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Legionella pneumophila Effector LpdA Is a Palmitoylated Phospholipase D Virulence Factor

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***Legionella pneumophila* is a bacterial pathogen that thrives in alveolar macrophages, causing a severe pneumonia. The virulence of *L. pneumophila* depends on its Dot/Icm type IV secretion system (T4SS), which delivers more than 300 effector proteins into the host, where they rewire cellular signaling to establish a replication-permissive niche, the *Legionella*-containing vacuole (LCV). Biogenesis of the LCV requires substantial redirection of vesicle trafficking and remodeling of intracellular membranes. In order to achieve this, several T4SS effectors target regulators of membrane trafficking, while others resemble lipases. Here, we characterized LpdA, a phospholipase D effector, which was previously proposed to modulate the lipid composition of the LCV. We found that ectopically expressed LpdA was targeted to the plasma membrane and Rab4- and Rab14-containing vesicles. Subcellular targeting of LpdA required a C-terminal motif, which is posttranslationally modified by S-palmitoylation. Substrate specificity assays showed that LpdA hydrolyzed phosphatidylinositol, -inositol-3- and -4-phosphate, and phosphatidylglycerol to phosphatidic acid (PA) *in vitro*. In HeLa cells, LpdA generated PA at vesicles and the plasma membrane. Imaging of different phosphatidylinositol phosphate (PIP) and organelle markers revealed that while LpdA did not impact on membrane association of various PIP probes, it triggered fragmentation of the Golgi apparatus. Importantly, although LpdA is translocated inefficiently into cultured cells, an *L. pneumophila* Δ*lpdA* mutant displayed reduced replication in murine lungs, suggesting that it is a virulence factor contributing to *L. pneumophila* infection *in vivo*.**

Legionella pneumophila is a bacterial pathogen which infects and replicates in protozoa, as well as in human alveolar macrophages and lung epithelial cells, causing a severe pneumonia called Legionnaires' disease (1). Several secretion systems allow *L. pneumophila* to export proteins into its environment and interact with host cells (1, 2). The *Legionella* type I and type II secretion systems (T1SS and T2SS) have been shown to contribute to *L. pneumophila* virulence (3, 4); however, only the Dot/Icm type IV secretion system (T4SS) is essential for intracellular replication and pathogenesis (5, 6). The Dot/Icm T4SS delivers more than 300 effector proteins into the host cell, where they manipulate cell signaling (7, 8). This enables the bacteria to evade phagolysosomal degradation and instead establish a replication-permissive endoplasmic reticulum (ER)-derived niche, the *Legionella*-containing vacuole (LCV) (9–11).

The biogenesis of the LCV is a multistep process and involves substantial remodeling of the membrane of the nascent *Legionella* phagosome (12, 13). Consequently, a large number of effectors secreted by *Legionella* are membrane-associated proteins which target cellular regulators of membrane trafficking, such as Rab GTPases (14, 15), or directly exploit and manipulate host lipids (16–18). To achieve membrane association, *Legionella* effectors highjack at least two common host cell mechanisms. Several effectors contain a classical C-terminal CAAX-box motif which is recognized by prenyltransferases and posttranslationally modified with a geranylgeranyl (20-carbon) or a farnesyl (15-carbon) lipid anchor (19, 20). After the lipid transfer, Ras-converting enzyme 1 (RCE-1) removes the amino acid residues after the modified cysteine and isoprenyl cysteine carboxyl methyltransferase (IcmT) methylates the new C terminus. Together, these irreversible modifications increase the hydrophobicity and membrane affinity of the effectors, facilitating their membrane association. *Legionella* ensures the efficient modification of effectors by recruiting host proteins, such as farnesyl transferase, RCE-1, and IcmT to the LCV in a T4SS-dependent manner (20).

Alternatively to using a covalently attached lipid anchor, several *Legionella* effectors associate with membranes by binding phosphatidylinositol phosphates (PIPs) (16, 21), negatively charged glycerophospholipids which contain a mono- or polyphosphorylated *myo*-inositol ring (22). PIPs are versatile signaling molecules, and specific enrichment of one subspecies gives identity to organelle membranes, leading to selective recruitment of PIP-binding proteins. *L. pneumophila* actively modulates the PIP composition of membranes. The effectors SidF and SidP are PIP phosphatases, which hydrolyze the D3 phosphate of different 3-phosphorylated inositol headgroups (23, 24). This conversion of PIP species is important for the transition of the phosphatidylinositol-3-phosphate (PI3P)-rich early *Legionella*-containing phagosome to the mature phosphatidylinositol-4-phosphate

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TABLE 1 Bacterial strains used in the study

Strain	Serogroup/genotype[a]	Source or reference(s)
Legionella pneumophila		
130b (AA100, ATCC BAA-74)	O1; clinical isolate	30, 31
130b Δ*dotA*	*dotA* gene disrupted with a Kan^r cassette	32
130b Δ*lpdA* (ICC1080)	*lpdA* (*lpw_19211*) gene disrupted with a Kan^r cassette	This study
Escherichia coli		
BL21(DE3)Star		Novagen
Top10		Invitrogen

[a] Kan^r, kanamycin resistance.

(PI4P)-rich LCV. In addition, VipD removes PI3P from early endosomes, contributing to the decoupling of the LCV from the normal phagosomal maturation pathway (25).

VipD belongs to the large family of phospholipases, which hydrolyze either the carboxyl ester (subfamilies A and B), the glycerol-oriented (subfamily C), or the alcohol-oriented (subfamily D) phosphodiester bonds of phospholipid substrates. *L. pneumophila* encodes at least 15 putative phospholipase A (PLA) enzymes (18, 25), but other than VipD and PlaB, the latter of which was implicated in virulence *in vivo* (26), their functions in infection are not well defined. Additional effectors belonging to the other phospholipase subfamilies exist. The effector PlcC/CegC1 is a novel Zn^{2+}-dependent phospholipase C (PLC) and, together with two homologues, PlcA and PlcB, contributes to virulence in the *Galleria mellonella* infection model (27). The effector LpdA was first recognized in a bioinformatic search for effectors with potential function in phospholipid biosynthesis due to its homology with eukaryotic PLDs (28). Eukaryotic PLDs use mainly phosphatidylcholine (PC) but can also accept other phospholipid substrates, to generate the free lipid headgroup and the important signaling molecule phosphatidic acid (PA) (29). LpdA localizes to the LCV and perturbs PA distribution upon ectopic expression in cells, suggesting that it might modulate PA levels on the LCV (28). However, important features of LpdA such as the substrate specificity and the consequences of its activity on host cell physiology remain unknown. In this study, we characterized the activity of LpdA, investigated its mode of membrane association, and determined its effect on host cells *in vitro* and during mouse infection *in vivo* to further develop our understanding of its function in *L. pneumophila* infection.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in the present study are listed in Table 1. *Escherichia coli* strains were cultured using Luria-Bertani broth or agar. *L. pneumophila* 130b (AA100) was grown at 37°C on buffered charcoal yeast extract (CYE) plates or in *N*-(2-acetamido)-2-aminoethanesulfonic acid buffered yeast extract (AYE) broth (30, 31). If required, *Legionella* media were supplemented with 25 μg of kanamycin/ml and 6 μg of chloramphenicol/ml. All chemicals and reagents were obtained from Sigma-Aldrich, Ltd., unless indicated otherwise.

Cell culture and transfection. Human alveolar epithelial A549 cells, cervical epithelial HeLa cells, and HEK293E cells were maintained at 37°C in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, GlutaMAX (Invitrogen), and nonessential amino acids in a humidified atmosphere of 5% CO_2. THP-1 human monocytic and RAW 264.7 murine macrophage-like cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and GlutaMAX (Invitrogen). THP-1 cells were differentiated by the addition of 50 ng of phorbol 12-myristate 13-acetate/ml for 72 h. A549, HeLa, THP-1, and RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC) and subjected to minimal passaging. HEK293E cells were kindly provided by Avinash R. Shenoy (Imperial College, London, United Kingdom). The transfection of HeLa and A549 cells with eukaryotic expression plasmids was performed according to the manufacturer's instructions using GeneJuice (Novagen) or jetPRIME (Polyplus Transfection) reagent, respectively.

Plasmid and strain construction. All plasmids, primers, and restriction enzymes (NEB, Inc.) used and created in this study are described in Tables 2, 3, and 4. Gene sequences were extracted from the draft genome sequence of *L. pneumophila* strain 130b (32; see also ftp://ftp.sanger.ac.uk/pub/pathogens/Legionella/pneumophila/130b/) or obtained by sequence homology search using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Unless indicated otherwise, chromosomal DNA of *L. pneumophila* 130b was used as the PCR template. The mutagenesis of the catalytic lysine residues of LpdA was performed sequentially with a QuikChange II site-directed mutagenesis kit (Agilent Technologies) and pRK5 Myc LpdA (pICC1565) as the starting template. The mutated gene was then subcloned in other plasmids. The expression plasmid for ER-targeted mCherry protein was obtained by introducing a C-terminal KDEL ER-targeting and an N-terminal calreticulin ER retention signal into pmCherry-C1 (Clontech) by reverse PCR. Standard molecular biology techniques were used, and the identities of all plasmids were confirmed by sequencing. Plasmids were transformed into *L. pneumophila* by electroporation (33).

To create the *L. pneumophila* 130b Δ*lpdA* mutant strain, a PCR deletion cassette consisting of the kanamycin resistance (Kan^r) cassette from pSB315 (34) embedded in the 3′ and 5′ chromosomal flanking regions of the *lpdA* gene was produced and transformed into *L. pneumophila* 130b by natural transformation (35). Transformants were selected by plating on CYE agar containing 25 μg of kanamycin/ml. Replacement of the *lpdA* gene with the Kan^r cassette and the integrity of the chromosomal flanking regions was confirmed by sequencing of the genomic region.

Generation of stable A549 cell lines by transduction. Viral transductions were performed as described previously (36). Briefly, HEK293E cells were transfected with pICC1583 and the pCMV-VSV-G envelope and pCMV-MMLV-gag-pol packaging plasmids using Lipofectamine 2000 (Life Technologies); the medium was replaced after 24 h, and the cell supernatant containing virions was harvested at 48 h posttransfection. The supernatant was passed through a 0.45-μm-pore-size filter and added to A549 cells. After 24 h, the cells were washed with Dulbecco's phosphate-buffered saline (D-PBS) and incubated with growth medium supplemented with 1.5 μg of puromycin (Gibco)/ml. After 1 week of selection, the expression of the green fluorescent protein (GFP) reporters throughout the cell population was confirmed by immunofluorescence (IF), and cells were frozen or further passaged without selection.

Lipase activity assay. The substrate specificity of recombinant His_6-tagged LpdA was assessed as described previously (27). Since solubility tests showed that His_6-tagged LpdA was poorly soluble, assays were carried out with bacterial lysates. For the generation of lysates, strains were grown until achieving exponential growth, and expression was induced by addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h. Expression of the recombinant proteins with the expected apparent molecular mass (52 kDa) was confirmed by SDS-PAGE. Subsequently, pelleted bacteria were suspended in 1/20 of the original culture volume of 10 mg of lysozyme/ml and 1 μl of Triton X-100/ml in 40 mM Tris-HCl (pH 7.5, 25°C) at 37°C for 30 min. After repeated passage through a 26-gauge needle, the lysates were resuspended in one-half of the original culture volume of 40 mM Tris-HCl (pH 7.5, 25°C). Then, 0.2 mM lipid substrates—dipalmitoyl-phosphatidylcholine (PC), 99% purity; dipalmitoyl-phosphatidylglycerol (PG), 99% purity; dipalmitoyl-phosphatidyle-

TABLE 2 Plasmids used and created in this study

Plasmid and derivative(s)	Description/function	Expressed protein	Primer sequence (5′–3′)	Restriction site	Source or reference
pRK5	Expression of proteins with N-terminal Myc tag in mammalian cells				Clontech
pICC1565		Myc-LpdA	CGCACTGAGGATCCATGCTCCAATTTTTTGAGAAAGGT GCGTCGCAGAATTCTCAAGAACAAACACAACAGGTTC	BamHI EcoRI	This study
pICC1566		Myc-LpdAΔC5aa	CGCACTGAGGATCCATGCTCCAATTTTTTGAGAAAGGT GCGTCGCAGAATTCTCAGGTTCTTTTAGGCGATATC	BamHI EcoRI	This study
pICC1585		Myc-SidC PI4P	GTCATAGGATCCAAATATTCCTCCAAGCCATTATTGG GATACCCTGCAGCTATTTCTTTATAACTCCCGTGTAC	BamHI PstI	This study
pICC1575		Myc-LpdA K165R	As for pICC1565, with pICC1574 as a template		This study
pICC1578		Myc-LpdA K376R	As for pICC1565, with pICC1577 as a template		This study
pICC1581		Myc-LpdA KK165/376RR	As for pICC1565, with pICC1580 as a template		This study
pICC1341	pRK5-HA; expression of proteins with N-terminal HA tag in mammalian cells				21
pICC1567		HA-LpdA	As for pICC1565		This study
pICC1568		HA-LpdAΔC5aa	As for pICC1566		This study
pICC1569		HA-LpdA C440F	CGCACTGAGGATCCATGCTCCAATTTTTTGAGAAAGGT GCGTCGCAGAATTCTCAAGAACAAACAAAACAGGTTC	BamHI EcoRI	This study
pICC1574		HA-LpdA K165R	See Table 3		This study
pICC1577		HA-LpdA K376R	See Table 3		This study
pICC1580		HA-LpdA KK165/376RR	See Table 3		This study
pEGFP-N1	Expression of proteins with C-terminal GFP tag in mammalian cells				Clontech
pICC1586		hs PLCδ1-PH-GFP	ATGCGCTCGAGATGGACTCGGGCCGGG AGCATGGATCCTCGATGTTGAGCTCCTTCAGGAAG	XhoI BamHI	This study
pICC562	pMMB207c-4HA; vector for the expression of proteins with four N-terminal HA tags in *L. pneumophila*				67
pICC1570		4HA-LpdA	TCGCACTGAGGTACCATGCTCCAATTTTTTGAGAAAGGT TCGCATCTAGATCAAGAACAAACACAACAGGTTC	KpnI XbaI	This study
pICC1576		4HA-LpdA KK165/376RR	As for pICC1570, with pICC1580 as a template		This study
pXDC61	Expression of proteins with N-terminal β-lactamase TEM1-tag in *L. pneumophila*				68
pICC1571		TEM1-LpdA	As for pICC1570		This study
pICC1579		TEM1-LpdA KK165/376RR	As for pICC1570, with pICC1580 as a template		This study
pXDC31	Expression of GFP in *L. pneumophila*				40
pET28a(+)	Expression of a His_6 tag fusion protein in *E. coli*				Novagen
pICC1572		His-LpdA	CGCACTGAGGATCCATGCTCCAATTTTTTGAGAAAGGT GCGTCGCAGAATTCTCAAGAACAAACACAACAGGTTC	BamHI EcoRI	This study
pICC1573		LpdA-His	CATGACCATGGGACTCCAATTTTTTGAGAAAGGT TCATGCTCGAGAGAACAAACACAACAGGTTC	NcoI XhoI	This study
pICC1582		LpdA KK165/376RR-His	As for pICC1573, with pICC1580 as a template		This study
pMXs-IRES-Puro	pMXs-IP; vector for viral transduction of mammalian cells				Clontech
pICC1583		GFPSPO reporter	CTAGGGATCCGCCACCATGGTGAGCAAGG GCATGAATTCTTAACTAGTCTTAGTGGCGTCATC		This study
pm-Cherry-C1	Expression of proteins with mCherry tag in mammalian cells				Clontech
pICC1584	pmCherryER	ER-targeted mCherry	See Table 3		This study

TABLE 3 Primers used for site-directed mutagenesis and reverse PCRs in this study

Description/function	Primer sequence (5′–3′)	Source
Introduction of a K165R mutation into LpdA by site-directed mutagenesis PCR	ATAATTCGATTGCGTCCAACCATAATAGAGCCATCTGGATTG CAATCCAGATGGCTCTATTATGGTTGGACGCAATCGAATTAT	This study
Introduction of a K376R mutation into LpdA by site-directed mutagenesis PCR	GAAAAAAGAGGTTATTCATATGAGATTTATGTCAATTGATGGGCAGG CCTGCCCATCAATTGACATAAATCTCATATGAATAACCTCTTTTTTC	This study
Fusion of a calreticulin ER retention signal to mCherry by reverse PCR	GAGCAGCAGCGGCACGGATAGCAGCATGGTGGCGACCGGTAGGG-CCTTCTTGGCCTAGCAGT AGCAGTGAGCAAGGGCGAGGAG	This study
Fusion of a KDEL ER targeting signal to mCherry by reverse PCR	GAGCTGTAACTGATCATAATCAGCCATACC ATCCTTTCTAGATCCGGTGGATCCC	This study

thanolamine (PE), 99% purity; dipalmitoyl-phosphatidylserine (PS), 99% purity; or 0.25 mM PI and PIPs (phosphatidylinositol diC16 [PI_{C16}], phosphatidylinositol 3-phosphate diC16 [PI3P], phosphatidylinositol 4-phosphate diC16 [PI4P], phosphatidylinositol 5-phosphate diC16 [PI5P], phosphatidylinositol 3,4-bisphosphate diC16 [PI3,4P], phosphatidylinositol 3,5-bisphosphate diC16 [PI3,5P], phosphatidylinositol 4,5-bisphosphate diC16 [PI4,5P], and phosphatidylinositol 3,4,5-trisphosphate diC16 ([PI3,4,5P])—were incubated with buffer (40 mM Tris-HCl [pH 7.5, 25°C]) or lysates of BL21 or BL21 expressing LpdA-His_6 or an LpdA KK165/376RR-His_6 inactive mutant in a 1:1 ratio. After 4 to 5 h of incubation at 37°C with agitation, the lipids were extracted (37) and analyzed by thin-layer chromatography (TLC) using chloroform-methanol-water (65:25:4 [vol/vol/vol]) as the running phase and pure lipid substrates and phosphatidic acid (1,2-dimyristoyl-*sn*-glycero-3-phosphate monosodium salt [PA]) as references. The TLC plate was developed using 0.2% naphthol blue-black. PI, PA, and PIPs were purchased from Echelon Biosciences; all other lipid substrates were purchased from Avanti Polar Lipids, Inc., or Sigma-Aldrich.

Metabolic labeling and isolation of palmitoylated proteins. HeLa cells (one well/condition in six-well plates) were transfected with expression plasmids for LpdA or its variants, grown overnight, and then incubated with 25 μM 17-octadecynoic acid (ODYA; Cambridge Biosciences). After 7 h, the medium was removed, and the cells were washed with PBS and scraped into 100 μl of lysis buffer containing 1% Triton X-100, 0.1% SDS, and complete EDTA-free protease inhibitor (Roche). After 15 min of incubation, large debris was removed by centrifugation at 20,000 × *g* for 10 min at 4°C, and the protein concentration in the supernatant was measured. Click-chemistry was then performed on equal amounts of protein. Briefly, 1 mM $CuSO_4$, 1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 100 μM Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA), and a 100 μM concentration of a trifunctional azido-TAMRA-PEG-biotin (AzTB) capture reagent (38, 39) were added to the clarified lysate, and the mixture was incubated for 45 min at room temperature. The reaction was stopped by adding 10 mM EDTA, and the proteins were precipitated by the addition of 1 ml of chilled methanol and centrifugation (20,000 × *g*, 30 min, 4°C). The pellet was washed three times with 1 ml of methanol, air dried, and subsequently resuspended in 100 μl of PBS (0.2% SDS, 1 mM EDTA). A 10-μl portion of the solution was kept as an input control. The biotinylated proteins were isolated using Dynabeads MyOne streptavidin C1 (Invitrogen; 50 μl of slurry per mg of protein), washed three times with at least 0.5 ml of PBS (0.2% SDS), and subsequently boiled in 50 μl of 1× SDS-gel sample buffer. Equal volumes of supernatant were subjected to SDS-PAGE and Western blotting. LpdA and control proteins were labeled with mouse anti-HA.11 (clone 16B12; MMs-101P, Cambridge Bioscience), mouse anti-Myc tag (catalog no. 05-724; Millipore), rabbit anti-calnexin (SPA-860F, Stressgen), and anti-tubulin (T6199; Sigma) antibodies in combination with appropriate horseradish peroxidase-labeled secondary antibodies (Jackson ImmunoResearch). Labeled proteins were visualized with a EZ-ECL detection reagent (Geneflow) and a Fuji Las3000 imager.

Infection of eukaryotic cells and intracellular growth assay. *L. pneumophila* strains were cultured for infection as described previously (32). The bacteria were suspended in cell culture media, supplemented with chloramphenicol (6 μg/ml) and 1 mM IPTG if required, and added to A549 cells at a multiplicity of infection (MOI) of 15 to 100. To synchronize infection, samples were centrifuged for 5 min at 600 × *g*.

For growth assays, individual wells of a black 96-well plate with translucent bottoms were seeded with 8.5×10^4 THP-1 cells. After differentiation for 72 h, the medium was replaced with 100 μl of growth medium supplemented with 6 μg of chloramphenicol/ml and 1 mM IPTG. The cells were infected with *L. pneumophila* strains containing the pXDC31 GFP expression plasmid (40) at an MOI of 0.1 to 0.5 in triplicate. At 1 h postinfection, the medium was replaced with one containing 100 μg of gentamicin/ml, and the cells were incubated for a further 1 h and then washed three times with PBS. The infected cells were incubated in 100 μl of supplemented growth medium without phenol red, and the GFP fluorescence was recorded over 92 h in a FLUOstar Omega plate reader (BMG Labtech) equipped with an atmospheric control unit. Readings were taken every 2 h. Fluorescence measurements were corrected for the background fluorescence signal of uninfected cells, and each curve was normalized to a zero starting value.

β-Lactamase (TEM1) translocation assay. The translocation of LpdA was tested as described previously (32). Briefly, RAW 264.7 cells were infected with *L. pneumophila* 130b expressing TEM1 fusions of LpdA, an inactive mutant of LpdA, or control proteins; then, after 1 h of incubation and washing, 20 μl of freshly prepared CCF2-AM β-lactamase substrate (LiveBLAzer FRET-B/G loading kit; Invitrogen) was added, and the fluorescence emission at 450 and 520 nm was measured from the bottom using a Fluostar Optima plate reader at 3 h postinfection. The transloca-

TABLE 4 Primers used for the construction of the *L. pneumophila* Δ*lpdA* strain ICC1080

Function	Primer sequence (5′–3′)	Source
Amplification of the 5′-flanking region	CATAATGACAGGTAAGGCTTC CAGTGGGTACCGATTCACTCATAAATTAATTTTACCTAAT	This study
Amplification of the 3′-flanking region	GCGACTCTAGATTTTAGGCTGTTTTGATCAAGCC ATCTCAATTACAGGAAATGCAAAC	This study
Sequencing of the *lpdA* genomic region	CTTGCCATGTTCTGCTATATATTTC GTGATATTGTATGCACTTCCATC	This study

tion rate was expressed as the fold increase in the 450/520-nm emission ratio of each infected cell sample versus the emission ratio of uninfected cells.

Immunofluorescence microscopy. Cells on coverslips were fixed with 3.2% paraformaldehyde (PFA), washed with D-PBS, incubated with 50 mM ammonium chloride, and washed and permeabilized with 0.1% Triton X-100. After blocking with PBS containing 2% (wt/vol) bovine serum albumin and 2% natural donkey serum, the samples were stained sequentially with primary and secondary antibodies and mounted using ProLong antifade reagent (Invitrogen). The primary antibodies (including catalog numbers) used were as follows: mouse anti-HA.11 (clone 16B12; MMs-101P; Cambridge Bioscience), mouse anti-Myc tag (05-724, Millipore), rabbit anti-*Legionella* lipopolysaccharide (PA1-7227; Affinity BioReagents), rabbit anti-Giantin (ab24586; Abcam), anti-c-Myc–fluorescein isothiocyanate conjugate (F2047; Sigma), and anti-HA-TRITC (H9037; Sigma). The secondary antibodies were donkey anti-rabbit or anti-mouse IgG Alexa Fluor 488 and donkey anti-rabbit or anti-mouse IgG rhodamine red X (RRX) (all from Jackson ImmunoResearch). The DNA was visualized with Hoechst 33342 dye. To visualize mitochondria, transfected cells were treated with MitoTracker Red (Life Technologies) prior to fixation. Samples were analyzed using an Axio Z1 imager microscope. Typically, 50 to 100 cells were assessed per condition, and at least two independent biological repeats were carried out per experimental series. Representative images were deconvoluted and processed using AxioVision software (Carl Zeiss).

Infection of mice. All mouse procedures were approved by the University of Melbourne Animal Ethics Committee and carried out as described previously (41). Briefly, groups of mice were inoculated separately with ~2.5 × 10^6 CFU of each *L. pneumophila* 130b strain under investigation. For consistency, *L. pneumophila* 130b wild-type (WT) or Δ*lpdA* mutant strains both carried the empty p4HA (pICC562) plasmid. At 72 h postinoculation, the mice were sacrificed, and their lung tissues were extracted. The tissue was homogenized, and complete host cell lysis was ensured by incubation in 0.1% saponin for 15 min at 37°C. Serial dilutions of the homogenate were plated onto plain CYE agar plates to determine the number of viable bacteria.

RESULTS

***L. pneumophila* infection causes drastic changes to the host cell PA distribution.** LpdA is conserved across the most commonly used sequenced prototype *L. pneumophila* isolates (130b [32], Philadelphia [42], Corby [43], Alcoy [44], Lens, Paris [45]; 97 to 98% identity; see Fig. S1 in the supplemental material). In order to investigate whether LpdA affects cellular distribution of PA, we generated an A549 lung epithelial cell line stably expressing a PA biosensor consisting of the PA-binding domain of the yeast protein Spo20 fused to GFP (A549-SPO) (46). In unchallenged cells GFP-Spo20 exclusively localizes to the nucleus (Fig. 1A), but upon stimulation of production it is rapidly recruited to sites where PA is generated (28, 46). A549-SPO cells were infected with *L. pneumophila* wild type, Δ*lpdA* mutant, or Δ*lpdA* mutant complemented with a plasmid encoding 4HA-tagged LpdA. Infection with the T4SS-deficient *L. pneumophila* Δ*dotA* strain was used as a control. IF microscopy at 6 h postinfection revealed that wild-type *L. pneumophila* but not the Δ*dotA* mutant induced redistribution of GFP-Spo20 from the nucleus, resulting in a diffuse signal throughout the cells (Fig. 1A). A similar redistribution of GFP-Spo20 was observed as early as 2 h, and remained after 24 h, postinfection (data not shown). The quantification of this effect by visual counting showed that at 6 h postinfection ca. 80% of cells infected with wild-type *L. pneumophila* had no nuclear GFP-Spo20 signal (Fig. 1B). In contrast, at the same time, >80% of the cells infected with the *L. pneumophila* Δ*dotA* mutant retained a

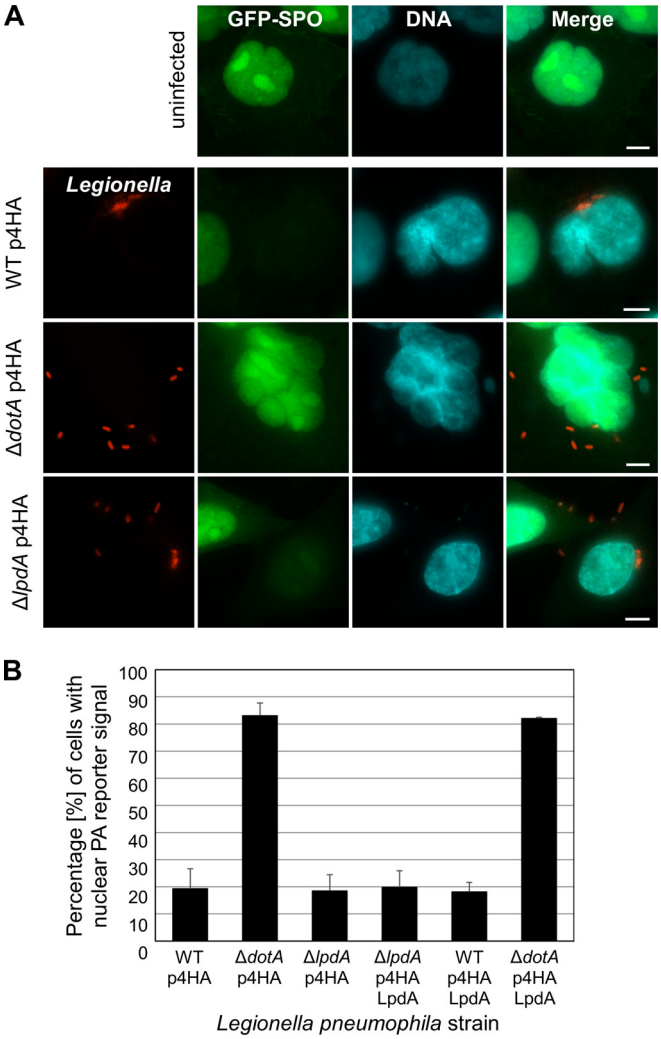

FIG 1 *L. pneumophila* infection causes drastic changes to the cellular PA distribution. A549 cells stably expressing the PA reporter GFP-SPO were left uninfected or infected for 6 h with an *L. pneumophila* 130b wild type (WT), Δ*dotA* mutant, or Δ*lpdA* mutant carrying the empty p4HA or p4HA LpdA plasmid and immunostained using an anti-*L. pneumophila* antibody (red). DNA was visualized with Hoechst DNA dye (blue). (A) Samples were examined by IF microscopy. Images are representative of at least three independent experiments. Scale bar, 5 μm. (B) Quantification of the effect of infection on the cellular distribution of the GFP-SPO PA reporter by IF counting. Error bars represent the mean standard deviations of three independent experiments.

clear nuclear GFP-Spo20 signal. Analysis and quantification of the PA redistribution in the cells infected with either the *L. pneumophila* Δ*lpdA* mutant or the complemented strain showed that both induced migration of GFP-Spo20 from the nucleus with the same kinetics and to the same extent as wild-type *L. pneumophila* (Fig. 1B and data not shown). These results show that upon infection, *L. pneumophila* induces rapid T4SS-dependent, but LpdA-independent, changes to the PA levels of host cells. The lack of obvious LpdA-dependent PA generation was not due to impaired translocation of the effector by *L. pneumophila* 130b into host cells, as TEM1-β lactamase translocation assays confirmed delivery of LpdA, and an inactive LpdA KK165/376RR mutant in which two catalytically important lysine residues were mutated (28), into

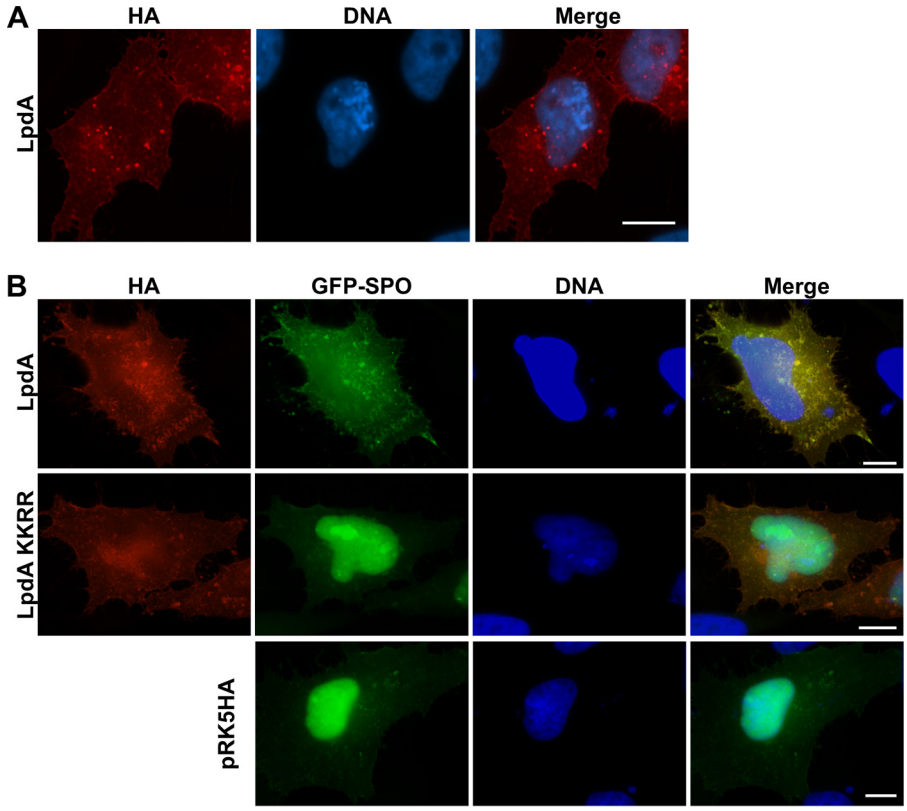

FIG 2 Ectopically expressed LpdA localizes to the plasma membrane and small vesicles, where it generates PA. IF images of HeLa cells transfected with pRK5 HA, pRK5 HA-LpdA WT, or LpdA KK165/376RR (KKRR) mutant alone (A) or together with the GFP-SPO PA reporter (B). The results are representative of at least three independent experiments. Scale bar, 10 μm.

host cells (see Fig. S2 in the supplemental material). This suggests that any impact of LpdA during infection might be subtle and masked by other effectors.

LpdA is targeted to membranes by posttranslational palmitoylation. Since the PA reporter assay (Fig. 1) suggested that the function of LpdA during infection is masked by redundant effectors, we continued our study using ectopic expression of hemagglutinin (HA)- or Myc-tagged LpdA or inactive mutants. In HeLa cells (Fig. 2A) and A549 lung epithelial cells (data not shown), HA-LpdA (Fig. 2A) or Myc-LpdA (data not shown) localized to small vesicular structures, which were distributed throughout the cell and, to a lesser extent, to the plasma membrane. Since similar distribution patterns for LpdA were observed in both cell lines, further experiments were carried out with HeLa cells because of their higher and more reproducible transfection efficiency. Cotransfection of these constructs with GFP-Spo20 showed redistribution of the reporter from the nucleus to vesicular structures and plasma membrane sites, which were enriched in LpdA. The catalytically inactive mutant LpdA KK165/376RR failed to induce the redistribution of GFP-Spo20, confirming that LpdA generates PA at the plasma membrane and vesicular structures (Fig. 2B).

Bioinformatic sequence analysis of LpdA using CSS-Palm 2.0 (47) revealed that the five C-terminal amino acid residues of LpdA contain three cysteines, which represent a potential site for posttranslational S-palmitoylation (Fig. 3A). Two of these cysteines are conserved across LpdA homologues of the most commonly used *L. pneumophila* isolates (see Fig. S1 in the supplemental material). In order to determine the role of these residues for the membrane association of LpdA, we created a truncation mutant (LpdAΔC5aa) and visualized its localization upon ectopic expression by IF. To examine the role of a nonconserved cysteine in the localization of LpdA, a C440F mutant corresponding to the amino acid replacement found in other LpdA homologues was included. Although full-length LpdA and LpdA C440F were indistinguishable and localized to vesicles and the plasma membrane, the LpdAΔ5aa mutant showed a diffuse cytoplasmic distribution (Fig. 3B). Notably, similarly to the wild-type effector, LpdAΔ5aa induced depletion of the GFP-Spo20 reporter from the nucleus (see Fig. S3 in the supplemental material), indicating that the truncated protein has lipase activity and that the loss of membrane association is not due to misfolding.

To elucidate whether the C-terminal membrane association motif of LpdA is palmitoylated, we performed bio-orthogonal metabolic labeling with a lipid reporter, an emerging strategy used to profile protein lipidation (48). HeLa cells expressing HA-LpdA or HA-LpdAΔC5aa were incubated with growth medium supplemented with 17-octadecynoic acid (ODYA), a palmitic acid analogue that is readily incorporated into proteins by palmitoyltransferases (Fig. 3C) (49). After cell lysis, the trifunctionalized AzTB capture reagent (38, 39) was ligated to the ODYA moiety in a Cu(I)-catalyzed cycloaddition, allowing the isolation and analysis of modified proteins by streptavidin pulldown (PD), Western blotting (WB) (Fig. 3D), and in-gel fluorescence detection (not

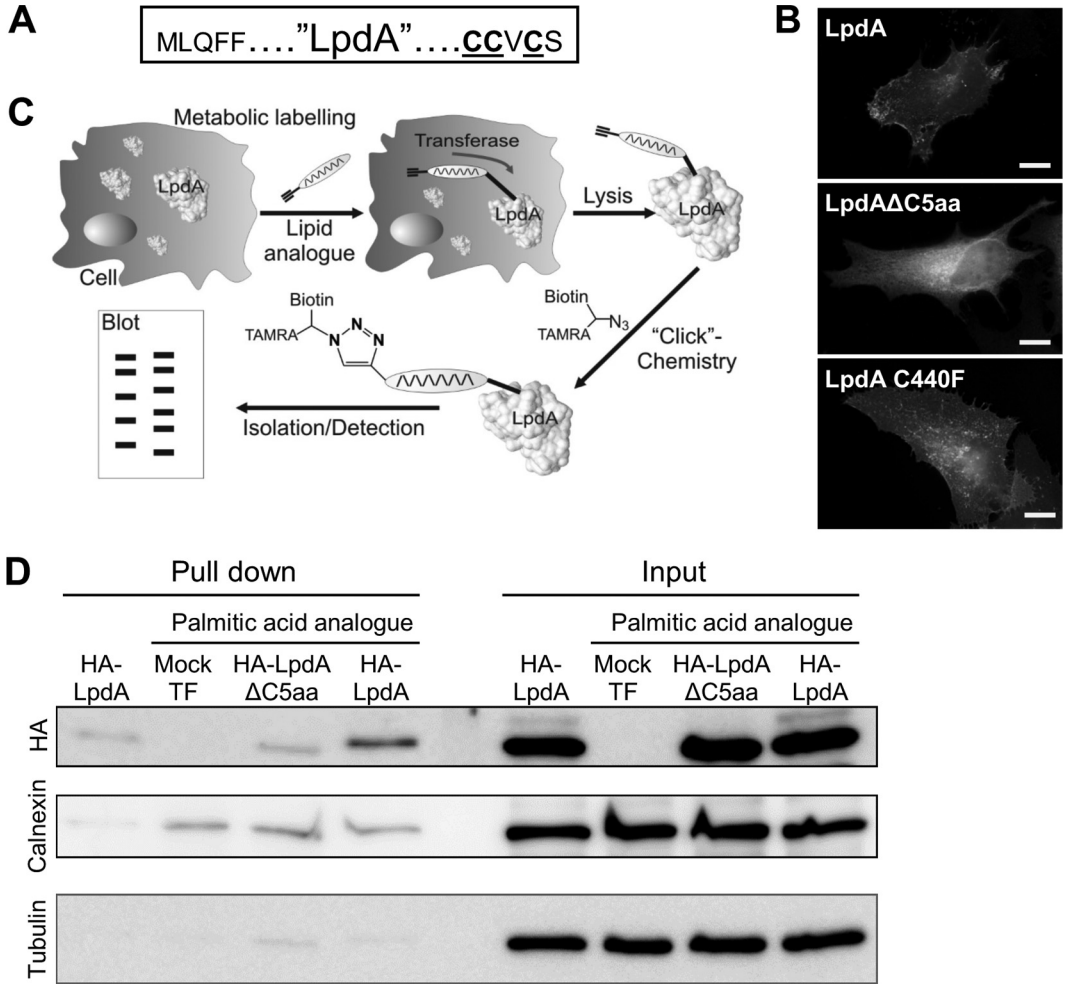

FIG 3 Posttranslational palmitoylation targets LpdA to membranes. (A) Putative C-terminal palmitoylation site of LpdA. The three cysteines predicted to be modified with a palmitic acid moiety are underlined. (B) HA-LpdA, HA-LpdA C440F, and HA-LpdAΔC5aa were ectopically expressed in HeLa cells and immunostained, and their localization was analyzed by IF microscopy. Shown are representative images of at least three independent experiments. Scale bar, 10 μm. (C) Metabolic labeling and protein isolation strategy. HeLa cells expressing LpdA were metabolically labeled with the palmitic acid analogue ODYA. After cell lysis, a biotin moiety and a fluorophore were ligated using click chemistry. Posttranslationally modified proteins were isolated using streptavidin pulldown (PD) and analyzed by Western blotting (WB). (D) Representative result of the WB analysis of PD and input fractions using HA-conjugated, calnexin (endogenous metabolic labeling control), and tubulin (separation control) antibodies.

shown). Anti-tubulin Western blotting confirmed equal concentrations of the PD input lysates and the effective elimination of unmodified proteins and cytosolic content from the isolated PD fractions. The detection of similar amounts of the endogenous, palmitoylated protein calnexin (Fig. 3D) (49) in all ODYA-treated samples, but not the unlabeled control sample, validated comparable and specific metabolic labeling and PD of modified proteins. Anti-HA WB revealed weak background binding of HA-LpdA in the absence of—and to a similar extent, HA-LpdAΔC5aa in the presence of—ODYA to the beads. Importantly, compared to HA-LpdAΔC5aa and the controls, HA-LpdA was enriched in the isolated PD fraction (Fig. 3D). This showed that LpdA is palmitoylated in the host cell and that the last five residues are critical for this modification.

LpdA localizes to Rab4- and Rab14-containing vesicles. To determine which subcellular membranes LpdA targets, we examined the colocalization of LpdA with a panel of marker proteins for different cellular membranes (GFP-Rab1, -Rab2, -Rab4, -Rab5, -Rab6, -Rab7, -Rab10, -Rab11, -Rab14, and -LC3) and the mitochondria. The vesicular structures formed by LpdA colocalized with GFP-Rab4- and GFP-Rab14-positive membranes (Fig. 4, insets). The association was lost when the C-terminal palmitoylation motif of LpdA was removed (see Fig. S4 in the supplemental material). Sporadic association of LpdA with GFP-Rab7- and -Rab5-positive late and early endosomes was also observed (see Fig. S5A in the supplemental material). Notably, expression of LpdA did not seem to change the distribution of Rab4- or Rab14-positive vesicles or early and late endosomes compared to cells transfected with the LpdA inactive mutant (data not shown), suggesting that LpdA localizes to, but does not compromise, these transport vesicles. No colocalization with the mitochondria or GFP-Rab1, -Rab2, -Rab6, -Rab10, -Rab11 or the autophagosome marker GFP-LC3 was observed (see Fig. S5A to C in the supplemental material). It is noteworthy that Rab1, Rab2, and Rab6, which are usually found at the Golgi apparatus, showed in some LpdA-expressing cells a more diffuse or vesicular distribution

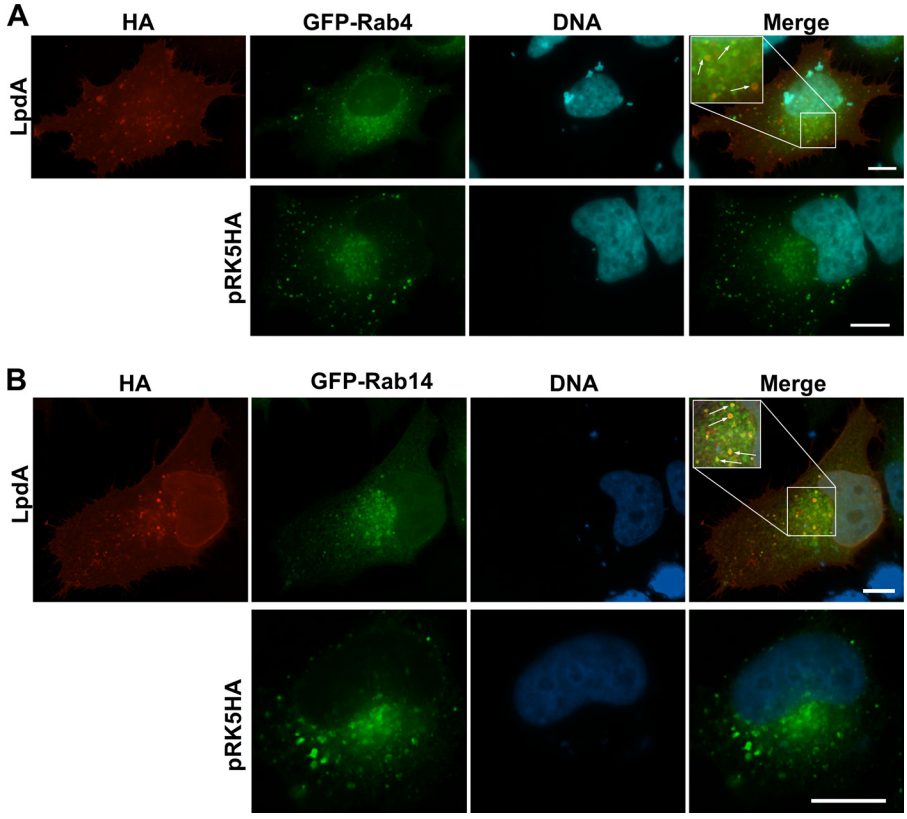

FIG 4 Vesicular LpdA localizes to Rab4 (A)- and Rab14 (B)-containing vesicles. HeLa cells were cotransfected with pRK5 HA or pRK5 HA-LpdA and plasmids for the expression of marker proteins of different cellular compartments and processed for IF microscopy. Rab4/Rab14- and LpdA-positive vesicles are highlighted with arrows in the magnified insets. Similar results were observed in at least two independent experiments. Scale bar, 10 μm.

rather than a compact perinuclear localization (data not shown). Our data show that vesicular LpdA mainly localizes to GFP-Rab4- and -Rab14-positive vesicles, which are involved in recycling transport processes between the plasma membrane and endosomes and endosomes and the Golgi apparatus.

LpdA hydrolyzes phosphatidylinositol, phosphatidylinositol-3- and phosphatidylinositol-4-phosphate, and phosphatidylglycerol. The plasma membrane and Rab4- and Rab14-positive vesicles, which are targeted by LpdA, are made up of several different lipid species (50). In order to define the substrate specificity of LpdA, we performed activity assays with a selection of purified lipids. We first attempted to purify recombinant His_6-tagged LpdA and the inactive mutant LpdA KK165/376RR. However, as the recombinant proteins were insoluble, PLD activity was determined using *E. coli* lysates containing wild-type or mutant LpdA. Bacterial lysates were incubated with phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG) for 5 h (Fig. 5A) or phosphatidylinositiol and phosphatidylinositiol-monophosphates (Fig. 5B) and -polyphosphates (data not shown) for 4 h, and the samples were analyzed by TLC as described previously (27). Incubation of PG but not PC (Fig. 5A), PE, or PS (data not shown) with lysates containing wild-type LpdA revealed PA generation. Furthermore, wild-type LpdA efficiently released PA from PI, PI3P, and PI4P but not PI5P or polyphosphorylated PIPs (Fig. 5B). Importantly, substrate turnover did not occur in incubations with lysates of bacteria containing the empty plasmid or expressing the LpdA KK165/376RR mutant. These results confirm that LpdA has PLD activity, specifically releasing PA from PI, PI3P, PI4P, and PG *in vitro*.

LpdA induces disruption of the Golgi apparatus. PI3P and PI4P are important determinants of the maturation of phagolysosomes, Golgi function, and LCV biogenesis (16, 51, 52). Since LpdA can hydrolyze these PIPs *in vitro*, we analyzed whether LpdA affects the distribution of PIP-binding reporter proteins in the cell. We coexpressed the active lipase or the inactive mutant with the PI3P and PI4P reporters 2×FYVE-GFP (Fig. 6A) or Myc-SidC PIP4 (Fig. 6B), respectively (21). 2×FYVE-GFP displayed strong association with LpdA-containing vesicles (Fig. 6A); however, its distributions were similar in mock-, LpdA- (Fig. 6A), or LpdA KK165/376RR (data not shown)-transfected cells. The Myc-SidC PIP4 reporter localized to perinuclear structures which were reminiscent of the Golgi and to very small vesicles throughout mock-transfected cells (Fig. 6B). Ectopically expressed LpdA did not colocalize with the PI4P reporter or induce its loss from membranes; however, in LpdA-expressing cells, the perinuclear Golgi body-like SidC PI4P structures seemed to have fragmented into small and medium sized vesicles, showing that LpdA's activity impacts PI4P-containing compartments.

In order to elucidate whether the action of LpdA has consequences for the integrity of the Golgi apparatus, we immunostained LpdA-transfected cells for the Golgi marker protein Giantin. This revealed that LpdA did not colocalize with the Golgi marker (Fig. 7A); however, in cells expressing the wild-type LpdA,

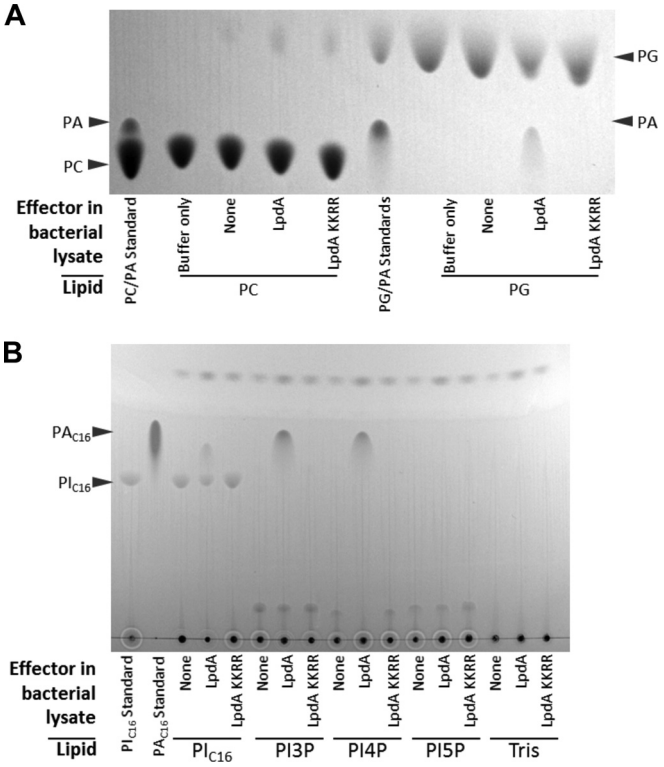

FIG 5 LpdA catalyzes the conversion of PG, PI, PI3P, and PI4P to PA. Lysates of *E. coli* with or without expression of His_6-tagged LpdA or LpdA KK165/376RR (KKRR) or a buffer control were incubated with 0.2 mM PC or PG for 5 h at 37°C (A) or 0.25 mM PI_{C16} or monophosphorylated PI_{C16} for 4 h at 37°C (B). Lipids were extracted and analyzed by TLC. Corresponding pure PC, PG, PI_{C16}, and PA standards were included. Similar results were obtained in at least two independent experiments.

but not in cells expressing the catalytically inactive mutant, the normal compact, tight stacks of Golgi cisternae had disappeared and, instead, disintegration into separated stacks or even small, vesicle-like fragments was apparent. At the same time, LpdA did not localize to or affect the integrity of the ER, indicating that the fragmentation of the Golgi apparatus was not due to action of the effector at the ER, which could interfere with early steps of the secretory pathway (see Fig. S6 in the supplemental material). Quantification of the Golgi damage revealed that ca. 90% of the LpdA KK165/376RR- or GFP-expressing cells, but only ca. 30% of the LpdA-expressing cells, retained an intact Golgi apparatus (Fig. 7B). Notably, the Golgi disruption induced by the LpdA mutant lacking the C-terminal palmitoylation motif was significantly higher (*, $P = 0.0243$, two-tailed, unpaired *t* test) than for the wild-type enzyme, suggesting that spatial control can restrict the activity of LpdA. IF analysis of infected A549 cells revealed a T4S-dependent, but LpdA-independent, disruption of the Golgi apparatus into ministacks and vesicles as early as 2 h 30 min postinfection (see Fig. S7 in the supplemental material), indicating that several effectors are involved in triggering the loss of integrity. Taken together, our data indicate that, although LpdA does not directly associate with Golgi membranes, its activity can impact on the integrity of the Golgi apparatus, most likely by perturbing PA levels and the lipid and protein transport by Rab14-positive vesicles between plasma membrane, endosomes, and the Golgi.

LpdA contributes to the virulence of *L. pneumophila* in A/J mice. The infection data (Fig. 1 and see Fig. S7 in the supplemental material) suggested that the activity of LpdA in cells might be masked by functionally redundant effectors. In line with this, an *L. pneumophila* LpdA-null mutant was previously reported to show no growth defect in the environmental host *Acanthamoeba castellanii* (28). Similarly, we found that the *L. pneumophila* 130b Δ*lpdA* mutant was not attenuated for growth in THP-1 macrophages (see Fig. S8 in the supplemental material). We reasoned, therefore, that the contribution of LpdA to virulence might only become apparent under the highly selective pressure encountered in the mammalian lung. In order to determine whether LpdA has a role in virulence, we infected A/J mice with *L. pneumophila* 130b, Δ*lpdA* mutant, or Δ*lpdA* mutant complemented with a plasmid, allowing expression of 4HA-tagged LpdA, and examined the bacterial growth in the murine lung after 72 h. The *L. pneumophila* Δ*lpdA* mutant showed a reduced bacterial load in murine lungs compared to *L. pneumophila* wild-type and Δ*lpdA* 4HA-LpdA strains in two independent experiments (Fig. 8). This difference between the Δ*lpdA* mutant and the complemented strain was statistically significant in both experiments (Fig. 8, $P = 0.0041$ and $P = 0.0355$; Mann-Whitney U test); however, due to a higher variation in the number of recovered wild-type bacteria, it was only significant between the Δ*lpdA* mutant and wild type once (Fig. 8A, $P = 0.0025$, Mann-Whitney U test). There was no statistically significant difference between the wild-type and the complemented strain. In order to test the role of the PLD activity of LpdA for its function in the murine infection model, an *L. pneumophila* Δ*lpdA* strain expressing the 4HA-LpdA KK165/376RR inactive mutant was included. This experiment showed that in contrast to the expression of wild-type 4HA-LpdA, the inactive LpdA mutant did not restore full virulence of the Δ*lpdA* mutant (Fig. 8B, $P = 0.0482$, Mann-Whitney U test). Taken together, our data indicate that, although functionally redundant effectors seem to exist, LpdA contributes to the optimal survival of *L. pneumophila in vivo* and that its lipase activity is required to fulfill this function.

DISCUSSION

L. pneumophila uses several effectors, such as SidF, LecE, and LegS2, which have enzymatic activities that directly modulate membrane lipid composition and metabolism (23, 28, 53). Moreover, many other effectors share homology with eukaryotic lipases (18). The high abundance of lipid-converting enzymes indicates that subversion of host cell lipids is crucial for *L. pneumophila* infection; however, our knowledge about the biology of most of these effectors remains limited. Here, we report that LpdA is an *L. pneumophila* effector that exploits host cell mediated posttranslational S-palmitoylation as membrane-targeting mechanism. To our knowledge, LpdA is the first palmitolylated *L. pneumophila* effector, although other T4SS effectors have previously been shown to exploit posttranslational prenylation for membrane targeting (19, 20). Bacterial type III secretion system effectors, such as *Salmonella* SspH2 and SseI or *Pseudomonas* AvrPphB, as well as several viruses, have been shown to use posttranslational palmitoylation for subcellular targeting (54–56), suggesting that its exploitation is an ancient strategy in the coevolution of pathogens and hosts.

LpdA is a lipolytic effector which has been proposed to be a PLD modulating phospholipid biosynthesis on the LCV (28). Our

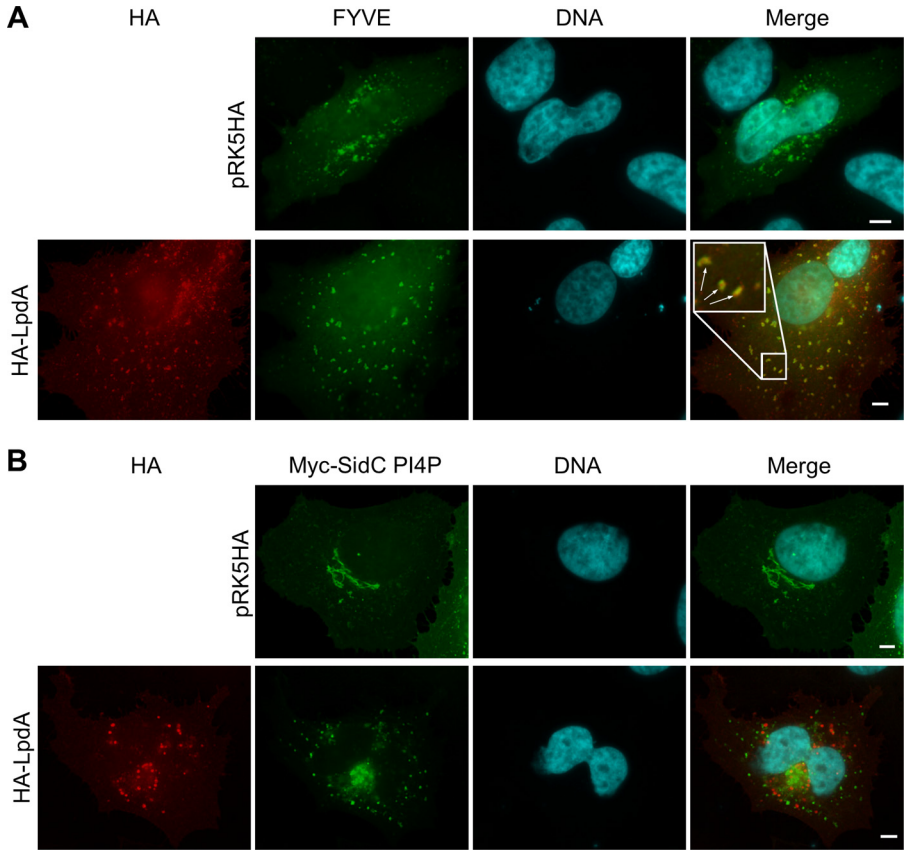

FIG 6 LpdA induces rearrangement of PI4P-containing compartments. HeLa cells were cotransfected with pRK5 HA or pRK5 HA-LpdA and a plasmid expressing the PI3P-binding protein FYVE fused to GFP (A) or the Myc-tagged PI4P binding domain of SidC (B), stained, and analyzed IF microscopy. Vesicles in which LpdA and the PI3P marker colocalize are highlighted with arrows in the magnified inset in panel A. Similar results were observed in at least three independent experiments. Scale bar, 5 μm.

study revealed that LpdA confers a moderate advantage for survival and replication in the lungs of A/J mice to wild-type *L. pneumophila* and the complemented strain compared to the Δ*lpdA* mutant. Importantly, our data indicate that this requires the catalytic activity of LpdA. The difference between the Δ*lpdA* and complemented Δ*lpdA* strains was more consistent across experiments than the one between Δ*lpdA* and wild-type *L. pneumophila*, since the latter showed larger variation in bacterial load compared to the complemented mutant. The increased variation could be due to higher heterogeneity of LpdA expression in the wild-type population. Notably, hardly any *L. pneumophila* effector null mutants, and not even strains lacking up to one-third of the known effectors, are attenuated in growth in most *L. pneumophila* cell infection models (57). An *L. pneumophila* JR32 Δ*lpdA* mutant was previously reported to replicate as well as the wild-type strain in amoebae (28). Similarly, we found that the *L. pneumophila* 130b Δ*lpdA* strain was not attenuated for replication in THP-1 macrophages. In the murine lung, the bacteria are confronted with the diverse antibacterial mechanisms of macrophages and neutrophils, creating a highly selective environment in which the impact of individual effectors, such as LpdA, might become more apparent than in monoculture infections.

To gain a deeper understanding of the function of LpdA on a molecular level, we performed *in vitro* substrate specificity assays. These showed that LpdA has PLD activity toward PI and its derivatives, PI3P and PI4P, which all are important signaling molecules. PI3P is also hydrolyzed by the PLA effector VipD, which leads to loss of the PI3P-binding proteins from the membrane (25). Ectopic coexpression of LpdA with PI3P and PI4P reporter proteins did not cause any loss of the reporters from the membrane, suggesting that LpdA does not hydrolyze these two lipids or that the amounts which are removed are too small to impact on membrane anchoring of the reporter proteins.

In vitro, LpdA also hydrolyzed PG, which is a minor lipid constituent of eukaryotic membranes but can amount to up to 20% of bacterial membranes. Overexpression of LpdA could therefore have adverse effects on the integrity of the bacteria and causes unspecific release of LpdA. However, using the inactive LpdA KK165/376RR mutant, we demonstrated that the translocation of LpdA occurs independently of its activity but requires a functional T4SS. In eukaryotic cells, PG is mainly found in mitochondrial membranes. However, neither our experiments nor the work of Viner et al. (28) provided any indication that LpdA is targeted to mitochondria or affects their integrity. In infected cells, LpdA localizes to the LCV (28). The exact lipid composition of the LCV is unknown. However, since *L. pneumophila* releases outer membrane vesicles (OMVs), which can fuse with the LCV membrane (58), it is likely that the LCV membrane also contains PG of bacterial origin, which could serve as the substrate for LpdA. OMVs released by *L. pneumophila* have been shown to interfere with

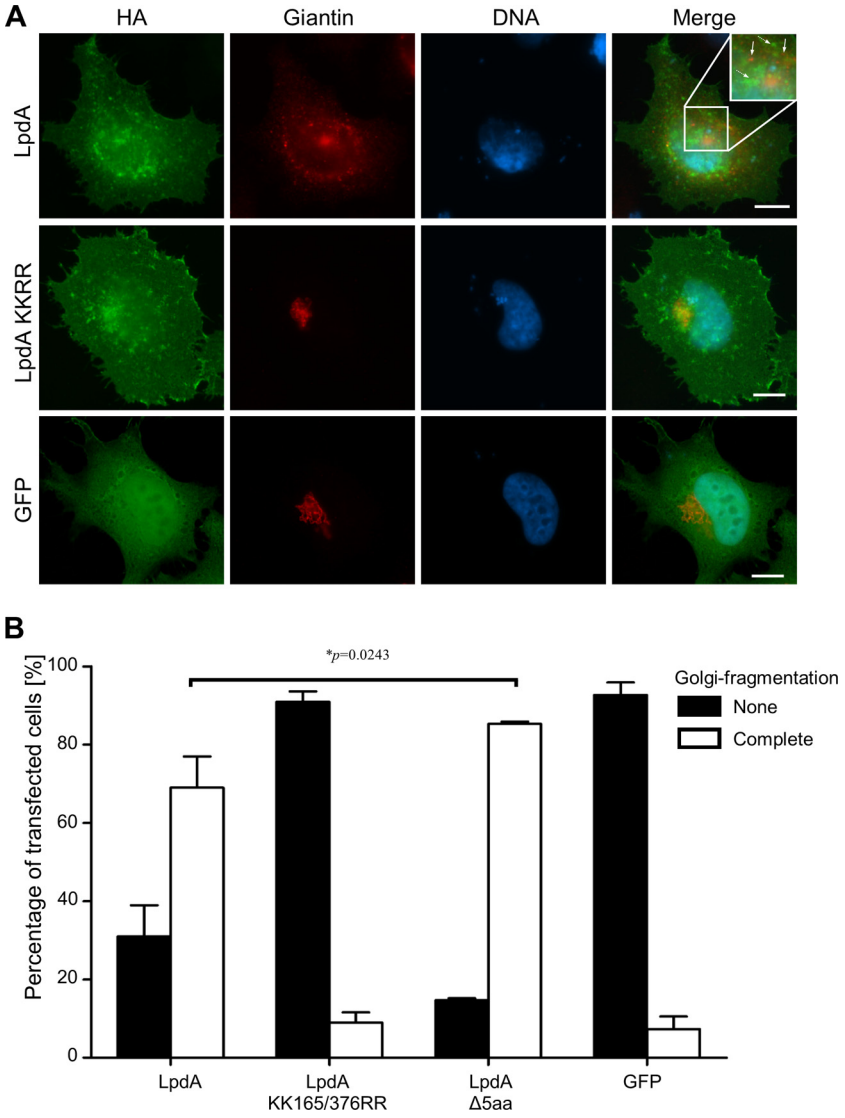

FIG 7 LpdA activity in the cell disrupts the Golgi apparatus. HeLa cells were transfected with pRK5 HA-LpdA WT, pRK5 HA-LpdA KK165/376RR (KKRR), pRK5 HA-LpdAΔ5aa, or pRK5 HA-GFP and immunostained for HA and the Golgi protein Giantin. Samples were imaged (A), and the integrity of the Golgi apparatus was assessed in 100 transfected cells (B). Full and dotted arrows in the panel A inset image highlight Giantin-positive fragments and LpdA-containing vesicles, respectively, which do not colocalize. The images are representative of at least three independent experiments. Scale bar, 10 μm. In panel B, the error bars represent the mean standard deviations of three independent experiments. Significance was analyzed using a two-tailed, unpaired *t* test.

phagosome-lysosome maturation and fusion of endosomes with the LCV (58), which is desirable for the bacteria at early stages of the infection. However, the maturation into the replication-permissive LCV relies on fusion of ER-derived vesicles with the LCV, for which the transition to a more ER-like lipid composition could be favorable. LpdA could function in this adjustment process.

Our data show that ectopic expression of LpdA induces drastic changes to cellular PA levels and has a detrimental effect on the integrity of the Golgi apparatus. Ectopic-expression levels of proteins might be substantially higher than during infection and therefore cause unspecific alterations of the cell physiology; however, our data show that *L. pneumophila* infection induces drastic redistribution of a PA reporter in host cells, as well as a rapid dispersal of the Golgi body, a phenomenon which was also reported earlier by Rothmeier et al. (59). Both phenotypes were T4S dependent, but not affected by deletion of LpdA, suggesting that LpdA has a small or very specific, tightly controlled role and that other redundant effectors mask its effect. A large number of effectors that were shown to target host cell mediators of the secretory pathway (60) could contribute to the Golgi disruption. Additional lipases encoded in *Legionella* genomes and effectors such as LecE, which activates host cell PA phosphatase, leading to the conversion of PA into diacylglycerol (28), could contribute to generation and turnover of PA, explaining why no accumulation of our PA reporter on any specific cellular membrane or the LCV was detected. In light of the involvement of redundant effectors during infection, only ectopic expression could be used to unravel the contribution of LpdA to these phenotypes in infection.

Using ectopic expression, the data show that the lipase activity of LpdA is required for PA generation and the disruption of the

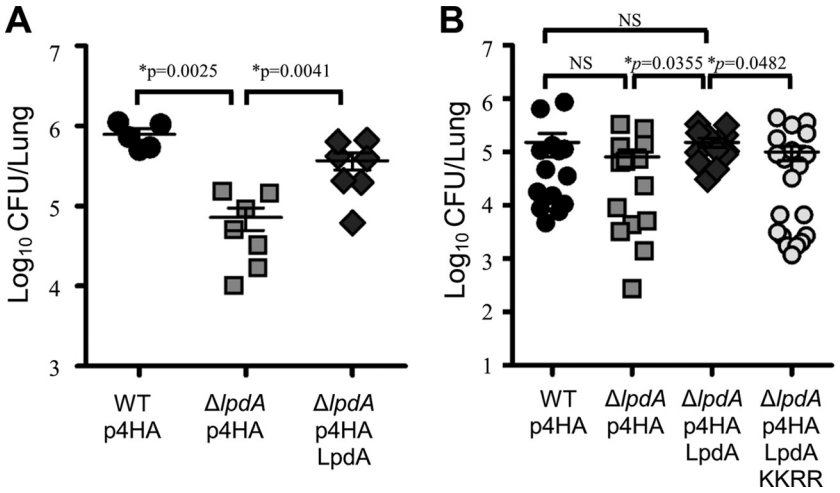

FIG 8 LpdA contributes to the fitness of *L. pneumophila* 130b during pulmonary infections of A/J mice. The results of two independent experiments (A and B) are shown. A/J mice were infected with *L. pneumophila* wild-type (WT) and Δ*lpdA* mutant strains by intranasal inoculation. At 72 h postinfection, the animals were sacrificed and the CFU in the lungs determined by plating. (A) Both the wild type and the complemented Δ*lpdA* strain grew significantly ($P = 0.0025$ and $P = 0.0041$, respectively [Mann-Whitney U test]) better than the mutant strain in the lungs of mice. (B) The Δ*lpdA* strain and the Δ*lpdA* strain expressing the inactive LpdA KK165/376RR (KKRR) mutant are significantly ($P = 0.0355$ and $P = 0.0482$, respectively [Mann-Whitney U test]) attenuated compared to the complemented *L. pneumophila* Δ*lpdA* p4HA LpdA strain.

Golgi apparatus. PA is an important signaling molecule and was implicated in the regulation of membrane tubulation and vesicle formation at recycling endosomes and the Golgi apparatus (61–63). In particular, the structure and function of the Golgi apparatus was shown to be sensitive to disturbance of the cellular PA homeostasis. The inhibition of phosphatidic acid synthesis was previously shown to disrupt the Golgi structure (64). Robust activation of human PLD, which generates PA and regulates membrane trafficking at various cellular sites, including the Golgi structure, is required for induction of Golgi fragmentation by the drug ilimaquinone (65, 66).

LpdA triggers Golgi disruption without localizing to the organelle, which suggests that changes in lipid composition and PA levels in distant membranes sites adversely affect membrane transport processes to and/or the lipid composition of the Golgi apparatus, which result in its breakdown. The main PA generation sites are the plasma membrane and Rab4- and Rab14-containing vesicles, to which LpdA localizes; however, the observation that LpdA lacking the palmitoylation motif, which does not specifically localize to these sites anymore, induces even more pronounced Golgi disruption, leads to the hypothesis that LpdA-mediated perturbation of the cellular PA homeostasis rather than the modulation of a specific vesicular trafficking pathway can cause this effect.

Taken together, we established here that the effector LpdA is a PLD that can hydrolyze several lipid substrates to modulate cellular PA levels and contributes to virulence of *L. pneumophila* in the murine lung. We discovered that *Legionella* exploits host cell-mediated posttranslational S-palmitoylation to exert spatial control over LpdA's activity in the cell. Moreover, our work revealed that *L. pneumophila* infection induces rapid disruption of the Golgi apparatus, as well as dramatic changes in host cell PA levels, with unknown benefits for the infection and involvement of unidentified effectors besides LpdA, warranting future research in the interdependence of these phenotypes and the role of phospholipase effectors in *L. pneumophila* pathogenesis.

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