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Listeria InlB Expedites Vacuole Escape and Intracellular Proliferation by Promoting Rab7 Recruitment via Vps34

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ABSTRACT Rapid phagosomal escape mediated by listeriolysin O (LLO) is a prerequisite for *Listeria monocytogenes* intracellular replication and pathogenesis. Escape takes place within minutes after internalization from vacuoles that are negative to the early endosomal Rab5 GTPase and positive to the late endosomal Rab7. Using mutant analysis, we found that the listerial invasin InlB was required for optimal intracellular proliferation of *L. monocytogenes*. Starting from this observation, we determined in HeLa cells that InlB promotes early phagosomal escape and efficient Rab7 acquisition by the *Listeria*-containing vacuole (LCV). Recruitment of the class III phosphoinositide 3-kinase (PI3K) Vps34 to the LCV and accumulation of its lipid product, phosphatidylinositol 3-phosphate (PI3P), two key endosomal maturation mediators, were also dependent on InlB. Small interfering RNA (siRNA) knockdown experiments showed that Vps34 was required for Rab7 recruitment and early (LLO-mediated) escape and supported InlB-dependent intracellular proliferation. Together, our data indicate that InlB accelerates LCV conversion into an escape-favorable Rab7 late phagosome via subversion of class III PI3K/Vps34 signaling. Our findings uncover a new function for the InlB invasin in *Listeria* pathogenesis as an intracellular proliferation-promoting virulence factor.

IMPORTANCE Avoidance of lysosomal killing by manipulation of the endosomal compartment is a virulence mechanism assumed to be largely restricted to intravacuolar intracellular pathogens. Our findings are important because they show that cytosolic pathogens like *L. monocytogenes*, which rapidly escape the phagosome after internalization, can also extensively subvert endocytic trafficking as part of their survival strategy. They also clarify that, instead of delaying phagosome maturation (to allow time for LLO-dependent disruption, as currently thought), via InlB *L. monocytogenes* appears to facilitate the rapid conversion of the phagocytic vacuole into an escape-conducive late phagosome. Our data highlight the multifunctionality of bacterial virulence factors. At the cell surface, the InlB invasin induces receptor-mediated phagocytosis via class I PI3K activation, whereas after internalization it exploits class III PI3K (Vsp34) to promote intracellular survival. Systematically elucidating the mechanisms by which *Listeria* interferes with PI3K signaling all along the endocytic pathway may lead to novel anti-infective therapies.

KEYWORDS *Listeria monocytogenes*, *Listeria* virulence, internalins, InlB, intracellular parasitism, intracellular survival, intracellular proliferation, endocytic trafficking, phagosome maturation, *Listeria*-containing vacuole, vacuole escape, subversion of phosphoinositide metabolism, phosphoinositide 3-kinase, class III PI3K, Vps34, phosphatidylinositol 3-phosphate, Rab5, Rab7

The Gram-positive pathogen *Listeria monocytogenes* is the causative agent of listeriosis, an invasive foodborne infection with severe clinical manifestations, including meningoenzephalitis, septicemia, stillbirth, and neonatal sepsis (1). *Listeria* virulence depends on the ability of these bacteria to proliferate within macrophages and a variety of nonphagocytic cells. In contrast to other major intracellular pathogens, such as

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Mycobacterium tuberculosis or *Salmonella*, *Listeria* replicates in the cytosol and not in a membrane-bound vacuole (2, 3).

Within 30 min of cell entry, *Listeria* bacteria escape from acidified vesicles (4) bearing characteristics of late endosomes prior to fusion with lysosomes (Rab5 negative, Rab7 positive, and lysosomal-associated membrane protein 1 [LAMP1] negative) (5). This process is mediated by the listerial *hly* gene product, the pore-forming toxin listeriolysin O (LLO), upon its activation at acidic pH (5.5 to 6.0), aided by phospholipases PlcA and PlcB (6). Rapid multiplication ensues, fueled by hexose phosphates, which *Listeria* take up from the cytosol via the Hpt permease (7). After replication, the listerial actin-polymerizing protein ActA induces actin-based motility and cell-to-cell spread (8), allowing cytosolic *Listeria* to escape from autophagy (9) and to disseminate throughout host tissues while remaining protected from extracellular defenses (10). InIC, a member of the listerial internalin family (11), cooperates with ActA in cell-to-cell spread (12) while dampening innate immunity by inhibiting NF- κ B responses (13).

Active invasion of epithelial and other normally nonphagocytic cells is critical for host barrier translocation and parenchymal tissue colonization by *Listeria*. Entry into these cells is mediated by the products of the *L. monocytogenes inlAB* operon, the surface-associated internalins InIA and InIB (14). InIA and InIB interact with specific host cell ligands, the junctional adhesion protein E-cadherin (15, 16) and the hepatocyte growth factor (HGF) receptor Met (17, 18), respectively, triggering phagocytosis. While both internalins synergize and are required for full *L. monocytogenes* invasiveness, InIA is thought to be specifically important for internalization into certain human epithelial cell subpopulations, whereas InIB promotes entry into a broader range of cell types (19, 20). The entry process begins with the ubiquitination of the InIA and InIB receptors, followed by recruitment of the actin cytoskeleton at clathrin-coated bacterial adhesion sites, with myosin VI providing the force for internalization (21–23). Subsequently, InIB reinforces the cortical actin polymerization cascade via activation of class I phosphoinositide 3-kinase (PI3K) downstream of Met (24), with accumulation of phosphatidylinositol 3,4,5-triphosphate (PI[3,4,5]P₃) in the cell membrane, local recruitment of small Rho family GTPases and actin-binding proteins, and phagosome formation (reviewed in reference 25).

The *Listeria* intracellular survival-promoting determinants (LLO, PlcA, PlcB, Hpt, ActA, InIC) are coordinately regulated by the transcriptional activator PrfA (26). Consistent with their primary intracellular function, PrfA-regulated genes are weakly expressed extracellularly and selectively activated within host cells (27). Interestingly, although having an established role in cell entry, the *inlAB* locus is also controlled by PrfA (28) and its expression is activated during intracellular infection (29, 30; our unpublished results), suggesting a potential function following internalization. We investigated the possible involvement of the *inlAB* products in *L. monocytogenes* intracellular proliferation in human epithelial cells. Here, we report that the invasin InIB promotes early phagosomal escape, and hence bacterial intracellular proliferation, by accelerating the formation of a late-endosomal Rab7-positive compartment via subversion of class III PI3K signaling.

RESULTS

InIB is required for efficient intracellular proliferation. Isogenic *L. monocytogenes* in-frame *inIA* and *inIB* deletion mutants were tested in human epithelial HeLa cells using gentamicin protection assays. Since the intracellular population at a given time point depends directly on the initial number of internalized bacteria, the multiplicity of infection (MOI) was adjusted such that each strain had comparable intracellular numbers at $t = 0$ (average of $\sim 5 \times 10^4$ colony forming units [CFU]/well). In addition, the intracellular proliferation data were normalized to the bacterial counts at $t = 0$ using an intracellular growth coefficient (IGC) (27) (see Materials and Methods).

As shown in Fig. 1A, while the $\Delta inIA$ mutant behaved as the wild type (WT), a distinct intracellular growth defect was observed for the $\Delta inIB$ mutant. This was reproduced in other cell lines (human intestinal Caco-2 cells, murine J774 macrophages) (see Fig. S1 in the supplemental material) and corroborated early observations in mouse hepatocytes (31).

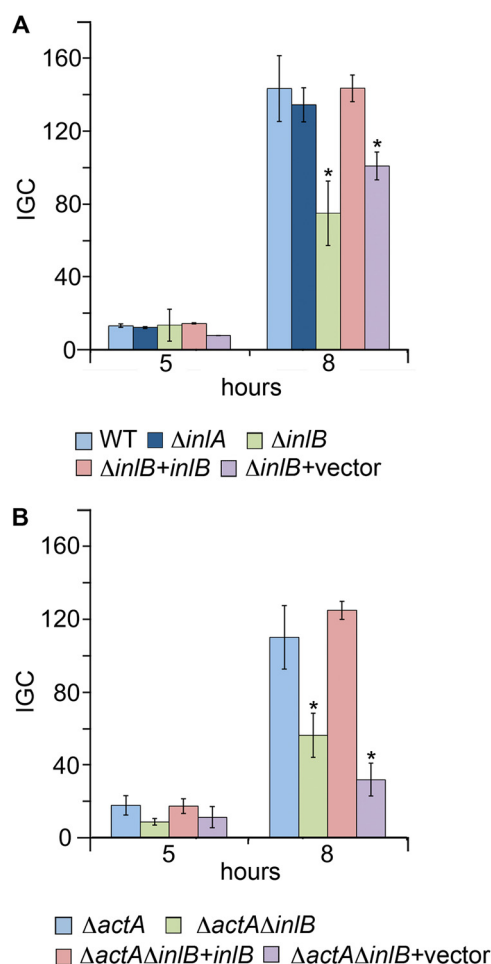


FIG 1 InIB is required for efficient intracellular proliferation of *L. monocytogenes*. Intracellular CFU of WT, ΔinA , and ΔinB in HeLa cells were normalized at each time point to the internalized bacterial CFU at $t = 0$ using an intracellular growth coefficient (IGC) (see Materials and Methods). *, significant difference from WT ($P < 0.05$). (A) Data for WT, ΔinA , ΔinB , and *inB*- or mock-complemented ΔinB bacteria. Corresponding mean CFU/well ($\times 10^4$) at $t = 0$ for the respective strains: 4.9 ± 1.8 , 1.4 ± 0.13 , 4.6 ± 1.7 , 6.1 ± 4.8 , and 3.9 ± 2.4 . (B) Experiments in the cell-to-cell spread-null ($\Delta actA$) background. Corresponding mean CFU/well ($\times 10^4$) at $t = 0$: $\Delta actA$, 4.4 ± 1.7 ; $\Delta actA \Delta inB$, 3.8 ± 2.4 ; $\Delta actA \Delta inB+inB$, 4.3 ± 2.6 ; $\Delta actA \Delta inB+vector$, 3.7 ± 2.6 .

The effect was not attributable to impaired bacterial fitness, since the ΔinB mutant showed no growth defect in broth (Fig. S1B). Complementation of ΔinB with the *inB* gene on a plasmid, but not empty vector, rescued InIB production (Fig. S1C), normal cell entry (Fig. S1D), and WT intracellular proliferation (Fig. 1A), linking the observed phenotype to InIB.

L. monocytogenes proliferation in a cell monolayer is dependent on both successful escape to the cytosol and actin-based spreading to adjacent cells. The contribution of cell-to-cell spread to the ΔinB proliferation defect can be tested using a strain lacking ActA (8). While, as expected, overall proliferation was generally reduced in the absence of ActA due to the spread deficiency, intracellular growth was significantly diminished in a $\Delta actA \Delta inB$ double mutant compared to the $\Delta actA$ single mutant. Again, complementation with *inB*, but not empty vector, reverted the proliferation defect (Fig. 1B). Together, these data indicated that the absence of InIB impairs *L. monocytogenes* intracellular proliferation due to effects upstream of cell-to-cell spread.

InIB promotes early vacuole escape. The ΔinB phenotype could be due either to a reduced ability to replicate within the cytoplasm or to an earlier defect in vacuole escape. To test this, the proportion of vacuole-associated and cytosolic bacteria was monitored over a 90-min infection time course in HeLa cells using two separate

microscopy-based assays. Vacuole-associated bacteria were identified by colocalization with FM 1-43FX, a fluorescent membrane probe that constitutively labels endosomes and phagosomes (32, 33). The vacuole marker was associated as early as 10 min after infection with WT *L. monocytogenes*, with a peak at 20 to 30 min before rapidly dropping to ~20% of the bacterial population by 90 min (Fig. 2A and B). Although entering in fewer numbers (Fig. S2B), the $\Delta inIB$ bacteria that successfully invaded the monolayer showed similar FM 1-43FX colocalization dynamics during the first 30 to 45 min of infection. Subsequently, however, the $\Delta inIB$ mutant remained membrane associated in high numbers, the percentage only dropping by 20% over the rest of the time course (Fig. 2A and Fig. S2A). To quantify the phenotype while controlling for variations in cell entry rate, vacuolar persistence (VP) was calculated based on the length of time between 50% initial bacterial association and 50% dissociation with the membrane probe relative to peak values. The VP values were 34.2 ± 8.0 and 69.6 ± 5.0 min for the WT and $\Delta inIB$ mutant, respectively (Fig. 2A, right), suggesting the latter had a vacuole escape defect. In parallel, cytosolic bacteria were identified both through ActA-mediated F-actin accumulation (8) and decoration with a transfected fluorescent probe that binds to *Listeria* after escape to the cytosol (CBD, a listeriophage endolysin cell wall-binding domain fused to yellow fluorescent protein [YFP]) (5). Consistent with the vacuole association data, significant decoration with the CBD probe was observed later for the $\Delta inIB$ mutant than the WT. The dynamics of actin accumulation around bacteria mirrored the CBD probe data (Fig. 2C and D). With both approaches, the mutant phenotype reverted to WT in the *inIB*-complemented $\Delta inIB$ mutant (Fig. 2A and C and Fig. S2A and B). These data indicated that InIB is required for efficient vacuole escape in HeLa cells.

InIB promotes Rab7 recruitment to the *Listeria*-containing vacuole (LCV). To explore the mechanism of $\Delta inIB$'s delayed escape, different endosomal trafficking markers were probed over the infection time course in HeLa cells. Consistent with previous findings in macrophages (5), the key regulator of early to late endosome maturation, Rab5 (34, 35), was not found to localize to WT LCVs, and the same was observed for $\Delta inIB$ (Fig. S3A). A positive signal was clearly visible on control bacteria-containing vacuoles known to associate with Rab5 (*Rhodococcus equi* and *Staphylococcus aureus*) (Fig. S4A), excluding a technical issue with the antibody. Early endosomal antigen-1 (EEA1) (36) could be seen for both WT and $\Delta inIB$ bacteria, but only at 10 min after infection and for a small proportion of LCVs (~10%) (Fig. 3A, left, and Fig. S3B). Thus, EEA1 might have a role during the initial stages of *Listeria* endocytosis, as previously observed (25), but apparently only transient and independent of InIB.

Differences between the WT and $\Delta inIB$ mutant were evident, however, for the Rab7 late endosomal marker. Rab7 associated with WT LCVs soon after internalization, as previously seen in macrophages (5). The Rab7 GTPase was detected on a large proportion of phagosomes 10 min after infection, with a peak at 20 min, while it was absent 25 min later, coinciding with vacuole escape. In contrast, $\Delta inIB$ LCVs showed late acquisition of Rab7, with a flatter peak at 30 min followed by prolonged association up to 90 min. The phenotype could be rescued using the *inIB*-complemented mutant, indicating that it was due to InIB expression (Fig. 3A, right, and Fig. S4B). This echoed the vacuole escape experiments showing that the $\Delta inIB$ mutant reached the cytoplasm much later than WT (Fig. 2). $\Delta inIB$ was not found to colocalize with the lysosomal-associated membrane protein 1 (LAMP1), implying that although delayed in escape, the mutant was still able to avoid lysosomal fusion (Fig. S3C). Collectively, these data indicated that InIB promotes Rab7 recruitment to the LCV, and this correlated with InIB-dependent early vacuole escape.

InIB-dependent PI3P accumulation and class III PI3K (Vps34) recruitment. The phosphoinositide PI3P plays a key role in the conversion of early endosomes/phagosomes into Rab7-positive vesicles (37, 38) and was previously detected in the LCV of macrophages (5). To determine whether InIB had an effect on PI3P levels in HeLa LCVs, infection time course experiments were conducted in cells expressing a fluorescent PI3P probe (GFP-PX, consisting of the PI3P-binding PX domain from the vacuolar NADPH oxidase subunit,

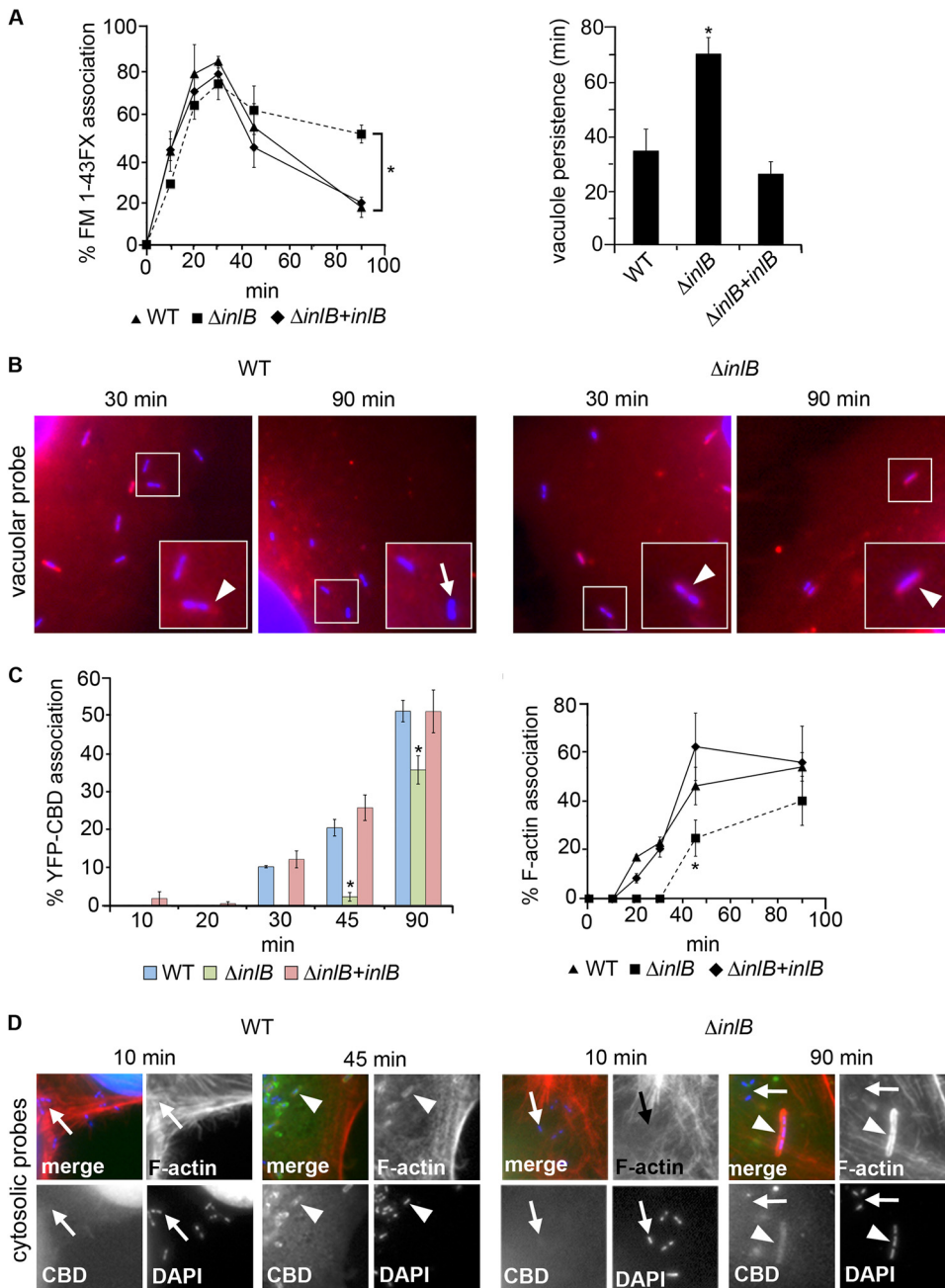


FIG 2 *InIB* promotes early vacuole escape. (A) (Left) Percentage of vacuole-associated WT, $\Delta inIB$, and *inIB*-complemented $\Delta inIB$ bacteria determined using the constitutive membrane probe FM 1-43FX; (right) vacuolar persistence (VP) for each strain (see the text). *, significant difference from WT ($P < 0.01$). (B) Illustrative fluorescence micrographs for data in panel A. Shown are merged images of FM 1-43FX (phagosome membranes [red]) and DAPI (cell nuclei and bacteria [blue]) stains. Represented are $\times 2.5$ enlarged areas of interest of images captured at $\times 630$ magnification (see Fig. S2A for a complete image composite). The inset shows a further $\times 2.5$ magnification of the area within the white rectangle (approximate total magnification, $\times 4,000$). Arrowheads and arrows indicate examples of vacuolar and cytosolic bacteria, respectively. (C) Percentages of cytosolic WT, $\Delta inIB$, and *inIB*-complemented $\Delta inIB$ bacteria as determined by decoration with CBD probe (left) or F-actin (right). *, significant difference from WT ($P < 0.01$ for 45 min on left panel and $P < 0.05$ for others). (D) Representative fluorescence micrographs for data in panel C. Shown are merged and individual channels for CBD (green), F-actin (Alexa Fluor 568-conjugated phalloidin [red]), and DAPI (bacteria and cell nuclei [blue]). Represented are $\times 2.5$ enlarged areas of interest of images originally captured at $\times 630$ magnification (complete image composite in Fig. S2B). Examples of YBD-CBD- and F-actin-associated and nonassociated bacteria are indicated by arrowheads and arrows, respectively.

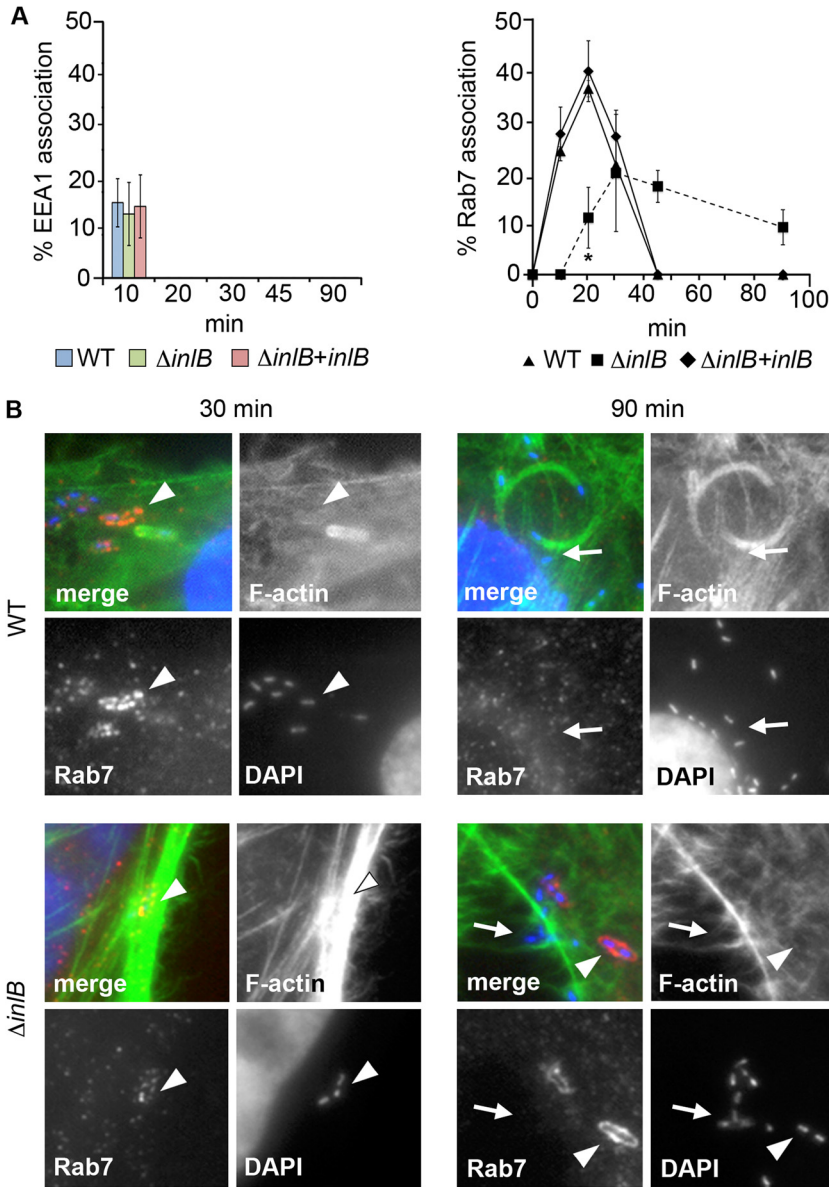


FIG 3 Transient recruitment of EEA1 and InIB-promoted accumulation of Rab7. (A) Percentage of EEA1- and Rab7-positive WT, $\Delta inIB$ and *inIB*-complemented $\Delta inIB$ LCVs. *, significant differences from WT ($P < 0.05$). (B) Representative fluorescence micrographs showing LCV association with Rab7 (for EEA1 see Fig. S3B). Shown are merged and individual channels for Rab7 (anti-Rab7 primary antibody and Alexa Fluor 568-conjugated anti-rabbit secondary antibodies [red]), F-actin (Alexa Fluor 488-conjugated phalloidin [green]), and bacteria/cell nuclei (DAPI [blue]). Shown are $\times 2.5$ enlarged areas of interest of images originally captured at $\times 630$ (complete image composite in Fig. S4B). Examples of Rab7-associated and nonassociated LCVs are indicated by arrowheads and arrows, respectively.

$p40^{phox}$, tagged with green fluorescent protein) (39). Accumulation of PI3P was observed after only 10 min in WT LCVs, with a peak by 20 min, declining by 30 min, and almost complete disappearance by 45 min after infection. In marked contrast, PI3P remained undetectable in $\Delta inIB$ LCVs during the 90-min time course. This again reverted to WT upon *inIB* complementation, indicating that, as for Rab7, PI3P accumulation was linked to InIB (Fig. 4A, left, Fig. 4B, and Fig. S5A). PI3P is primarily generated in endosomes and phagosomes by vacuolar protein sorting-34 (Vps34), the sole mammalian class III PI3K isoform (40, 41). Parallel experiments in which HeLa cells were stained with a Vps34 antibody showed Vps34 localizing to WT and *inIB*-complemented $\Delta inIB$ LCVs as early as 10 min after infection, while remaining absent from $\Delta inIB$ LCVs (Fig. 4A, right, Fig. 4C,

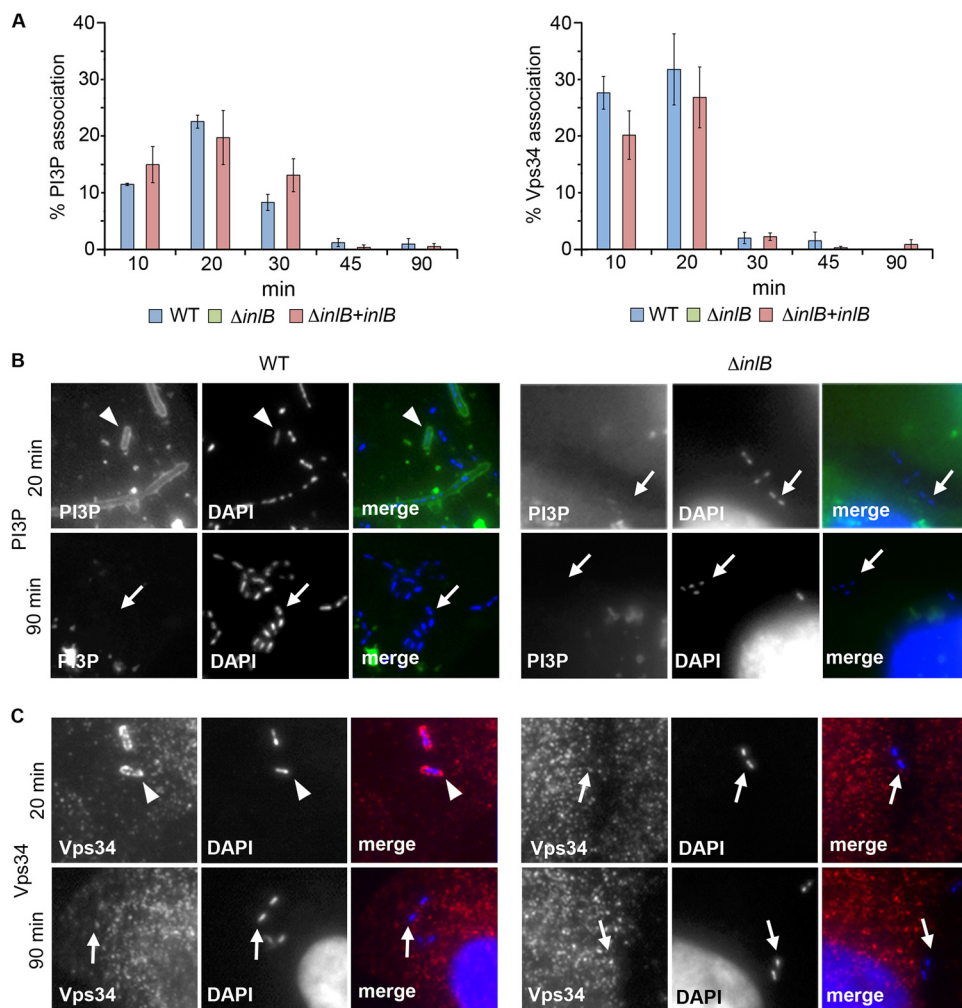


FIG 4 InIB-dependent Vps34 recruitment and PI3P accumulation. (A) Percentage of (left) PI3P (GFP-PX)-positive and (right) Vps34-positive WT, $\Delta inIB$ and *inIB*-complemented $\Delta inIB$ LCVs. (B and C) Representative single channel and merge fluorescence micrographs illustrating association with (B) the transfected PI3P-binding GFP-PX probe (green) or (C) Vps34 (monoclonal primary and Alexa Fluor 568-conjugated secondary antibodies [red]). Cell nuclei and bacteria were stained with DAPI (blue). Examples of PI3P- or Vps34-associated LCVs are indicated by arrowheads and nonassociated bacteria by arrows. See Fig. S5 for complete image composite.

Fig. S5B). This pattern mirrored the PI3P data, consistent with the absence of PI3P being due to a lack of Vps34 recruitment. Thus, the phagosomal escape deficiency caused by absence of InIB was associated with a lack of effective Vps34 recruitment and accumulation of its lipid product on the LCV.

Vps34 supports InIB-dependent intracellular proliferation but not entry. If InIB-dependent intracellular proliferation involves class III PI3K Vps34 activity, a pan-PI3K inhibitor such as wortmannin (42) should abolish it. Wortmannin treatment of preinfected HeLa cells inhibited *L. monocytogenes* intracellular proliferation to an extent similar to that seen in the absence of InIB. The effect was clearly dependent on InIB, as wortmannin did not affect the intracellular proliferation of the $\Delta inIB$ mutant, while significant inhibition was observed with the *inIB*- but not mock-complemented mutant (Fig. 5A, left). Wortmannin had a similar InIB-dependent inhibitory effect in Caco-2 cells and J774 macrophages (Fig. S6A).

To pinpoint the specific PI3K isoform involved, we first used isoform-specific drugs that inhibit class I PI3K. HeLa cells were treated with PIK75 (PI3K α), TGX-221 (PI3K β), AS252524 (PI3K γ) and PI-103 (PI3K α,β,δ) (42–44), and PI3K inhibition efficacy was confirmed by Western blotting for pAktS473 (45) (Fig. S6B). None of these inhibitors

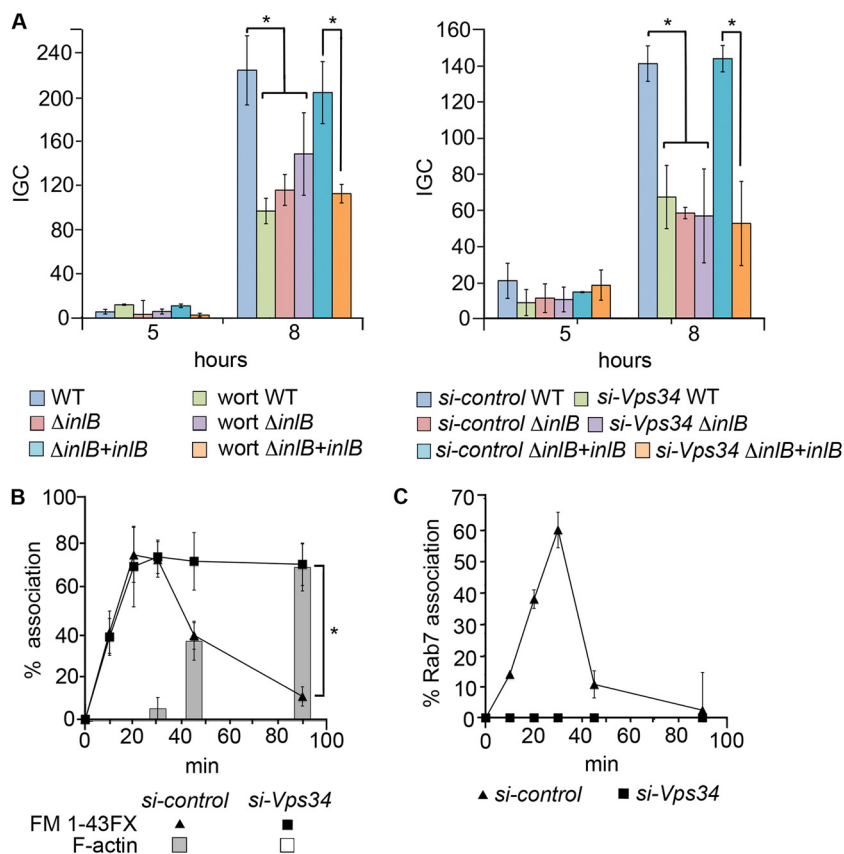


FIG 5 Vps34 supports InIB-dependent intracellular proliferation and vacuole escape. (A) Intracellular proliferation of WT, $\Delta inIB$, and *inIB*-complemented $\Delta inIB$ *L. monocytogenes* in (left) wortmannin-treated HeLa cells (100 nM, nontreated cells with a corresponding amount of DMSO vehicle added; mean CFU/well at $t = 0$ [$\times 10^4$]: 3.4 ± 1.3 to 8.9 ± 3.6) or (right) cells treated with control or Vps34-specific siRNA (mean CFU/well at $t = 0$ [$\times 10^4$]: 1.7 ± 0.31 to 6.1 ± 0.47). IGC, intracellular growth coefficient. *, significant difference ($P < 0.05$). (B) Vacuole escape dynamics of *L. monocytogenes* WT in cells treated with control or Vps34-specific siRNA determined using FM 1-43FX membrane probe (vacuole-associated bacteria [line graph]) and F-actin staining (cytosolic bacteria [bars]). *, $P < 0.01$. Illustrative fluorescence micrographs are shown in Fig. S7A. (C) Same as panel B for Rab7 association (Fig. S7B).

significantly affected *Listeria* proliferation (Fig. S6C), indicating that the effect caused by wortmannin might be linked to non-class I PI3K. We next tested the involvement of class II and class III PI3K using small interfering RNA (siRNA) knockdown experiments. The siRNA treatments resulted in effective depletion of class II (PIK3C2 α and PIK3C2 β ; PIK3C2 γ did not appear to be expressed in HeLa) and class III (Vps34) PI3K. Similar targeting of the class I PI3K regulatory subunit PIK3R1 as a control resulted in a partial knockdown of p85 and a complete loss of class I PI3K activity as determined by pAktS473 Western blotting (Fig. S6D). Transfection with siRNA to the Met receptor required for InIB-mediated entry (17) was also used as an additional control. Both *Listeria* invasion and intracellular proliferation were investigated in the siRNA-transfected HeLa cells. While as expected (17, 24), both siPIK3R1 and siMet significantly reduced cell invasion (60% and 80%, respectively), siRNAs to class II and class III PI3Ks had no effect, indicating they were not involved in the entry process (Fig. S6E). Conversely, in Vps34-, but not class I or class II PI3K-depleted cells, intracellular proliferation was significantly impaired for WT *Listeria* (Fig. 5A, right, and Fig. S6F), indicating that class III PI3K did indeed have a role in cell infection downstream entry. None of the siRNAs, including siVps34, had any effect on the already reduced intracellular proliferation of the $\Delta inIB$ mutant. As seen with wortmannin, complementation with *inIB* rescued the proliferation inhibition by siVps34 (Fig. 5A, right). Overall, these data indicated that Vps34 required InIB

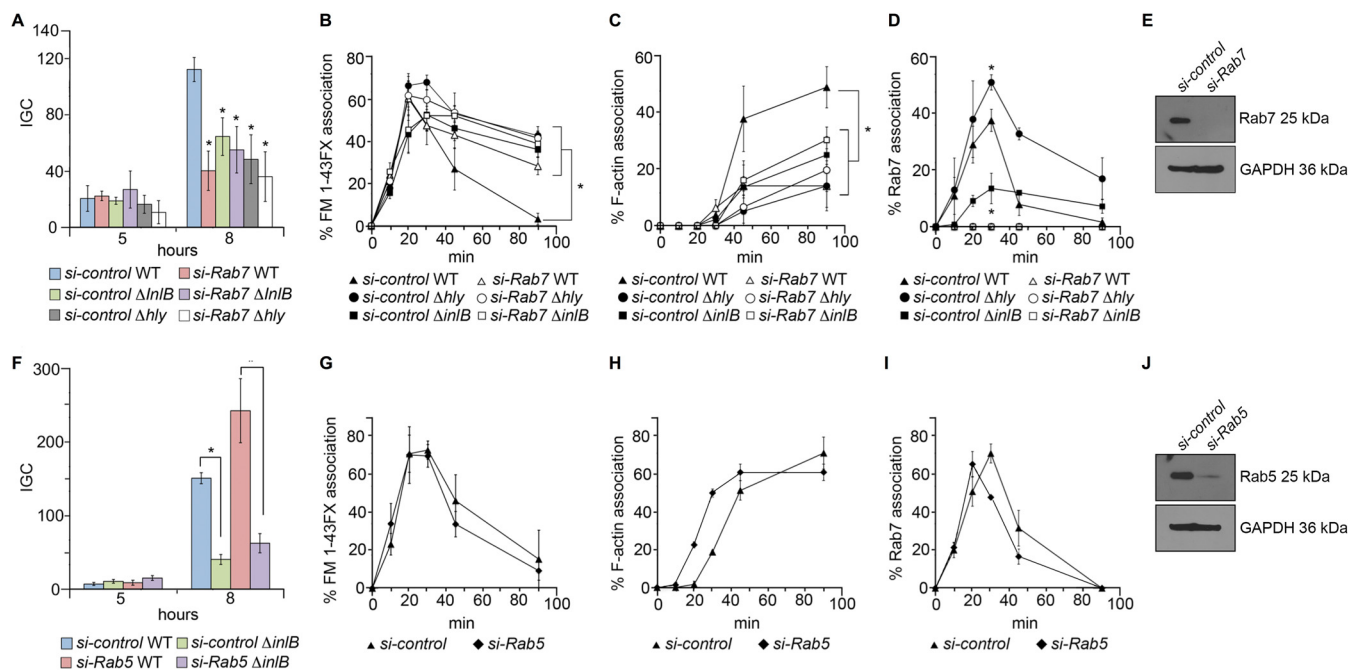


FIG 6 Rapid vacuole escape requires Rab7 and is not hindered by Rab5 inhibition in HeLa cells. (A) Intracellular proliferation of WT, $\Delta inIB$, and Δhly *L. monocytogenes* in Rab7-depleted or control siRNA-treated cells. IGC, intracellular growth coefficient. Mean bacterial CFU at $t = 0$ ($\times 10^4$): 2.7 ± 0.6 to 5.6 ± 1.2 . *, significant difference from WT in control cells ($P < 0.05$). While capable of LLO-independent growth in HeLa cells, the early escape-defective Δhly mutant (Fig. S8, left) shows significantly impaired intracellular proliferation, as seen with the $\Delta inIB$ mutant. (B, C, and D) Percentages of association for bacteria in panel A with membrane probe FM 1-43FX, F-actin, and Rab7. Illustrative fluorescence micrographs are shown in Fig. S8. *, significant differences ($P < 0.01$). (F) Intracellular proliferation of WT and $\Delta inIB$ *L. monocytogenes* in Rab5-depleted or control siRNA-treated cells. Bacterial CFU at $t = 0$ ($\times 10^4$): 3.8 ± 1.0 to 8.2 ± 1.2 . *, significant differences ($P < 0.01$). (G, H, and I) Same as in panels B, C, and D for control and Rab5-depleted cells. Illustrative fluorescence micrographs are shown in Fig. S9C and D. (E and J) Depletion of Rab7 and Rab5 in siRNA-treated HeLa cells. Western immunoblotting with GAPDH was used as a loading control.

to exert its effect, suggesting that InIB and the class III PI3K act together to promote *Listeria* intracellular proliferation.

Vps34 is required for Rab7 recruitment and efficient vacuole escape. To establish whether the (InIB-dependent) effect of Vps34 on intracellular proliferation was linked to vacuole escape efficiency, siVps34- or control siRNA-treated HeLa cells were infected with WT *L. monocytogenes* and LCVs were monitored over 90 min using either FM 1-43FX or Rab7 antibody. Whereas bacteria appeared to rapidly disengage with LCVs in control cells (as determined by dissociation with the membrane probe and association with F-actin), they remained membrane associated for up to 90 min after infection in Vps34-depleted cells (Fig. 5B and Fig. S7A). Additionally, in contrast to control cells, no Rab7 could be seen around LCVs at any time point in siVps34-treated cells (Fig. 5C and Fig. S7B). These effects were essentially similar to, albeit more severe than, those observed with the *L. monocytogenes* $\Delta inIB$ mutant (Fig. 3A, right, and Fig. 3B), supporting the notion that InIB promotes vacuole escape by subverting LCV membrane trafficking via Vps34. These data also demonstrated the importance of Vps34 in Rab7 recruitment to the *Listeria* phagosome.

Rapid escape requires Rab7 and is not hindered by Rab5 inhibition. *Listeriae* appear to escape from Rab7-positive phagosomes (see reference 5 and data herein), and InIB/Vps34-promoted vacuole escape correlated with Rab7 recruitment to the LCV (this study). To test the role of Rab7 in InIB-facilitated escape, HeLa cells were transfected with control or Rab7-specific siRNAs and infected with WT or $\Delta inIB$ *L. monocytogenes* (Fig. 6A to E). Since the acidic pH of the maturing Rab7-positive phagosome is thought to be critical for efficient LCV disruption by LLO (5, 6), an isogenic Δhly mutant was also included to determine whether Rab7 recruitment is linked to LLO-mediated escape. Although inefficiently, escape may occur in human epithelial cells in the absence of LLO, resulting in LLO-independent intracellular growth (46). We therefore verified that LLO was required for rapid vacuole escape under our conditions during the infection time course experiments in HeLa cells (Fig. 6A to D and Fig. S8A and B).

Rab7 depletion correlated with significantly reduced intracellular growth of WT *L. monocytogenes* (Fig. 6A), while it did not affect internalization (not shown). Similarly reduced intracellular growth was observed for both $\Delta inlB$ and Δhly mutants in control cells, with no significant further change in Rab7-depleted cells (Fig. 6A). Proliferation defects correlated with significantly impaired vacuole escape, as determined by fluorescence microscopy using FM 1-43FX (Fig. 6B) and F-actin staining (Fig. 6C). As expected, Rab7 was not detected in siRab7-treated cells, while in control cells, the Rab7 acquisition pattern of WT and $\Delta inlB$ LCVs reproduced our previous results (Fig. 6D; see also Fig. 3A, right). No defect in Rab7 acquisition, but rather a general increase, was observed for Δhly LCVs, followed by a protracted association consistent with a vacuole escape deficiency (Fig. 6D). Together, these data suggest that the effects of InlB on intracellular proliferation depend on Rab7 recruitment, in turn promoting rapid, LLO-mediated vacuole escape.

Although acquisition of the early endosomal Rab GTPase Rab5 normally precedes that of Rab7 in the endosome/phagosome maturation sequence (34, 37), Rab5 could not be recognized on HeLa (our data) or mouse macrophage (5) LCVs even immediately after infection. While this may reflect only poor or transient recruitment, evidence in macrophages suggested that Rab5 might be excluded from the LCV (5). We therefore explored in a final set of experiments the role of Rab5 in *Listeria* intracellular infection. Rab5 was inhibited in HeLa cells prior to infection either by transfection with Rab5-S34N, a dominant-negative mutant in which the GTPase is locked in GDP-bound inactive form (47), or by using an siRNA pool targeting Rab5 isoforms A, B, and C (Fig. 6J and Fig. S9A). Proliferation of WT *L. monocytogenes* was not impaired, suggesting that entry into the cytosol was not significantly affected when Rab5 function was perturbed. The defective proliferation phenotype of the $\Delta inlB$ mutant also remained unaffected by Rab5 inhibition (Fig. 6F and Fig. S9B). Microscopic analysis of siRNA-treated cells confirmed that vacuole escape was not delayed in Rab5-depleted cells (Fig. 6G and H). Furthermore, no interference with Rab7 recruitment was observed in siRab5-treated cells (Fig. 6I), indicating that Rab5 does not seem to play a significant role in LCV's maturation to the Rab7-positive stage.

DISCUSSION

The InlB protein is a well-known *Listeria* invasin that promotes entry into nonphagocytic cells via hijacking of the ligand-dependent Met receptor endocytic pathway and downstream activation of type I PI3K (18, 24, 25, 48). Here, we provide evidence in HeLa cells that InlB has an additional key function at later stages of intracellular infection, accelerating vacuole escape, presumably through subversion of class III PI3K signaling. InlB is sufficient to mediate this process, as shown using gene complementation analysis and pharmacological and siRNA inhibition studies. The role of InlB in LCV maturation and vacuole escape is not unique to HeLa cells as similar effects were observed in Caco-2 human enterocytes (see Fig. S10). InlB has been reported to support *Listeria* infection in epithelial cells by modulating transcriptional host cell responses via SIRT2-mediated histone H3K18 deacetylation (49). Recently, InlB-induced c-Met/class I PI3K (PI3K α) signaling has been also found to facilitate *L. monocytogenes* *in vivo* persistence and invasive (e.g., brain) infection by inhibiting Fas-mediated killing of infected macrophages (50). Together with these studies, our findings highlight the growing evidence that bacterial virulence factors are multifunctional, encapsulating multiple key host-cell modulatory activities to support infection.

Cell invasion permits *Listeria* to evade extracellular host defenses while vacuolar escape is essential for intracellular survival (3, 6). Since cytosolic replication relies on successful vacuole escape, optimization of the latter by InlB maximizes listerial intracellular proliferation. *In vivo* survival also crucially depends on the ability of *L. monocytogenes* to avoid autophagy and the cytotoxic immune response directed against infected cells via a "runaway" strategy involving ActA-mediated actin-based cell-to-cell spread after cytosolic replication (3, 10). The rapid amplification of the bacterial load is clearly an essential component of this strategy because it increases the chances of

spreading before the infected cell is targeted by host defenses. Indeed, *L. monocytogenes* possesses a dedicated virulence factor to promote rapid intracellular replication, the hexose phosphate transporter Hpt (7). The vacuolar escape-accelerating activity of InlB here identified (together with its recently reported role in protecting infected monocytes from CD8⁺-mediated killing) (50) can therefore be seen as contributing, alongside Hpt, to provide *L. monocytogenes* a competitive edge against host immunity.

Strikingly, InlB targets both extracellularly and intracellularly the endocytic pathway through interference with PI3K signaling. It seems unlikely that InlB-dependent class III PI3K (Vps34)-promoted escape is directly linked to the stimulatory effect of InlB on class I PI3K during entry because Vps34 is not known to be downstream of the Met receptor. Indeed, our data show that Vps34 supports InlB-dependent intracellular proliferation but not entry. Moreover, InlB/Vps34-promoted vacuole escape was resistant to class I PI3K isoform inhibition. Rather, these two distinct InlB functions appear to be spatiotemporally coordinated, since the hijacking of the endocytic pathway occurs at two sequential levels involving stage-specific PI3K isoforms (51) coincident with consecutive steps in *Listeria* intracellular pathogenesis. After Met/class I PI3K-mediated internalization and phagocytic cup closure, a receptor-free fraction of InlB, or InlB in complex with Met before sorting for lysosomal degradation (21, 52), might direct Vps34 recruitment to the early LCV. Cotransfection experiments demonstrated colocalization of Vps34 and PI3P in InlB-positive vesicles, but we could not detect binding between InlB and Vps34 by immunoprecipitation (data not shown). This may reflect either an interaction too weak to be captured or that InlB acts upstream of Vps34.

The catalytic subunit of class III PI3K Vps34 is an effector of the small GTPase Rab5 in the endocytic pathway and localizes to nascent endosomes in complex with several regulatory subunits through interaction with membrane-bound GTP-Rab5 (41, 51). Via Vps34, Rab5 controls recruitment of PI3P-binding proteins necessary for early endosome sorting and progression to the Rab7 late endosome stage (35, 41, 51, 53, 54). Intriguingly, however, although Vps34 was obviously present, we could not detect Rab5 in the HeLa LCVs, recapitulating previous observations in macrophages (5). The FYVE-domain (PI3P-binding) Rab5 effector EEA1 requires not only PI3P but also corecognition by GTP-Rab5 for targeting to early endosomes (35, 51, 55). EEA1 was barely detectable in the *Listeria* phagosomes regardless of significant PI3P accumulation, consistent with Rab5 being only transiently or poorly recruited to the LCV. Indeed, in contrast to Vps34 knockdown, inhibition of Rab5 did not impede Rab7 acquisition by the LCV (or *Listeria* escape/intracellular proliferation), suggesting that Rab5 has a marginal or accessory role in the process.

Our findings do not seem to support the previous proposition that *L. monocytogenes* actively recruits and inhibits Rab5 to block LCV maturation and delay phagolysosome fusion (56, 57). Indeed, the late endosomal marker Rab7 is rapidly recruited to the LCV after *Listeria* entry in human epithelial cells (our data) and murine macrophages (5), inconsistent with phagocytic vacuole maturation being arrested. On the contrary, *L. monocytogenes* appears to expedite the process via InlB-promoted recruitment of Vps34, driving LCVs to a Rab7-positive, prelysosome (LAMP1-negative) stage. This might be facilitated by diminished Rab5 levels/EEA1-mediated fusion with other (bacterium-free, Rab5-loaded) early endosomes (34, 54, 58), as this would contribute to isolating LCVs from normal endosomal traffic. The experiments using *inlB* and *hly* mutants in Rab7-depleted cells indicated that rapid (InlB-promoted) escape takes place from Rab7-positive vacuoles and that Rab7 facilitates LLO-mediated escape, likely because of the acidified intraluminal environment of the maturing endosome (6). Consequently, InlB could be seen as acting intracellularly in concert with the low-pH-activated LLO toxin to achieve efficient vacuole escape and avoid lysosomal killing.

The apparent lack of Rab5 recruitment to the LCV is puzzling, as the coordinated replacement of Rab5 by Rab7 appears to be key in the early-to-late endosome conversion process (34, 54, 59, 60). According to the current model, upon accumulation of PI3P, the Mon1/Sand1-Ccz1 protein complex displaces the Rab5-activating guanine nucleotide

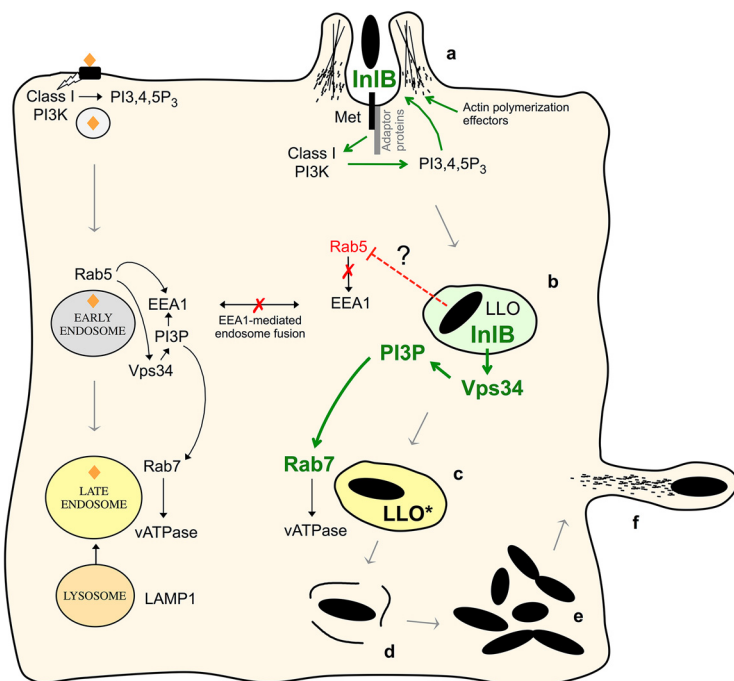


FIG 7 Model of sequential PI3K subversion by *L. monocytogenes* InIB along the endocytic pathway. At the cell surface, InIB first induces Met receptor-dependent class I PI3K activation by mimicking the natural role of HGF, leading to phagosome formation. The process involves InIB-induced autophosphorylation of the Met tyrosine kinase receptor, recruitment of adaptor proteins involved in class I PI3K recruitment/activation (Gab1, Shc, Crkl), and local accumulation of PI(3,4,5)P₃ leading to Rac1 activation, recruitment of actin polymerization promoting proteins (Ena/VASP, WAVE, N-WASP), and activation of the Arp2/3 actin-polymerizing complex (reviewed in reference 25). After entry, InIB next modulates endosomal trafficking via activation of class III PI3K (Vps34) signaling to accelerate LLO-mediated phagosome disruption, promoting intracellular bacterial survival and proliferation. Depicted on the left is the canonical endocytic pathway leading to an acidified late endosome via recruitment of the vATPase proton pump and fusion with LAMP1-positive lysosomes; on the right is depicted the “*Listeria* pathway” with the different steps of the intracellular infection cycle. (a) InIB-dependent entry. (b) Formation of a *Listeria*-containing vacuole (LCV), typically Rab5-negative, Rab7-positive, and LAMP1-negative. Lack of detection of Rab5 in the LCV, due to either defective/transient recruitment or active exclusion (5) by unknown mechanisms, impairs EEA1 acquisition and EEA1-mediated tethering and fusion with early endosomes, contributing to isolate the LCV from normal endocytic traffic. InIB bypasses the regulatory role of Rab5 in phagosome maturation by promoting the recruitment of the Rab5 downstream effector Vps34 (class III PI3K). (c) Accumulation of the Vps34 product PI3P promotes the rapid conversion of the LCV into an acidified Rab7 late endosome-like compartment in which the pore-forming toxin LLO becomes active (LLO*). (d) Vacuole disruption occurs followed by (e) rapid bacterial proliferation in the cytosol (e) and actin-based cell-to-cell spread (f). InIB-stimulated effects are indicated in green.

exchange factor (GEF) Rabex5, causing membrane dissociation of Rab5, and recruits/activates Rab7 through interaction with the HOPS (homotypic fusion and vacuole protein sorting) complex in association with the Rab7 GEF, Ccz1 (38, 54, 59, 61). Although GTP-Rab5 interacts with Mon1 and HOPS components (34, 38, 62), it is unclear whether Rab5 is strictly needed in the process once PI3P is generated by its downstream effector Vps34, because the Mon1-Ccz1 complex binds to PI3P (63, 64). Conceivably, therefore, *L. monocytogenes* might directly promote Rab7 accumulation without a sustained Rab5 input via InIB-modulated Vps34 recruitment and concomitant PI3P generation (Fig. 7). It is worth noting, however, that even in the absence of InIB, and thus of detectable Vps34 and PI3P accumulation, Rab7 acquisition still occurred in the LCV, albeit more slowly. This observation is consistent with previous data showing significant Rab7 recruitment to phagosomes in wortmannin-treated cells (37), implying that the phagosome maturation process can be, at least partially, independent of Vps34 activity (65). Nevertheless, our siRNA experiments support a crucial role for Vps34 because its depletion appeared to prevent Rab7 recruitment to the LCV. Since via a regulatory loop Vps34 negatively regulates Rab5 during endosome maturation (66), InIB-promoted Vps34 recruitment itself might have a role in the lack

of detection of Rab5 in the LCV. Multiple mediators and interactors are involved in the intricate regulation of endosome trafficking (41, 51, 54, 59, 60), and further research is needed to clarify the mechanism underlying the apparent Rab5 exclusion and how InIB promotes Vps34 and Rab7 recruitment in the LCV.

Avoidance of lysosomal killing by vacuolar pathogens often involves interference with class III PI3K/PI3P signaling, but the specific underlying mechanism differs for each pathogen (67, 68). *M. tuberculosis*, for example, causes early phagosome maturation arrest through depletion of PI3P by the secreted SapM phosphatase as well as inhibition of Vps34 by mycobacterial cell envelope components. Rab5 is retained, but EEA1 and Rab7 are excluded, blocking acquisition of the acidifying vacuolar proton pump ATPase and lysosomal fusion (69–72). *Salmonella*, in contrast, promotes homotypic fusion of PI3P-enriched, Rab5- and EEA1-positive vesicles to form a large vacuole in which the bacteria proliferate. This is mediated by the type III secreted effector SopB, which promotes PI3P generation by dephosphorylating phosphatidylinositol di- and triphosphates and through recruitment of Rab5 and Vps34 activation (73, 74). Here, we show that *L. monocytogenes*, via InIB, promotes the recruitment of Vps34 and induces PI3P generation on the LCV, boosting the formation of an escape-favorable Rab7 late phagosome. Our data offer a novel example of bacterial modulation of endosomal trafficking, unexpectedly in a cytosolically replicating pathogen that only very transiently interacts with the phagocytic vacuole after internalization.

MATERIALS AND METHODS

Bacteria, plasmids, antibodies, and reagents. Isogenic derivatives of *L. monocytogenes* serovar 4b clinical isolate P14 with constitutive *in vivo*-like virulence gene expression (75) were used in this study. The construction of $\Delta inIA$ and $\Delta inIB$ in-frame deletion mutants is described below, the $\Delta actA$ and Δhly mutants have been reported elsewhere (27, 76). The *R. equi* 103S and *S. aureus* USA300 strains used as controls for Rab5 detection in bacterial phagosomes were from our bacterial isolate collection and R. Fitzgerald's laboratory (Roslin Institute, University of Edinburgh), respectively. Mammalian expression plasmids pEYFP-CBD, encoding the *Listeria* cell wall-binding cytosolic probe, and pGFP-iPX, encoding the PI3P-binding probe, were kindly provided by J. Swanson's lab (University of Michigan Medical School, Ann Arbor, MI, USA) and the L. Stephen/P. Hawkins's lab (Babraham Institute, Cambridge, United Kingdom), respectively (5, 39). The plasmid pHinIB used for complementation of the $\Delta inIB$ mutant is a derivative of the *E. coli* Gram-positive shuttle vector pHPS9 (77) containing the *inIB* gene. The insert was prepared by PCR amplification of *inIB* and upstream *inIB* operon regulatory regions from *L. monocytogenes* PAM14 $\Delta inIA$ genomic DNA using oligonucleotides main113Sall13 (ACGCGTCGACGAACATAAAGGGTAGAGG) and inIBBamHI (CGGGATCCCGATTCTGCTAGACCACC), which carry Sall and BamHI restriction sites (underlined), respectively. After cloning into the pTOPO cloning T-vector (Invitrogen), the Sall/BamHI insert was transferred to pHPS9.

Reagents, antibodies, and oligonucleotides. Chemicals were from Sigma-Aldrich unless stated otherwise. Class I PI3K inhibitors PIK-75, TGX-221, AS-604850, and PI-103 were from Cayman Chemicals. FM 1-43FX membrane probe was from Life Technologies. Antibodies to Akt, pAktS473, Rab7, Rab5, EEA1, and Vps34 were from Cell Signaling Technology, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) from Chemicon, p85 from Millipore, PI3K α and PI3K β from BD Biosciences, PI3K γ from Abgent, LAMP1 (H4A3) from Santa Cruz, and horseradish peroxidase (HRP)-conjugated-mouse and rabbit antibodies from Amersham. Specific antibodies to InIA and InIB were kindly provided by P. Cossart, Institut Pasteur, Paris, France. Oligonucleotides for siRNA knockdowns were from Dharmacon. Pools of four individual siRNA oligonucleotides were used to target each of the human PI3K genes PIK3R1 (D-003020-10, D-003020-11, D-003020-26, D-003020-27) PIK2CA (D-006677-01, D-006677-02, D-006677-03, D-006677-04), PIK2CB (D-006677-01, D-006677-02, D-006677-03, D-006677-04), PIK2CG (D-006677-01, D-006677-02, D-006677-03, D-006677-04) and PIK3C3 (D-005250-01, D-005250-02, D-005250-03, D-005250-04). Pools of four oligonucleotides were also used for Rab5A (D-004009-01, D-004009-02, D-004009-03, D-004009-04), Rab5B (D-004010-1, D-004010-2, D-004010-3, D-004010-4), Rab5C (D-004011-1, D-004011-2, D-004011-3, D-004011-4), and Rab7 (D-010388-01, D-010388-02, D-010388-03, D-010388-04), while the HGF receptor Met was targeted with oligonucleotide D-003156-13. Oligonucleotide primers for PCR or genetic constructs were purchased from Sigma or Metabion (Martinsried, Germany).

General DNA techniques. Restriction enzymes were used according to the manufacturer's instructions (Promega and NEB). Chromosomal DNA from *Listeria* was extracted and purified as previously described (75). Plasmid DNA was extracted from *E. coli* using the Qiagen plasmid purification kit and was introduced into *E. coli* or *L. monocytogenes* by electroporation using a GenePulserXcell apparatus (Bio-Rad). PCR was carried out with *Taq* DNA polymerase (Biotools, Madrid, Spain) for detection/mapping purposes or high-fidelity ProofStart DNA polymerase (Qiagen) for plasmid or mutant constructions. The reaction mixtures contained 100 ng of DNA template, 200 μ M deoxynucleoside triphosphates (dNTPs), 0.25 μ g oligonucleotide primers, 2.5 mM MgCl₂, a suitable amount of polymerase buffer, and 1 U of polymerase per kb in a 25- μ L volume. The standard amplification program was 3 min at 94°C, followed by 30 cycles of 15 s at 95°C, 30 to 60 s at 48 to 58°C, and 1 to 3 min at 72°C, and a final 3 min at

72°C. PCR products were purified with the Qiagen PCR purification kit. DNA sequences were determined on both strands.

Construction of *L. monocytogenes inIA* and *inIB* in-frame deletion mutants. Chromosomal gene deletions were generated by allelic exchange via homologous recombination as previously described (76). For the $\Delta inIA$ mutant, the recombinogenic plasmid was constructed by in-frame ligation of two PCR fragments containing small portions of the 5'- and 3'-terminal regions of the *inIA* gene plus part of the upstream and downstream flanking regions, respectively. Oligonucleotides inIA1 (ATTGGATCCTAAAGGGTAGAGG) and inIA2 (CATACCCCGGGCCAAATACT) carrying BamHI and XmaI/SmaI sites, respectively (underlined), were used to PCR amplify a 643-bp fragment from the 5' region of *inIA*, including the first 72 bp of the gene. Similarly, oligonucleotides inIA3 (TCACCCGGGAATTCAGCTAGC) and inIA4 (TGAACGGATCCAATATCACTATTAT) carrying XmaI/SmaI and BamHI sites, respectively, were used to amplify a 709-bp fragment from the 3' region of *inIA*, including the last 174 nucleotides of the gene. The two PCR products were digested with XmaI and ligated, and the ligation product was used as a template for PCR amplification with oligonucleotides inIA1 and inIA4. The resulting amplicon was inserted into the pTOPO T-vector (Invitrogen) and then the BamHI fragment containing the $\Delta inIA$ construct was transferred to the thermo-sensitive shuttle vector pMAD (78) to give the recombinogenic plasmid pM $\Delta inIA$. The in-frame-deleted allele for the $\Delta inIB$ mutant was generated by "recombinant PCR" (79). Oligonucleotide primers inIB1 (TCCTGTGGATCCACCAACAACACT) and inIB2R (TACCGGAACCTTTGTCTAGATCCGTCACAC) amplified a 573-bp fragment from the 5' region of *inIB*, including the first 213 bp of the gene, and primers inIB3R (GATCTAGTGACAAGTCCGGTAGTAGATAGC) and inIB4 (GTGATGGATCCACATTTTGGC) were used to amplify the 3' region of *inIB*. Primers inIB1 and inIB4 carried BamHI sites, and primers inIB2R and inIB3R had complementary sequences (underlined). The two PCR products were fused by splicing overlap extension using oligonucleotides inIB1 and inIB4, and the recombinant PCR product was processed as described above to produce the pMAD-based recombinogenic plasmid pM $\Delta inIB$.

Mammalian cell cultures and transfections. HeLa cells (and Caco-2 and J774 cells for some control experiments) were sourced from ATCC, passaged at no more than 70% confluence, and used between passages 3 and 15. Cells were cultured in 24-well plates or on 13-mm glass coverslips at 37°C under 5% CO₂ in DMEM (Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum [FCS] and L-glutamine [Invitrogen]) without antibiotics, as previously described (45). HeLa cells were seeded at a density of 3.5×10^4 or 5×10^4 cells per well (24-well plates) or coverslip 24 h prior to transfection with siRNA or plasmid, respectively. Plasmid DNA or siRNAs diluted in optiMEM without FCS were premixed with optiMEM-diluted Lipofectamine 2000 reagent (Invitrogen) as per the manufacturer's instructions. Cells were transfected for 12 h in 1 mL DMEM, giving a final concentration of 80 to 200 nM oligonucleotide or 1 to 5 μ g/mL plasmid DNA. Transfected cells were incubated for 24 h prior to infection, fixation for immunofluorescence microscopy, or production of cell lysates. Plasmid transfection efficiency was typically >85%.

Cell infections. Culture plates containing $\approx 1 \times 10^5$ cells per well were inoculated with bacterial suspensions in DMEM and immediately centrifuged ($180 \times g$, 3 min) to synchronize infection. Internalization and intracellular proliferation assays were carried out using a gentamicin protection assay as previously described (27). After centrifugation, inoculated plates were incubated for 30 min (15 min for J774 macrophages) and washed several times with Dulbecco's phosphate-buffered saline (DPBS) (Invitrogen) followed by a further incubation in DMEM containing 100 μ g/mL gentamicin for 30 min to kill the remaining extracellular bacteria. Samples were taken at this point to determine invasion ($t = 0$), and medium was replaced with fresh medium containing 10 μ g/mL gentamicin to prevent extracellular bacterial growth. Where applicable, PI3K inhibitors were added at this stage diluted in 0.1% dimethyl sulfoxide (DMSO). At the sampling time points, cells were washed in DPBS, lysed in 10 mM Tris-HCl (pH 7.4), 1% Triton X-100, and viable bacteria plate counted. Intracellular proliferation data were normalized using an intracellular growth coefficient to correct for variation in cell entry using the formula $IGC = (CFU_m - CFU_0)/CFU_0$ (27).

Western blotting. HeLa cells were processed for immunoblotting as detailed by Cain et al. (45), and bacterial proteins were prepared as previously described (76). Immunoreactive proteins were visualized using the ECL enhanced chemiluminescence detection system (Amersham).

Fluorescence microscopy. Coverslips for time course quantifications were collected 10, 20, 30, 45, and 90 min after plate centrifugation, thoroughly washed four times in warm PBS to remove extracellular bacteria, fixed with 3.7% (wt/vol) paraformaldehyde, and processed as described by Cain et al. (45). For time points 45 and 90 min, 100 μ g/mL gentamicin was added to the medium 30 min after infection to prevent extracellular growth. Alexa Fluor 488- or 568-conjugated anti-rabbit or anti-mouse IgG secondary antibodies (1 h) were used for immunofluorescence staining, followed by Alexa Fluor 568- or 488-conjugated phalloidin (Invitrogen) and DAPI (4',6-diamidino-2-phenylindole) (Sigma) (20 min) to visualize F-actin and bacterial DNA/cell nuclei, respectively. To determine vacuole-associated bacteria, cells were incubated with 2 μ M of the fixable fluorescent membrane probe FM 1-43FX (Life Technologies) in DMEM for 45 min, washed twice, and incubated with fresh DMEM for 30 min before infection. Bacteria that escaped from the vacuole were identified based on their association with either F-actin (46) or a transfected listerial cell wall-binding fluorescent cytosolic probe (yellow fluorescent protein [YFP]-CBD) (5). Images were acquired at room temperature using a Leica CTR-6000 fluorescence microscope with a 63 \times oil immersion objective, collecting data as a z-stack, which were later flattened by deconvolution. Images were processed using Leica LAF software (Leica) and ImageJ (NIH; <http://rsbweb.nih.gov/ij/>), and figures were assembled using Adobe Photoshop.

Statistics. In all cases, data are shown as the mean \pm standard error of the mean (SEM) of values from a minimum of three independent experiments performed in triplicate. Data for image-based quantifications were collected from 60 to 70 bacteria/vacuoles across five randomly selected microscopic

fields per time point per condition in each of three independent experiments. The statistical significance of data was determined by Student *t* tests using Prism software (<http://www.graphpad.com/scientific-software/prism/>).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 0.9 MB.

FIG S2, TIF file, 2.6 MB.

FIG S3, TIF file, 2.6 MB.

FIG S4, TIF file, 2.7 MB.

FIG S5, TIF file, 2.6 MB.

FIG S6, TIF file, 1.3 MB.

FIG S7, TIF file, 2.8 MB.

FIG S8, TIF file, 2.9 MB.

FIG S9, TIF file, 2.6 MB.

FIG S10, TIF file, 2.8 MB.

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