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Targeting of Host Rab GTPase Function by the Intravacuolar Pathogen *Legionella pneumophila*

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Summary

The intracellular pathogen *Legionella pneumophila* replicates in a vacuole that recruits material from the host cell endoplasmic reticulum (ER). Biogenesis of this unique vacuole depends on the bacterial Dot/Icm type IV secretion system that translocates proteins across host cell membranes. Here, we show that two translocated substrates, SidM and LidA, target host cell Rab1, a small GTPase regulating ER-to-Golgi traffic. SidM is a guanosine nucleotide exchange factor for Rab1 that recruits Rab1 to *Legionella*-containing vacuoles, a process that is enhanced by LidA. Expression of *sidM* in mammalian cells interferes with the secretory pathway and causes Golgi fragmentation. Consistent with a collaborative relationship between the two proteins, immobilized SidM and LidA synergize to promote Rab1-dependent binding of early secretory vesicles. These results indicate that proteins translocated into the host cell by the intravacuolar pathogen *L. pneumophila* are able to recapitulate events involved in host secretory trafficking.

Introduction

Many bacterial pathogens grow in a membrane bound vacuole within host cells (Meresse et al., 1999). These microorganisms manipulate target cell secretory and endocytic traffic to promote close association of their replication compartment with specific host organelles. This intimate relationship probably facilitates expansion of the membranous vacuole surrounding the bacterium and prevents association with antimicrobial compartments. *Legionella pneumophila*, the causative agent of Legionnaires' pneumonia, is one such intravacuolar pathogen (Fraser et al., 1977; McDade et al., 1977). Disease involves uptake of aerosolized bacteria by alveolar macrophages; after uptake occurs, *L. pneumophila* becomes sequestered in a membrane bound vacuole that bypasses fusion with the lysosomal network (Horwitz, 1983a). Instead, the *Legionella*-containing vacuole (LCV) recruits early secretory vesicles from the host endoplasmic reticulum (ER) and transforms its vacuole into a ribosome-studded compartment that morphologically resembles host cell rough ER (Horwitz, 1983b; Swanson and Isberg, 1995; Tilney et al., 2001; Kagan and Roy, 2002). Within this specialized vacuole, *L. pneumophila* replicates to a high density, lyses the host cell, and infects neighboring macrophages.

Intracellular growth of *L. pneumophila* is strictly dependent on a bacterial type IV secretion system (T4SS), encoded by the *dot/icm* gene clusters (Vogel et al., 1998; Segal et al., 1998). This T4SS mediates translocation of bacterial effector molecules into the host cell cytosol (Conover et al., 2003; Nagai et al., 2002; Chen et al., 2004; Luo and Isberg, 2004). Mutations in *dot/icm* genes result in defective targeting of the LCV into an endocytic compartment (Berger and Isberg, 1993; Sadosky et al., 1993; Berger et al., 1994; Andrews et al., 1998; Roy et al., 1998; Wiater et al., 1998) and an inability to associate with the rough ER (Swanson and Isberg, 1995; Tilney et al., 2001).

Little is known about the identity and function of the translocated substrates of the *L. pneumophila* Dot/Icm system. RalF recruits and activates ADP-ribosylation factor 1 (Arf1), a small GTPase involved in retrograde vesicle transport from the Golgi compartment to the ER (Nagai et al., 2002). LidA is translocated by the *L. pneumophila* Dot/Icm system immediately after uptake by host cells and associates with the cytosolic surface of the LCV (Conover et al., 2003). Several of these effectors, including LidA, have been shown to disrupt secretory traffic when produced in either yeast or mammalian cells, indicating that they manipulate host cell vesicle trafficking during intracellular growth, allowing formation of the LCV (Shohdy et al., 2005; Derré and Isberg, 2005; Campodonico et al., 2005). Deletion of individual genes encoding translocated substrates, however, results in little or no growth defect of *L. pneumophila* in macrophages, suggesting that bacterial effectors have redundant activities. One explanation for this redundancy is that there appear to be multiple vesicle trafficking pathways located between the ER and the Golgi that promote formation of the LCV (Dorer et al., 2006). *L. pneumophila* mutants that are unable to hijack vesicles derived from one pathway may still be able to utilize membrane material provided by other pathways, as translocated effectors potentially interact with a subset of these vesicle transport routes. Thus, the identification of translocated effector mutants in the absence of detectable growth phenotypes has become a challenging task.

ER proteins, such as Sec22b and calnexin, as well as Rab1, are recruited to LCVs shortly after uptake of *L. pneumophila* into target cells (Derré and Isberg, 2004; Kagan et al., 2004). Rab1 is a small guanosine nucleotide binding protein essential for ER-to-Golgi vesicle trafficking (Tisdale et al., 1992; Plutner et al., 1991; Wilson et al., 1994; Nuoffer et al., 1994; Allan et al., 2000; Moyer et al., 2001). Upon activation, GTP-Rab1 specifically interacts with downstream effectors such as the tethering protein p115 as well as other *cis*-Golgi proteins, thereby programming ER-derived vesicles for docking and fusion with the Golgi (Allan et al., 2000; Moyer et al., 2001; Weide et al., 2001; Satoh et al., 2003; Diao et al., 2003; Beard et al., 2005). As Rab1 colocalizes with the LCV within 10 min of bacterial uptake, manipulation of this host GTPase may be one of the first activities promoted by *L. pneumophila*-translocated

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effectors after cell contact. These effector proteins may be missing links in the process of vesicle recruitment to the LCV.

In the present study, we demonstrate that two Dot/Icm-translocated *L. pneumophila* proteins specifically interact with host cell Rab1 and modulate its function. Both proteins may mimic host factors involved in ER-derived vesicle trafficking and may facilitate vesicle binding and integration of the LCV into the host cell secretory pathway.

Results

SidM from *L. pneumophila* Directly Interacts with Mammalian Rab1

Formation of the replicative vacuole involves rapid recruitment of host cell proteins such as Sec22b and the small GTPase Rab1 (Derré and Isberg, 2004; Kagan et al., 2004). As Rab1 is a key regulator of early secretory vesicle trafficking, we looked directly for *L. pneumophila* proteins that could manipulate its activity. To identify bacterial Rab1 ligands, glutathione S-transferase (GST) fusion proteins of GDP-locked Rab1(S25N) or GTP-locked Rab1(Q70L) bound to agarose beads were incubated with *L. pneumophila* lysate (Figure 1A). A 73 kD protein bound beads coated with inactive GST-Rab1(S25N), but not beads coated with the constitutively active mutant GST-Rab1(Q70L) or with GST alone. The isolated bacterial protein was identified by mass spectrometry analysis (Experimental Procedures) as a 647 amino acid protein of unknown function that we named SidM (substrate of Icm/Dot; *lpg2464*). The *L. pneumophila* genome encodes two proteins (*lpg1101* and *lpg2603*) with weak homology to SidM (13% and 11% identity, respectively). Remarkably, the open reading frame encoding SidM is located immediately upstream of the open reading frame encoding SidD, a recently identified substrate of the Dot/Icm translocation system (Luo and Isberg, 2004).

We tested whether SidM directly binds Rab1. Purified recombinant SidM was incubated with agarose beads crosslinked to wild-type Rab1 loaded with either GDP, GTPγS, or no nucleotide, and the amount of SidM bound to Rab1-coated beads was analyzed (Figure 1B). SidM preferentially bound nucleotide-free Rab1, whereas binding of SidM to GTP-Rab1 was almost undetectable. Rab1 shares significant sequence identity (up to 54%) with other members of the Rab protein subfamily. However, bead-immobilized SidM did not bind any other Rab GTPases from U937 cell lysate besides Rab1 (Figure 1C), even after SidM-coated beads were used to deplete Rab1 from the extract to reduce competition (first versus second SidM pull-down). Thus, SidM specifically binds Rab1 and preferentially associates with the inactive conformation of this GTPase.

SidM Functions as a GDP/GTP Exchange Factor for Rab1

High-affinity binding to the nucleotide-free form of GTPases is a characteristic feature of guanosine nucleotide exchange factors (GEFs) (Lai et al., 1993). Therefore, we tested SidM for its GEF activity by monitoring the association of Rab1 with radiolabeled [^{3}H]GDP

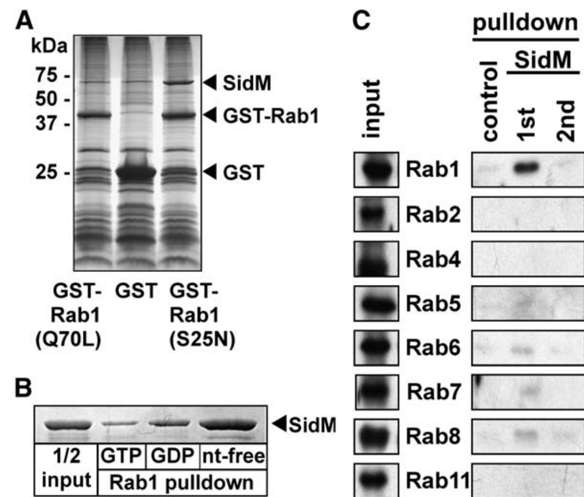

Figure 1. SidM Specifically Binds the Inactive Form of Rab1

(A) Pull down of SidM with GST-Rab1-coated beads. Glutathione-Sepharose beads coated with GST-Rab1(Q70L), GST-Rab1(S25N), or GST alone were incubated with lysate of *L. pneumophila*. Bacterial proteins retained by the beads were separated by SDS-PAGE and were visualized by silver staining.

(B) Rab1 binding to SidM is specific and guanosine nucleotide dependent. Affigel beads coated with Rab1 loaded with GTPγS (a nonhydrolyzable GTP analog), GDP, or no nucleotide (nt-free) were incubated with purified SidM, and SidM binding was analyzed in Coomassie-stained gels.

(C) SidM specifically binds Rab1. SidM- or BSA-coated agarose beads were incubated with U937 cell lysate, and proteins retained by the beads were analyzed by Western blot with antibodies specific for the indicated Rab GTPases.

(Figure 2A). Without SidM, incubation of [^{3}H]GDP-Rab1 with unlabeled GTP only resulted in a slow loss of radiolabel from Rab1. In contrast, in the presence of both SidM and GTP, radioactivity was rapidly lost from Rab1, indicating [^{3}H]GDP release from Rab1. No [^{3}H]GDP dissociation was observed in the absence of unlabeled GTP, showing that SidM-induced GDP release from Rab1 was dependent on the presence of GTP. Efficient nucleotide exchange took place even when Rab1 was present in a 100-fold molar excess relative to SidM, indicating that the GEF activity of SidM that we observed was catalytic (Figures 2B and 2C). SidM also triggered incorporation of [γ^{35}S]GTP into Rab1, and rates were dependent on the concentration of SidM (Figure 2D). SidM did not promote GDP/GTP exchange in Rab2 (data not shown), and this finding is in agreement with the absence of a direct interaction between SidM and Rab2 or any other Rab GTPase besides Rab1 (Figure 1C).

The catalytic activity of SidM requires that GTP-Rab1 be released from the GEF after activation in order to allow binding and activation of another GDP-Rab1 molecule. Consistent with a transient interaction between both proteins, we found that GDP-Rab1, but not GTPγS-Rab1, was efficiently coprecipitated with SidM (Figure 2E), indicating that activated Rab1 is released by SidM once the nucleotide exchange reaction is completed. Taken together, these experiments demonstrate that a single SidM molecule can efficiently activate multiple Rab1 proteins in vitro by stimulating exchange of GDP against GTP.

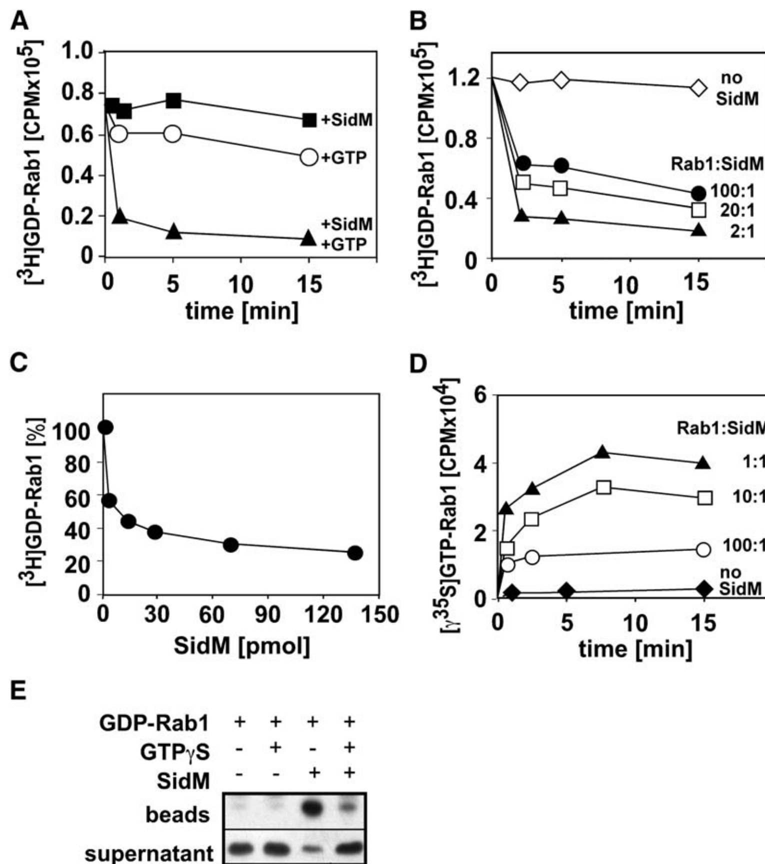

Figure 2. SidM Is a Nucleotide Exchange Factor for Rab1

If not otherwise indicated, equimolar protein concentrations were used. Samples were removed at the indicated time points, and the amount of radiolabeled Rab1 was determined in a nitrocellulose filter assay (Experimental Procedures). Each graph shows a representative experiment of at least two repetitions.

(A) SidM stimulates release of [^{3}H]GDP from Rab1 only in the presence of GTP. [^{3}H]GDP-loaded Rab1 (50 pmol) was incubated in the presence of either SidM, unlabeled GTP (5 mM), or both.

(B) Concentration dependence of SidM-mediated nucleotide release from Rab1. [^{3}H]GDP-loaded Rab1 was incubated with SidM in the indicated molar ratios and unlabeled GTP (5 mM).

(C) SidM stimulates Rab1 GDP/GTP exchange in a dose-dependent manner. [^{3}H]GDP-Rab1 (5 pmol) was incubated with the indicated amounts of SidM. Exchange reactions were started by the addition of unlabeled GTP. Shown is the percentage of GDP-Rab1 after a 2 min incubation with the indicated amount of SidM.

(D) SidM mediates incorporation of [γ^{35}S]GTP into Rab1. GDP-loaded Rab1 (50 pmol) was incubated with SidM in the indicated molar ratios. GDP/GTP exchange was started by the addition of [γ^{35}S]GTP.

(E) Coimmunoprecipitation of Rab1. GDP-Rab1 was incubated for 30 min at 25ºC in the presence or absence of GTPγS or SidM (as indicated). The reaction was transferred to 4ºC, and SidM was precipitated by agarose beads coated with antibody directed against SidM. Rab1 in the supernatant or bound to bead-immobilized SidM was detected by Western blot analysis with polyclonal anti-Rab1 antibody.

SidM Is Translocated by the *L. pneumophila* Dot/Icm Secretion System and Localizes to the Vacuolar Surface

As SidM directly interacts with Rab1 (Figure 1B), we tested whether SidM is injected into the host cell cytoplasm during *L. pneumophila* infection. Bone marrow-derived macrophages (BMMs) from A/J mice were incubated for 1 hr with wild-type *L. pneumophila* (Lp02; intact Dot/Icm system) or with Lp03 (*dotA*$^-$; defective T4SS) and were probed for SidM by fluorescence microscopy with an antibody raised against full-length SidM (Figure 3A). SidM was clearly associated with LCVs containing wild-type bacteria, but not with vacuoles harboring *L. pneumophila dotA*$^-$, demonstrating that SidM secretion is Dot/Icm dependent. To discriminate between SidM secretion and translocation, we probed for SidM association with LCVs isolated from mechanically lysed U937 cells in the absence of membrane-permeabilizing detergents (Figures 3B and 3C). SidM was detected on the external surface of LCVs containing wild-type *L. pneumophila*, but not on vacuoles containing *L. pneumophila dotA*$^-$ or *ΔsidM*. Complementation of *L. pneumophila ΔsidM* with a plasmid encoding SidM restored vacuole association of this protein (Figures 3B and 3C). Therefore, SidM is an effector protein from *L. pneumophila* that associates with the cytoplasmic surface of the LCV after translocation by the Dot/Icm system.

SidM Interferes with the Host Secretory Pathway

As several *L. pneumophila* proteins were found to interfere with host cell processes after ectopic expression in eukaryotic cells (Campodonico et al., 2005; Shohdy et al., 2005), we examined the effects of SidM on the host cell secretory pathway. Green fluorescence protein (GFP)-tagged SidM was produced in COS1 cells, and Golgi integrity in transfected cells was analyzed by fluorescence microscopy with an antibody against GM130, a Golgi-resident protein (Figures 3D and 3E). GFP alone had no detectable effect on Golgi morphology, whereas GFP-SidM caused Golgi fragmentation in COS1 cells (97% ± 2%) even at low production levels, with GFP-SidM accumulating in a perinuclear locale. In contrast, the morphology of the ER appeared to remain unaffected by GFP-SidM (data not shown). Thus, SidM efficiently interfered with the host cell secretory pathway when ectopically expressed.

Rab1 Interacts with LidA from *L. pneumophila*

Mammalian cells overproducing LidA, another *L. pneumophila* Dot/Icm substrate of unknown function, show disruption of the Golgi apparatus in a fashion similar to

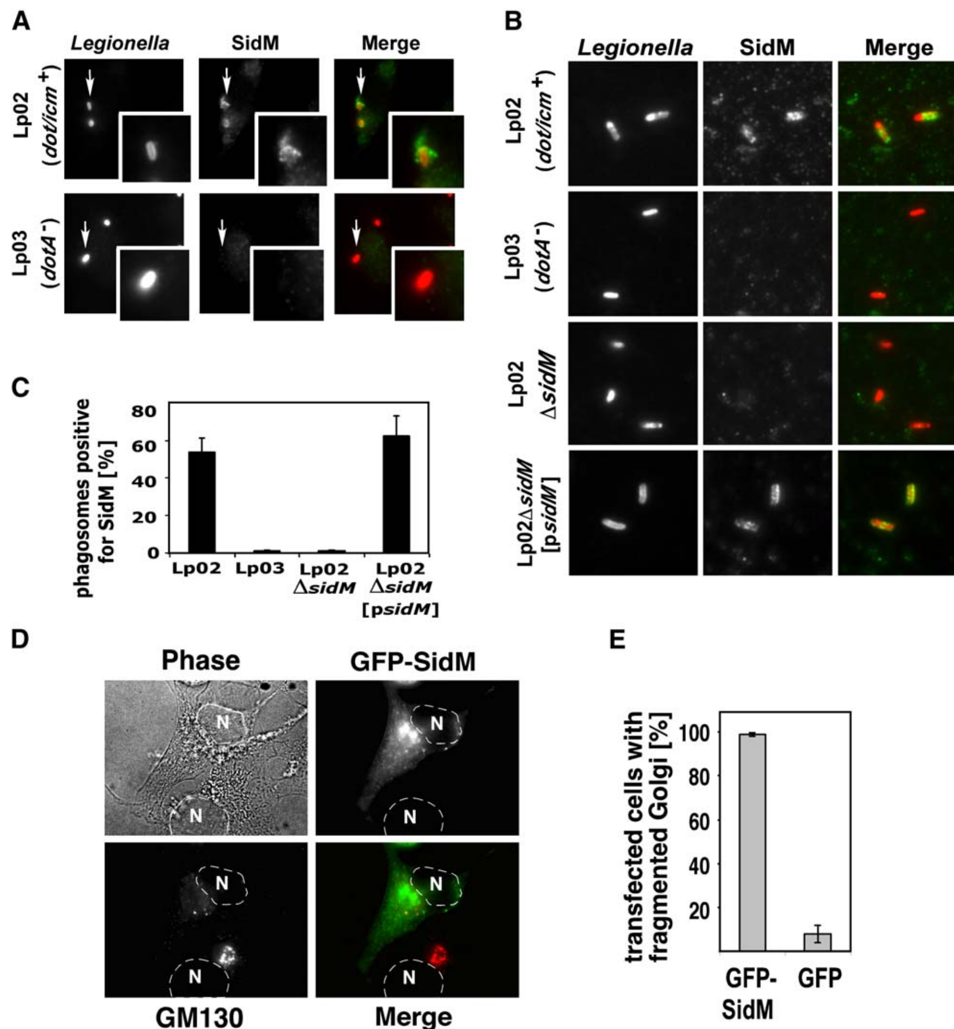

Figure 3. SidM Is a Translocated Substrate of the *L. pneumophila* Dot/Icm System that Causes Golgi Fragmentation When Overproduced

(A) Dot/Icm-dependent secretion of SidM by intracellular *L. pneumophila*. BMMs were infected for 1 hr with Lp02 (wild-type *L. pneumophila*) or Lp03 (Lp02 *dotA*$^{-}$ with a defective T4SS), followed by fixation and membrane permeabilization. Left panels: intracellular *L. pneumophila* (Experimental Procedures). Middle panels: SidM staining with affinity-purified antibody. Right panels: merged images showing bacteria (red) and SidM (green). Arrows indicate the location of the LCVs that are magnified in the inset of each panel.

(B) SidM staining on isolated LCVs. U937 cells were infected for 30 min with Lp02, Lp03, Lp02Δ*sidM*, or Lp02Δ*sidM* (p*sidM*). Isolated vacuoles were probed with anti-SidM antibody prior to permeabilization (left column) and anti-*Legionella* antibody after permeabilization (middle). Right column: merged images showing *Legionella* (red) and SidM (green).

(C) Quantification of intact LCVs staining positive for translocated SidM 30 min postinfection. The graph represents the average ± SD of two independent experiments.

(D) SidM causes Golgi fragmentation. COS1 cells producing low levels of GFP-SidM were stained with antibody specific for GM130, a Golgi-resident protein, to determine Golgi integrity. N, cell nucleus.

(E) Percentage of cells from (D) with fragmented Golgi after low-level production of either GFP or GFP-SidM. Results are the mean ± SD of two independent experiments.

that observed in SidM-producing cells (Figures 3D and 3E) (Conover et al., 2003; Derré and Isberg, 2005). Given that SidM and LidA display similar phenotypes when synthesized in COS1 cells, we determined whether LidA also bound a host protein involved in ER-to-Golgi transit. Purified LidA was immobilized on agarose beads and incubated with postnuclear supernatant (PNS) of macrophage-like U937 cells (Figure 4A). An ~22 kD protein was retained by LidA-coated beads, but not by uncoated control beads. This protein was identified by mass spectrometry analysis as Rab1, which was previously shown to bind SidM (Figure 1). To confirm a direct interaction between LidA and Rab1 and to determine whether LidA preferentially bound the active or inactive form of Rab1, an enzyme-linked immunosorbent assay (ELISA) was performed (Figure 4B). Immobilized LidA directly interacted with both GTP-locked Rab1(Q70L) and GDP-locked Rab1(S25N), with a higher affinity for the active GTP bound form of Rab1.

When analyzing the effect of LidA on Rab1 activity in vitro, we found no evidence for LidA being either a GEF or a GTPase-activating protein (GAP) for Rab1 (data not shown). However, LidA also bound to Rab6 and Rab8 from U937 cell lysate, but it did not associate with Rab2, Rab4, Rab5, Rab7, or Rab11, even after depletion of Rab1, Rab6, and Rab8 from the extract

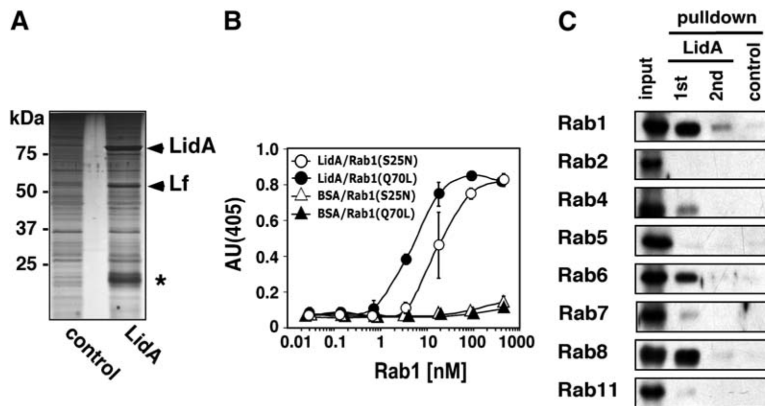

Figure 4. Identification of LidA as a Rab1 Ligand

(A) Pull down of Rab1 by LidA. LidA-coated beads or uncoated control beads were incubated with postnuclear supernatant (PNS) from U937 cells. Proteins retained by the beads were separated by SDS-PAGE and visualized by silver staining. Rab1 (asterisk) was identified by HPLC/mass analysis; Lf, LidA degradation fragment.

(B) LidA interacts preferentially with GTP-Rab1. ELISA plates were coated with LidA or BSA and probed with Rab1(S25N) or Rab1(Q70L). The data points represent the average ± SD of two independent experiments performed in duplicate.

(C) Western blot analysis of Rab proteins bound by LidA. PNS from U937 cells was incubated twice with LidA-coated beads (first and second incubations; to allow depletion of all LidA-interacting Rab proteins) or BSA-coated control beads, and proteins retained by the beads were analyzed by Western blot with antibodies specific for the indicated Rab GTPases.

(Figure 4C, first versus second LidA pull-downs). Thus, LidA may allow *L. pneumophila* to simultaneously target a subset of Rab GTPases involved in different membrane trafficking routes.

SidM Is Required for Rab1 Recruitment to the *L. pneumophila*-Containing Vacuole

Formation of LCVs involves rapid recruitment of host cell proteins of the early secretory pathway, a process dependent on a functional Dot/Icm system (Derré and Isberg, 2004; Kagan et al., 2004). As both LidA and SidM associate with the vacuolar surface after translocation and directly interact with Rab1 in vitro, we tested whether these two proteins are involved in Rab1 recruitment in vivo (Figures 5A and 5B). Rab1 colocalized with 50% of LCVs containing wild-type *L. pneumophila* 30 min after uptake by BMMs, and it colocalized with 58% of the LCVs at 2 hr postinfection (hpi). High-level Rab1 recruitment was dependent on SidM, since *L. pneumophila* mutants lacking *sidM* displayed a severe defect in Rab1 recruitment (<3%). This defect was fully complemented by plasmid-encoded SidM. The absence of LidA caused a kinetic defect in Rab1 recruitment, with fewer Rab1-positive vacuoles 30 min after uptake, but not 2 hpi, compared to wild-type bacteria. Thus, Rab1 recruitment to the LCV is mediated by SidM and is supported by LidA.

Growth Requirements for SidM and LidA during Infection

Recent work demonstrated that several host cell membrane trafficking pathways contribute to the formation of the LCV (Dorer et al., 2006). As both SidM and LidA seem to target the Rab1-regulated early secretory traffic, we analyzed the role of both effector proteins during intracellular replication of *L. pneumophila* (Figure 5C). Bacteria harboring a deletion in *sidM* showed no reduction in the establishment of large, replicative vacuoles, consistent with the model that shows that loss of a single vesicle trafficking pathway is not sufficient to interfere with intracellular *L. pneumophila* growth. In contrast, a strain lacking LidA, which targets three Rab GTPases (Figure 4C), showed the previously reported targeting defect in BMMs (Conover et al., 2003), with about twice as many bacteria failing to replicate intracellularly compared to a wild-type strain (Figure 5C). This defect could be reversed by complementation of Lp02Δ*lidA* with plasmid-encoded LidA. *L. pneumophila* deficient for both SidM and LidA showed an efficiency in replicative vacuole formation 13 hpi, comparable to that of a Δ*lidA* strain (data not shown). This result shows that deletion of *sidM* did not further increase the growth defect caused by the lack of LidA. Similar results were obtained in a 3-day growth curve in BMMs (data not shown), with mild growth defects of *L. pneumophila* strains lacking either LidA or LidA and SidM and with a replication efficiency of Lp02Δ*sidM* equal to that of wild-type *L. pneumophila*.

SidM and LidA Collaborate for Recruitment of Early Secretory Vesicles

Rab1 regulates targeting and fusion of ER-derived vesicles with the Golgi complex (Tisdale et al., 1992; Plutner et al., 1991; Wilson et al., 1994; Nuoffer et al., 1994; Allan et al., 2000). Since Rab1 recruitment to LCVs is dependent on SidM and is supported by the presence of LidA (Figure 5B), we asked whether particles coated with SidM or LidA can bind ER-derived vesicles. Latex beads coated with SidM, LidA, or both proteins were incubated with PNS from U937 cells, and the association of host cell vesicles with beads was analyzed by transmission electron microscopy (Figures 6A–6C). LidA-coated beads showed vesicle recruitment efficiencies only slightly higher than those of BSA-coated control beads (10.2 ± 2 versus 6.3 ± 0.4 vesicles/bead) (Figure 6D). In contrast, beads coated with SidM showed considerable binding activity to host cell vesicles (18.9 ± 4.5). However, the simultaneous presence of both SidM and LidA on beads resulted in a large increase in the vesicle recruitment efficiency (37.8 ± 9.1), showing that LidA stimulates the vesicle binding activity of SidM. Preincubation of SidM + LidA-coated beads with GDP-locked Rab1(S25N) led to a strong reduction in vesicle binding (Figure 6E), demonstrating that vesicle recruitment by SidM + LidA is dependent on activated Rab1. The addition of soluble SidM in *trans* to U937 cell lysate had no stimulatory effect on vesicle binding by LidA-coated beads (Figure 6F), showing that efficient recruitment of vesicles in vitro

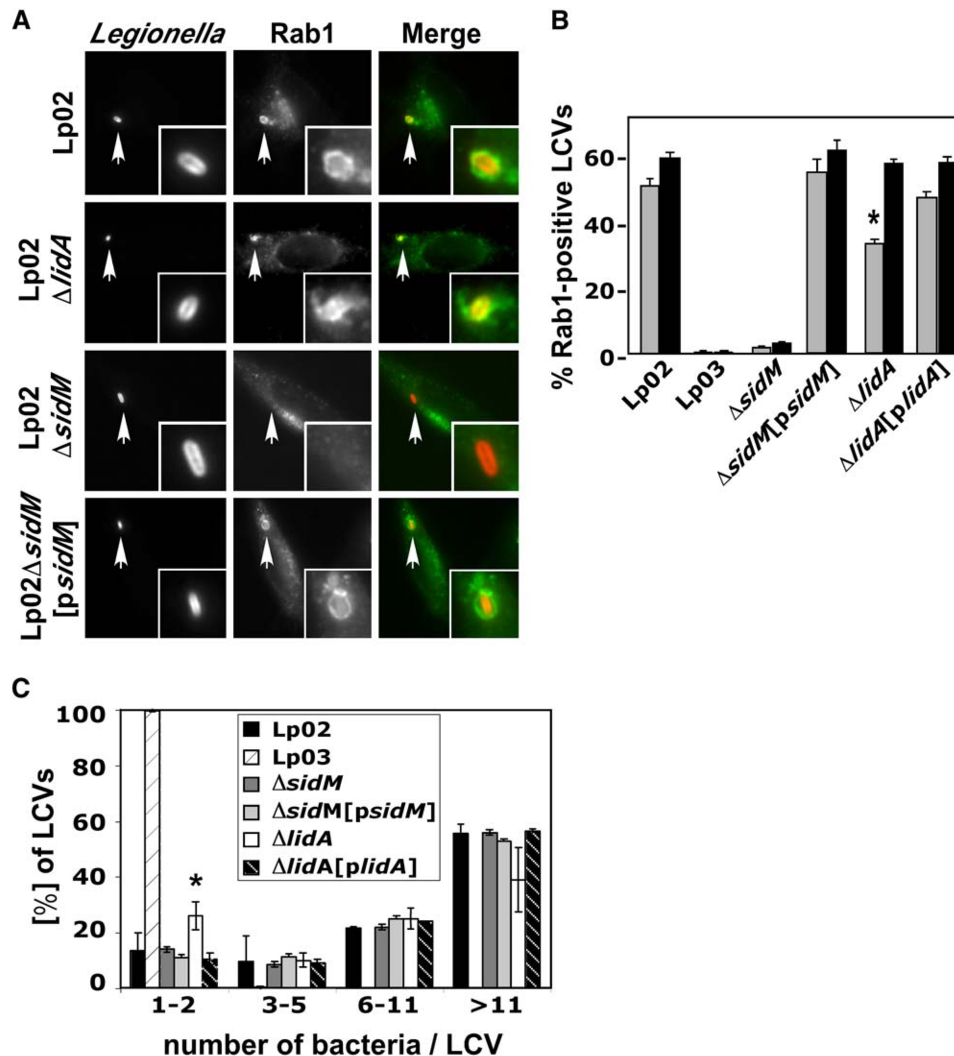

Figure 5. SidM Is Required for Recruitment of Rab1 to LCVs

(A) BMMs were infected for 30 min with the indicated *L. pneumophila* strains, and association of LCVs with cellular Rab1 was visualized by indirect immunofluorescence microscopy with Rab1-specific antibody (middle column). Intracellular bacteria were detected by using an anti-*Legionella* antibody (left column). Right column: merged images showing intracellular *L. pneumophila* (red) and Rab1 (green). Arrows indicate the location of the LCVs that are magnified in the inset of each image.

(B) Quantification of LCVs colocalizing with Rab1 30 min or 2 hr after bacterial uptake by BMMs visualized by immunostaining. *, $p < 0.001$ (t test).

(C) Efficiency of large, replicative vacuole formation of *L. pneumophila* strains. BMMs were incubated for 1 hr at an MOI of 1 with the indicated *L. pneumophila* strains. Extracellular bacteria were removed, and infected cells were incubated for an additional 12 hr at 37ºC. The number of bacteria per vacuole 13 hpi was visually determined for at least 300 vacuoles per strain. *, $p = 0.013$ (t test).

Results in (B) and (C) represent the mean ± SD of two independent experiments.

requires the combined local activity of SidM and LidA on the beads.

To confirm that SidM and LidA specifically target vesicles of the early secretory pathway, we determined the protein composition of vesicles bound to SidM + LidA-coated beads by Western blot analysis (Figure 6G). Proteins of the secretory pathway such as Rab1, Syntaxin-5, Sec22, or p115 were associated with beads coated with SidM + LidA, whereas Rab5 (a marker for early endosomes), βCOP (a component of the COPI protein coat complex), or GM130 (a Golgi-resident tethering factor) were almost undetectable. Taken together, these studies show that SidM and LidA combine to specifically intercept host cell vesicles of the Rab1-regulated early secretory pathway.

Discussion

Shortly after internalization by macrophages, *L. pneumophila* recruits ER-derived membrane components as well as the host GTPase Rab1 to its surrounding vacuole. Here, we show that two secreted effector proteins from *L. pneumophila* modulate Rab1 function and promote binding of host cell vesicles.

To our knowledge, SidM and LidA are the first translocated effectors encoded by intravacuolar pathogens shown to target a host cell GTPase of the Rab family. Furthermore, to our knowledge, SidM is the only GEF from any source known to act on mammalian Rab1, the key regulator of ER-to-Golgi trafficking. SidM stimulates nucleotide exchange with much higher efficiency

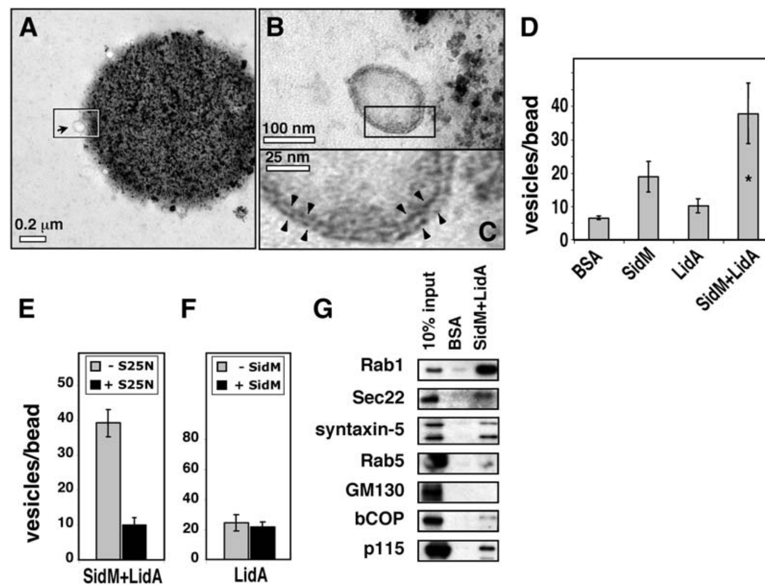

Figure 6. SidM and LidA Synergize during Recruitment of Early Secretory Vesicles

Paramagnetic beads coated with SidM, LidA, or both proteins were incubated with PNS from U937 cells. Beads were harvested, fixed, and prepared as described (Experimental Procedures), and cut sections were analyzed by transmission electron microscopy (TEM).

(A) Example of a vesicle (arrow) bound to the surface of a SidM-coated bead.

(B and C) Magnification of the vesicle shown in (A). The lipid bilayer of the vesicle is indicated by arrowheads (C).

(D) Quantification of vesicles from U937 cell lysate associated with beads coated with the indicated proteins.

(E) Effect of Rab1(S25N) on vesicle recruitment by SidM + LidA-coated beads. Paramagnetic beads coated with both SidM and LidA were incubated with a molar excess of recombinant Rab1(S25N) prior to incubation with PNS from U937 cells. Vesicle recruitment efficiency was determined by TEM as described above.

(F) Effect of soluble recombinant SidM on vesicle recruitment to LidA-coated beads. Paramagnetic beads coated with LidA were incubated with PNS from U937 cells supplemented with purified recombinant SidM (1 nM). Vesicle recruitment efficiency was determined by TEM as described above.

(G) Western blot analysis of SidM + LidA-coated beads after incubation with PNS of U937 cells with antibodies specific for the indicated proteins. Results in panels (D–F) are the mean ± SD of two independent experiments.

than that observed for other bacterially encoded exchange factors such as the Rac1-GEF SopE (Hardt et al., 1998) or the Arf-GEF RalF (Nagai et al., 2002). To avoid trafficking into the degradative pathway, *L. pneumophila* modifies host cell signaling processes immediately after uptake by the host (Roy et al., 1998). The high activity of SidM may allow the bacterium to compete with a host GEF for Rab1, or to accelerate the slow intrinsic activation of Rab1 by cellular GEFs, leading to efficient recruitment of Rab1 to the LCV.

The analysis of Rab1 colocalization with LCVs showed that vacuoles containing *L. pneumophila* Δ*sidM* mutants were defective for Rab1 recruitment at each time point examined, demonstrating that Rab1 recruitment is dependent on SidM. Mutations in *lidA* resulted in a delay in large-scale Rab1 recruitment compared to wild-type *L. pneumophila* (Figure 5B), consistent with a complementary role of LidA during SidM-mediated Rab1 recruitment. LidA may accelerate SidM-promoted accumulation of Rab1 about the LCV in a number of fashions. LidA could (1) inhibit the intrinsic GTP hydrolysis activity of Rab1, (2) interfere with the binding and inactivation of GTP-Rab1 by GAPs, or (3) bind GDP-Rab1 after GTP hydrolysis, thereby preventing membrane extraction of GDP-Rab1 by GDP dissociation inhibitors (GDIs). The mammalian Rabex-5/Rabaptin-5 complex involved in the endocytic pathway provides a precedent for SidM/LidA cooperation (Horiuchi et al., 1997; McBride et al., 1999). Rabex-5 catalyzes Rab5 GDP/GTP exchange and forms a complex with Rabaptin-5 (Horiuchi et al., 1997). Rabaptin-5 stabilizes GTP-Rab5 and promotes binding to the tethering factor early endosome antigen (EEA) 1 (McBride et al., 1999).

LidA may have many roles in supporting *L. pneumophila* intracellular replication, since it is able to bind three known Rab proteins (Figure 4C). The observation that this protein cooperates with SidM to promote Rab1-dependent vesicle binding to synthetic beads indicates that one of these roles may involve vesicle recruitment to the LCV (Figure 6). Several studies predict a regulatory role for Rab1 in tethering or docking of vesicles during ER-to-Golgi transport, possibly through the interaction of active GTP-Rab1 with proteins in the donor and/or target membrane, such as the tethering protein p115 (Allan et al., 2000). Rab1 may play a similar role during SidM/LidA-induced vesicle binding. LidA presumably supports vesicle binding only in the presence of SidM because, in the absence of SidM, there is not sufficient activated Rab1 available to allow vesicle binding in our system. Consistent with this, a GDP-locked Rab1 mutant efficiently blocked vesicle association with protein-coated beads, indicating that active Rab1 is directly involved (Figure 6E). It should be noted that although vesicle binding by beads was largely dependent on Rab1, some vesicle association was probably independent of Rab1 function, since beads coated solely with SidM showed higher vesicle binding efficiencies than control beads (Figure 6D). SidM probably recognizes a vesicle-associated factor in addition to Rab1, since Rab1 binding by SidM is only transient and is limited to the inactive form of Rab1 (Figure 2E).

As Rab1 is involved in recruitment of early secretory vesicles to the LCV, it may seem unexpected that a Δ*sidM* mutant showed no intracellular growth defect in BMMs, while mutants lacking LidA were reduced in the formation of mature replicative vacuoles in BMMs (Figure 5) (Conover et al., 2003). These observations most likely reflect the recent discovery that several host trafficking pathways deliver membrane material to the LCV, and that elimination of only one of these trafficking pathways by RNA interference is not sufficient to cause severe defects in intracellular replication of *L. pneumophila* in *Drosophila* cells (Dorer et al., 2006). The contribution of multiple pathways must be

considered when analyzing mutations affecting SidM and LidA. SidM exclusively binds Rab1 (Figure 1C), whereas LidA also interacts with Rab6 and Rab8 (Figure 4C), two GTPases that regulate the transport of vesicles from the Golgi complex to the ER and to the plasma membrane, respectively (Huber et al., 1993; White et al., 1999). Multiple sites of action for LidA may be the reason why a mutation affecting this protein caused a growth defect in BMMs, and they may also increase the likelihood that additional disruptions in the secretory pathway could further depress intracellular growth of this mutant. In support of this, RNA interference studies indicate that the intracellular growth of a Δ*lidA* mutant can be further reduced when this strain is introduced into *Drosophila* cells depleted for single proteins involved in vesicle trafficking between the ER and the Golgi (M.S. Dorer and R.R.I., unpublished data). *L. pneumophila* Δ*sidM* mutants, on the other hand, were much more tolerant of disruption of individual membrane trafficking pathways in these studies. Thus, compensatory trafficking pathways exist that may mask defects caused by individual mutations in *sidM* or other effector proteins of *L. pneumophila*, and, as a result, simultaneous disruption of several different host cell membrane transport steps may be required to observe a depression in intracellular growth.

Taken together, our data show that *L. pneumophila* secretes molecular mimics of host cell proteins involved in vesicle trafficking that facilitate integration of the LCV into the secretory pathway. Although SidM and LidA from *L. pneumophila* are the first, to our knowledge, bacterial effector proteins known to target host cell Rab GTPases in this particular fashion, it seems likely that other intravacuolar pathogens have developed similar strategies for utilizing Rab GTPases for their survival in host cells. Further analyses of the molecular function of SidM and LidA and pathways for vesicle recruitment by *L. pneumophila* should provide detailed insight into how pathogens hijack host cell secretory traffic.

Experimental Procedures

Strains, Media, and Plasmids

L. pneumophila strains were grown and maintained as described (Feeley et al., 1979; Gabay et al., 1985). *L. pneumophila* strains Lp02 (*thyA hsdR rpsL*) and Lp03 (Lp02 *dotA3*) are thymine-auxotroph derivatives of Philadelphia-1 (Berger and Isberg, 1993; Berger et al., 1994). The strain Lp02Δ*lidA* was a kind gift of Dr. Zhao-Qing Luo (Purdue University, Indiana). Lp02Δ*sidM* harboring an in-frame deletion in *sidM* was constructed as described previously (Luo and Isberg, 2004; Dumenil and Isberg, 2001).

Plasmids for production of recombinant SidM (pGEX-*sidM*), human Rab1A (pGEX-*rab1*), or human Rab2 (pGEX-*rab2*) in *E. coli* were generated by subcloning PCR fragments into pGEX-6P-1 (Amersham Pharmacia). Rab1A mutants Rab1(S25N) and Rab1(Q70L) were generated by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The complementation plasmid p*sidM* for production of SidM in *L. pneumophila* under the control of its native promoter was created by subcloning a PCR fragment containing the *sidM* open reading frame and its upstream promoter sequence into pJB908 (J. Vogel, Washington University School of Medicine, St. Louis). The plasmid p*lidA* for production of LidA in *L. pneumophila* under the control of the *sidM* promoter was created by digesting p*sidM* with XhoI and SphI, followed by ligation with a XhoI/SphI-digested *lidA* PCR product generated with primers 5′-AATCCTCG AGTACATGGCAAAAGATAACAAATCAC-3′ and 5′-TTGAGCATGCG GTGGGGAGGGGTAGAGC-3′ that encode full-length LidA. pEGFP-*sidM* for production of green fluorescent protein (GFP)-labeled SidM in COS1 cells was created by subcloning a *sidM* PCR fragment into pEGFP-C1 digested with BglII/SalI (Clontech).

Bone marrow-derived macrophages (BMMs) were prepared and cultured as described previously (Celada et al., 1984; Swanson and Isberg, 1995). Macrophage-like U937 cells were cultured as described elsewhere (Alrutz and Isberg, 1998; Berger and Isberg, 1993). Antibodies were purchased from Santa Cruz Biotechnology (antibodies against Rab1B, Rab2A, Rab4, Rab5A, Rab6, Rab7, Rab8B, and Sec23), Transduction Laboratories (anti-Rab11), or Calbiochem (anti-GM130). Antibodies against Sec22b and syntaxin-5 were kindly provided by Jesse Hay, University of Montana, and antibody against p115 was a kind gift of G. Watters, Merck, Inc.

Recombinant Protein Production and Purification

LidA was purified as described previously (Conover et al., 2003). SidM, Rab1, Rab1(S25N), Rab1(Q70L), and Rab2 were produced as GST fusion proteins in *E. coli* BL21(DE3) (Stratagene). For protein production, bacteria were grown at 37ºC in LB medium (100 μg/ml ampicillin) to an OD_{600} of 0.5, shifted to 20ºC, induced with 0.5 mM isopropylthio-*D*-galactopyranoside (IPTG), and cultivated for an additional 18 hr at 20ºC. Cells were harvested and lysed by French press, and soluble proteins were incubated with glutathione Sepharose resin (Amersham Pharmacia) equilibrated with PreScission Protease cleavage buffer (50 mM Tris/HCl, [pH 7.0], 150 mM NaCl, 1 mM DTT) to separate recombinant GST-tagged proteins from bacterial proteins. The GST tag was removed by incubation with PreScission Protease (100 U) (Amersham Pharmacia) for 2 days at 4ºC, and recombinant proteins were eluted with PBS. If required, GST-tagged Rab1 and Rab2 were washed with cleavage buffer + EDTA (20 mM) prior to the addition of PreScision Protease to remove protein bound guanosine nucleotides.

Immunofluorescence Microscopy

Immunofluorescence microscopy studies on BMMs were performed and processed as described (Conover et al., 2003; Swanson and Isberg, 1995). LidA (Conover et al., 2003) and SidM were labeled by using affinity-purified polyclonal antibody followed by FITC-conjugated goat anti-rabbit antibody (Zymed). Rabbit anti-SidM antiserum was generated by immunizing a rabbit with purified recombinant full-length SidM according to standard protocols (Pocono Rabbit Farm and Laboratory). Rab1 was detected by affinity-purified rabbit anti-Rab1B antibody (sc-599, Santa Cruz Biotechnology).

Postnuclear supernatants (PNSs) from infected U937 cells were prepared, and the integrity of the vacuolar membranes was confirmed as described (Derré and Isberg, 2004).

Affinity Chromatography from Cell Lysates

Purified recombinant LidA was immobilized on Affigel beads according to the manufacturer's recommendation (BioRad). U937 cells were resuspended in ice-cold lysis buffer (PBS, 5 mM DTT, protease inhibitor cocktail [Roche]) and lysed in a Dounce homogenizer. Unbroken cells and nuclei were pelleted by centrifugation at 10,000 × g for 10 min at 4ºC. The PNS was added to beads and incubated for 12 hr at 4ºC. Beads were washed five times with lysis buffer, resuspended in SDS sample buffer, and analyzed by SDS-PAGE and silver staining (BioRad) or by Western blot analysis. Individual protein gel bands were isolated, digested with trypsin, and analyzed by high-performance liquid chromatography/mass spectrometry (HPLC/MS). To isolate Rab1 binding proteins, a culture of *L. pneumophila* Δ*lidA* was grown to postexponential phase and lysed by French Press, and soluble proteins were incubated for 12 hr at 4ºC with glutathione Sepharose beads linked to noted proteins. Bound proteins were identified as described above.

Assay for Binding of Purified Proteins

Microtiter plates (96-well, Linbro/Titertek) were coated overnight at 4ºC with purified LidA (2 μg/well) and blocked for 1 hr with 1% BSA in PBS prior to probing with biotinylated Rab1(S25N) or Rab1(Q70L) for 4 hr at room temperature. Wells were washed five times with PBS and incubated for 1 hr with streptavidin conjugated with alkaline phosphatase (Zymed) and were analyzed as described (Leong et al., 1995).

For coimmunoprecipitation of Rab1 with SidM, 50 pmol GDP-Rab1 was incubated for 30 min at room temperature in reaction

buffer (PBS, 15 mM $MgCl_2$, 100 μl total volume) in the presence or absence of equimolar amounts of SidM and 1 mM GTPγS. The reaction was transferred to 4ºC and incubated with Affiprep beads (Biorad) coated with antibody directed against SidM. After 1 hr of incubation, the supernatant containing unbound proteins was removed, and beads were washed five times in reaction buffer and resuspended in SDS sample buffer. Rab1 in the supernatant (SN) or bound to bead-immobilized proteins was detected by Western blot analysis by using polyclonal anti-Rab1 antibody (Calbiochem).

GDP/GTP Exchange Reaction

Proteins were dialyzed against loading buffer (Rab1) or reaction buffer (SidM), and all guanine nucleotide exchange experiments were performed at room temperature. For the [^{3}H]GDP release assay, 50 pmol EDTA-washed, nucleotide-free Rab1 was incubated for 2 hr with 1 nmol [^{3}H]GDP in loading buffer (50 mM Tris/HCl [pH 7.5], 50 mM NaCl, 1 mM DTT, 0.1 mM EDTA). Nucleotide binding was stabilized by the addition of $MgCl_2$ (5 mM final concentration), and SidM was added to the reaction along with 10 nmol unlabeled GTP. Aliquots of the reaction were removed at indicated time points and passed through nitrocellulose membrane filters (HAWPO2500, Millipore). Filters were washed twice with 1 ml reaction buffer (50 mM Tris/HCl [pH 7.5], 1 mM DTT, 50 mM NaCl, 5 mM $MgCl_2$), transferred to scintillation vials containing 8 ml scintillation fluid (Fisher Biotech), and analyzed in a scintillation counter.

For [γ^{35}S]GTP incorporation assays, EDTA-washed, nucleotide-free Rab1 (50 pmol) was loaded with unlabeled GDP (5 mM) for 2 hr in loading buffer. Nucleotide binding was stabilized by the addition of $MgCl_2$ (5 mM final concentration), and nucleotide exchange was started by transferring GDP-Rab1 into loading buffer plus 5 mM $MgCl_2$ containing 50 pmol indicated proteins in the presence of 1 nmol [γ^{35}S]GTP. Aliquots of the reaction were removed and analyzed by scintillation counting as described above.

Vesicle Binding Assays

Paramagnetic beads (Dynal) were coated with recombinant SidM, LidA, or both proteins according to the manufacturer's recommendation. U937 cells were mechanically lysed in PBS (250 mM sucrose, 0.5 mM DTT, protease inhibitor cocktail tablet [Roche]), followed by centrifugation for 10 min at 10.000 × g to generate PNS. A total of 1 × 10^7 beads were incubated for at least 4 hr at 4ºC with PNS (containing 1 nM soluble SidM when indicated), pelleted, and processed for electron microscopy (Li et al., 2005). For immunological analysis of vesicle composition, Affigel beads coated with SidM, LidA, or both proteins were incubated for 5 hr at 4ºC with PNS (prepared as described above) of U937 cells. Beads were pelleted by centrifugation (<200 × g), washed three times with PBS (250 mM sucrose, 0.5 mM DTT, protease inhibitor), and boiled in sample buffer, and proteins retained by the beads were analyzed by SDS-PAGE and Western blot analysis. If indicated, beads coated with SidM + LidA or with LidA alone were incubated for 2 hr at 4ºC with purified Rab1(S25N) (50 μM) prior to incubation with PNS.

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