Typhimurium can dynamically modify the Rab network associated with phagosome maturation. Bacterial T3SS effectors may play a critical role in this function, but further experimentation is required to test this hypothesis. Several hours after invasion, bacteria induce the expression of the SPI-2-encoded T3SS, which mediates the delivery of a distinct complement of effector proteins into the host cell across the SCV membrane. These effectors modify endosome transport globally in the host cell (195) and promote the formation of Salmonella-induced filaments (Sifs) (25). Sifs associate with microtubules and extend centrifugally away from bacteria, which are localized at the MTOC (69). The function of Sifs is not clear, but the effectors involved in their formation and regulation are all known virulence factors, and their formation is associated with maximal intracellular growth by bacteria (19). Our screen revealed only two Rab GTPases associated with Sifs: Rab7 and Rab9. Dominant negative mutants of these Rabs inhibited Sif formation (176). This finding suggested that late endosomal fusion gives rise to Sif formation. In support of this model, the late endosome markers LBPA, NPC-1, and syntaxin 7 accumulate on late SCVs and on Sifs (25, 176). The SCV may also receive secretory cargo from the trans-Golgi network, possibly providing nutrients for bacteria in vacuoles. EE, early endosome; RE, recycling endosome; LE, late endosome; Lys, lysosome.

FIG. 4. S. enterica serovar Typhimurium. S. enterica serovar Typhimurium cells replicate in SCVs in host cells. The SCV undergoes a multistep interaction with the host endosomal system but blocks fusion with lysosomes. Up to 18 Rabs are present on the SCV during its maturation sequence. Those Rabs enriched on SCVs containing wild-type bacteria are indicated. Some Rabs are enriched on model phagosomes containing noninvasive bacteria that traffic to and are killed in lysosomes (ΔinvA/Inv). Importantly, these Rabs are excluded from wild-type SCVs, suggesting that the bacteria actively modulate Rab localization to prevent SCV fusion with lysosomes. Several hours after infection, the SCV undergoes extensive fusion with late endosomes. This gives rise to the formation of membrane tubes that extend away from SCVs, known as Sifs. Rab7 and Rab9 are both required for Sif formation. The late SCV may also receive cargo from the trans-Golgi network, possibly providing nutrients for bacteria in vacuoles. EE, early endosome; RE, recycling endosome; LE, late endosome; Lys, lysosome.
the SPI-2 T3SS, was found to be at least partially responsible for uncoupling Rab7 on Sifs from RILP. The expression of a SifA-GFP fusion induced the formation of Lgp-positive “Sif-like tubules” in transfected cells that were positive for Rab7 but negative for RILP (85). In vitro experiments revealed an interaction of SifA with Rab7 (85). Together, these data suggest that by disengaging RILP from Rab7, SifA enables the centrifugal extension of tubules from the SCV (Sif formation). How SifA modulates Rab7’s interaction with RILP (and perhaps its other effectors) remains undetermined.

A notable exception to the model described above is presented by the work of Hashim and colleagues (86). Those authors utilized a clinical isolate of *S. enterica* serovar Typhimurium to infect J774E mouse macrophages and isolated phagosomes using a sucrose density gradient method (86). Phagosomes containing live bacteria were found to retain Rab5 for up to 90 min p.i. and did not acquire Rab7, despite the recruitment of Lgp (86). Their subsequent studies suggested that the SPI-1 T3SS effector SopE interacts with Rab5 and acts as a GEF to promote Rab5 activation and the recruitment of nonprenylated Rab5 to SCVs (128). At present, it is not clear why the results of that group are apparently at odds with those of other investigators. However, it is increasingly apparent that differences in cell type, bacterial strain, or infection conditions can give rise to different outcomes with in vitro bacterial infections. Furthermore, it is now appreciated that intracellular bacterial pathogens can have multiple fates within host cells and that quantitative analysis of different populations is required (20). In a broader context, the nature of SCVs in vivo is not known and needs to be addressed.

**CHLAMYDIA SPECIES**

*Chlamydia* species are obligate intracellular bacteria that are important pathogens of ocular, urogenital, and pulmonary mucosal surfaces (165). Of the recognized *Chlamydia* species, *Chlamydia trachomatis*, the leading cause of sexually transmitted disease and preventable blindness, and *Chlamydia pneumoniae*, an important cause of community-acquired pneumonia, are the two major human pathogens (165). The bacterium is characterized by a unique biphasic developmental cycle that alternates between infectious metabolically inactive elementary bodies and noninfectious metabolically active reticulate bodies (126). Differentiation and replication of the bacterium occur solely within a membrane-bound vacuole termed the inclusion. Chlamydiae enter nonprofessional phagocytes by multiple mechanisms, but irrespective of the specific uptake mechanism, chlamydiae actively mediate the remodeling of the nascent vacuole by processes that are dependent on chlamydial gene expression (59, 167). As a result, the inclusion avoids the recruitment of Rab11 and secretory (Rab1, Rab6, and Rab10) Rabs, are recruited to lipid bodies. Multiple Rabs, including both endocytic (Rab4 and Rab11) and secretory (Rab4, Rab6, and Rab10) Rabs, are recruited to the inclusion in a guanine nucleotide-dependent fashion. For at least one Rab, Rab4, recruitment to the inclusion is mediated by a chlamydia-encoded inclusion membrane protein, Inc CT229. Neither the functional role nor the host source of inclusion-localized Rabs has been identified. EE, early endosome; RE, recycling endosome; LE, late endosome; Lys, lysosome.

**FIG. 5. Chlamydia species.** Chlamydiae replicate within a nonacidified inclusion that is actively remodeled by chlamydiae during the first 2 h p.i. by processes that require chlamydial protein synthesis. As a result of this chlamydia-mediated remodeling, fusion with lysosomes is inhibited, and the inclusion is trafficked to the peri-Golgi region, where it intercepts Golgi apparatus- and MVB-derived vesicles as well as lipid bodies. Multiple Rabs, including both endocytic (Rab4 and Rab11) and secretory (Rab6, Rab6, and Rab10) Rabs, are recruited to the inclusion in a guanine nucleotide-dependent fashion. For at least one Rab, Rab4, recruitment to the inclusion is mediated by a chlamydia-encoded inclusion membrane protein, Inc CT229. Neither the functional role nor the host source of inclusion-localized Rabs has been identified. EE, early endosome; RE, recycling endosome; LE, late endosome; Lys, lysosome.

Chlamydiae exploit host trafficking pathways to obtain essential nutrients and biosynthetic precursors (167). Eukaryotic lipids such as sphingomyelin (82, 83), cholesterol (30), and glycerophospholipids (209) are delivered to the inclusion by multiple mechanisms including Golgi apparatus-dependent vesicular trafficking pathways (82, 83), MVB-dependent trafficking pathways (14), and recruitment of lipid bodies (111). In addition, recycling endosomes containing Th are recruited to but do not fuse with the inclusion (168, 197). However, the molecular mechanisms by which chlamydiae exploit these host trafficking pathways have not been identified.

The potential role of Rabs in the biogenesis or trafficking of the inclusion was investigated by examining the intracellular localization of Rab GTPases in infected HeLa cells transiently expressing a collection of GFP-Rabs (161). From those studies, it was shown that an unexpectedly large number of GFP-Rabs were localized to the 18-h inclusion, including Rab1, Rab4, Rab6, Rab10, Rab11, and Rab14 (161; M. A. Scidmore, unpublished data) (Fig. 5). Even though a large number of Rab GTPases were found to decorate the inclusion, several, including Rab5, Rab7, and Rab9, were shown to be absent from the inclusion, thus demonstrating the specificity of Rab recruitment to the inclusion (161). The absence of Rab5, Rab7, and Rab9 from the inclusion confirms that the inclusion does not interact with the default endocytic/lysosomal pathway. In addition, recruitment of at least one endogenous Rab, Rab14, has been confirmed by laser scanning confocal microscopy, suggesting that the localization of GFP-tagged Rabs reflects the localization of their endogenous counterparts (M. A. Scidmore, unpublished data). Unfortunately, elucidating the functional relevance of the recruitment of Rabs to the inclusion has been difficult. Inclusions develop normally in cells expressing a
variety of different guanine nucleotide mutants, including constitutively active GTPase-defective, dominant negative, GDP-restrictive, non-nucleotide-binding, and effector binding Rab mutants (125, 160). The fact that the expression of any single guanine nucleotide binding mutant fails to inhibit inclusion development suggests that, similar to Legionella (51), chlamydiae may target multiple functionally related trafficking pathways and that the inhibition of a single pathway or single step in a pathway may not alter chlamydial development or inclusion formation. Alternatively, chlamydiae may recruit multiple Rabs to the inclusion not to exploit specific trafficking pathways but rather to camouflage the inclusion as either a recycling endosome or secretory vesicle such that it is not targeted to the lysosome for degradation.

Although biological functions of Rabs during chlamydial infection have not been identified, several Rab effectors, including BICD1 (125), have been localized to chlamydial inclusions. Since Rab effectors interact with the GTP-bound active Rabs, the presence of effectors at the inclusion suggests that active GTP-bound Rabs decorate the inclusion membrane and that they are likely performing some function through their interaction with specific effectors. The recycling kinetics of inclusion-localized Rabs have not yet been analyzed, so it is unknown whether chlamydiae secrete GEFs or GAPs that may alter the activation status or recycling kinetics of inclusion-localized Rabs.

The mechanism by which chlamydiae target and recruit Rabs to the inclusion is just beginning to be examined. By analyzing the recruitment of GFP-tagged guanine nucleotide binding mutants, it was shown that only wild-type and constitutively active or GTP-bound Rabs, but not inactive GDP-bound Rabs, localized to the inclusion (125, 160). These data demonstrate that Rabs associate with the inclusion in a manner similar to how they interact with their respective effector molecules. Therefore, the guanine nucleotide-dependent localization to the inclusion suggests that either a Rab effector that is also localized to the inclusion or a chlamydial protein that mimics a Rab effector may recruit Rabs to the inclusion. Chlamydiae secrete both TSSS-dependent proteins (141), including inclusion membrane proteins (Inc) (153), and TSSS-independent proteins into the host cytosol or inclusion membrane. Because of their intracellular localization, some of these secreted proteins may function to recruit or modulate the activity of Rabs at the inclusion membrane. Incs are a large family of highly diverse chlamydia-specific proteins that are incorporated into the growing inclusion membrane (153). Yeast two-hybrid analysis identified a specific guanine nucleotide-dependent interaction between Rab4 and the C. trachomatis-specific Inc CT229 (160). Although loss-of-function mutants cannot be generated in Inc CT229, GFP-Rab4 was shown to colocalize with CT229 at the inclusion membrane, suggesting that CT229 functions to recruit Rab4 to the inclusion (160). A second interaction between the C. pneumoniae inclusion membrane Cpn0585 and multiple Rabs including Rab1, Rab10, and Rab11 has also been reported (B. Wizel and M. A. Scidmore, unpublished data). Collectively, these data demonstrate that chlamydiae modify the inclusion membrane through the secretion of chlamydia-specific Incs to promote interactions with host secretory pathways through the specific recruitment of Rab GTPases.

Although it is known that the inclusion receives cargo from both Golgi apparatus-derived vesicles and MVB-derived vesicles, it is unclear whether Rabs are delivered to the inclusion as a result of fusion with these or other vesicles or, alternatively, are sequestered from a cytosolic pool. However, GFP-Rab6 as well as its effector, BICD1, are still recruited to the inclusion in the presence of brefeldin A (BFA), a fungal metabolite that inhibits endoplasmic reticulum (ER)-to-Golgi apparatus trafficking by inactivating Arf1 (125). These data suggest that neither protein is trafficked to the inclusion through a Golgi apparatus-derived intermediate or via an ER-to-Golgi apparatus-dependent trafficking pathway and are consistent with chlamydiae recruiting at least some Rabs from a cytosolic pool. Without the ability to genetically knock out chlamydial genes that mediate the recruitment of Rabs to the inclusion, the elucidation of the biological role of Rabs will ultimately have to rely on gene-silencing techniques to deplete infected cells of individual Rabs or subsets of Rabs.

**LEGIONELLA PNEUMOPHILA**

Legionella pneumophila is a gram-negative, facultative, intracellular bacterium that is the causative agent of “Legionnaires’ disease” (62, 117). In its natural environment, the bacterium resides within freshwater amoebas such as Acanthamoeba and Hartmanella, but the organism can also infect humans upon the inhalation of contaminated aerosols (2). Invasion and replication in alveolar macrophages can lead to pneumonia or “Legionnaires’ disease” in individuals who are immunocompromised or have some underlying conditions that reduce the efficiency of their lung defense mechanisms (2).

Within the macrophage, L. pneumophila promotes its own survival by replicating within an ER-derived phagosome that avoids fusion with the default endocytic/lysosomal pathway (93, 190) (Fig. 6). The Icm/Dot-encoded secretion system, which functions to deliver macromolecules (DNA and protein) into the eukaryotic and protozoan host (171), is essential for determining the intracellular fate of the organism as shown by the rapid trafficking of icm/dot-containing phagosomes to late endosomes (16, 100, 157) and the decreased intracellular survival of icm/dot mutants (23). The formation, but not the maintenance, of the Legionella-containing vacuole (LCV) is dependent on the Icm/Dot type IV secretory system (35, 157). Maturation of the nascent LCV into the replicative vacuole (RV) occurs during the first several hours p.i. By 5 min p.i., the vacuole containing wild-type Legionella avoids interactions with the default endocytic/lysosomal pathway. By 15 min p.i., the limiting vacuolar membrane is decorated with smooth vesicles. After 4 h, the vacuolar membrane has thickened and is decorated with ribosomes, at which time the organism has begun to replicate and is thus termed the RV at this stage (93). Within this specialized RV, the bacteria replicate to high densities, resulting in host cell lysis and infection of neighboring macrophages. In primary macrophages derived from bone marrow of the permissive A/J mouse, the interaction with the lysosomal pathway is only delayed, as the RV eventually matures into an acidified vacuole in a process that resembles autophagy (6, 187).

Recent studies proposed that the maturation of the LCV into the RV occurs in at least two stages (152). The first stage
is the recruitment and fusion of ER-derived early secretory vesicles with the nascent LCV. The second stage, which is dependent on the first stage, involves fusion with the ER to form the RV. In support of this model, coincident with the appearance of vesicles attached to the LCV, proteins that cycle between the ER and the Golgi apparatus are detected at the LCV within the first 30 min p.i. (104). Electron microscopy studies have confirmed the presence of several ER markers including protein disulfide isomerase, KDEL-containing proteins, and glucose-6-phosphatase within vesicles attached to the phagosome by 1 h p.i. (152). In addition, immunofluorescence microscopy studies demonstrated the presence of Arf1 (131), Rab1 (48, 104), and Sec22b (48, 104) by 1 h p.i. as well as the resident ER marker calnexin by 4 h p.i. (103). However, separate studies that examined the presence of resident ER markers on density-purified LCVs by indirect immunofluorescence microscopy demonstrated the recruitment of the resident ER marker calnexin and yellow fluorescent protein-KDEL as early as 30 min p.i. (48). The discrepancies relating to the timing of recruitment of different ER markers may be a result of different sensitivity levels of the assays utilized (48).

How does Legionella target the early secretory pathway? ER-Golgi apparatus trafficking is regulated by Rab1A/Rab1B (153, 208), Rab2 (193), and at least one SNARE complex composed of one v-SNARE (Sec22b) and three cognate t-SNAREs (syntaxin 5, membrin, and Bet1) (87, 210). Rab1 promotes the fusion of ER-derived vesicles with pre-Golgi and Golgi compartments by recruiting effectors, such as p115 (3) and GM130 (127), that are necessary for the tethering and fusion of vesicles and facilitating the pairing of v-SNAREs with cognate t-SNAREs. In addition, the sequential interactions of two additional small GTPases, Sar1 and Arf1, which regulate the formation of COPII- and COPI-coated vesicles, respectively, are also required for the production of early secretory vesicles (7, 53, 164).

Both Rab1 and Arf1, which are found on vesicles that cycle between the ER and the Golgi apparatus, are localized to the LCV by 30 min p.i. (48, 104, 131). In addition, the v-SNARE Sec22b, but not any of its cognate t-SNAREs, is also delivered to the LCV (48, 104). Interestingly, prior remodeling of the vacuole with ER-derived vesicles is not required for the delivery of either Rab1 or Arf1 to the vacuole, since both are recruited to the vacuole in the presence of BFA or in cells expressing the dominant interfering Sar1(H179G) mutant (103, 104). These data suggest that Legionella is able to recruit both proteins from a cytosolic pool and activate them at the vacuolar membrane. On the other hand, the delivery of Sec22b is inhibited by BFA and by the expression of dominant interfering Arf1 and Sar1 mutants, demonstrating that ER-Golgi apparatus trafficking is required for the delivery of Sec22b to the LCV (104). In addition, the expression of the dominant negative Rab1 mutant delays the delivery of Sec22b to the vacuole (104). Collectively, these data are consistent with a two-stage maturation model and suggest that Rab1 is required for an early step in remodeling of the LCV and that the subsequent recruitment of Sec22b is dependent on prior Rab1-mediated remodeling of the LCV.

Remodeling of the LCV with ER-derived vesicles is an early event required for the formation of the RV. The expression of dominant interfering mutants in all three small GTPases (Sar1, Rab1, and Arf1) inhibits the formation of the RV and decreases the intracellular survival of the organism (48, 104, 152). Also, time course studies done with BFA demonstrated that BFA blocked the maturation of the vacuole only if it was added prior to or during the first 30 min p.i. (103). The addition of BFA after 30 min had no effect on the formation of the RV, demonstrating that ER-derived vesicle remodeling was required only during the initial stages of infection and that once remodeling has occurred, the inhibition of ER-to-Golgi apparatus trafficking is no longer required to maintain the RV. Collectively, these data suggest that the delivery of ER-derived vesicles is crucial for the remodeling of the LCV and that Legionella recruits these vesicles by targeting key regulatory proteins in this pathway (152). However, in no case was replication inhibited completely (48, 104), consistent with recent data demonstrating that Legionella targets functionally redundant early secretory pathways and that more severe replication defects are observed only when more than one pathway is inhibited (51).

The intracellular survival of the organism, the initial remodeling of the vacuole, and the recruitment of early secretory proteins are all dependent of the Icm/Dot-encoded secretion apparatus. These data suggest that Legionella secretes type IV effectors into the host cell cytosol that function to target specific host trafficking pathways in order to remodel the nascent vacuole (171). Although over 100 potential type IV effectors have been identified, the function of only several effectors has been determined. This includes RalF, which acts as an Arf1-specific GEF that functions to recruit Arf1 to the LCV (131).
Additionally, LidA and DrrA/SidM regulate the activity of Rab1 (see below) (114, 130).

As mentioned above, Rab1 is recruited to the LCV in an Icm/Dot-dependent but BFA-independent manner, suggesting that Legionella secretes a type IV effector that plays a role in the cytosolic recruitment and/or modulation of Rab1 activity (48, 104). Consistent with these data, two groups independently identified a type IV effector, DrrA/SidM, which localizes to the cytosolic face of the LCV and functions to recruit and regulate Rab1’s activity at the LCV. In one study, SidM (substrate of Icm/Dot) was identified as a result of its ability to bind purified glutathione S-transferase–Rab1 (114), and in the other study, DrrA (defect in Rab recruitment) was identified in a mutant screen designed to identify Legionella proteins that were necessary for the recruitment of Rab1 to the LCV (130). In vitro pull-down assays demonstrated that DrrA/SidM preferentially bound to the GDP-restrictive or no-nucleotide form of Rab1, which is a characteristic of GEFs (114, 130). Consistent with the in vitro binding data, both groups confirmed that DrrA/SidM exhibits a Rab1-specific GEF activity (114, 130), although weak activity for Rab38, a Rab1-related GTPase, was also detected (130).

Although the deletion of DrrA/SidM prevented the recruitment of Rab1 to the LCV, no decrease in bacterial replication resulted from a loss of this protein (114, 130). Again, these data support the hypothesis that Legionella targets multiple functionally redundant trafficking pathways and that the inhibition of only a single trafficking pathway results in little or no intracellular growth defects (51). Ecotropic expression of DrrA/SidM in eukaryotic tissue culture cells was shown to interfere with ER-to-Golgi apparatus trafficking and induced the dispersal of the Golgi apparatus (114, 130). In addition, GFP-DrrA localized to and recruited endogenous Rab1 to the plasma membrane (130), suggesting that DrrA/SidM may compete with endogenous GEFs to redirect Rab1 from its normal secretory intracellular localization to plasma membrane-derived vesicles such as the nascent LCV.

A second type IV effector, LidA, which is secreted into the host cytosol and localizes to the cytosolic face of the LCV, was also identified and shown to play a cooperative role in the recruitment and activation of Rab1 (114). Similarly to DrrA/SidM, the ecotropic expression of LidA interfered with cytosolic trafficking, and LidA interacted with Rab1 (49). In contrast to DrrA/SidM, LidA preferentially bound to the GTP-bound form of Rab1 (114). Additionally, LidA was also shown to be more promiscuous in its binding to Rab GTPases, as it also interacted with Rab6 and Rab8 (114). Although lidA mutants still recruited Rab1 to the LCV, these mutants displayed a much more dramatic decrease in intracellular replication than did the drrA/sidM mutant (114). These data may be explained by observation that LidA targets multiple trafficking pathways, as opposed to DrrA, which targets only a single Rab1-dependent trafficking pathway. Although lidA mutants were not defective in Rab1 recruitment, in vitro data suggest that LidA cooperates with DrrA/SidM to recruit ER-derived vesicles. In those in vitro experiments, it was shown that SidM, but not LidA, immobilized to beads facilitated the recruitment of secretory vesicles in a Rab1-dependent manner (114). Interestingly, although LidA was unable to recruit secretory vesicles on its own, the presence of both SidM/DrrA and LidA on the beads increased the efficiency of secretory vesicle recruitment. Collectively, these data suggest that LidA acts with SidM/DrrA in a cooperative manner. Since LidA binds Rab1 but does not display any GEF or GAP activity, LidA may increase the activity of Rab1 by inhibiting the intrinsic GTPase activity of either Rab1 or a Rab1-GAP. Alternatively, LidA may inhibit the membrane recycling of Rab1 by preventing the GDI-mediated removal of Rab1-GDP from the vacuolar membrane.

CONCLUDING REMARKS

Although this review focuses on only a subset of intracellular pathogens, many more are likely to exploit Rab-dependent trafficking pathways to generate replication-competent intracellular niches. For example, similarly to Legionella, Brucella abortus also targets the early secretory pathway by a mechanism that is regulated by the activity of Rab5 (31, 75), while Rhodococcus equi replicates in nondegradative phagosomes that are blocked somewhere between early and late endosomes (58). Listeria monocytogenes grows rapidly in the cytosol (146). However, recent data suggest that these bacteria can modify Rab5 recruitment to phagosomes prior to their escape into the cytosol (90). Conversely, host upregulation of Rab5 may prevent L. monocytogenes escape from phagosomes (4, 5, 147). Further studies are needed to determine whether these and other bacterial pathogens specifically target Rab proteins to modulate the identity of their vacuoles.

As discussed above, bacterium-containing vacuoles retain or exclude Rab proteins to alter their identity. However, except for Legionella, where there is substantial evidence that secreted bacterial proteins interact with and directly regulate the activity of Rab proteins (114, 130), it is unclear whether the other bacterial pathogens use virulence factors to interact directly or indirectly with Rabs. Future challenges in the field include the identification of additional bacterial secreted proteins that interact with or regulate the interaction of the bacterium-containing vacuoles with specific Rab proteins. An open question in cell biology is how Rab proteins are targeted to and maintained at specific intracellular localizations. Future studies of how pathogens recruit or exclude Rabs to compartments that they occupy may provide insights into this problem and may further the understanding of how Rab proteins coordinate distinct trafficking pathways.

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