The *Legionella pneumophila* phosphatidylinositol-4 phosphate-binding type IV substrate SidC recruits endoplasmic reticulum vesicles to a replication-permissive vacuole

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Summary

*Legionella pneumophila*, the causative agent of Legionnaires’ disease, uses the intracellular multiplication/defective organelle trafficking (Icm/Dot) type IV secretion system to establish within amoebae and macrophages an endoplasmic reticulum (ER)-derived replication-permissive compartment, the *Legionella*-containing vacuole (LCV). The Icm/Dot substrate SidC and its parologue SdcA anchor to LCVs via phosphatidylinositol-4-phosphate [PtdIns(4)P]. Here we identify the unique 20 kDa PtdIns(4)P-binding domain of SidC, which upon heterologous expression in *Dictyostelium* binds to LCVs and thus is useful as a PtdIns(4)P-specific probe. LCVs harbouring *L. pneumophila*ΔsidC-sdcA mutant bacteria recruit ER and ER-derived vesicles less efficiently and carry endosomal but not lysosomal markers. The phenotypes are complemented by supplying sidC on a plasmid. *L. pneumophila*ΔsidC-sdcA grows at wild-type rate in calnexin-negative LCVs, suggesting that communication with the ER is dispensable for establishing a replicative compartment. The amount of SidC and calnexin is directly proportional on isolated LCVs, and in a cell-free system, the recruitment of calnexin-positive vesicles to LCVs harbouring ΔsidC-sdcA mutant bacteria is impaired. Beads coated with purified SidC or its 70 kDa N-terminal fragment recruit ER vesicles in *Dictyostelium* and macrophage lysates. Our results establish SidC as an *L. pneumophila* effector protein, which anchors to PtdIns(4)P on LCVs and recruits ER vesicles to a replication-permissive vacuole.

Introduction

The Gram-negative bacterium *Legionella pneumophila* parasitizes various species of environmental predatory amoebae, including the social amoeba *Dictyostelium discoideum* (Fields *et al.*, 2002; Steinert and Heuner, 2005; Hilbi *et al.*, 2007). *L. pneumophila* is an accidental human pathogen, which is transmitted only via contaminated aerosols but not between infected humans, where it may cause the severe pneumonia Legionnaires’ disease. The bacteria replicate in alveolar macrophages (Nash *et al.*, 1984), using a mechanism likely adopted through their long-standing evolutionary interaction with amoebae (Greub and Raoult, 2004; Molmeret *et al.*, 2005; Hilbi *et al.*, 2007). An intimate relationship between *L. pneumophila* and its phagocytic host cells is further reflected by the presence of many eukaryotic-like genes in the genome (Cazalet *et al.*, 2004; Chien *et al.*, 2004; de Felipe *et al.*, 2005; Brüggemann *et al.*, 2006).

Within macrophages and amoebae, *L. pneumophila* forms a replicative compartment, the *Legionella*-containing vacuole (LCV). LCVs avoid fusion with lysosomes (Horwitz, 1983a), and instead intercept the early secretory trafficking pathway at endoplasmic reticulum (ER) exit sites (Kagan and Roy, 2002) and other trafficking routes (Dorer *et al.*, 2006). In the course of their maturation, LCVs associate and fuse with ER-derived vesicles, followed by attachment to and fusion with ER in macrophages (Horwitz, 1983b; Swanson and Isberg, 1995; Tilney *et al.*, 2001; Derre and Isberg, 2004; Robinson and Roy, 2006) and *D. discoideum* (Solomon *et al.*, 2000; Lu and Clarke, 2005). Thus, LCVs accumulate the early secretory v-SNARE Sec22b, the resident ER protein calnexin and the cytoplasmic small GTPases Arf1 and Rab1, the activity of which is required for trafficking between the ER and the Golgi, as well as for the formation of the replicative niche of *L. pneumophila* (Kagan and Roy, 2002; Derre and Isberg, 2004; Kagan *et al.*, 2004).

The formation of LCVs in macrophages and amoebae depends on a type IV secretion system (T4SS), the intracellular multiplication (Icm)/defective organelle trafficking (Dot) apparatus (Segal *et al.*, 1998; Vogel *et al.*, 1998; Segal and Shuman, 1999; Solomon *et al.*, 2000;
Otto et al., 2004). The Icm/Dot T4SS also modulates the phosphoinositide-3 kinase-independent uptake of L. pneumophila (Hilbi et al., 2001; Khelef et al., 2001; Watarai et al., 2001; Weber et al., 2006). More than 50 Icm/Dot substrates ("effector" proteins) have been identified, some of which interfere with host cell processes by subverting GTPases, phosphoinositide metabolism or apoptotic pathways (Segal et al., 2005; Brüggemann et al., 2006; Hilbi, 2006; Ninio and Roy, 2007).

Two Icm/Dot substrates have been characterized as guanine nucleotide exchange factors (GEFs) for small eukaryotic GTPases. RaIF recruits the host GTPase Arf1 to LCVs and acts as a GEF for the Arf GTPase family (Nagai et al., 2002). SidM/DrrA is a Rab1 GEF and recruits this small GTPase to LCVs (Machner and Isberg, 2006; Murata et al., 2006). Interestingly, SidM/DrrA also functions as a GDP dissociation inhibitor displacement factor, whose activity is required to activate Rab1 (Ingmundson et al., 2007; Machner and Isberg, 2007). The Icm/Dot-translocated effector protein LidA also localizes to LCVs, where it binds to GDP dissociation inhibitor-free Rab1 and thus synergizes with SidM/DrrA to promote the recruitment of early secretory vesicles (Conover et al., 2003; Derre and Isberg, 2005; Machner and Isberg, 2006; 2007; Murata et al., 2006).

LepA and LepB, two Icm/Dot substrates that show weak similarity to eukaryotic SNAREs and tethering factors are dispensable for intracellular replication. However, these effectors seem to promote the non-lytic egress of L. pneumophila from amoebae (but not macrophages) via a novel pathway, leading to bacteria-filled respirable vesicles (Chen et al., 2004; 2007). LepB recently was found to have GTPase-activating protein activity for Rab1, thus contributing to Rab1 membrane cycling as an antagonist of SidM/DrrA (Ingmundson et al., 2007).

SdhA and its two paralogues are required for intracellular replication in macrophages and, to a much lower extent, in D. discoideum (Laguna et al., 2006). These effectors do not affect the formation of LCVs, but prevent a mitochondria-dependent apoptotic pathway in macrophages infected by L. pneumophila. Thus, in absence of the effectors, intracellular replication of L. pneumophila is attenuated due to a "premature" killing of the host cell. Another effector modulating host cell apoptosis is SidF, which by binding to and inactivating pro-apoptotic members of the mitochondrial Bcl-2 family inhibits the cell death programme (Bang et al., 2007).

Some Icm/Dot substrates targeting host cell lipids have also been characterized. VipD is a putative phospholipase and a member of a family of four paralogues that inhibit trafficking in yeast and assist in the establishment of LCVs (Ninia et al., 2005; Shohdy et al., 2005). SidC and its parologue SdcA localize to the LCV membrane (Luo and Isberg, 2004), where the proteins specifically bind to the host cell lipid phosphatidylinositol-4 phosphate (PtdIns(4)P) (Weber et al., 2006). Phosphoinositides are pivotal players in eukaryotic receptor-mediated signal transduction, actin remodelling and membrane dynamics (De Matteis and Godi, 2004; Di Paolo and De Camilli, 2006). The inositol headgroup of these lipids can be reversibly phosphorylated at three different positions, leading to seven different molecules, which recruit specific effector proteins to distinct membranes. PtdIns(4)P is found on the cytoplasmic membrane, but localizes preferentially to the trans Golgi network.

Here we analyse the recruitment and functions of the PtdIns(4)P-binding Icm/Dot substrate SidC. We identify a novel 20 kDa PtdIns(4)P-binding domain and a 70 kDa N-terminal fragment that binds calnexin-positive vesicles. LCVs harbouring ΔsidC-sdcA mutant L. pneumophila are characterized by endosomal but not lysosomal markers and are replication-permissive without acquiring ER or ER-derived vesicles.

Results

Identification of the PtdIns(4)P-binding domain of SidC

The 106 kDa protein SidC selectively binds PtdIns(4)P in vitro (Weber et al., 2006). To map the PtdIns(4)P-binding domain, we produced and affinity-purified N-terminal GST fusion proteins of SidC fragments (Fig. 1A). In protein-lipid overlay experiments a 36 kDa C-terminal fragment of SidC (SidC_{609-917}), as well as a 20 kDa part of this fragment (SidC_{609-776}), specifically bound to PtdIns(4)P spotted onto nitrocellulose membranes (Fig. 1B). Compared with full-length SidC, the binding affinity of SidC_{609-917} and SidC_{609-776} to PtdIns(4)P appeared to be higher. The SidC_{609-776} fragment is predicted to adopt a largely helical conformation (> 80%) but does not share any homology with known eukaryotic phosphoinositide-binding domains. Thus, the 20 kDa SidC_{609-776} fragment represents a unique prokaryotic PtdIns(4)P-binding domain, which we term ‘P4C’ (PtdIns(4)P-binding of SidC).

Larger C-terminal fragments of SidC (56, 73 kDa) also specifically bound to PtdIns(4)P (data not shown). In contrast, the 70 kDa N-terminal fragment of SidC (SidC_{1-608}) or the 16 kDa C-terminal fragment (SidC_{777-917}) did not bind to any of the immobilized phosphoinositides (Fig. 1B; data not shown). A 50- to 100-fold weaker binding of the 36 kDa SidC_{609-917} fragment and the 20 kDa SidC_{P4C} (SidC_{609-776}) fragment to PtdIns(3)P was also observed, matching the binding characteristics of full-length SidC (Weber et al., 2006). Pulldown assays using phospholipid (PL) vesicles containing 5% of the individual phosphoinositides revealed that SidC_{609-917} and SidC_{P4C} preferentially bind to PtdIns(4)P, much weaker to PtdIns(3)P and...
Fig. 1. Identification of the PtdIns(4)P-binding domain of SidC.

A. Map of the 106 kDa Icm/Dot T4SS substrate SidC. Numbers indicate the amino acids delineating a predicted coiled-coil domain ('CC', amino acids 76–128), N-terminal 70 kDa fragment (1–608), 20 kDa PtdIns(4)P-binding domain ('P4C', 609–776) and a 36 kDa translocated fragment (609–917).

B. Protein-lipid overlays with phosphoinositides immobilized on nitrocellulose membranes (twofold serial dilutions, 100 – 1.56 pmol).

C. Pulldown assays with PL vesicles (20 μl, 1 mM lipid) containing 5% (1 nmol) PtdIns(4)P, PtdIns(3)P or PtdIns were performed using affinity-purified GST fusion proteins of SidC609-917, SidC609-776 and SidC1-608 (200 pmol). Binding was visualized by Western blot with an anti-GST antibody.

D. Confocal laser scanning micrographs of *D. discoideum* strain AX3 constitutively expressing GFP–SidCP4C infected at an MOI of 50 for 1 h with *L. pneumophila* wild-type JR32, ΔsidC-sdcA or ΔicmT mutant strains producing the red fluorescent protein DsRed (Mampel et al., 2006). Bar, 2 μm. The data shown are representative of approximately 200 LCVs analysed in three independent experiments.
not at all to PtdIns, while the N-terminal SidC\textsubscript{1-608} fragment did not bind to any of the vesicles (Fig. 1C).

Upon constitutive ectopic expression in \textit{D. discoideum}, the 20 kDa SidC\textsubscript{P4C} fragment fused to GFP bound to approximately 80% of the LCVs in amoeba infected with either wild-type or \textbullet{\textit{\textbullet}}\textsubscript{sidC-sdcA} \textit{L. pneumophila} (Fig. 1D). Ectopically produced full-length GFP–SidC also colocalized with LCVs in \textit{D. discoideum} infected with wild-type \textit{L. pneumophila} (data not shown). In \textit{D. discoideum} infected with \textit{L. pneumophila} \textbullet{\textit{\textbullet}}icmT, the GFP–SidC\textsubscript{P4C} fragment never localized to bacteria-containing vacuoles, but rather accumulated on the plasma membrane. Similarly, in uninfected amoeba the GFP–SidC\textsubscript{P4C} fragment localized preferentially to the plasma membrane, suggesting that this membrane contains PtdIns(4)\textsubscript{P}. As PtdIns(4)\textsubscript{P} is a lipid component of LCVs harbouring wild-type but not \textbullet{\textit{\textbullet}}icmT mutant \textit{L. pneumophila} (Weber \textit{et al}, 2006), these results indicate that SidC\textsubscript{P4C} binds to PtdIns(4)\textsubscript{P} not only in vitro but also \textit{in vivo}.

In summary, we identified a novel prokaryotic protein domain that specifically binds PtdIns(4)\textsubscript{P} \textit{in vitro} and \textit{in vivo}. The SidC\textsubscript{P4C} fragment can be easily produced in high quantities in \textit{Escherichia coli} and similarly expressed in eukaryotic cells. Thus, this protein is useful as a PtdIns(4)\textsubscript{P} ologous probe in cell biological and biochemical experiments.

\textit{L. pneumophila} \textbullet{\textit{\textbullet}}sidC-sdcA is defective for the recruitment of calnexin-GFP to LCVs

To analyse the function of \textit{L. pneumophila} SidC, the corresponding gene and its adjacent parologue sdcA were deleted from the chromosome by double homologous recombination. The \textbullet{\textit{\textbullet}}sidC-sdcA deletion mutant (strain CR01) replicated in AYE broth at the same rate as wild-type \textit{L. pneumophila} and was not impaired for efficient phagocytosis by \textit{D. discoideum} or for intracellular replication in \textit{D. discoideum}, \textit{Acanthamoeba castellanii} and macrophages (Fig. S1; Luo and Isberg, 2004).

During maturation LCVs associate with ER and acquire the ER resident protein calnexin-GFP (Lu and Clarke, 2005). To determine whether SidC and SdcA are required for the interaction with the ER, we infected calnexin-GFP producing \textit{D. discoideum} with \textit{L. pneumophila} \textbullet{\textit{\textbullet}}sidC-sdcA and visually scored for calnexin-positive LCVs (Fig. 2A). While in \textit{D. discoideum} infected for 1 h with wild-type \textit{L. pneumophila} more than 70% of the LCVs stained positive for calnexin-GFP, less than 30% of LCVs harbouring the \textbullet{\textit{\textbullet}}sidC-sdcA mutant strain acquired the ER marker protein under the same conditions (Fig. 2B). These results indicate that SidC and SdcA promote the acquisition of calnexin-positive ER by LCVs.

The recruitment of calnexin-GFP to LCVs was restored to wild-type levels by plasmid-encoded SidC or SdcA, indicating that the proteins are functionally redundant (Fig. 2B). The overexpression of SidC from a multi-copy plasmid yielded even a slightly higher percentage of calnexin-positive LCVs compared with \textit{D. discoideum} infected with wild-type \textit{L. pneumophila} harbouring the vector control. This finding corresponds to the significant higher amount of SidC produced in \textit{L. pneumophila} strains harbouring a SidC-encoding plasmid (Fig. S2).

The M45-SidC\textsubscript{609-917} fragment produced in a \textbullet{\textit{\textbullet}}sidC-sdcA mutant strain was still translocated into \textit{D. discoideum} and bound to the LCV membrane (Fig. 2), in agreement with the notion that Icm/Dot substrates share a C-terminal secretion/translocation signal (Nagai \textit{et al}., 2005). However, the production of SidC\textsubscript{609-917} did not complement the \textbullet{\textit{\textbullet}}sidC-sdcA mutant with regard to the recruitment of calnexin-positive ER (Fig. 2B), indicating a functional role in this process for the N-terminal fragment SidC\textsubscript{1-608} (see below). As expected, the translocation of the M45-SidC\textsubscript{609-917} fragment required a functional Icm/Dot T4SS and did not occur in a \textbullet{\textit{\textbullet}}icmT mutant strain (data not shown).

To assess whether in absence of \textit{sidC} and \textit{sdcA} calnexin is recruited to LCVs more slowly and/or in lower amounts, the kinetics of calnexin-GFP recruitment to LCVs were followed during a 4 h infection (Fig. 2C). Within 2 h post infection, a maximum of approximately 80% calnexin-GFP-positive LCVs harbouring wild-type \textit{L. pneumophila} was reached, which stayed constant for at least another 2 h. In contrast, within 2 h only 20% of LCVs harbouring \textbullet{\textit{\textbullet}}sidC-sdcA mutant bacteria turned calnexin-GFP-positive, and this low level did not increase throughout the infection. These results indicate that not only the acquisition rate but also the total amount of calnexin-GFP on LCVs are lower in absence of \textit{sidC} and \textit{sdcA}.

\textit{L. pneumophila} \textbullet{\textit{\textbullet}}sidC-sdcA is defective for the transition from ‘tight’ to ‘spacious’ LCVs

Calnexin-positive LCVs undergo a morphological transition from ‘tight’ to ‘spacious’ vacuoles (Fig. 3A), where the vacuolar membrane is detached from the ER (Li \textit{et al}., 2005; Lu and Clarke, 2005; Weber \textit{et al}., 2006). To correlate LCV dynamics with the presence of \textit{sidC} and/or \textit{sdcA}, the morphology of calnexin-GFP-positive LCVs was assessed in \textit{D. discoideum} infected with DsRed-labelled \textit{L. pneumophila} (Fig. 3B). One hour post infection 33% spacious LCVs harbouring wild-type \textit{L. pneumophila} were scored, compared with only 13% spacious LCVs harbouring the \textbullet{\textit{\textbullet}}sidC-sdcA mutant strain, indicating that SidC and SdcA not only promote the acquisition of the resident ER protein calnexin but subsequently also affect the dynamics of calnexin-positive LCVs. The transition phenotype was
**Fig. 2.** *L. pneumophila* ΔsidC-sdcA is defective for the recruitment of calnexin-GFP to LCVs.

A. Confocal laser scanning micrographs of calnexin-GFP-labelled *D. discoideum* AX3 (green), infected at an MOI of 50 for 1 h with *L. pneumophila* wild-type strain JR32 labelled with a serogroup-specific antibody (blue) and immuno-stained for SidC/SdcA with an anti-M45 antibody (red). Bar, 2 μm.

B. Quantification of calnexin-GFP recruitment to LCVs in *D. discoideum* infected with *L. pneumophila* JR32, ΔsidC-sdcA or ΔicmT harbouring plasmid pCR39 (vector), pCR34 (M45-SidC), pCR78 (M45-SdcA) or pCR55 (M45-SidC<sub>609-917</sub>). **P < 10<sup>-4</sup>.

C. Kinetics of calnexin-GFP recruitment to LCVs in *D. discoideum* infected at an MOI of 50 with *L. pneumophila* JR32 (▲) or ΔsidC-sdcA (●). The data shown are the means and standard deviations of three (B) or two (C) independent experiments.
containing question and characterize membrane markers of LCVs containing wild-type acquire other organelle markers differentially than LCVs lack the resident ER protein calnexin and thus might LCVs containing D. discoideum but not lysosomal markers L. pneumophila (see below).

Taken together, compared with LCVs containing wild-type bacteria, LCVs harbouring \( \Delta sidC-sdcA \) mutant strain were defective for the acquisition of calnexin, as documented above (Fig. 2).

Similar results were obtained for LCVs in RAW264.7 cells, a murine macrophage-like cell line permissive for intracellular replication of \( L.\) pneumophila (Fields, 1996; Albers et al., 2005), which after infection was stained for the lysosomal marker LAMP-1 or Rab1, a marker of ER and ER-derived vesicles (Fig. 4C). While only 20% LCVs containing either \( \Delta sidC-sdcA \) or wild-type \( L.\) pneumophila acquired LAMP-1, more than 60% of LCVs harbouring either strain were positive for Rab1 (Fig. 4D). In contrast, only 5% LCVs containing \( \Delta icmT \) mutant bacteria carried Rab1, while 60% of these LCVs were LAMP-1-positive. L. pneumophila \( \Delta sidC-sdcA \) is defective for the recruitment of GFP-HDEL to LCVs

In addition to calnexin-GFP, LCVs also acquire the ER retention signal GFP-HDEL, which labels the ER as well as ER exit vesicles (Lu and Clarke, 2005). Infections of GFP-HDEL-producing \( D.\) discoideum with \( L.\) pneumophila revealed that the mutant is impaired for the acquisition of the HDEL peptide (Fig. 5A). While more than 60% of LCVs harbouring wild-type \( L.\) pneumophila acquired the HDEL peptide within 2 h of infection, only approximately 30% of LCVs harbouring the \( \Delta sidC-sdcA \) mutant strain acquired the peptide within the same time frame, and the percentage of HDEL-positive LCVs remained at this lower level throughout a 6 h infection period (Fig. 5B). These results indicate that SidC and SdcA are Icm/Dot substrates promoting the acquisition of ER as well as ER-derived vesicles by LCVs.

L. pneumophila \( \Delta sidC-sdcA \) replicates at wild-type level in calnexin-negative LCVs

\( L.\) pneumophila \( \Delta sidC-sdcA \) replicates intracellularly at wild-type rate (Fig. S1; Luo and Isberg, 2004) and scored for LCVs positive for the lysosomal protein common antigen-1, the endosomal marker p80 and calnexin-GFP (Fig. 4A).

Interestingly, 1 h post infection approximately 80% of LCVs harbouring either wild-type or \( \Delta sidC-sdcA \) mutant \( L.\) pneumophila carried the endosomal marker p80 (Fig. 4B), a putative copper transporter (Ravanel et al., 2001). At the same time, the lysosomal marker common antigen-1 was absent from the majority of LCVs harbouring these strains, but was present on approximately 70% of LCVs containing \( \Delta icmT \) mutant bacteria. LCVs harbouring the \( \Delta sidC-sdcA \) mutant strain were defective for the acquisition of calnexin, as documented above (Fig. 2).

LCVs harbouring \( L.\) pneumophila \( \Delta sidC-sdcA \) carry endosomal but not lysosomal markers

LCVs containing \( \Delta sidC-sdcA \) mutant \( L.\) pneumophila lack the resident ER protein calnexin and thus might acquire other organelle markers differentially than LCVs containing wild-type \( L.\) pneumophila. To address this question and characterize membrane markers of LCVs containing \( \Delta sidC-sdcA \) mutant \( L.\) pneumophila, we used immuno-fluorescence and determined whether the LCVs acquire endosomal and lysosomal markers in \( D.\) discoideum or murine RAW264.7 macrophages. In addition to calnexin-GFP, LCVs also acquire the ER retention signal GFP-HDEL, which labels the ER as well as ER exit vesicles (Lu and Clarke, 2005). Infections of GFP-HDEL-producing \( D.\) discoideum with \( L.\) pneumophila revealed that the mutant is impaired for the acquisition of the HDEL peptide (Fig. 5A). While more than 60% of LCVs harbouring wild-type \( L.\) pneumophila acquired the HDEL peptide within 2 h of infection, only approximately 30% of LCVs harbouring the \( \Delta sidC-sdcA \) mutant strain acquired the peptide within the same time frame, and the percentage of HDEL-positive LCVs remained at this lower level throughout a 6 h infection period (Fig. 5B). These results indicate that SidC and SdcA are Icm/Dot substrates promoting the acquisition of ER as well as ER-derived vesicles by LCVs.

L. pneumophila \( \Delta sidC-sdcA \) replicates at wild-type level in calnexin-negative LCVs

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in LCVs apparently lacking calnexin to a large extent (Fig. 2). Therefore, the acquisition of ER does not seem to be required to form replication-permissive LCVs. To address this question, we used confocal fluorescence microscopy to correlate intracellular replication of L. pneumophila (defined as more than two bacteria per LCV) with the presence of calnexin on LCVs (Fig. 6A). Fourteen hours post infection, approximately 80% of D. discoideum infected with either wild-type or ΔsidC-sdcA mutant L. pneumophila were found to harbour replicating bacteria (data not shown). Interestingly, while the number of intracellularly replicating wild-type or ΔsidC-sdcA mutant L. pneumophila was similar, 85% of the growing wild-type bacteria were found in calnexin-positive vacuoles, while 80% of the replicating ΔsidC-sdcA mutant bacteria localized to calnexin-negative vacuoles (Fig. 6B). These results clearly indicate that the acquisition of calnexin, and as a corollary, the interaction with ER vesicles, are dispensable to form a vacuolar compartment, which is replication-permissive for L. pneumophila.
As the membrane integrity of LCVs might be compromised in absence of SidC and SdcA, we analysed by electron microscopy the appearance of LCVs harbouring \( \Delta \text{sidC-sdcA} \) mutant \( L. \text{pneumophila} \). We found that upon infection of \( D. \text{discoideum} \) (Fig. 7A) or murine RAW264.7 macrophages (Fig. 7B) the membrane of LCVs harbouring the mutant strain looked intact and morphologically similar to LCVs containing wild-type \( L. \text{pneumophila} \). Therefore, SidC and SdcA apparently do not play a role in maintaining the membrane integrity of LCVs.

Quantitative correlation of SidC and calnexin on LCVs in \( D. \text{discoideum} \) lysates

Next, we analysed the recruitment of calnexin to LCVs by SidC or SdcA in homogenates of calnexin-GFP-labelled \( D. \text{discoideum} \) infected with DsRed-labelled \( L. \text{pneumophila} \). To this end, the infected amoebae were lysed with a ball homogenizer, and the fluorescence intensity of calnexin-GFP and immuno-labelled SidC on LCVs was analysed by flow cytometry or by immuno-fluorescence microscopy (Fig. S3; Weber et al., 2006). Using flow cytometry, fewer DsRed\textsuperscript{high} LCVs were found to be GFP\textsuperscript{high} in the absence of \( \text{sidC} \) and \( \text{sdcA} \) (Fig. 8A). Using immuno-fluorescence microscopy, the average GFP fluorescence intensity of individual LCVs was found to be three to four times lower (\( P < 10^{-27} \)) compared with LCVs from \( D. \text{discoideum} \) infected with wild-type \( L. \text{pneumophila} \) (Fig. 8B). This defect in the recruitment of calnexin was observed throughout the course of a 4 h infection (data not shown), similar to what was observed with intact infected amoebae (Fig. 2C).

The presence or absence of SidC and SdcA did not affect the membrane integrity of LCVs in infected phagocytes (Fig. 7), but still might affect the stability of isolated LCVs. This proved to be not the case, as the percentage of calnexin-positive LCVs containing wild-type or \( \Delta \text{sidC-sdcA} \) mutant \( L. \text{pneumophila} \) was similar in intact infected amoebae.

**Fig. 5.** \( L. \text{pneumophila} \ \Delta \text{sidC-sdcA} \) is defective for the recruitment of GFP-HDEL to LCVs.

A. Confocal laser scanning micrographs of GFP-HDEL-labelled \( D. \text{discoideum} \) AX3 (green), infected at an MOI of 50 for 1 h with DsRed-labelled \( L. \text{pneumophila} \) wild-type strain JR32 (red). Bar, 2 \( \mu \text{m} \).

B. Kinetics of GFP-HDEL recruitment to LCVs in \( D. \text{discoideum} \) infected at an MOI of 50 with \( L. \text{pneumophila} \) JR32 (\( \Delta \)) or \( \Delta \text{sidC-sdcA} \) (\( \square \)). The data shown are the means and standard deviations of two independent experiments.

**Fig. 6.** \( L. \text{pneumophila} \) replicates at wild-type level in calnexin-negative LCVs.

A. Confocal laser scanning micrographs of calnexin-GFP-labelled \( D. \text{discoideum} \) AX3 (green), infected at an MOI of 10 for 14 h with DsRed-labelled \( L. \text{pneumophila} \) wild-type JR32 or the \( \Delta \text{sidC-sdcA} \) mutant strain (red). Bar, 2 \( \mu \text{m} \).

B. Quantification of percentage of calnexin-positive and calnexin-negative LCVs harbouring one, two to five or more than five wild-type or \( \Delta \text{sidC-sdcA} \) mutant \( L. \text{pneumophila} \). Means and standard deviations of three independent experiments are shown.
amoebae (Fig. 2B) and in homogenates of infected D. discoideum (Fig. S4). In summary, SidC and SdcA apparently do not play a role for the stability or integrity of the LCV membrane of infected phagocytes or isolated LCVs.

Upon expression of M45-SidC in ΔsidC-sdcA mutant L. pneumophila, the acquisition of calnexin-GFP by LCVs was complemented not only in infected intact D. discoideum (Fig. 2B), but also on isolated LCVs (Fig. 8B). For isolated LCVs, the average GFP fluorescence intensity of the ΔsidC-sdcA mutant producing M45-SidC was even significantly higher than the intensity of LCVs harbouring wild-type L. pneumophila (P < 10^-2). Similarly, expression of M45-SidC in wild-type L. pneumophila significantly increased the average GFP fluorescence on LCVs (P < 10^-7). These findings suggest that the amount of SidC directly correlates with the amount of calnexin on LCVs. Indeed, a comparison of the fluorescence intensity of calnexin-GFP and M45-SidC on individual LCVs revealed a direct correlation between the amounts of the two proteins present on LCVs (Fig. 8C). This finding mirrors the increased levels of SidC produced in L. pneumophila strains encoding the protein on a plasmid (Fig. S2). We also observed a predominant localization of M45-SidC in distinct LCV membrane regions of high calnexin-GFP content (Fig. 8D). This finding is in agreement with a direct role for SidC in recruiting calnexin-positive vesicles (see below).

Association of LCVs with calnexin-GFP-positive vesicles in D. discoideum lysates

SidC and SdcA promote the recruitment of calnexin-positive vesicles to LCVs in infected D. discoideum (Fig. 2) and on isolated LCVs (Fig. 8). Therefore, we attempted to reconstitute the recruitment of ER vesicles to LCVs in a cell-free system. To this end, a homogenate of D. discoideum strain AX3 infected with DsRed-labelled L. pneumophila was mixed with a lysate of uninfected, calnexin-GFP-labelled D. discoideum, and the association of LCVs with calnexin-GFP-positive vesicles was observed by fluorescence microscopy (Fig. 9A).

This biochemical association assay was rather inefficient, and only approximately 7% of LCVs harbouring wild-type L. pneumophila were found to associate with calnexin-GFP-positive vesicles (Fig. 9B). However, compared with LCVs harbouring ΔsidC-sdcA or ΔicmT mutant strains, approximately twice as many LCVs harbouring wild-type L. pneumophila associated with calnexin-GFP-positive ER vesicles in a statistically significant manner (P < 10^-2). The addition of purified SidC to lysates of D. discoideum infected with the ΔsidC-sdcA mutant, or providing plasmid-encoded M45-SidC (data not shown), restored the recruitment of calnexin-GFP-positive vesicles to LCVs. Taken together, these experiments demonstrate that purified SidC protein added to lysates of L. pneumophila-infected D. discoideum promotes the recruitment of ER vesicles to LCVs.

Fig. 7. Membrane integrity of LCVs harbouring L. pneumophila ΔsidC-sdcA. Transmission electron micrographs of (A) D. discoideum strain AX3 or (B) murine RAW 264.7 macrophages infected at an MOI of 50 with wild-type or ΔsidC-sdcA L. pneumophila. Representative images of LCVs that associate with smooth vesicles (D. discoideum/ΔsidC-sdcA; middle panel) or ER (RAW 264.7/JR32; left panel) are shown. No differences in the membrane integrity of LCVs containing wild-type or ΔsidC-sdcA L. pneumophila was observed. Bars, 1 μm.
Recruitment of ER vesicles to LCV by an N-terminal fragment of SidC

SidC and SdcA do not harbour any obvious catalytic or other known domain, yet both proteins contain an N-terminal region, which is predicted to form coiled-coils possibly involved in protein–protein interactions (Fig. 1A). Thus, SidC and SdcA might directly engage in the recruitment of ER vesicles or serve as adaptors for other host cell or \textit{L. pneumophila} effectors subverting host cell vesicle trafficking.

To address the question whether SidC alone interacts with vesicles, we purified SidC, its 70 kDa N-terminal fragment as well as the 36 kDa C-terminal fragment, and we covalently linked these proteins to paramagnetic beads. Binding of the proteins to the beads was verified by using an affinity-purified polyclonal anti-SidC antibody (Fig. 10A). The protein-coated beads were incubated with lysate of calnexin-GFP-labelled \textit{D. discoideum}, retained by a magnet and washed several times. The eluate was then separated by SDS-PAGE, and the retention of vesicles was visualized by Western blot with antibodies against resident ER marker proteins (calnexin, protein disulfide isomerase) or against the late endosomal/lysosomal compartment (common antigen-1).

Using this pulldown assay, full-length SidC and the N-terminal SidC\textsubscript{1-608} fragment were found to specifically retain ER vesicles, but not lysosomal compartments, while the C-terminal PtdIns(4)\textsubscript{P}-binding SidC\textsubscript{609-917} fragment apparently did not bind to any subcellular compartment (Fig. 10B). In contrast, if beads coated with full-length SidC or SidC fragments were incubated with the cytoplasmic fraction of \textit{D. discoideum} lysate obtained after ultracentrifugation, no calnexin-GFP was retained (data not shown). This result confirms that SidC and its N-terminal fragment bind to membrane-associated (vesicular) components rather than to soluble cytoplasmic components.

Similar results were obtained using lysate from RAW 264.7 macrophages (Fig. 10C). Full-length SidC and the
N-terminal SidC<sub>1-608</sub> fragment specifically retained ER and ER-derived vesicles staining positive for calnexin or Rab1 respectively, but not late endosomes/lysosomes or Golgi fragments staining positive for LAMP-1 or giantin respectively. In contrast, the C-terminal PtdIns(4)<sub>P</sub>-binding SidC<sub>609-917</sub> fragment did not specifically bind to any vesicles tested. Thus, the N-terminus of SidC is sufficient to recruit ER vesicles to the LCV membrane by anchoring to PtdIns(4)<sub>P</sub>.

Discussion

A number of intracellular bacterial pathogens interfere with host cell phosphoinositide metabolism (Pizarro-Cerda and Cossart, 2004; Hilbi, 2006). *Shigella flexneri*, *Salmonella enterica* and *Mycobacterium tuberculosis* secrete phosphoinositide phosphatases, which facilitate pathogen-triggered uptake, activate anti-apoptotic pathways and promote the formation of replication-permissive vacuoles respectively. Recently, *L. pneumophila* was found to replicate more efficiently in *D. discoideum* in absence of phosphoinositide-3 kinases and to exploit
PtdIns(4)P on LCVs to anchor the Icm/Dot substrates SidC and SdcA (Weber et al., 2006), suggesting that these ‘effector’ proteins interfere with host cell vesicle trafficking. Here we show that SidC is indeed a bifunctional effector protein, which is translocated into host cells by means of a C-terminal signal (Fig. 2), anchors to LCVs via a 20 kDa PtdIns(4)P-binding domain close to the C-terminus (Figs 1 and 2) and recruits ER vesicles to LCVs through a 70 kDa N-terminal fragment (Fig. 10).

SidC does not share significant homology to any other known protein. Specifically, the 20 kDa PtdIns(4)P-binding domain SidC609–776 is not similar to eukaryotic phosphoinositide recognition folds, such as the pleckstrin homology (PH), phagocyte oxidase homology (PX), Fab1-YotB-Vac1-EEA1 (FYVE), epsin/AP180 N-terminal homology (ENTH/ANTH), band 4.1-ezrin-radixin-moesin (FERM) or lysine/arginine (KR) domains (Lemmon, 2003; De Matteis and Godi, 2004; Downes et al., 2005; Varnai and Balla, 2006).

The 20 and 36 kDa fragments of SidC, SidC40C and SidC609–917 selectively bind PtdIns(4)P with high affinity in vitro (Fig. 1), are readily expressed in E. coli as GST fusion proteins, can be affinity-purified with high yield and are stable even upon repeated freezing/thawing steps. Therefore, the fragments might be useful to identify and quantify PtdIns(4)P in biochemical experiments. Moreover, upon heterologous expression in D. discoideum, the GFP–SidC40C fusion protein accumulated on LCVs containing wild-type but not icmT mutant L. pneumophila (Fig. 1), indicating that translocation through the Icm/Dot T4SS is not required for binding to PtdIns(4)P in eukaryotic cells. Given that SidC40C is functionally produced in D. discoideum, the fragment might prove useful to analyse the dynamics of PtdIns(4)P in other protozoan as well as metazoan model systems, including mammalian cells, fruit flies, nematodes or zebra fish.

To date, more than 50 Icm/Dot substrates have been identified in L. pneumophila, some of which form families of paralogues. The functional characterization of these putative effector proteins is not straightforward, as deletion of even multiple family members barely affects intracellular replication of L. pneumophila (Luo and Isberg, 2004; Ninio et al., 2005). Thus, a pool of redundant effectors seems to ensure robust intracellular replication of L. pneumophila. An L. pneumophila ∆sidC-sdcA mutant strain is not defective for intracellular replication in D. discoideum, A. castellanii and macrophages (Fig. S1; Luo and Isberg, 2004), yet LCVs harbouring the mutant strain are impaired for the acquisition of HDEL-positive (Fig. 5) and calnexin-positive vesicles (Figs 2 and 8), as well as for the transition from tight to spacious calnexin-positive vacuoles (Fig. 3). These results suggest that SidC and SdcA play a role in three consecutive steps during the maturation of LCVs, namely the interaction with early secretory vesicles, the recruitment of ER vesicles and the membrane dynamics of the LCVs. The recruitment and trafficking defects of the L. pneumophila ∆sidC-sdcA mutant were complemented by supplying either sidC or sdcA on a plasmid (Figs 2 and 3), indicating that the paralogues SidC and SdcA are indeed functionally redundant.

The amount of calnexin on LCVs is directly proportional to the amount of SidC (Fig. 8). Moreover, in cell-free assays SidC and its 70 kDa N-terminal fragment SidC1–608 were shown to promote the recruitment of calnexin-positive vesicles (Figs 9 and 10). These results suggest that SidC is itself a tethering factor for ER vesicles or might bind to a host cell tethering factor. SidC and its N-terminal fragment were found to interact with membrane-associated (but not soluble) calnexin, PDI as well as Rab1. Given that SidC apparently interacts with a number of different host factors, it is unlikely that the protein binds to these targets directly. Thus far, we failed to identify the direct SidC binding partner(s), which might be a host cell factor on target vesicles, or another Icm/Dot substrate. Recently, the Icm/Dot substrate SidJ was also shown to contribute to the recruitment of ER to LCVs (Liu and Luo, 2007). However, in contrast to SidC, SidJ was not detected on LCV membranes and thus, the protein promotes intracellular replication of L. pneumophila in primary macrophages and amoebae by an unknown mechanism.

In agreement with the proposed function of the 70 kDa N-terminus of SidC as a ‘tethering domain’ for ER vesicles, the C-terminal fragment SidC609–917 did not complement the mutant phenotypes, even though the fragment was still translocated and accumulated on LCVs (Figs 2 and 3). These results further indicate that SidC harbours a C-terminal secretion/translocation signal, as previously identified for the Icm/Dot substrates RalF (Nagai et al., 2005) and SdhA (Laguna et al., 2006). Note-worthy, the requirements for secretion by the Icm/Dot T4SS might differ among different classes of substrates, as the C-terminus is apparently dispensable for translocation of LepA and LepB (Chen et al., 2007).

The analysis of intracellular replication of L. pneumophila is further hampered by the fact that multiple host cell vesicle trafficking pathways seem to contribute to the formation of replication-permissive LCVs (Kagan and Roy, 2002; Dorer et al., 2006). Early secretory vesicles and the ER communicate with LCVs and thus likely are membrane sources for the growing LCVs harbouring increasing numbers of replicating bacteria. However, other trafficking pathways also seem to contribute to LCV formation and enlargement. LCVs harbouring a ∆sidC-sdcA mutant strain do not acquire the ER resident protein calnexin, and thus, barely interact with the ER during the first 4 h post infection (Fig. 2), are defective for the expansion of tight to spacious vacuoles (Fig. 3) and remain calnexin-
negative for at least up to 14 h (Fig. 6). At this point, we cannot rule out that LCVs harbouring ΔsidC-sdcA mutant \textit{L. pneumophila} interact with a distinct subset of calnexin-negative ER vesicles.

In absence of \textit{sidC} and \textit{sdcA}, the communication of LCVs with ER (Fig. 2) and, although less pronounced, with ER-derived vesicles (Fig. 5), is apparently impaired. Thus, under these conditions alternative trafficking pathways seem to communicate with LCVs and provide the vacuolar membranes that allow intracellular replication of \textit{L. pneumophila} in a growing vacuole. LCVs harbouring wild-type or ΔsidC-sdcA mutant \textit{L. pneumophila} do not acquire lysosomal markers (Fig. 4) ruling out an interaction of the LCV with this compartment. Interestingly, however, LCVs harbouring either strain acquire the endosomal marker p80 (Ravanel et al., 2001), suggesting that LCVs are characterized by endosomal features previously not appreciated.

In agreement with early endosomal characteristics of LCVs, the small GTPase Rab5 was found to be functionally involved in the formation of a replicative vacuole (Dorer et al., 2006). Similarly, LCVs formed upon infection of \textit{D. discoideum} with \textit{Mycobacterium marinum} recently have been shown to acquire p80 (Hagedorn and Soldati, 2007).

Compared with LCVs harbouring wild-type \textit{L. pneumophila}, LCVs harbouring ΔsidC-sdcA mutant bacteria were equally stable upon homogenization of infected \textit{D. discoideum} (Fig. 2B, Fig. S4) and not impaired with regard to membrane integrity (Fig. 7). The ΔsidC-sdcA mutant strain replicated at the same rate as wild-type \textit{L. pneumophila}, while localizing mainly in calnexin-negative LCVs (Fig. 6). These results indicate that calnexin-negative LCVs are replication-permissive vacuoles for \textit{L. pneumophila} and the interaction with the ER might not be a prerequisite to form such a compartment. In agreement with these results, an analysis by RNA interference of intracellular replication in \textit{Drosophila} phagocytes suggested that \textit{L. pneumophila} recruits vesicles from multiple compartments or trafficking pathways, each of which alone is apparently dispensable for the formation of a replication-permissive vacuole (Dorer et al., 2006). Yet, intracellular replication of \textit{L. pneumophila} was inhibited to different extents by dominant-negative forms of the small GTPases Arf1, Rab1 or Sar1 (Kagan and Roy, 2002; Derre and Isberg, 2004; Kagan et al., 2004), or by preventing expression of Arf1 or Sar1 using RNA interference (Dorer et al., 2006). All of these small GTPase are implicated in transport between the ER and Golgi. Inhibition of Arf1, known to be a small GTPase with pleiotropic functions, was by far most effective in preventing the formation of replication-permissive LCVs. Therefore, it is likely that inhibition of this GTPase abolishes multiple host cell vesicle trafficking pathways communicating with LCVs.

**Experimental procedures**

**Growth of bacteria and phagocytes**

The bacteria and plasmids used in this study are listed in Table S1. \textit{L. pneumophila} was grown on CYE agar plates (Feeley et al., 1979) or in AYE broth; \textit{E. coli} was cultured in Luria–Bertani medium. Antibiotics were added at the following concentrations: chloramphenicol (5 μg ml⁻¹) or kanamycin (Kan; 50 μg ml⁻¹) for \textit{L. pneumophila}, chloramphenicol (30 μg ml⁻¹) or ampicillin (100 μg ml⁻¹) for \textit{E. coli}. \textit{A. castellanii} (ATCC 30234) was grown in PYG medium at 30°C (Moffat and Tompkins, 1992; Segal and Shuman, 1999) using protease from Becton Dickinson Biosciences and yeast extract from Difco. \textit{D. discoideum} wild-type Ax3 strains (Zhou et al., 1995) was grown axenically in HL-5 medium at 23°C as described, supplemented with 20 μg ml⁻¹ G418 when necessary (Weber et al., 2006). Murine RAW 264.7 macrophages were grown in RPMI 1640 medium supplemented with 2 mM l-glutamine and 10% fetal calf serum at 37°C in 5% CO₂ (Albers et al., 2005). All reagents were from Sigma, if not specified otherwise.

**Cloning and protein purification**

DNA manipulations were performed according to standard protocols, and plasmids were isolated using commercially available kits from Qiagen or Macherey-Nagel. Translational N-terminal \textit{gst} and M45 fusions of \textit{sdcA} and fragments of \textit{sidC} were constructed by PCR amplification of the corresponding DNA using the primers listed in Table S2. The PCR fragments were cut with BamHI and Sall and ligated into plasmid pGenEX-4T-1, pGenEX-6P-1 and pCR33 yielding the plasmids listed in Table S1. All constructs were sequenced. Production of the fusion proteins was performed as described in Weber et al. (2006). \textit{E. coli} BL21(DE3) harbouring pGenEx constructs were induced at a cell density (OD₆₀₀) of 0.6 with 0.5 mM IPTG for 3 h at 30°C in Luria–Bertani medium. In all cases, this protocol resulted in a significant portion of soluble fusion protein of the expected size. The fusion proteins were purified from lysates prepared by French press using glutathione-sepharose beads in a batch procedure according to the manufacturer’s recommendations (Amersham Pharmacia).

To remove the GST tag from SidC fragments expressed with pGenEX-6P-1 vectors, glutathione-sepharose containing the fusion protein was equilibrated with PreScission Protease cleavage buffer (50 mM Tris/HCl, 150 mM NaCl, 1 mM DTT, pH 7.0), 40 U Prescission protease (Amersham Pharmacia) per ml sepharose bed volume was added and incubated for 24 h at 4°C. Eluted protein was dialysed in 2.5 l PBS for 2 x 1 h. Purity of the protein preparations was analysed by SDS-PAGE.

To construct the \textit{Dictyostelium} expression vector pSU01, the GFP gene was amplified by PCR using the primer pair oSU01 and oSU02. The product was cloned into the NsiI site of pDXA-GFP, which was previously blunted by Mung bean Nuclease. The sequences of the plasmids were confirmed by sequencing.

The chromosomal deletion of \textit{sidC-sdcA} was performed as described previously (Tiaden et al., 2007). The 700 bp upstream
and downstream fragments were amplified by PCR using the primer pairs oCR67/68 and oCR69/70. Both fragments were inserted by a four-way ligation into a pGEM-T easy vector with a Kan-resistance cassette in-between using BamHI sites and adenine overhangs. Clones were analysed by restriction digestion and sequencing. The Kan-resistance cassette flanked by upstream and downstream fragments was transferred into the pLAW344 suicide plasmid using NotI, yielding plasmid pCR49. Wild-type *L. pneumophila* JR32 was transformed with pCR49, and colonies were selected for Kan^R^ and Suc^+^. Positive clones were tested by PCR and Western blot (Fig. S2).

**Binding of SidC fragments to phosphoinositides in vitro**

The binding affinity of SidC fragments was tested in a protein-lipid overlay assay (Weber et al., 2006; Dowler et al., 2000). The lipid compounds bound to nitrocellulose membranes were incubated with GST fusion proteins expressed from pGEX-4T-1 plasmids, which were constructed and purified as described above. All experiments were done with commercially available 'PIP arrays' (Echelon). The membranes were blocked with 4% fat-free milk powder in TBST [50 mM Tris, 150 mM NaCl, 0.1% Tween-20 (v/v), pH 8.0] for 1 h at room temperature (RT) and incubated with the fusion proteins (120 pmol ml^-1^ blocking buffer) overnight at 4°C. Binding of the GST fusion proteins to lipids was visualized by ECL (Amersham) using a monoclonal anti-GST antibody (Sigma) and a secondary goat anti-mouse peroxidase-labelled antibody (Jackson Laboratories). Positive controls were performed using GST-SidC fragments (40 pmol) in a total of 1 ml of binding buffer (50 mM Tris, 150 mM NaCl, 0.05% Nonidet P40, pH 7.6). The liposomes were subsequently centrifuged at 1 ml of binding buffer (50 mM Tris, 150 mM NaCl, 0.05% Nonidet P40, pH 7.6). The liposomes were subsequently centrifuged at 250 mM sucrose, 0.5 mM EGTA). The infected phagocytes were subsequently centrifuged on eight poly-L-lysine pre-treated coverslips in 24-well plates at 2.5 ¥ 10^5^ per well in 0.5 ml of HL5 medium and allowed to adhere and grow overnight. The amoebae were infected at an MOI of 50 with 50 μl of *L. pneumophila* grown for 21 h in AYE broth and diluted in HL5 medium. The infection was synchronized by centrifugation (470 g, 10 min, RT). 1 h post infection, the amoebae were washed three times with cold SorC buffer (2 mM Na2HPO4, 15 mM KH2PO4, 50 μM CaCl2, pH 6.0), fixed with 4% paraformaldehyde (30 min, 4°C), washed three times, permeabilized (0.1% Triton X-100, 10 min, RT) and blocked with 2% normal human AB serum in SorC (30 min, RT). SidC or the M45 tag was visualized by using affinity-purified polyclonal rabbit anti SidC antibody or monoclonal mouse anti M45 hybridoma supernatant, respectively, followed by C5s-conjugated goat anti rabbit or anti mouse antibody (Jackson Laboratories). If necessary, *L. pneumophila* was stained by a monoclonal rhodamine-conjugated rabbit anti *Legionella* Philadelphia-1 serogroup 1 antibody (m-Tech). Finally, the coverslips were mounted using Vectashield (Vector Laboratories) containing DAPI (1 μg ml^-1^) to stain DNA. The samples were viewed with an inverted confocal microscope (Axiovert 200M; Zeiss), equipped with an ‘Ultrapview’ LCI confocal spinning disk head (PerkinElmer), a krypton/argon laser (643-RYB-A01; Melles Griot) and a ×100 phase contrast objective (Plan Neofluar; Zeiss, aperture 1.3, oil). Data processing was performed with Volocity 2.6.1 software (Improvement). 80–110 vacuoles were counted per sample in a blinded manner. ‘Spacious’ vacuoles were defined as vacuoles where the calnexin-GFP-labelled membrane surrounding *L. pneumophila* was clearly detached; all other LCVs observed were scored as ‘tight’.

**Quantification of LCV components in lysates**

Calnexin-GFP, other LCV marker proteins and SidC were quantified on LCVs in homogenates of infected Dictyostelium or macrophages. 2 ¥ 10^7^ calnexin-GFP-labelled *D. discoideum* or RAW264.7 macrophages were infected (MOI 100) with the *L. pneumophila* wild-type strain JR32, the SidC-sdcA or SciCmut mutant bacteria harbouring a plasmid expressing M45-SidC, the corresponding empty vector or pSW001 (DsRed-Express) (Mampel et al., 2006), washed with SorC buffer and suspended in 4 ml of homogenization buffer (20 mM Hepes-KOH, pH 7.2, 250 mM sucrose, 0.5 mM EGTA). The infected phagocytes were lysed by nine passages through a ball homogenizer (Isubiotech) using an exclusion size of 8 μm in the presence a protease inhibitor cocktail (Roche). 300–500 μl of the homogenate was subsequently centrifuged on eight poly-L-lysine pre-treated coverslips. The samples were fixed (4% paraformaldehyde, 30 min, 4°C) and blocked (2% normal human AB serum in SorC, 30 min, RT). The following antibodies were used to stain LCV components: anti- *D. discoideum* common antigen-1 and p80 (undiluted; Ravanel et al., 2001), anti-LAMP-1 (1:500; Abcam), anti-Rab1 (1:500; Santa Cruz) and anti-SidC (see above). The samples were viewed as described above, and all images were acquired with the same exposure time. For calnexin-GFP and SidC quantifications only LCVs containing rod-shaped and non-

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permeabilized bacteria were considered. The calnexin-GFP fluorescence intensity of an area identical for all samples and covering the LCV was quantified using the QuantityOne software (Bio-Rad) after background correction for each picture as shown in Fig. S3. Templates from GFP images were transferred on Cy5 images, and SidC intensities were measured and plotted against the corresponding calnexin-GFP signals.

**Quantification of calnexin-GFP in dependence of SidC on LCV in lysates by flow cytometry**

Calnexin-GFP-labelled D. discoideum (2 × 10⁷) was infected withDsRed-labelled L. pneumophila JR32 or ΔsidC-sdcA (MOI 100), suspended in 4 ml of homogenization buffer and subsequently lysed as described above. A sample was immobilized on coverslips coated with poly-L-lysine to confirm the quality of the preparation. Homogenized samples were analysed using a FACSCalibur flow cytometer (Becton Dickinson). The LCVs were gated for GFP and DsRed double positive events and quantified using the FlowJo software (Treestar). DsRed- and GFP-expressing phages were used as controls.

**Recruitment of calnexin-GFP to LCVs in lysates**

Wild-type D. discoideum (2 × 10⁷ cells per dish) was infected withDsRed-labelled JR32, ΔicmT or ΔsidC-sdcA (MOI 100), each suspended in 4 ml of homogenization buffer and subsequently lysed as described above. One homogenate containing ΔsidC-sdcA LCVs was mixed with purified SidC (see above; end concentration of 5 μM) and incubated for 30 min at 4°C. Calnexin-GFP-labelled D. discoideum (8 × 10⁷ cells) was suspended in 8 ml of homogenization buffer and lysed by nine passages through a ball homogenizer (Isobiotech), using an exclusion size of 4 μm in the presence of a protease inhibitor cocktail (Roche), followed by centrifugation for 10 min at 10 000 g to generate post-nuclear supernatants (PNS). Subsequently, the LCVs were mixed with 2 ml of the calnexin-GFP homogenate and incubated 2 h at 4°C or RT. Different experiments were performed with or without an ATP-regenerating system with final concentrations of 1 mM MgCl_, 1 mM ATP, 15 mM creatine phosphate and 50 IU ml⁻¹ rabbit muscle creatine phosphokinase (Goda and Pfeffer, 1988). Samples were fixed on coverslips pre-treated with poly-L-lysine and fixed (4% paraformaldehyde, 30 min, 4°C). Finally, the coverslips were mounted using Vectashield (Vector Laboratories). The samples were viewed with an inverted confocal microscope, and calnexin-GFP-positive LCVs were counted.

**Recruitment of ER vesicles with purified SidC or fragments bound to beads**

To prepare protein-coated beads for precipitation experiments, paramagnetic beads (Dynabeads MyOne Carboxylic Acid) were coated with purified SidC, its fragments or BSA in a two-step coating procedure using PBS buffer and N-hydroxysuccinimide ester as recommended by the manufacturer. Equimolar and limiting amounts of the proteins were used, and the binding efficiency and stability were monitored at every step using SDS-PAGE and staining with Coomassie Brilliant Blue. Thus, SidC, its fragments and BSA were found to quantitatively bind and remain stably attached to the beads. No protein eluted from the beads upon repeated washing prior to the precipitation experiments.

The PNS from 4 ml of calnexin-GFP-labelled D. discoideum or RAW 264.7 macrophages (2 × 10⁷ each) in homogenization buffer was prepared as described above. Approximately 5 × 10⁷ beads were incubated with 1 ml of the D. discoideum PNS for 5 h at 4°C with slow overhead rotation. The beads were washed at least four times with homogenization buffer and subsequently re-suspended in SDS sample buffer. Proteins retained by the beads were analysed by SDS-PAGE and Western blot using primary antibodies against GFP (Roche), D. discoideum protein disulfide isomerase and common antigen 1 (P. Cosson, University of Geneva), LAMP-1 (Abcam), giantin (Abcam), calnexin (Santa Cruz) or Rab1b (Santa Cruz). Peroxidase-labelled secondary antibodies were visualized by ECL (Amersham).

**LCV membrane integrity in absence of SidC and SdcA**

To assess the integrity of the membranes of LCVs containing L. pneumophila JR32 or ΔsidC-sdcA, infected phagocytes were analysed by transmission electron microscopy (Tilney et al., 2001). Briefly, 2.5 × 10⁷ D. discoideum or RAW 264.7 macrophages were seeded onto sterile, charcoal-coated coverslips in 24-well plates and infected at an MOI of 50 with L. pneumophila JR32 or ΔsidC-sdcA. The infection was synchronized by centrifugation (470 g, 10 min, RT), and 1 h post infection, the infected cells were fixed for 45 min with a freshly made solution of 1% glutaraldehyde, 1% OsO₄. Samples were washed with water and stained en bloc with uranyl acetate overnight. After ethanol dehydration, the plates were transferred to hydroxypropyl methacrylate and embedded in epoxy resin. Samples on the resin surface were cut in thin sections. Sections were placed on grids, stained with uranyl acetate and lead citrate and examined with a FEI Morgagni 268 transmission electron microscope.

**Bioinformatic and statistical analysis**

Homology searches were performed using the following software packages: BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), PHyre (Protein Homology/analogy Recognition Engine, Imperial College London; http://www.sbg.bio.ic.ac.uk/~phyre/), ELM (Eukaryotic Linear Motif resource; http://elm.eu.org/) and ScanSite (http://scansite.mit.edu/). For statistical analysis the two-tailed Student’s t-test was used, considering P < 0.05 as significant.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Growth characteristics and phagocytosis of an *L. pneumophila* ΔsidC-sdcA mutant strain. For *L. pneumophila* wild-type strain JR32 (■), a ΔsidC-sdcA mutant strain (▲) or an ΔicmT mutant strain (●) the following phenotypes were determined: (A) growth in AYE broth, (B) phagocytosis by *D. discoideum* (MOI of 100, 40 min), or intracellular growth in (C) *D. discoideum* (MOI 0.1), (D) *A. castellanii* (MOI 1) or (E) murine RAW264.7 macrophages (MOI 0.01). The data shown are means and standard deviations of triplicates (B–E) and representative of at least two independent experiments.

**Fig. S2.** Quantification of endogenous and overexpressed *sidC* in *L. pneumophila*. The *L. pneumophila* wild-type strain JR32 or a ΔsidC-sdcA mutant strain harbouring the plasmid pCR33 (vector) or pCR34 (M45-SidC) were grown in presence of 1 mM IPTG for 21 h, and production of SidC was analysed by Western blot using an affinity-purified polyclonal anti-SidC antibody. Upon overexpression of *sidC* from plasmid pCR34, approximately 10 times more protein is produced.

**Fig. S3.** Quantification of calnexin-GFP associated with LCVs in *D. discoideum* lysates. Calnexin-GFP-labelled *D. discoideum* wild-type strain AX3 was infected at an MOI of 50 for 1 h with the *L. pneumophila* wild-type strain JR32 or a ΔsidC-sdcA mutant strain harbouring the plasmid pCR33 (vector) or pCR34 (M45-SdC). The infected amoebae were lysed with a ball homogenizer, and the fluorescence intensity on LCV was quantified by subtracting the averaged fluorescence intensity within background areas (‘B1-3’) from the intensity of the sample area (‘S’). Within a view field, all LCVs were assessed for calnexin-GFP; representative examples are shown (S1-S7). Bar, 2 μm.

**Fig. S4.** Quantification of calnexin-positive LCVs in homogenates of *D. discoideum* infected with *L. pneumophila* JR32 or ΔsidC-sdcA harbouring plasmid pCR33 (vector) or pCR34 (M45-SidC). The data shown are the means and standard deviations of two independent experiments.

**Table S1.** Bacterial strains and plasmids.

**Table S2.** Oligonucleotides used in this study.

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