VipD is a Rab5-activated phospholipase A₁ that protects Legionella pneumophila from endosomal fusion

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A crucial step in the elimination of invading microbes by macrophages is phagosomal maturation through heterotypic endosomal fusion. This process is controlled by the guanine nucleotide binding protein Rab5, which assembles protein microdomains that include the tethering protein early endosomal antigen (EEA) 1 and the phosphatidylinositol (PI) 3-kinase hVps34, which generates PI(3)P, a phospholipid required for membrane association of EEA1 and other fusion factors. During infection of macrophages, the pathogen Legionella pneumophila bypasses the microbial endosomal compartment by an unknown mechanism. Here, we show that the effector protein VipD from L. pneumophila exhibits phospholipase A₁ activity that is activated only upon binding to endosomal Rab5 or Rab22. Within mammalian cells, VipD localizes to endosomes and catalyzes the removal of PI(3)P from endosomal membranes. EEA1 and other transport and fusion factors are consequently depleted from endosomes, rendering them fusion-incompetent. During host cell infection, VipD reduces exposure of L. pneumophila to the endosomal compartment and protects its surrounding vacuoles from acquiring Rab5. Thus, by catalyzing PI(3)P depletion in a Rab5-dependent manner, VipD alters the protein composition of endosomes thereby blocking fusion with Legionella-containing vacuoles.

Phagosomal maturation is a fundamental innate immune mechanism of eukaryotic cells that facilitates the killing and degradation of ingested microbes. After separation from the plasma membrane, the nascent phagosome rapidly undergoes a series of fusion events with early endosomes, late endosomes, and eventually lysosomes, resulting in the gradual acidification of the phagosome’s lumen and the destruction of its contents (1). The various stages of phagosome maturation are interconnected through, and mainly controlled by, small guanine nucleotide binding proteins (GTPases) of the Rab (ras genes from rat brain) family. Rab5 is a signature protein of early endosomes and crucial for the initial fusion with nascent phagosomes. Once activated by the guanine nucleotide-exchange factor Rabex-5, GTP-loaded Rab5 orchestrates the recruitment of several downstream ligands, such as the tethering protein early endosomal antigen (EEA) 1 and the type III phosphatidylinositol (PI) 3-kinase vacuolar protein sorting 34 (hVps34) and their assembly into membrane microdomains capable of instigating lipid bilayer fusion (2). hVps34 plays a supporting yet critical role in the assembly of these multimeric fusion protein complexes by catalyzing the phosphorylation of PI at the 3’ hydroxyl position, thereby generating PI 3-phosphate (PI(3)P), which in turn enhances association of EEA1, rabenosyn-5, sorting nexins (SNX), and other proteins that possess PI(3)P-specific binding modules with the endosomal surface (3). Failure of cells to generate endosomal PI(3)P, for example after chemical inhibition of the PI 3-kinase activity of hVps34, results in the reduced accumulation of EEA1 on early endosomes and an attenuated maturation of phagosomes into phagolysosomes (4, 5).

The ability to delay or block a particular step during phagosomal maturation is a key virulence trait of a variety of intracellular pathogens. The Gram-negative bacterium Legionella pneumophila, the causative agent of Legionnaires’ pneumonia, possesses the remarkable ability to block phagosomal maturation at the earliest stage in the endocytic pathway. Upon uptake by macrophages, the Legionella-containing vacuole (LCV) does not acquire any early or late endosomal markers (6). Instead, the pathogen attracts proteins and vesicles from the early secretory pathway and transforms its surrounding phagosome into a specialized replication compartment that morphologically resembles host-cell rough endoplasmic reticulum (7).

Endolysosomal avoidance and intracellular proliferation of L. pneumophila relies on more than 250 bacterial proteins, so called “effectors,” which are delivered through the Dot/Icm type IV secretion system (T4SS) into the infected host cell (8). Although several L. pneumophila effector proteins have been functionally characterized over the past decade, surprisingly few have been ascribed a function in manipulating endolysosomal maturation.

One effector known to target host cell endolysosomal trafficking, and the focus of this study, is L. pneumophila VPS inhibitor protein D (VipD) (9, 10). The C-terminal part of VipD lacks sequence homology to other proteins but, when overproduced in the surrogate host Saccharomyces cerevisiae, interferes with protein sorting to the vacuole (9), the yeast equivalent of mammalian lysosomes. A possible explanation for this phenomenon came from a recent study showing that the C-terminal domain of VipD (residues 316–621) binds endosomal Rab5 and Rab22 (11), another GTPase regulating endosomal trafficking. Ku et al. (11) consequently

Significance

The pathogen Legionella pneumophila replicates within human alveolar macrophages, causing a potentially fatal pneumonia known as Legionnaires’ disease. We identified that the effector protein VipD, which is injected into infected cells by L. pneumophila, localizes to degradative host organelles called endosomes. There, VipD binds to the endosomal regulator Rab5, an event that triggers the hydrolytic phospholipase A₁ activity in VipD, which causes the removal of the lipid phosphatidylinositol 3-phosphate. Without this key lipid, endosomes can no longer execute their function and L. pneumophila is protected from their harmful effect. Our finding opens new avenues for the development of therapeutics that interfere with the activation of VipD and with endosomal avoidance by L. pneumophila.
proposed that VipD overproduction interferes with endosomal maturation in transiently transfected mammalian cells by competing with EEA1 and other cellular ligands for Rab5 binding. However, it was inconclusive from their studies whether VipD is in fact involved in endosomal avoidance by \textit{L. pneumophila}. The N-terminal region of VipD shows significant sequence homology to the patatin-like phospholipase domain of \textit{Pseudomonas aeruginosa} ExoU (Fig. L4), a type III-translocated toxin with phospholipase \( \text{A}_2 \) (PLA\(_2\)) activity (12). Despite the obvious homology to ExoU, several laboratories failed over the past decade to underpin endosomal avoidance by this effector. Hence, the role of the catalytic domain for the biological function of this effector.

Despite the aforementioned efforts, the precise mechanisms underlying endosomal avoidance by \textit{L. pneumophila} remained elusive. Our study now provides detailed insight into this key virulence process of \textit{L. pneumophila} by revealing that a previously unrecognized phospholipase activity in VipD plays a major role in altering the lipid composition of endosomes, thereby interfering with their ability to target LCVs during infection. Our findings also provide a remarkable example for the emerging concept of spatiotemporal regulation of microbial effectors by coupling their catalytic activity to the arrival in a certain subcellular location.

**Results**

**VipD Interacts with Active Rab5 via its C-Terminal Domain.** The finding that \textit{L. pneumophila} VipD binds mammalian Rab5 (11) led the authors of a recent study to suggest that competitive binding between VipD and cellular Rab5 ligands was the reason for the block in endosomal maturation observed in transiently transfected mammalian cells overproducing VipD. Given that most microbial pathogens translocate only trace amounts of their effector proteins into infected host cells, and that stoichiometric binding, as opposed to catalysis, is a very inefficient mechanism of manipulating host proteins, it seemed unlikely that such a strategy would allow \textit{L. pneumophila} VipD to successfully interfere with endosomal fusion during host cell infection. In fact, we determined that on average, each \textit{L. pneumophila} bacterium delivers no more than \( 3.85 \times 10^{-7} \) fmol (or 232 molecules) of VipD into an infected mammalian cell, but that each host cell contains on average \( 5.31 \times 10^{-4} \) fmol (or 319,771 molecules) of active Rab5. Thus, assuming the most likely scenario of a single bacterium infecting a single mammalian macrophage, active Rab5 outnumbers VipD by a factor 1,379 (Fig. S1). This large stoichiometric difference between VipD and Rab5 strongly argues against VipD manipulating the early endosomal pathway solely by competitive binding to Rab5. As a result, we reevaluated the functional relation between \textit{L. pneumophila} VipD and host-cell Rab5.

Given that the VipD fragments used in our study differed in length and composition from those used by Ku et al. (11) (Fig. L4 and Fig. S24), we first confirmed the interaction between Rab5 and our VipD and its truncated variants in protein-protein binding studies. As expected, we found that binding of Rab5 to full-length VipD (residues 2–621; VipD\(_{2-621}\)) was nucleotide-dependent, with a strong preference of VipD for the constitutively active conformation of Rab5 [Rab5a(Q79L) or Rab5c(Q80L)] compared with the constitutively inactive form [Rab5a(S34N) or Rab5c(S35N)] (Fig. 1 B and C). VipD\(_{2-621}\) binding was specific for Rab5 but active variants of other endosomal Rab GTPases, such as Rab7, Rab9, or Rab10, were not precipitated from 293T cell lysate by bead-immobilized VipD\(_{2-621}\) (Fig. 1D). Moreover, using our truncated VipD variants, we confirmed that Rab5 binding was mediated by the C-terminal domain of VipD (VipD\(_{311-621}\)) (Fig. 1F), with the shortest of our fragments capable of Rab5 binding being VipD\(_{311-456}\) (Fig. S2 B and C). Thus, the VipD proteins used in this study exhibited a behavior similar to those described previously (11).

**L. pneumophila**, in addition to VipD, encodes three paralogs named VpdA, VpdB, and VpdC. These paralogs all possess sequence homology to the N-terminal phospholipase domain of VipD but show little homology to its C-terminal region (Fig. L4). Upon closer examination, we found that, unlike VipD, neither VpdA nor VpdB interacted with recombinant Rab5 (Fig. 1F and S2D). Taken together, these findings illustrated that the \textit{L. pneumophila} effector VipD specifically targets active Rab5 through its C-terminal region, a feature that is not shared by its paralogs.

**Inhibitory Effect of VipD on Yeast Growth Is Exacerbated by Rab5.** Translocated bacterial effectors often manipulate conserved host-cell pathways that are essential for eukaryotic cell growth and survival. To decipher the functional relationship between VipD and Rab5 we studied the two proteins in a yeast sensitivity assay. When overproduced in \textit{Saccharomyces cerevisiae}, VipD\(_{2-621}\) caused a slow-growth phenotype both on media plates (Fig. 2, row 4) and in liquid media (Fig. S3), a phenomenon not observed in cells containing the empty vector (Fig. 2, row 1). Synthesis of VipD\(_{311-621}\) in yeast also resulted in a slow-growth phenotype, most likely by binding to and interfering with the function of yeast Rab (Ypt) GTPases.
VipD Is a Rab5-Activated Phospholipase A1. We consequently tested if VipD possessed Rab5-dependent phospholipase activity using a fluorometric in vitro assay. PLA1 and PLA2 enzymes hydrolyze the carboxylester bond of phospholipids at the sn-1 or sn-2 position, respectively, releasing lysophospholipids and free fatty acids that can either be further degraded or function as signaling molecules (Fig. 3A). Although VipD2–621 alone exhibited neither PLA1 (Fig. 3B) nor PLA2 activity (Fig. S4A), we detected robust PLA1 activity in VipD2–621 upon addition of active Rab5c(Q80L) (Fig. 3B). The stimulatory effect of active Rab5 was concentration-dependent, with the strongest PLA1 activity at an equimolar or higher ratio of Rab5c(Q80L) to VipD2–621 (Fig. 3C), suggesting a 1:1 stoichiometry for the VipD–Rab5 complex. Addition of constitutively inactive Rab5a (S34N) to VipD2–621 failed to stimulate PLA1 activity (Fig. 3D), consistent with the lack of interaction between both proteins (Fig. 1B and C). Similarly, no other constitutively active Rab protein tested here (Rab7a, -10a, -11a, -20a, -40a) had a stimulatory effect on the PLA1 activity of VipD2–621, with the exception of Rab22a(Q64L) (Fig. 3D), the only other GTPase shown to interact with VipD (11). Consistent with its inability to bind Rab5 (Fig. 1F), L. pneumophila VpdB did not exhibit PLA1 activity, even in the presence of Rab5c(Q80L) (Fig. S4B), suggesting that this paralog requires a different host factor for activation. Recent reports (11, 13) that VipD exhibited PLA2 activity were not supported by our study (Fig. S4A).

Unlike full-length VipD2–621, neither the N- nor the C-terminal domain on its own exhibited notable PLA1 activity in vitro when incubated with Rab5c(Q80L) (Fig. 3E). Similar results were obtained in a yeast sensitivity assay where overproduction of VipD2–310 alone or together with Rab5c(Q80L) did not reduce cell growth (Fig. 2, rows 2 and 8, and Fig. S3). Similarly, the intermediate growth reduction caused by VipD2–621 in yeast was not exacerbated by Rab5c(Q80L) (Fig. 2, rows 3 and 9) showing that active Rab5 stimulates phospholipase activity only in full-length VipD.
The ability of VipD<sub>2–621</sub> to efficiently attenuate yeast growth depended on its PLA<sub>1</sub> activity because substitution of individual residues within the consensus motifs predicted to be essential for substrate hydrolysis (Fig. 1A) rendered VipD mutant proteins less toxic to yeast (Fig. 3F and Fig. S3). Similarly, we found that the in vitro PLA<sub>1</sub> activity of purified VipD<sub>2–621</sub> mutant proteins was strongly attenuated (Fig. 3G), confirming the importance of the active site residues for the catalytic activity of VipD. The loss of PLA<sub>1</sub> activity was likely not because of a folding defect of the VipD<sub>2–621</sub> point mutants because they interacted with Rab5<sub>c</sub>(Q80L) to an extent comparable to wild-type VipD<sub>2–621</sub> (Fig. S4C).

Enzymes with PLA<sub>1</sub> activity, although present in many organisms and cell types, are not studied and understood as well as PLA<sub>2</sub> proteins, and the biological function for most—if not all—PLA<sub>1</sub> enzymes has yet to be defined. To characterize VipD in greater detail, we determined its sensitivity toward phospholipase inhibitors. Because inhibitors specific for PLA<sub>2</sub> enzymes have not been identified, we analyzed a variety of PLA<sub>2</sub> inhibitors for their ability to interfere with VipD activity (Fig. 3J). Bromoeno lactone (BEL), Cay10502, Cay10650, AACOCF<sub>3</sub>, or aristolochic acid (10, 11, 13) were unable to detect any notable phospholipase activity in VipD activity is triggered upon binding of the C-terminal domain of PLA1 activity was likely not because of a folding defect of the active site residues for the catalytic activity of VipD. The loss of PLA1 activity requires activation through the C-terminal Rab5-binding domain. To reduce any secondary effects attributable to cytotoxicity caused by overproduction of VipD or its variants, all subsequent analyses were limited to transfected cells with intermediate or low protein production levels.

It has been reported that VipD heterologously produced in HeLa cells colocalizes with Rab5 and Rab22 on early endosomes, and that this colocalization required its C-terminal domain (11). We analyzed the intracellular distribution pattern of our VipD variants and obtained similar results (Fig. 4C). Although VipD<sub>2–310</sub> showed a primarily cytosolic localization in COS-1 cells (Fig. S5), VipD<sub>2–621</sub> and VipD<sub>311–621</sub> colocalized with enlarged endosomal compartments enriched in Rab5a(Q79L) (Fig. 4C and Fig. S5), consistent with these fragments binding active Rab5 (Fig. 1E). A similar distribution was observed for catalytically inactive VipD(S73A), showing that PLA<sub>1</sub> activity was dispensable for endosomal localization.

Concomitant with these findings, we also noticed that endogenous EEA1, unlike Rab5a(Q79L), assumed a primarily cytoplasmic localization in VipD<sub>2–621</sub>-producing cells, whereas in untransfected cells or control cells producing mCherry, EEA1 displayed a vesicular distribution in accordance with its accumulation on

![Fig. 4. VipD alters the protein and lipid composition of endosomes.](image-url)
endosomes (Fig. 4 D and E). This finding was in stark contrast to earlier reports (11), which claimed that EEAl was still associated with endosomes in VipD-producing cells. We found that EEAl displacement from endosomes was also observable in cells producing either VipD311–621 or VipD(S73A) but not in cells producing only VipD3–110 (Fig. 4 D and E), indicating that the phenomenon of EEAl displacement was, at least in part, mediated by the C-terminal domain of VipD, most likely a consequence of its ability to compete with EEAl and other cellular ligands for Rab5 binding (Fig. S6A).

We also found that SNX2, a component of the retromer and marker of recycling endosomes, was redistributed to the cytosol in COS-1 cells producing either VipD2–621 or VipD311–621 (Fig. S6B). Because recruitment of SNX2 to endosomes does not directly depend on Rab5 (16), it seemed unlikely that competitive binding of VipD to Rab5 caused the displacement of SNX2 from endosomes. Instead, given that both EEAl and SNX2 require PI(3)P to associate with endosomal membranes, we hypothesized that VipD affected the pool of endosomal PI(3)P, thereby causing the loss of PI(3)P ligands from membranes.

Using the FYVE domain of EEAl (GFP-2xFYVE) as a PI(3)P sensor, we found that PI(3)P was highly enriched on endosomal structures both in untransfected COS-1 cells or in control cells producing mCherry. In contrast, production of even low levels of VipD2–621 resulted in the redistribution of GFP-2xFYVE to the cytosol (Fig. 4 F and G), indicating that PI(3)P had been efficiently removed from endosomal membranes by VipD2–621. PI(3)P displacement from endosomes was less pronounced in cells producing either VipD311–621 or the catalytically inactive mutant VipD(S73A), demonstrating that the PLA1 activity of VipD2–621 was primarily responsible for PI(3)P removal. VipD311–621 depleted PI(3)P more slowly from endosomes, most likely by blocking Rab5 from recruiting PI (3) kinases like hVps34, thus preventing the de novo synthesis of PI(3)P (Fig. 4 F and G and Fig. S6B). Notably, VipD2–621 did not alter the distribution of other organelle markers such as Gia1 (Golgi compartment), Sec61b (endoplasmic reticulum), Hoechst (DNA), TOM70 (mitochondria), or lysosomal-associated membrane protein-2 (LAMP-2, lysosomes) (Fig. S7A). Similarly, the localization of GFP-SidM51–647, a PI(4)P probe (17), was not affected by VipD (Fig. S7 B and C), demonstrating that its PLA1 activity was directed only against Rab5-containing endosomes. Taken together, these findings define a unique role for the PLA1 domain of VipD in manipulating the lipid and, consequently, protein composition of endosomal membranes.

**VipD Is Required for Efficient Endosomal Avoidance by L. pneumophila.**

Given the striking effect of exogenous VipD on the molecular assembly of endosomes (Fig. 4), we subsequently examined the involvement of this effector protein in early endosomal avoidance by L. pneumophila. We used CHO-FcγRII cells challenged with L. pneumophila to monitor, by fluorescence microscopy, the acquisition of endosomal markers on LCVs shortly after bacterial uptake. The parental strain L. pneumophila Lp02 avoided endosomal fusion with much greater efficiency than the avirulent strain Lp03, with only 45 ± 3% Rab5a(Q79L)-positive Lp02-containing vacuoles as opposed to 80 ± 6% Rab5a(Q79L)-positive LCVs observed for Lp03 (Fig. 5). A L. pneumophila mutant lacking VipD (Lp02ΔvipD) showed a significant increase (61 ± 4%) in the colocalization with Rab5a(Q79L) compared with Lp02, and this phenotype was efficiently rescued (29 ± 2%) by complementing Lp02ΔvipD with a plasmid encoding VipD (Fig. 5). On the other hand, catalytically inactive VipD(S73A) and VipD(G288A) were unable to protect Lp02ΔvipD from Rab5a(Q79L)-positive compartments, confirming the biological significance of the PLA1 activity of VipD for endosomal avoidance by L. pneumophila.

**Discussion**

The ability of L. pneumophila to avoid endolysosomal trafficking is a key virulence feature. Despite recent observations pointing toward a role of the T4SS-translocated effector protein VipD in this process, the true biological function of VipD and the molecular mechanisms underlying endosomal avoidance by L. pneumophila remained unclear. Our study now shows that VipD possesses robust PLA1 (but not PLA2) activity, which alters the lipid and, consequently, protein composition of endosomal membranes, thereby preventing them from fusing with LCVs. The truly unexpected finding, however, was that VipD, to exhibit PLA1 activity, has to be bound and activated by host-cell endosomal Rab GTPases, namely Rab5 and Rab22.

To our knowledge, VipD is the first T4SS-translocated effector with phospholipase activity. Unlike ExoU and most secreted bacterial phospholipases, VipD alters the composition of endosomes in a bimodal manner (Fig. S8). The PLA1 domain catalyzes the removal of PI(3)P from the endosomal compartment by either directly hydrolyzing this phospholipid or by targeting its precursor molecules (Fig. 4 F and G), the C-terminal domain competes with cellular ligands like EEAl or hVps34 for Rab5 binding (Fig. S6A), thereby preventing their de novo recruitment to endosomes. Simultaneously depleting the existing pool of PI(3)P and interfering with its replenishment through PI 3-kinase exclusion may explain how VipD can efficiently transform endosomes into membrane structures that lack many of their endosome-defining features, most likely rendering them fusion-incompetent. Given that the amount of VipD translocated during infection is very low (Fig. S1), we do not expect VipD to target the entire cellular pool of endosomes but rather only those organelles in the immediate vicinity of the LCV.

The finding that L. pneumophila VipD does not simply de-activate Rab5, for instance by functioning as a GTPase-activating protein, but rather exploits Rab5 for the activation of its PLA1 activity may seem extraneous at first. However, given that endosomal function is controlled by several Rab GTPases (18), their collective deactivation by L. pneumophila would require numerous effectors with GTPase-activating protein activity. PI(3)P, on the other hand, is the only phospholipid known to be essential for the assembly of tethering and fusion protein complexes into endosomal microdomains, and its removal can be efficiently accomplished by a single bacterial phospholipase, as shown here.

The strategy of removing endosomal PI(3)P is an efficient tactic used by other intracellular pathogens to combat the host’s bactericidal defenses. For example, Mycobacterium tuberculosis (MtB) secretes SapM, a phosphatase that removes PI(3)P from MtB-containing vacuoles by converting it to PI, thereby arresting endosomal maturation (19). VipD differs from SapM because it targets PI(3)P within the endosomal membrane rather than the
pathogen-containing vacuole, which may explain why earlier attempts to detect VipD on the surface of LCVs were unsuccessful (10). Despite the importance of VipD for endosomal avoidance, *L. pneumophila* mutants lacking VipD, either individually or in combination with other VipD paralogs, were not attenuated for growth in mammalian or amoeboid cells (10), arguing for the existence of additional effector proteins that interfere with endosomal maturation by a yet unknown mechanism. This hypothesis may also explain why the defect in endosomal avoidance observed for *L. pneumophila* mutants lacking VipD, although statistically significant, was subtle (Fig. 5).

The involvement of VipD in endosomal avoidance has recently been challenged by a study proposing that VipD stimulates caspase-3 activation by targeting the mitochondrial membrane and causing cytochrome *c* release (13). Several points of evidence argue against such a role of VipD. First, VipD does not colocalize with mitochondria (or the plasma membrane) but is highly enriched on endosomal membranes of transfected mammalian cells (Fig. 4C) (11). Second, VipD requires endosomal Rab GTAPases for the activation of its PLA2 domain (Fig. 3D), and because these GTAPases are absent from mitochondrial membranes, it seems difficult to envision how they could logistically be involved. Finally, caspase-3 activation was blocked by the PLA2 inhibitor BEL (13), yet our study clearly demonstrates that the PLA2 activity of VipD is insensitive even to high concentrations of BEL (Fig. 3H), suggesting that other contaminating phospholipases caused the observed effects on caspase-3. This convincing evidence against such a role of VipD in endosomal avoidance and causing caspase-3 activation combined with our own data prompt us to favor a model in which the primary role of this *L. pneumophila* effector is to attenuate endosome function by altering their protein and lipid composition (Fig. S8).

This finding that the PLA2 activity of VipD is entirely dependent on the binding of active endosomal Rab GTAPases adds an intriguing case to a very short list of related examples in which microbial proteins require a host-cell GTAPase as a trigger for their catalytic activity (20–22). Linking catalytic activity to subcellular localization most likely allows *L. pneumophila* and other microbial pathogens to remotely control the activity of their effectors and toxins after their translocation into the host cell and to prevent them from indiscriminately targeting other host compartments or even the bacterium's own physiology (Fig. S8). We hypothesize that the VipD paralogs and probably a variety of other effectors from *L. pneumophila* and related pathogens possess safety mechanisms similar to that described here for VipD. Understanding their mechanism of activation not only provides valuable insight into microbial virulence strategies, but also opens the way for the discovery of novel therapeutics designed to interfere with the activation processes.

**Experimental Procedures**

**Phospholipase Assays.** PLA2, or PLA3 activities were monitored using EnzChek Phospholipase Assay Kits (Invitrogen) according to the manufacturer's specifications. For the assays, 400 nM of each recombinant protein was used in 100-μL reactions in black 96-well microtiter plates for 40 min at room temperature. In protein complex reactions involving Hiα reagent and GST-Rab5, 400 nM of each protein (unless otherwise indicated) was preincubated at 4 °C for 2 h to allow protein complex formation following the addition of fluorescent substrates. Phospholipase inhibitors were added after protein complex formation at room temperature for 1 h before the addition of fluorescent substrates. Fluorescence intensities, representing substrate cleavage, were detected after 40 min at an emission wavelength of 515 nm using a microplate reader (BioTek Synergy 4). We used the following concentrations: EEA1 (1:200), SNX2 (1:1,250), LAMP-2 (1:2,000), Sec61β (1:200), Giantin (1:3,000), and anti-rabbit FITC (1:1,000). All images were analyzed on a Zeiss Axio Observer.Z1 inverted light microscope using a Zeiss Plan-Apochromat 63×/oil M27 objective (CO2I imaging) or a Zeiss Plan-Apochromat 100×/1.4 OIL DIC M27 objective (CHO infections) and processed with Zeiss AxioVision 4.7.2 software.

**Legionella Infections.** CHO-FcgRII cells producing GFP-Rab5a(Q79L) were challenged with opsonized *L. pneumophila* at a multiplicity of infection of 15 for 10 min at 37 °C. Cells were chemically fixed, and intracellular and extracellular bacteria were differentially stained (23). Statistical representation of GFP-endosomal markers localizing to LCVs is derived from the means of four independent experiments (n = 25).

See Tables S1 and S2 for lists of plasmids and oligos used.

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