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Calnexin, calreticulin and cytoskeleton-associated proteins modulate uptake and growth of *Legionella pneumophila* in *Dictyostelium discoideum*

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The haploid amoeba *Dictyostelium discoideum* is a versatile host system for studying cellular aspects of *Legionella* pathogenicity. Previous studies have shown that the internalization of *L. pneumophila* leads to an endoplasmic reticulum (ER)-derived organelle that supports intracellular replication of the bacteria. In this study a roadmap of host-cell factors involved in this process was developed. Phagocytosis assays with specific cellular inhibitors and the effects of well defined host-cell mutants revealed that cytoplasmic calcium levels, cytoskeleton-associated proteins and the calcium-binding proteins of the ER, calreticulin and calnexin, specifically influence the uptake and intracellular growth of *L. pneumophila*. Confocal microscopic time series with green fluorescent protein (GFP)-tagged calnexin and calreticulin demonstrated the accumulation of both proteins in the phagocytic cup of *L. pneumophila*-infected host cells. In contrast to the control experiment with *Escherichia coli*-containing phagosomes, both proteins decorated the replicative vacuole of *L. pneumophila* during the entire growth phase of the bacteria. The cumulative effects of cytosolic calcium levels, the spatial distribution of calnexin and calreticulin, and the defective invasion and replication of *L. pneumophila* in calnexin- and calreticulin-minus cells suggest that these factors are part of a regulatory system that leads to the specific vacuole of *L. pneumophila*.

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INTRODUCTION

Legionella pneumophila is the aetiological agent of severe bacterial pneumonia called Legionnaires' disease. The Gram-negative bacterium is naturally found in fresh-water habitats where it parasitizes within protozoa (Steinert *et al.*, 2002). During human infection, *L. pneumophila* invades and grows within alveolar macrophages and epithelial cells (Abu Kwaik, 1998).

In human macrophages, phagocytosis of *L. pneumophila* occurs through binding to complement receptors CR1, CR3 and other host-cell receptors. The coating of *L. pneumophila* with specific antibodies results in FcR-mediated

phagocytosis (Jacob *et al.*, 1994; Payne & Horwitz, 1987). In the host amoeba *Hartmannella vermiformis* the bacteria attach to a 170 kDa galactose/*N*-acetylgalactosamine-inhibitable lectin which results in the dephosphorylation of paxillin, vinculin and pp125^{FAK} (Venkataraman *et al.*, 1998). In addition, it was found that 33 amoebal proteins were induced and 11 other amoebal proteins were repressed upon contact with *L. pneumophila* (Abu Kwaik *et al.*, 1994). The resulting phagosomes which sequentially associate with vesicles and other organelles undergo neither acidification nor phagolysosomal fusion. Within this reprogrammed and maturation-blocked phagosome intracellular legionellae down-regulate a number of virulence traits including those that prevent the fusion with the lysosomal compartment. Evidence that the vacuole in which *L. pneumophila* replicates has characteristics of the endoplasmic reticulum

Abbreviations: ER, endoplasmic reticulum; GFP, green fluorescent protein; PLC, phospholipase C.

(ER) came from electron microscopy and the detection of the ER protein BiP (Abu Kwaik, 1996; Swanson & Isberg, 1995; Roy, 2002). During later phases of infection the bacteria-containing vacuoles merge with the lysosomal compartment (Swanson & Hammer, 2000).

In recent years much progress has been made toward the characterization of how *L. pneumophila* modifies the phagosome maturation. Since the reprogramming of the phagocytic pathway occurs between the initial attachment and the replicative phase of the bacteria, it is highly likely that cell-surface components and secreted factors are involved in this process. Accordingly it has been shown that the Dot/Icm secretion system plays a pivotal role in macrophages and protozoa (Hilbi *et al.*, 2001). Factors which are exported via this secretion system obviously separate the *L. pneumophila*-containing phagosome from the endosomal pathway (Segal *et al.*, 1999).

The molecular analysis of host-cell functions during *L. pneumophila* infection is much less understood. So far it has been shown that early *L. pneumophila*-containing phagosomes of macrophages lack major histocompatibility complex (MHC) class I and class II molecules, alkaline phosphatases and other membrane proteins. Further markers which are excluded from the phagosome during the course of intravacuolar growth of *L. pneumophila* are CD63, LAMP-1, LAMP-2, lysosomal cathepsin D, transferrin receptors and Rab7 (Hammer *et al.*, 2002; Swanson & Hammer, 2000). During the uptake by *H. vermiformis*, tyrosine-phosphorylated proteins including a 170 kDa receptor and various cytoskeleton-associated proteins are dephosphorylated (Abu Kwaik, 1998; Venkataraman *et al.*, 1998). In macrophages, wild-type *L. pneumophila* induce phosphorylation of a 76 kDa protein (Yamamoto *et al.*, 1992). Also the caspase 3-dependent apoptosis in mammalian cells has been reported to be induced during early stages of infection (Müller *et al.*, 1996; Zink *et al.*, 2002). Nevertheless, an integrated view of how bacterial and host proteins interact and how the signalling cascade of the host is initiated and manipulated is still missing.

Due to its experimental versatility, *Dictyostelium discoideum* has proven to be a representative host-model system to analyse cellular aspects of *L. pneumophila* infection (Eichinger *et al.*, 1999; Hägele *et al.*, 2000; Skriwan *et al.*, 2002; Solomon *et al.*, 2000). It has been reported that the sequence of events involved in particle uptake by *D. discoideum* resembles that of macrophages. The initial signal transduction to the actin system requires a heterotrimeric G-protein (Peracino *et al.*, 1998) and, as in mammalian cells, the phospholipase C (PLC) pathway is involved (Cardelli, 2001; Seastone *et al.*, 1999). The phagosome matures by sequential transient fusion events with early and late endosomal compartments. A recent study demonstrated that macroautophagy is dispensable for the intracellular replication of *L. pneumophila* in *D. discoideum*. The *L. pneumophila* replicative vacuole is rather transformed by other means to resemble the rough ER (Otto *et al.*, 2004).

In this study we focused on the role of calcium, specific cytoskeleton-associated proteins and the two calcium-binding proteins calnexin and calreticulin during *L. pneumophila* infection of *D. discoideum*. Calreticulin is an ER luminal protein supplied with a K(H)DEL recognition signal and calnexin is an ER-specific type I transmembrane protein. Both proteins have been characterized as calcium storage proteins and several studies have indicated that changes in the concentration of calcium affect ER functions. Since *L. pneumophila* replicates in an ER-derived organelle, we set out to define the involved cell-signalling processes by the use of specific cellular inhibitors in phagocytosis assays. To examine host functions required for uptake and intracellular growth of *L. pneumophila*, a collection of well-defined *D. discoideum* mutants was investigated. Furthermore, by using green fluorescent protein (GFP)-transformed host cells we were able to localize the calcium-binding proteins calreticulin and calnexin during the infection process.

METHODS

Bacterial strains and growth conditions. *L. pneumophila* Corby (serogroup 1) was grown on buffered charcoal-yeast (BCYE) agar at 37 °C in 5% CO₂ atmosphere for 3 days (Wintermeyer *et al.*, 1995). The *Escherichia coli* K-12 DH5 α strain was cultivated on Luria-Bertani (LB) agar (Sambrook *et al.*, 1989). Colony-forming units (c.f.u.) were determined by plating serial dilutions on agar plates.

***D. discoideum* strains and growth conditions.** The *Dictyostelium discoideum* wild-type strain AX2, the mutant strains G β ⁻ (Peracino *et al.*, 1998), coronin⁻ (Maniak *et al.*, 1995), α -actinin/ABP120 AGHR2 (Rivero *et al.*, 1996), DAip1⁻ (Konzok *et al.*, 1999), LimC⁻, LimD⁻, LimC⁻/D⁻ (Khurana *et al.*, 2002), villidin⁻ (Gloss *et al.*, 2003), synexin⁻ (Döring *et al.*, 1995), calnexin⁻, calreticulin⁻ and the *gfp*-transformed cells HG1738 and HG1767 (Müller-Taubenberger *et al.*, 2001) were grown at 23 °C either in shaking culture or in 75 cm² cell-culture flasks with 10 ml HL5 medium [5 g yeast extract, 10 g glucose, 10 g proteose peptone (Oxoid), 0.64 g Na₂HPO₄, 0.48 g KH₂PO₄ dissolved in 1 l H₂O, pH 7.5], G418 (20 μ g ml⁻¹) and/or blasticidin S (10 μ g ml⁻¹) was added to the mutants and GFP-transformed cells. Generation of spores and storage of *D. discoideum* cells were performed as described previously (Hägele *et al.*, 2000).

Infection of *D. discoideum* cells and effect of cellular inhibitors on phagocytosis. For infection, *D. discoideum* cells were harvested and resuspended in a 1:1 solution of HL5 medium and Sørensen phosphate buffer, pH 6.0, as described recently (Skriwan *et al.*, 2002). The experiments were performed at 24.5 °C in 25 cm² cell-culture flasks. In order to determine the effect of cellular inhibitors and host-cell mutations on bacterial uptake 1 ml of 10 \times 10⁶ host cells ml⁻¹ were co-incubated with *L. pneumophila* Corby at a m.o.i. of 10. For the inhibitor studies, *D. discoideum* AX2 cells were pretreated with various drugs (Sigma, ICN Biochemicals, Calbiochem) for 30 min (see Table 1). According to previous publications, a broad range of different drug concentrations was used for each inhibitor (Peracino *et al.*, 1998): neomycin sulfate (0.1–100 μ M, IC₅₀ 1–10 μ M), U73122 (0.5–50 μ M, IC₅₀ 1–2.1 μ M), U73343 (0.5–50 μ M), EDTA (1–1000 μ M), EGTA (1–1000 μ M), BAPTA AM (50–1000 μ M), thapsigargin (1–500 nM). The drugs were tested for antibiotic effects and maintained during the entire experiment. Following an invasion period of 2 h, the remaining extracellular bacteria were killed by a gentamicin treatment

Table 1. Effects of cellular inhibitors on the uptake of *L. pneumophila* Corby by *D. discoideum* AX2

All drugs were added to *D. discoideum* cells 30 min before the addition of bacteria. Extracellular bacteria were killed by gentamicin treatment, and the c.f.u. of intracellular bacteria were determined by plating serial dilutions on agar plates. The asterisks denote those drugs that had a significant effect on phagocytosis (Student's unpaired two-tail *t* test; $P < 0.05$, $n = 12$). Neomycin sulfate, U73122 and thapsigargin exhibited effects in a dose-dependent manner (data not shown). The value for neomycin sulfate takes 16% antibiotic killing background of the bacteria into account.

Drug	Concn (μM)	Effect	Phagocytosis (% of control)
Control	–		100 \pm 9.8
PLC pathway			
Neomycin sulfate	5	Inhibits PLC via binding to inositol phospholipids	40.9 \pm 7.4*
U73122	5	Inhibits agonist induced PLC activation	49.1 \pm 13.9*
U73343	6	Negative control for U73122	101.2 \pm 4.3
Calcium			
EDTA	500	Extracellular Ca^{2+} and Mg^{2+} chelator	84.2 \pm 10.5
EGTA	500	Extracellular Ca^{2+} chelator	63.7 \pm 8.9*
BAPTA AM	200	Intracellular Ca^{2+} chelator	45.7 \pm 13.3*
Thapsigargin	0.1	Inhibits Ca^{2+} ATPase of ER, leading to depletion of intracellular Ca^{2+} stores	68.6 \pm 4.7*

(100 $\mu\text{g ml}^{-1}$); the *D. discoideum* cells were washed three times with Sørensen buffer and finally resuspended in a 1:1 solution of HL5 medium and Sørensen buffer. After this treatment, *D. discoideum* cells were removed from the culture flasks, and cell lysates were plated on BCYE agar.

In order to determine the growth of *L. pneumophila* at different time points, 5 ml of a 1×10^6 cells ml^{-1} suspension were seeded into 25 cm^2 cell-culture flasks and *L. pneumophila* were added to the cells at a m.o.i. of 10. After 2 h co-incubation, extracellular bacteria were killed by gentamicin and washed (see above). This was defined as time 0. After different time intervals (0, 24, 48, 72 h), cell lysates were plated on BCYE agar (see above). The determination of the c.f.u. ml^{-1} represents the number of intracellularly grown *L. pneumophila*, since the bacteria were not able to grow in the liquid medium. Host-cell viability at the given m.o.i. was ensured by trypan blue exclusion. In brief, cell suspensions were incubated with trypan blue for 5 min at room temperature. The cells were counted and trypan blue-negative cells were considered as viable cells. Moreover we ensured comparable metabolic activities of the mutant and wild-type host cells by using the WST-1 and almar blue assay (Ahmed *et al.*, 1994; Ishiyama *et al.*, 1996).

Transmission electron microscopy, *in vivo* microscopy and fluorescence imaging. Transmission electron microscopy was performed as described previously (Hägele *et al.*, 2000). Confocal imaging with or without the agar overlay technique (Köhler *et al.*, 2000; Yumura *et al.*, 1984) was performed with a Zeiss LSM 510 laser scanning microscope equipped with a $63 \times /1.4$ Plan-Neofluar objective. GFP-transformed *D. discoideum* cells were harvested and washed twice with Sørensen buffer to remove antibiotics from the medium. The cells were allowed to adhere to glass coverslips for 5 min. For *in vivo* monitoring, bacteria were added at a m.o.i. of 10. For labelling of *L. pneumophila* and *E. coli* K-12 DH5 α , bacteria were washed three times with PBS, pH 7.8, and resuspended in a solution of 0.3 mM 5- (and 6-) carboxytetramethylrhodamine, succinimidyl ester [5(6) TAMRA, SE] (Molecular Probes) in PBS. Bacteria were rotated slowly in the dark at room temperature for 30 min, washed four times with Tris/HCl (Applchem), pH 7.5, and finally resuspended in Sørensen buffer. Rhodamine-labelled *L. pneumophila* and *E. coli* were then added to the cells and incubated for 0–24 h and 0–6 h at 25 °C, respectively.

RESULTS

Effect of cellular inhibitors on bacterial uptake

The necessity for calcium in phagocytosis and phagolysosome fusion has been demonstrated in various cell lines (Jaconi *et al.*, 1990; Dewitt & Hallett, 2002). Moreover, the ER has been shown to be the major calcium store of the cell (Berridge *et al.*, 2000). In order to identify the host-cell signalling processes that lead to *L. pneumophila* uptake and an ER-derived replicative vacuole, several specific cellular inhibitors were added to *D. discoideum* AX2 wild-type cells. According to the current working model that describes the signalling cascade of phagocytosis (Cardelli, 2001) we analysed the involvement of the PLC pathway and the effect of intracellular calcium levels. The effects of previously well-established inhibitor concentrations on the bacterial internalization were determined by plating host-cell lysates on agar (Table 1).

The activation of PLC during *L. pneumophila* uptake was investigated by the inhibitors neomycin sulfate (inhibits PLC via binding to inositol phospholipids), U73122 (inhibits agonist-induced PLC activation) and U73343 (negative control for U73122). This analysis revealed that neomycin and U73122 reduced the uptake of *L. pneumophila* in a dose-dependent manner. Control experiments showed that the effect of neomycin sulfate was partly due to killing of the bacteria (16%). Nevertheless, the PLC-related effect of neomycin sulfate remained significant (Table 1).

The role of calcium was determined by adding EDTA (extracellular chelator of calcium and magnesium), EGTA (extracellular chelator of calcium), BAPTA AM (intracellular calcium chelator) and thapsigargin (induces release of intracellular calcium by inhibition of the ER calcium ATPase). EDTA and EGTA exhibited an inhibitory effect on

bacterial uptake at concentrations above 500 μM (84.2% and 63.7% phagocytosis of control, respectively). The differences between EDTA and EGTA can be explained by the lower affinity of EDTA for calcium (Bers, 1982; Shelling & Sykes, 1985). BAPTA AM inhibited the uptake of *L. pneumophila* in a dose-dependent manner. The depletion of intracellular calcium stores by the addition of calcium ATPase inhibitor thapsigargin also resulted in a decrease of bacterial uptake. In summary, these results suggest that *L. pneumophila* uptake involves the PLC pathway and is modulated by changes of cytosolic calcium levels.

Effect of host-cell mutations on bacterial uptake and growth

A number of different proteins are known to regulate the phagocytic process. However, many interactions remain to be tested as to how cell signalling ultimately results in spatial and temporal changes of the cytoskeleton and membrane trafficking. In order to develop a roadmap of host-cell factors leading to the ER-associated replicative vacuole of *L. pneumophila*, we analysed the effects of three different classes of host-cell mutants: (i) $G\beta$ subunit of heterotrimeric G-proteins, (ii) cytoskeleton-associated proteins, as well as (iii) calcium-binding proteins. In order to differentiate between uptake and growth, we analysed intracellular bacterial numbers after 2 h post-infection (% of wild-type) and compared the growth rates

during the exponential growth phase (24–48 h post-infection) with the wild-type phenotype (Table 2).

Since the attachment of *L. pneumophila* initiates a signalling cascade that possibly activates PLC via heterotrimeric G-proteins, we analysed a knock-out mutant of the $G\beta$ subunit. In our phagocytosis assay, uptake of *L. pneumophila* was reduced to 38.3% when compared to wild-type host cells. The growth rate of intracellular *L. pneumophila* was also affected (Fig. 1). This result confirms a previous hypothesis in which the reduced growth of *L. pneumophila* in a $G\beta$ mutant was explained by a reduced efficiency of uptake (Solomon *et al.*, 2000).

Actin-associated proteins contribute to the formation and dynamics of the growing phagocytic cup and the replicative vacuole. Therefore we analysed *D. discoideum* mutants lacking specific cytoskeleton-associated proteins. This set included the knock-out mutants coronin⁻ (Maniak *et al.*, 1995), α -actinin/ABP-120 AGHR2, DAip1⁻ (F-actin fragmenting and capping protein), LimC⁻, LimD⁻, LimC⁻/D⁻ (cytoskeleton-membrane interface protein) and villidin⁻ (F-actin-associated protein that associates with the Golgi apparatus and the ER) (Eichinger *et al.*, 1999; Noegel & Schleicher, 2000; Konzok *et al.*, 1999; Prassler *et al.*, 1998; Khurana *et al.*, 2002; Vardar *et al.*, 2002; Gloss *et al.*, 2003). In contrast to a previous report, we observed a reduced uptake of *L. pneumophila* (63.9%) into coronin null

Table 2. Effects of host-cell mutations in *D. discoideum* on uptake and growth of *L. pneumophila* Corby

Extracellular bacteria were killed by gentamicin treatment, and the c.f.u. of intracellular bacteria were determined by plating serial dilutions on agar plates. The asterisks denote those mutants that had a significant effect on uptake of bacteria (Student's unpaired two-tail *t* test; $P < 0.05$, $n = 12$). For growth data of impaired infections, see Fig. 1. p.i., post-infection.

Mutant	Intracellular bacteria in <i>D. discoideum</i> mutants	
	Uptake (% of wild-type 2 h p.i.)	Intracellular growth [log (c.f.u. at 48 h/c.f.u. at 24 h)]
AX2 (control)	100 \pm 4.8	1.47
Heterotrimeric G protein		
$G\beta$ ⁻	38.3 \pm 7.9*	0.74
Cytoskeleton-associated proteins		
Coronin	63.9 \pm 11.6*	1.62
α -Actinin/ABP-120 AGHR2	55.7 \pm 2.6*	0.5
DAip1 ⁻	63.7 \pm 4.2*	1.05
LimC ⁻	84.8 \pm 4.9	1.22
LimD ⁻	82.5 \pm 11.4	1.1
LimC ⁻ /D ⁻	51.0 \pm 5.4*	0.5
Villidin	50.1 \pm 8.3*	0.95
Calcium-binding proteins		
Synexin ⁻	86.1 \pm 8.1	1.23
Calnexin ⁻	66.4 \pm 6.9*	0.49
Calreticulin ⁻	43.34 \pm 5.43*	0.86

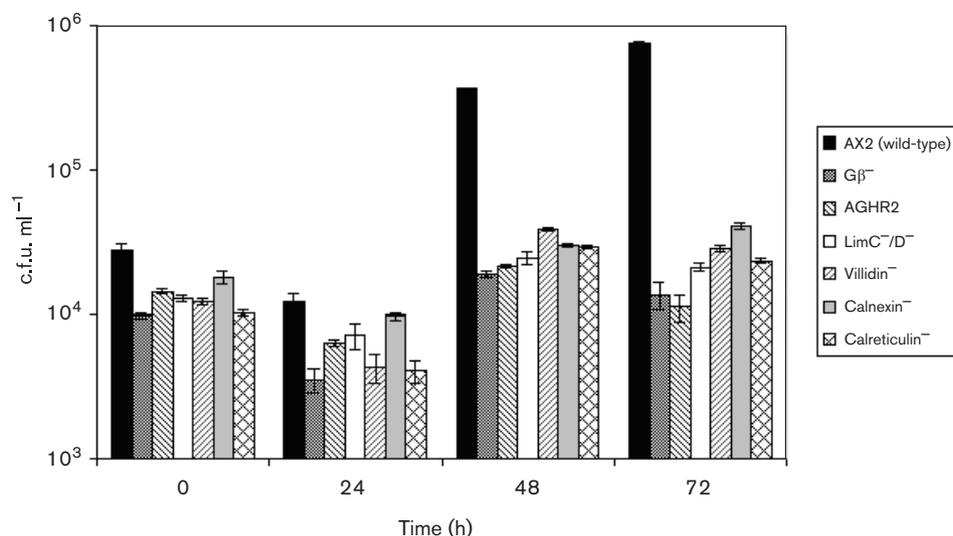


Fig. 1. Intracellular growth of *L. pneumophila* Corby in *D. discoideum* AX2 wild-type, $G\beta^-$, α -actinin/ABP-120 AGHR2, LimC⁻/D⁻, villidin⁻, calnexin⁻ and calreticulin⁻ minus cells. Each gentamicin infection experiment was performed three times in a 1 : 1 solution of HL5 medium and amoebal saline at 24.5 °C. The values shown are the mean numbers of c.f.u. determined at 0, 24, 48 and 72 h after co-culture was started. The standard deviation is indicated by an error bar for each time point after infection.

mutants (Solomon *et al.*, 2000). The growth rate of intracellular legionellae within this mutant, however, was higher compared to legionellae grown in wild-type host cells. The double mutant α -actinin/ABP-120 AGHR2 showed a 55.7% reduction in uptake of *L. pneumophila* and also the intracellular growth of *L. pneumophila* was inhibited in this mutant (Fig. 1). The knock-out mutation of the F-actin fragmenting and capping protein DAip1 had a moderate effect on phagocytosis (63.7%) when compared to wild-type host cells. Intracellular growth of *L. pneumophila* in the DAip1 mutant was similar to the wild-type phenotype. Lim proteins are thought to act as adapters at the cytoskeleton–membrane interface, where they might be involved in a receptor-mediated rac1-signalling pathway that leads to actin polymerization in lamellipodia (Prassler *et al.*, 1998). Moreover, LimC and LimD of *D. discoideum* were shown to directly bind to F-actin and affect actin-related cellular processes (Khurana *et al.*, 2002). Mutations in LimC and LimD had minimal effects on bacterial uptake. However, a double mutation resulted in a reduction of *L. pneumophila* uptake (51.0%). The growth rate of *L. pneumophila* was also only affected in the LimC⁻/D⁻ double mutant (Fig. 1). The protein villidin is primarily associated with membranes of the ER and the Golgi apparatus but it can also associate with phagocytic membranes of *D. discoideum*. Interestingly, the corresponding knock-out mutant exhibited a reduced uptake (50.1%) and a reduced growth of *L. pneumophila* (Fig. 1).

The third group of mutants were lacking the calcium-binding proteins synexin/annexin C1, calnexin and calreticulin, respectively. *D. discoideum* synexin/annexin C1 is

characterized by its ability to bind certain phospholipids in a calcium-dependent manner. The synexin-minus mutant showed a slight reduction in uptake of *L. pneumophila* (86.1%) in our phagocytosis assay. In the gentamicin infection assay, *L. pneumophila* exhibited wild-type growth rates in synexin-minus host cells. Calreticulin and calnexin are calcium-binding proteins with chaperone activity in the ER (Müller-Taubenberger *et al.*, 2001). The phagocytosis of *L. pneumophila* into calnexin-minus cells was reduced (66.4%) and the intracellular growth rate of the bacteria was significantly lower (Fig. 1). The calreticulin-minus cells showed an even more severe reduction in *L. pneumophila* uptake (43.3%). The intracellular growth rate in this mutant was also reduced compared to wild-type host cells. Confocal laser scanning microscopy after different time points confirmed that the reduced bacterial numbers in the mutants were not only due to a defect in phagocytosis. Calreticulin- and calnexin-minus cells contained fewer bacteria within a single phagosome compared to phagosomes of wild-type cells. This indicates an intracellular growth defect of the bacteria within the phagosomes of the mutants. Taken together, these data suggest that both proteins have modulatory functions during phagocytic cup formation and the establishment of the replicative vacuole.

Intracellular distribution of GFP-tagged calnexin and calreticulin during *L. pneumophila* infection

Transmission electron microscopy of calnexin-minus and calreticulin-minus host cells demonstrated the association of the *L. pneumophila*-containing phagosomes with the rough ER (data not shown). This observation indicates that

cellular trafficking of the bacteria in the mutant host cells is similar to the wild-type host cells (Solomon & Isberg, 2000). Since the intracellular calcium homeostasis is maintained primarily by the ER, we further analysed the distribution of calreticulin and calnexin upon infection. The previous visualization of GFP-tagged calnexin and calreticulin in *D. discoideum* revealed a physical connection of these proteins to the phagocytic cup during uptake of yeast particles and *E. coli* (Müller-Taubenberger *et al.*, 2001). To visualize the spatial and temporal distribution of both proteins during *L. pneumophila* uptake, we recorded phagocytosis in a confocal time series of wild-type cells using GFP-tagged calnexin or calreticulin. The phase-contrast sequence in Fig. 2(a) (upper panel) shows the first engagement of the cell with *L. pneumophila*. The corresponding fluorescence images (lower panel) reveal a specific accumulation of GFP-tagged calnexin in the phagocytic cup which embraces the bacterium. A similar result with green fluorescence present in the protrusions on both sites of the cup was observed with GFP-tagged calreticulin (Fig. 2b).

In a previous study with yeast particles and *E. coli* it was demonstrated that contacts of the ER and the phagosome were transient (Müller-Taubenberger *et al.*, 2001). In accordance with that study, we observed the disappearance of calnexin or calreticulin shortly after the complete internalization of *E. coli* (Fig. 3a, b). In contrast, *L. pneumophila*-containing phagosomes were permanently decorated by GFP-tagged calnexin and calreticulin (Fig. 3c, d). Also the quantification of *L. pneumophila*-containing phagocytic cups and phagosomes confirmed the permanent decoration with calnexin and calreticulin over time. Decorated phagosomes containing *E. coli* were not observed after 5 h co-incubation (Fig. 4). Taken together, the requirement of calcium for phagocytosis, the recruitment and tight association of calnexin and calreticulin to the phagocytic cup, the decoration of the replicative vacuole with both ER proteins, and the defective infection of *L. pneumophila* in calnexin- and calreticulin-minus cells suggest that calnexin and calreticulin modulate uptake and the formation of an ER-derived replicative vacuole of *L. pneumophila*.

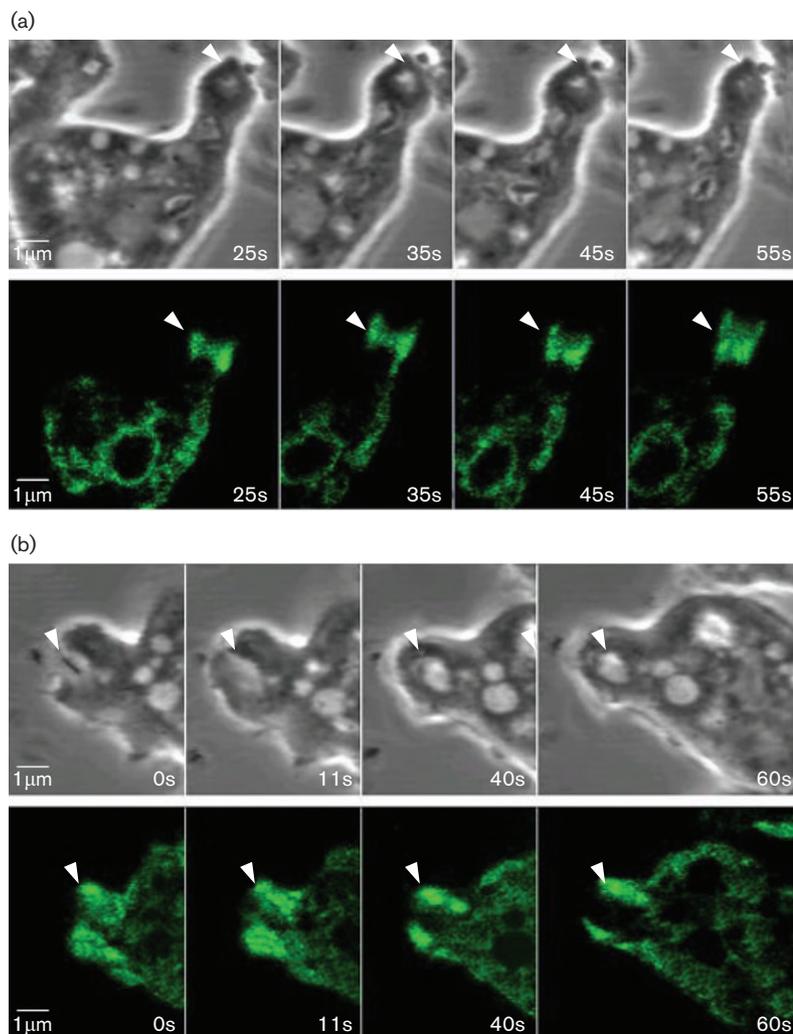


Fig. 2. Confocal *in vivo* monitoring of phagocytic cup formation during *L. pneumophila* uptake by *D. discoideum*. The phase-contrast sequence (upper panel) shows the internalization of a single bacterium (arrow) from the first contact (0 s) until the closure of the phagocytic cup (55 s). Green fluorescence of the corresponding images (lower panel) indicate the respective GFP-tagged host proteins calnexin (a) and calreticulin (b). Both proteins (arrows) accumulate at the basis of the protrusions and extend into the outgrowing phagocytic cup. Size bar as indicated.

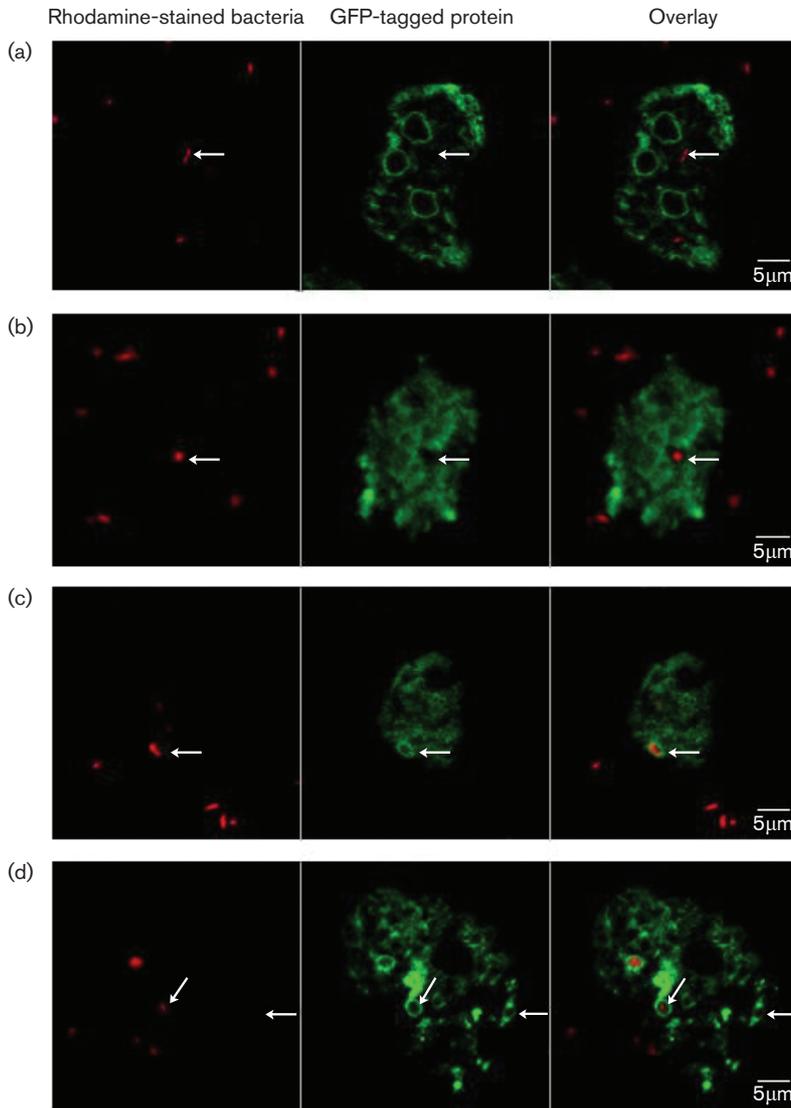


Fig. 3. Confocal *in vivo* monitoring of the *D. discoideum* proteins calnexin and calreticulin during *E. coli* uptake and *L. pneumophila* infection. Red fluorescence indicates rhodamine-labelled bacteria (left), and green fluorescence indicates the respective GFP-tagged host proteins (middle). The double exposure (red and green fluorescence) shows the association of bacteria with calnexin or calreticulin (right). The ER-specific type I transmembrane protein calnexin (a) and the luminal ER-specific protein calreticulin (b) do not surround the *E. coli* phagosome. In contrast, the phagosomes of *L. pneumophila* are surrounded by calnexin (c) and calreticulin (d) during the entire infection. Arrows indicate the location of the accumulated Ca^{2+} -binding proteins. Size bar as indicated.

DISCUSSION

The dynamics of phagocytosis in *D. discoideum* and macrophages are very similar. In both cases particle adhesion to the cell surface induces local polymerization of the actin

cytoskeleton, pseudopodial extension, formation of a phagocytic cup, and internalization of the particle into a phagosome (Müller *et al.*, 1996). The alteration of the phagosomal maturation which is required for intracellular growth of *L. pneumophila* appears to be the result of the

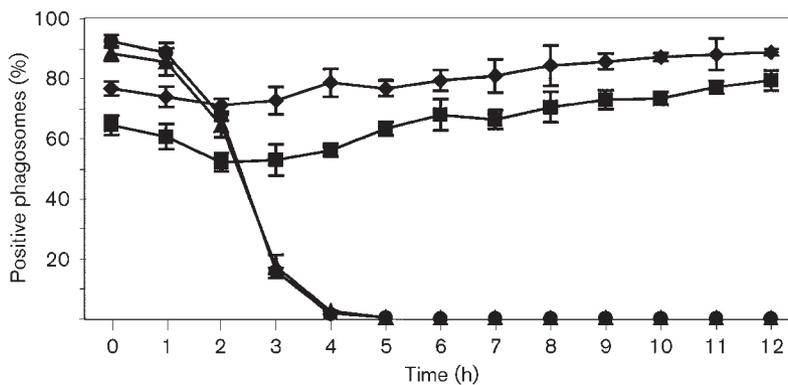


Fig. 4. Presence of GFP-tagged calnexin and calreticulin in phagocytic cups and phagosomes of *L. pneumophila* and *E. coli* at different time points of coincubation. Values are means of three independent experiments. A total of 40 phagosomes were analysed for each protein. ◆, Calreticulin/*L. pneumophila*; ■, calnexin/*L. pneumophila*; ▲, calreticulin/*E. coli*; ●, calnexin/*E. coli*.

presence or absence of special signals at the onset of infection. In a recent publication it was shown that *L. pneumophila* replicates normally in *D. discoideum* macroautophagy mutants and produces replicative vacuoles that are morphologically indistinguishable from those in wild-type host cells (Otto *et al.*, 2004).

In the present study we focused on early processes of *L. pneumophila* infection by using the *Legionella*–*Dictyostelium* infection model (Hägele *et al.*, 2000; Skriwan *et al.*, 2002; Solomon *et al.*, 2000). Phagocytosis in *D. discoideum* and macrophages is known to be regulated by a heterotrimeric G-protein-linked signal transduction. Inactivation of the gene encoding the G β subunit inhibits signal-induced actin polymerization (Damiani & Colombo, 2001; Garin *et al.*, 2001). We were able to demonstrate that *L. pneumophila* uptake into *D. discoideum* cells involves the β -subunit of heterotrimeric G-proteins and the PLC pathway (Fig. 5). These results suggest a conventional uptake of *L. pneumophila* by *D. discoideum*. This finding does not exclude the possibility that phagocytosis may be stimulated by secreted bacterial effectors (Hilbi *et al.*, 2001). In addition, our data also explain previous findings of increased bacterial growth of *L. pneumophila* in profilin-minus *D. discoideum* cells (Hägele *et al.*, 2000; Haugwitz *et al.*, 1994). Profilin up-regulates macropinocytosis and inhibits the PLC-regulated phagocytosis. In profilin-minus cells this regulation is shifted towards phagocytosis, which results in a higher rate of *L. pneumophila* uptake via phagocytosis (Cardelli, 2001).

The process of phagocytic cup and phagosome formation also depends on the coordinated interplay of certain actin-binding proteins (May & Machesky, 2001). Therefore, special signals at the onset of invasion may reorganize cytoskeletal components and provide mechanical means for the *L. pneumophila*-specific vacuole. One of the main obstacles in the analysis of these processes, however, is the

substantial redundancy of the actin system, with multiple proteins sharing overlapping functions. Accordingly, the mutations in LimC and LimD had minimal effects on bacterial uptake, while the double mutation (LimC/D) resulted in a strongly reduced uptake. Both proteins are unique in that they interact directly with F-actin and might thereby regulate lamellipodia protrusion and phagocytosis (Khurana *et al.*, 2002). The DAip1 protein plays a regulatory role in the rapid remodelling of the cortical actin meshwork (Konzok *et al.*, 1999). DAip1-minus cells are reduced in their capacity of fluid-phase uptake and phagocytosis but show minimal effects on uptake of *L. pneumophila*. Similar effects on *L. pneumophila* uptake were observed with the mutant affected in the F-actin and membrane-associated protein villidin. This is especially interesting since GFP-fusion constructs suggest that the protein associates with the membranes of the ER as well as the Golgi and that the WD-repeat domain can bind to the actin cytoskeleton.

Calcium is one of the most important signalling molecules of the cell. Changes in calcium levels appear to be essential for the induction of certain genes as well as for the activation of cytoskeletal components and enzymes. In our study the general role of calcium during *L. pneumophila* infection was determined by adding extracellular and intracellular chelators of calcium to phagocytosis assays. The extracellular chelators EDTA and EGTA exhibited inhibitory effects on bacterial uptake. BAPTA AM, a chelator of intracellular calcium, inhibited the uptake of *L. pneumophila* in a dose-dependent manner. The depletion of intracellular calcium stores by the addition of calcium ATPase inhibitor thapsigargin resulted in a moderate decrease of bacterial uptake. These results suggest that *L. pneumophila* uptake is modulated by changes of cytosolic calcium levels. Since calcium-mediated signal transduction is known to be essential for the activation of the phagocytic respiratory burst, production of nitric oxide, secretion of microbicidal

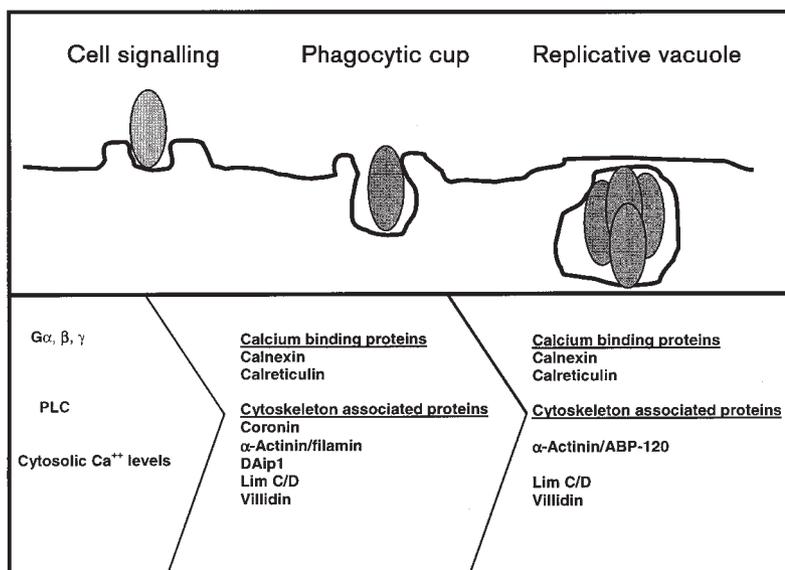


Fig. 5. Roadmap of *D. discoideum* host-cell factors which significantly contribute to the uptake of *L. pneumophila* and the establishment of an ER-associated replicative vacuole.

granule constituents, and synthesis of proinflammatory mediators, it will be important to analyse the spatial alterations of cellular calcium concentrations (Malik *et al.*, 2000). To obtain more specific insights into the role of calcium we chose different calcium-binding proteins for further investigations.

It was hypothesized that the calcium-buffering capacity of calreticulin and calnexin in the ER directly influences the calcium concentration in the narrow cytoplasmic zone of the phagocytic cup (Müller-Taubenberger *et al.*, 2001). The release and reuptake of calcium between the cytosol and the ER also affect functions of the ER itself, including protein synthesis, protein secretion and chaperone activity. Since the *L. pneumophila* phagosome associates with the ER, the changes in calcium levels may be involved in this interaction. Our infection assays with calreticulin- and calnexin-minus mutants and the *in vivo* monitoring of GFP-tagged calreticulin and calnexin are a first indication in this respect. Another possibility is that calnexin and calreticulin play a role in the proper folding of proteins involved in infection. The firm association of the *L. pneumophila* phagosome and the GFP-tagged proteins extended beyond the uptake phase and was observed throughout the entire infection. The reasons as to why only the *L. pneumophila*-containing phagosome remained enveloped by calnexin and calreticulin, while the association of these proteins with the phagocytic cup of *E. coli* was transient remains to be elucidated. A recent study with bone marrow-derived macrophages from A/J mice suggests that *L. pneumophila* creates an ER-derived organelle by a process that involves intercepting early secretory vesicles exiting from transitional ER (Kagan & Roy, 2002; Roy & Tilney, 2002). The authors state that transport of *L. pneumophila* to the ER depends on ARF1 function and is distinct from ER-mediated phagocytosis.

Taken together our results show that *L. pneumophila* is internalized by phagocytosis which involves heterotrimeric G-proteins, the PLC pathway, and specific cytoskeleton-associated and calcium-binding proteins (Fig. 5). The observed effects of (i) the intracellular calcium concentrations on bacterial uptake, (ii) the reduced phagocytosis of *L. pneumophila* into calnexin- and calreticulin-minus cells, (iii) the impaired growth of *L. pneumophila* in both mutant host cells, and (iv) the permanent decoration of the replicative vacuole with calnexin and calreticulin suggest a strong modulatory function of these factors on *L. pneumophila* infection. The roadmap presented here lays the foundation for understanding the link of calcium signals, phagocytic cup formation and the establishment of the *L. pneumophila*-specific vacuole. Clearly, further work will be required to identify how *L. pneumophila* determines this intracellular route.

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