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Topical Review

Phagosome Maturation: A Few Bugs in the System

C.C. Scott, R.J. Botelho, S. Grinstein
Programme in Cell Biology, The Hospital for Sick Children and Department of Biochemistry, University of Toronto, 555 University Avenue, Toronto, Ontario, Canada

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Abstract. Cells of the innate immune system ingest and destroy invading microorganisms by initially engulfing them into a specialized vacuole, known as the phagosome. The membrane of the forming phagosome is similar to the plasmalemma and its contents resemble the extracellular milieu. As such, the nascent phagosome is not competent to kill and eliminate the ingested microorganisms. However, shortly after sealing, the phagosome undergoes a series of rapid and extensive changes in its composition, the result of a sophisticated sequence of membrane fusion and fission reactions. Understanding the molecular basis of these events is of particular importance, since they are often the target of disruption by intracellular parasites such as *Mycobacterium*, *Salmonella* and *Legionella*. The objective of this review is to summarize the current knowledge of the molecular mechanisms underlying phagosomal maturation and its subversion by parasitic microorganisms.

Key words: Phagosome — Macrophage — Rab — SNARE — Phosphoinositides — *Salmonella* — *Mycobacterium*

Introduction

Macrophages and neutrophils are sentinels that seek and destroy invading pathogens. Elimination of invading microorganisms is effected primarily by phagocytosis, a process whereby the pathogens are engulfed into a plasma membrane-derived vacuole or phagosome. However, destruction of the pathogen is not the consequence of internalization *per se*, but of a complex ensuing process termed phagosome maturation. Following formation, the phagosome sequentially fuses with a series of endomembrane compartments and thereby acquires microbicidal and degradative properties (Beron et al., 1995; Aderem & Underhill, 1999; Tjelle, Lovdal & Berg, 2000). Paradoxically, phagocytosis is also a portal of infection. Some of the most vicious bacterial pathogens such as *Mycobacterium* and *Leishmania* take advantage of the phagocytic machinery to gain access to the intracellular milieu where, by subverting the maturation process, they become intracellular parasites (Meresse et al., 1999b; Duclos & Desjardins, 2000; Hackstadt, 2000). Alternatively, some pathogens, typified by *Salmonella* and *Shigella*, promote their own entry into host cells by a process known as invasion. Intracellular residence is advantageous because it provides the parasite a niche wherein it can hide from circulating immune factors.

In this review, we will briefly summarize the salient features of the process of phagosome maturation and will then focus on how some pathogens can circumvent phagosome maturation by interfering with cellular signaling events. Other aspects of phagocytosis and phagosome maturation have been recently reviewed (Rabinovitch, 1995; Franc, White & Ezekowitz, 1999; Kwiatkowska & Sobota, 1999; Bangs, Franc & White, 2000; Cardelli, 2001; May, 2001; May & Machesky, 2001; Aderem, 2002; Stephens, Ellson & Hawkins, 2002; Underhill & Ozinsky, 2002; Vieira, Botelho & Grinstein, 2002).

Phagosome Maturation: From Plasmalemma to Lysosomes

Phagocytosis is initiated by the clustering of surface receptors upon interaction with their cognate ligand. A variety of phagocytic receptors can recognize innate components of the pathogen surface, or pathogen-associated molecular patterns, effecting non-opsonic phagocytosis. Included in this category

Correspondence to: S. Grinstein; email: sga@sickkids.ca

Table 1. Molecular markers of endomembrane organelles thought to interact with phagosomes or invasion vacuoles

Organelle	Markers
Sorting endosome; early phagosome	EEA-1, Rab5, PI(3)P, syntaxin-13, transferrin receptor, VAMP3
Late endosome; late phagosome	Rab7, Rab9, mannose-6-phosphate receptor, syntaxin-7, LAMPs, LBPA
Lysosome; phagolysosome	LAMPs, mature cathepsin D; fluid-phase markers chased for ≥2 hr
Endoplasmic reticulum	Calnexin, calreticulin, GRP78, UDPGT, VAP33

are the mannose receptors that bind to mannyl and fucosyl residues on the surface of bacteria and fungi, and the promiscuous scavenger receptors, which recognize numerous ligands including lipopolysaccharides and lipoproteins (Ofek et al., 1995; Kwiatkowska & Sobota, 1999; Schutt, 1999; Hoffmann et al., 2001). Alternatively, several phagocytic receptors recognize an exogenous, host-derived ligand, or opsonin, that adheres to the pathogen surface. Complement receptors, which include members of the integrin family (CR3 and CR4), recognize primarily complement-coated pathogens, although these receptors exhibit some degree of promiscuity towards ligands (Diamond et al., 1993; Le Cabec et al., 2002; Thornton et al., 1996). In contrast, the best characterized phagocytic receptor class, the Fcγ receptors (FcγRs), bind to IgG-opsonized pathogens. Detailed reviews on the structure and function of these receptors can be found elsewhere (Daeron, 1997; Gessner et al., 1998; Petty & Todd, 1993; Yefenof, 2000).

The signaling cascades activated during phagocytosis depend on the specific receptor-ligand pair. For instance, proximal signaling in FcγR-mediated phagocytosis entails the activation of Src family and Syk tyrosine kinases. In contrast, integrin-family and mannose receptors are thought to operate independently of Syk kinase (Allen & Aderem, 1996). This is of significance since the receptor-ligand pair seems to influence the path of phagosome maturation, i.e., not all phagosomes are created equal. The current list of proteins implicated in the complex process of particle engulfment is long and grows longer by the month, at present including not only protein kinases, but also phosphatases, GTPases, lipid-modifying enzymes, adaptor complexes, actin-binding proteins and membrane fusion and fission mediators (Kwiatkowska & Sobota, 1999; Lennartz, 1999; May & Machesky, 2001). The concerted action of all these proteins and co-factors causes an exquisite localized remodeling of the membrane, driven largely by the actin cytoskeleton and culminating in the formation of the phagosome.

As mentioned above, newly formed phagosomes are relatively inert, unable to digest their microbial contents. However, after its scission from the plasmalemma, the phagosome appears to have acquired information that directs it to interact with endomembrane organelles. It fuses sequentially with sorting endosomes (often referred to as early endosomes), late endosomes and lysosomes (Desjardins et al., 1994). As a result of these fusion events the phagosomal lumen becomes a highly acidic and oxidizing environment, endowed with a variety of hydrolytic enzymes that can effectively digest its contents (Hampton, Kettle & Winterbourn, 1998; Tapper, Furuya & Grinstein, 2002). While a brief description of endocytic organelles follows, expert reviews on endocytosis and endocytic traffic can be found elsewhere (Mellman, 1996; Mukherjee, Ghosh & Maxfield, 1997; D'Hondt, Heese-Peck & Riezman, 2000; Gruenberg, 2001). For reference, Table 1 lists a number of markers for each compartment of the endocytic pathway, which are often used to identify the stage of progression of phagosome maturation.

Phagosome age, i.e., the time elapsed after fission of the vacuole from the plasmalemma, dictates its preference to fuse with the individual subcompartments of the endocytic pathway: early phagosomes selectively fuse with sorting/recycling endosomes, intermediate phagosomes with late endosomes and late phagosomes with lysosomes (Mayorga, Bertini & Stahl, 1991; Pitt et al., 1992a; Desjardins et al., 1994; Desjardins et al., 1997) (Table 1). This suggests the sequential acquisition of fusion determinants during the course of maturation. Desjardins and colleagues (Desjardins et al., 1994; Desjardins, 1995) have proposed that fusion between phagosomes and endo/lysosomes does not result in amalgamation of the organelles. Instead, they propose a "kiss-and-run" mechanism, where endosomes or lysosomes and phagosomes form transient hybrid organelles connected by fusion pores that allow selective exchange of some membranes and luminal components, followed by fission. Regardless of the detailed mechanism involved, there is general agreement that it is the orderly interaction with elements of the endocytic pathway that confers onto phagosomes their highly acidic and protease-rich features.

Recent experiments suggest that, shortly after formation, phagosomes merge with the endoplasmic reticulum (Garin et al., 2001; Gagnon et al., 2002). If confirmed, these observations would represent a paradigm shift and the resulting implications to phagosome composition and maturation would need to be explored in detail.

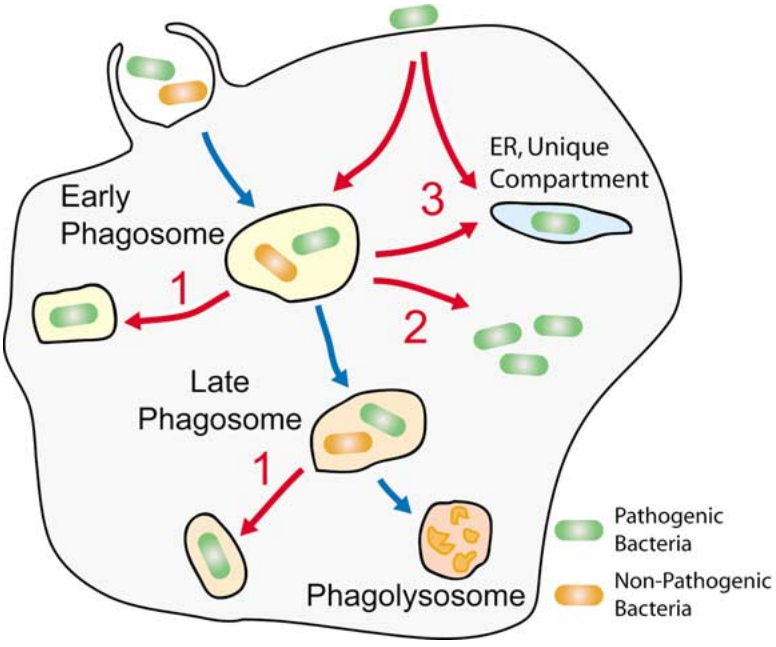

Fig. 1. Typical phagosome maturation and strategies of intracellular pathogen survival. Normally, internalized non-pathogenic bacteria (represented by the orange oval) are enclosed in a membrane-bound structure, the phagosome, which, by sequential interaction with the endocytic pathway, matures through several distinct stages (indicated by the blue arrows). The nascent phagosome is similar in composition to the plasma membrane but soon acquires markers of early endosomes. The resulting early phagosome then interacts with late endosomes to produce the late phagosome, which eventually fuses with lysosomes producing the phagolysosome where the internalized particle is degraded. Various pathogens (represented by the green ovals) have evolved mechanisms to subvert phagosome maturation (indicated by the red arrows) in order to provide a suitable niche for their survival within cells. In addition to uptake by phagocytic cells, which is used as the primary mechanism of entry by parasites like *Mycobacterium*, many bacteria such as *Salmonella* have developed specialized systems to enter non-phagocytic cells. Once inside, successful pathogens use one of three general strategies to avoid host cell defenses: (1) arrest maturation of the phagosome or vacuole, (2) disrupt their enclosing vacuole to escape into the host cell's cytosol, or (3) manipulate membrane traffic to take up residence in a unique membrane structure or an existing organelle such as the endoplasmic reticulum.

An overview of the molecular mechanisms regulating phagosome maturation at the various stages is presented below, followed by a discussion of how these may be subverted by pathogens (Fig. 1). Particular attention will be paid to the role of *SNARE* (soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptor) complexes, Rab GTPases and phosphoinositides (PIs).

Regulation of Sorting Endosome-Phagosome Fusion

Sorting endosomes are mildly acidic (pH 6.1) and poor in hydrolytic activity. They are the recipients of cargo internalized by endocytosis, which they acquire upon fusion with endocytic vesicles and then aptly segregate for either recycling or degradation. Cargo intended for recycling is restricted to tubules and/or vesicles that pinch off the sorting endosome and are targeted to recycling endosomes and/or directly back to the plasma membrane. The remaining endosomal components are transformed into a multivesicular body (MVB) that fuses with late endosomes (Mukherjee et al., 1997; Gruenberg, 2001).

Rab5 is a critical regulator of sorting endosome dynamics. This GTPase has been implicated in myriad functions, including pinocytosis, microtubule-dependent endosomal motility, sorting endosome-endocytic vesicle fusion and homotypic fusion of sorting endosomes (Somsel Rodman & Wandinger-Ness, 2000). The effector molecules responsible for these functions are not always known, but the p150/hVPS34 complex is of prominence. Originally characterized in yeast as Vps15, p150 is a regulatory Ser/Thr kinase subunit of the complex. The catalytic subunit is hVPS34, a class III PI 3-kinase that phosphorylates PI to synthesize phosphatidylinositol-3-phosphate [PI(3)P] (Volinia et al., 1995; Panaretou et al., 1997; Vanhaesebroeck & Waterfield, 1999). Inhibition of hVPS34 results in enlargement of sorting endosomes, mistargeting of vacuolar/lysosomal proteins and arrest of endosome maturation (Schu et al., 1993; Shpetner et al., 1996; Futter et al., 2001; Row et al., 2001). PI(3)P, the product of hVPS34, binds to the FYVE or PX domains of effector proteins, thereby anchoring them to the cytosolic face of sorting endosomes (Burd & Emr, 1998; Gaullier et al., 1998; Kanai et al., 2001; Misra, Miller & Hurley, 2001; Xu et al., 2001). An important and well characterized PI(3)P effector is EEA1, which also interacts directly with Rab5 (Lawe et al., 2000). EEA1 dimers are thought to tether or bridge sorting endosomes with incoming endocytic vesicles, a prerequisite for the coalescence of these compartments (Callaghan et al., 1999).

Complementing its tethering function, EEA1 also interacts with syntaxin 13, a SNARE molecule, and with NSF (McBride et al., 1999; Mills, Urbe & Clague, 2001). SNARE molecules are catalysts of membrane fusion. The current paradigm envisions the formation of a four-helix bundle, or SNAREpin, where an R-SNARE (R: arginine) on one membrane contributes one coiled-coil domain and

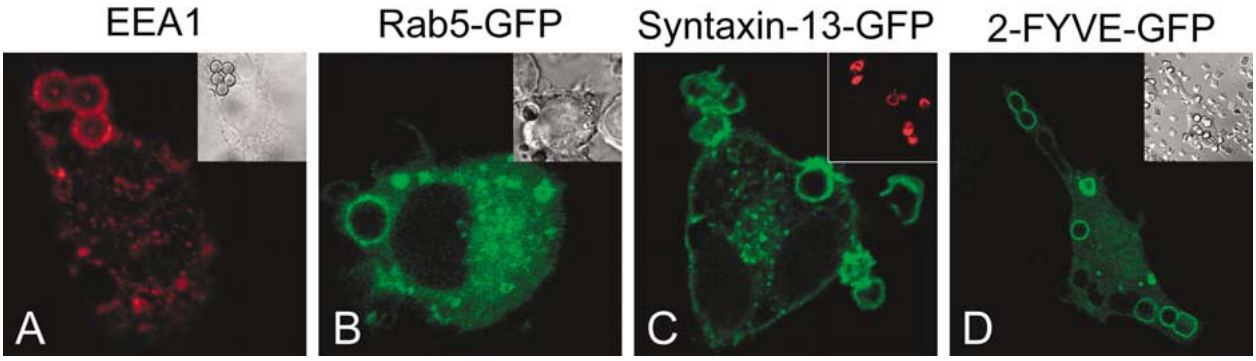

Fig. 2. Early phagosome markers. Several early endocytic markers are found on the early phagosome. Latex beads or sheep red blood cells were opsonized with IgG and added to RAW264.7 cells to induce phagocytosis. After allowing the phagosomes to mature to an early phagosome stage (2.5 to 10 min), cells were fixed and stained with antibodies to Early Endosome Autoantigen 1 (EEA1) (*A*). In *B–D* the cells had been transfected with chimeric constructs of GFP with endosomal markers: (*B*) cells transfected with Rab5a-GFP; (*C*) cells transfected with syntaxin 13-GFP; (*D*) cells transfected with a probe for phosphatidylinositol-3-phosphate (2-FYVE-GFP). Insets in *A*, *B* and *D* show corresponding bright-field images. Inset in *C* shows immunostained red blood cells.

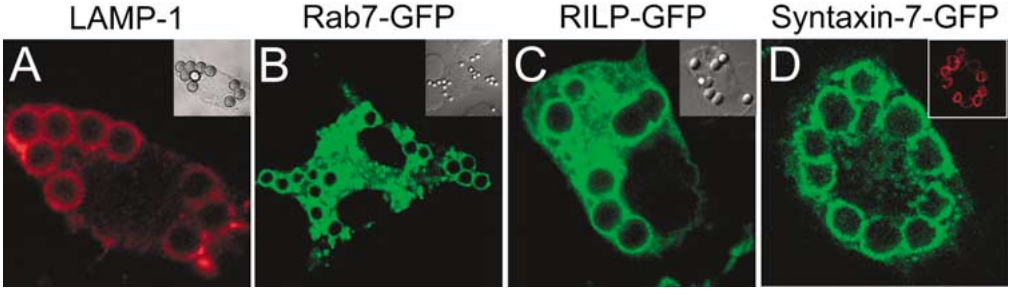

Fig. 3. Late phagosome markers. Several late endosomal markers are found on the late phagosome. Latex beads were opsonized with human IgG and added to RAW264.7 cells to induce phagocytosis. After maturing to a late phagosome stage (30 to 60 min), cells were fixed and stained with antibodies to Lysosomal Associated Membrane Protein 1 (LAMP-1) (*A*). In *B-D* the cells had been transfected with chimeric constructs of GFP with endosomal markers: (*B*) cells transfected with Rab7-GFP; (*C*) cells transfected with RILP-GFP; (*D*) cells transfected with Syntaxin 7-GFP. Insets *A–C* show corresponding bright-field images. Inset *D* shows immunostained red blood cells.

three Q-SNAREs (Q: glutamine) present on the opposite membrane, contribute three coiled-coils (SNAP-25-like SNAREs contribute two coiled-coils and therefore only require an additional Q-SNARE) (Hay, 2001). SNAREpins bring opposing bilayers in very tight apposition, a critical step in membrane coalescence. After fusion, SNAREpins are disassembled by NSF, a hexameric chaperone ATPase, and can then be recycled for another round of fusion (Sollner et al., 1993; Swanton et al., 2000; Littleton et al., 2001). Inhibition of Rab5, or of its effectors, EEA1, hVPS34, NSF or syntaxin-13, ablates sorting endosome maturation and traffic to lysosomes (Bucci et al., 1992; Christoforidis et al., 1999a, 1999b; McBride et al., 1999). In summary, Rab5 integrates the targeting, tethering and fusion of endosomes, and a similar job description likely applies also to other Rab GTPases.

Newly formed phagosomes rapidly gain the properties of sorting endosomes as revealed by phagosomal acquisition of Rab5, EEA1, syntaxin-13 and PI(3)P (Alvarez-Dominguez et al., 1996; Sciamanico et al., 1999; Ellson et al., 2001; Vieira et al., 2001; Collins et al., 2002) (Table 1, Fig. 2). The presence of PI(3)P in phagosomes was visualized using green fluorescent protein (GFP) chimeras of the FYVE or PX domains, and was shown to start shortly after sealing and to be transient (Ellson et al., 2001; Vieira et al., 2001). Early phagosomes have a propensity to fuse with sorting endosomes while being refractory to fusion with lysosomes, as demonstrated by experiments in vitro as well as in vivo (Mayorga et al., 1991; Pitt et al., 1992a; Desjardins et al., 1994, 1997). Like early endosomes, early phagosomes also have the ability to recycle molecules to the plasma membrane (Muller, Steinman & Cohn, 1980; Pitt et al., 1992a), a process partly enabled by COPI and ARF GTPases (Beron et al., 1995; Botelho et al., 2000).

Many of the components that dictate endosome fusion appear to be essential also for early phagosome maturation. Thus, expression of Rab5 dominant-negative alleles (Funato et al., 1997; Mukherjee et al., 2000; Fratti et al., 2001; Vieira

et al., 2001; Collins et al., 2002), microinjection of EEA1 antibodies (Fratti et al., 2001), inhibition of hVPS34 (Vieira et al., 2001), treatment with NSF antagonists (Mukherjee et al., 2000) or expression of inhibitory fragments of syntaxin-13 (Collins et al., 2002) all arrested phagosome maturation. It is not clear whether arrest was due to impairment of phagosome fusion with sorting endosomes, or to the inability of early phagosomes to fuse with late endosomes/lysosomes. While these factors are known to act at discrete steps, we are currently unable to integrate their function and to reconstruct the entire fusogenic sequence. The cognate SNAREs ligands that form a complex with syntaxin-13 are not yet known. Moreover, while EEA1 inhibition hinders phagosome maturation (Fratti et al., 2001), it is unclear whether this is the only PI(3)P-dependent component of the maturation sequence, since a variety of other proteins can potentially also interact with PI(3)P. Of these, Hrs, PIKfyve and sorting nexins have been implicated in membrane traffic events and therefore constitute potential modulators of phagosome maturation.

The early, sorting endosomal-like state of the phagosome is transient. This is indicated by the loss of Rab5, EEA1 and PI(3)P from the phagosomal membrane, followed immediately by the acquisition of late endosomal markers (*see* below and Table 1). Whether divestment of sorting endosomal components is required for transition into the next phase of maturation, or a consequence thereof, remains undefined. Notably, inhibition of PI 3-kinases, which impedes phagolysosome formation, also delays removal of Rab5, suggesting a negative feedback loop between Rab5 and its effector (O.V. Vieira and S. Grinstein, unpublished observations).

Regulation of Fusion of Phagosomes to Late Endosomes and Lysosomes

Late endosomes are characterized by the presence of multivesicular structures, acidic luminal pH (5.5–6.0) and active proteases. Late endosomes are identified by the expression of Rab7, syntaxin-7, mannose-6-phosphate receptors (M6PR) and lysobisphosphatidic acid (LBPA), (Table 1, Fig. 3). These organelles are also enriched in many lysosomal-associated membrane proteins (LAMPs) and proteases (Mukherjee et al., 1997; Gruenberg, 2001). There are two major avenues of traffic into late endosomes: vesicles with de novo lysosomal components that arrive from the Golgi and the MVBs derived from maturing sorting endosomes (Gruenberg, 2001). Like sorting endosomes, late endosomes can undergo homotypic fusion and in addition form hybrid compartments by transiently fusing with lysosomes by a "kiss-and-run" mechanism (Luzio et al., 2000).

Lysosomes are more acidic (pH 4.5–5.5) than late endosomes and are enriched in mature proteases. Presently, however, there are no known markers that would unequivocally and exclusively identify lysosomes. Nevertheless, pulse-chase protocols using fluid-phase markers can be designed to label lysosomes virtually uncontaminated by other organelles (Table 1).

Little is known about the mechanisms regulating late endosomal dynamics. Rab7 was originally thought to regulate early to late endosome traffic (Feng, Press & Wandinger-Ness, 1995) but recent work suggests a role in late endosome to lysosome traffic instead (Bucci et al., 2000). Exaggeration of Rab7 function results in enlarged late endosomes/lysosomes, while its down-regulation precludes traffic to lysosomes (Bucci et al., 2000). To date there is only one identified effector of Rab7, namely RILP. Expression of inhibitory (truncated) forms of RILP impedes transport to lysosomes, resembling the phenotype induced by dominant-negative Rab7 (Cantalupo et al., 2001; Jordens et al., 2001). RILP seemingly promotes the movement of late endosomes and/or lysosomes on microtubules by a dynein/dynactin-mediated process (Jordens et al., 2001). Unlike sorting endosomes, there are currently no known molecules that tether late endosomes to lysosomes to mediate their fusion. However, some of the SNAREs involved in the process are known. Syntaxin-7, which forms a complex with mVti1b/Syntaxin 6, VAMP7 or VAMP8, was previously demonstrated to regulate late endosome homotypic fusion and late endosome-lysosome fusion (Antonin et al., 2000; Wade et al., 2001).

The transition of the early phagosome into a more mature, late endosome-like stage, is signaled by the acquisition of LBPA, Rab7 (Pitt et al., 1992b; Via et al., 1997; Fratti et al., 2001; Harrison & Grinstein, 2002) and RILP (R. Harrison and S. Grinstein, unpublished observations) (*see* Table 1 and Fig. 3). Phagolysosome formation is completed subsequently and involves divestment of these components and additional enrichment with lysosomal components such as LAMPs, mature proteases and additional V-type ATPases (proton pumps) that greatly acidify the phagosomal lumen (Desjardins et al., 1994; Clemens & Horwitz, 1995; Via et al., 1997; Botelho et al., 2000) (Table 1). Consistent with these properties, late phagosomes or phagolysosomes coalesce in vivo and in vitro with lysosomes but are refractory to fusion with sorting endosomes (Desjardins et al., 1997; Jahraus et al., 1998).

Expression of dominant-negative Rab7 hinders phagosome fusion with lysosomes. In addition, RILP is required for phagosome maturation (R. Harrison and S. Grinstein unpublished observations), in a manner akin to that described for endosome matu-

Table 2. Survival strategies of intracellular pathogens

Strategy	Pathogen	Known mechanisms of intracellular survival	References
Maturation Arrest	*Salmonella enterica* sv. Typhimurium	SPI-2-mediated inhibition of vesicle fusion Remodeling of the *Salmonella* vacuole by SifA Cholesterol accumulation in the *Salmonella* vacuole?	Beuzon et al., 2000; Brumell et al., 2002a; Brumell et al., 2001a; Brumell et al., 2001b; Catron et al., 2002; Garner et al., 2002; Ruiz-Albert et al., 2002; Uchiya et al., 1999
	Mycobacteria	Modulation of SNARE proteins Inhibition of phagosomal EEA1 acquisition by ManLAM	Fratti et al., 2002; Perskvist et al., 2002; Fratti et al., 2001
	Neisseria gonorrhoeae	Porin (PorB) expression permeabilizes the phagosome and inhibits maturation	Lorenzen et al., 2000; Mosleh et al., 1998
	Leishmania donovani	Inhibition of actin depolymerization from phagosome Direct inhibition of phagosome-endosome fusion by lipophosphoglycan (LPG)	Holm et al., 2001 Dermine et al., 2000; Scianimanico et al., 1999
Escape into the Cytoplasm	*Shigella flexneri*	Disruption of vacuole by IcsB, IpaB, IpaC (and others?)	Allaoui et al., 1992; Page et al., 1999
	Listeria monocytogenes	Listeriolysin O expression permeabilizes the vacuole Delivery of PI-PLC to the host cytoplasm disrupts vacuole Activation of host cell PLC and PLD activity disrupts vacuole	Gaillard et al., 1987 Goldfine et al., 2000; Portnoy et al., 2002
	Rickettsia prowazekii	Secreted phospholipase A2 destabilizes the phagosome	Walker, Feng & Popov, 2001
	Trypanosoma cruzi	Direct recruitment of lysosomes during/after invasion Rapid lysis of vacuole by Tc-TOX	Wilkowsky et al., 2002 Andrews, 1993
Other/Unique compartment	*Chlamydia trachomatis*	Formation of unique inclusion vacuole Permeabilization of the inclusion vacuole	Hackstadt et al., 1996 Grieshaber et al., 2002
	Legionella pneumophila	Association with endoplasmic reticulum vesicles Recruitment of ARF-1 by RalF	Tilney et al., 2001 Nagai et al., 2002
	Afipia felis	Directly enters non-endocytic compartment	Luhrmann et al., 2001
	Toxoplasma gondii	Forms non-fusogenic vacuole which associates with mitochondria and endoplasmic reticulum	Carruthers, 2002

ration (Cantalupo et al., 2001; Jordens et al., 2001). Specifically, RILP mediates dynein-dependent centripetal displacement of phagosomes from the cell periphery to the perinuclear region, a process required for their fusion with lysosomes. Importantly, PI 3-kinase antagonists do not prevent acquisition of Rab7 and RILP by the phagosome, even though phagosome maturation is arrested (R. Harrison and S. Grinstein unpublished observations). This suggests that, while necessary, Rab7 and RILP are not sufficient for fusion of phagosomes with lysosomes. Finally, expression of syntaxin-7 dominant-negative mutants attenuated phagosome maturation, although the precise role of this SNARE was not defined (Collins et al., 2002).

Survival Strategies of Intracellular Parasites

The remainder of this review will encapsulate our current understanding of how some pathogens co-opt phagosome maturation to their advantage, thereby becoming intracellular parasites. Intracellular parasites can be grouped into three general classes:

1) Those that arrest normal phagosome maturation; maturation arrest can occur at both an early phagosome-like stage, as in the case of *Mycobacterium*, or at a late phagosome-like stage, as in the case of *Salmonella*.
2) Those which escape from the phagosome; mechanisms to escape from the phagosome or vacuole are employed by bacteria such as *Shigella*, *Rickettsia*, and *Listeria*.
3) Those which manipulate the traffic of the host cell membranes to take up residence in a non-phagosomal organelle; one such parasite is *Legionella*, which is secluded in an endoplasmic reticulum-like compartment. Others, such as *Chlamydia*, can produce their own unique compartment, distinct from any of those of the host cell.

Organisms employing any of these strategies are able to avoid prolonged exposure to the harsh environment of the phagolysosome (Fig. 1). A list of intracellular parasites and an overview of the mechanisms they use to survive within host cells is provided in Table 2. Individual representative examples of some of these intracellular parasites are discussed below.

Maturation Arrest

Salmonella

The gram-negative enteropathogenic bacteria *Salmonella* is a well studied example of an organism that can inhibit normal phagosomal maturation in order to survive within an infected cell. Pathogenic strains of *Salmonella* are capable of proliferation in both macrophages and in epithelial cells after invasion (Steele-Mortimer et al., 2000). The intracellular fate of *Salmonella* in both cell types, as well as others, has been extensively studied and although significant cell-type dependent differences in maturation are becoming apparent (Garcia-Del Portillo et al., 2000), certain commonalities do exist. Upon internalization, the *Salmonella*-containing vacuole (SCV) begins to mature as a typical phagosome, but maturation is arrested before reaching the phagolysosomal stage. It is within this compartment with altered maturation that the bacterium is able to take up residence and eventually escape the host cell to infect neighboring cells.

Salmonella enterica serovar Typhimurium has two specialized type III secretion systems, which are both required for virulence (Cirillo et al., 1998; Galan & Curtiss, 1989). The first secretion system, which is encoded on a region of the *Salmonella* genome termed the pathogenicity island 1 (SPI-1), delivers to the host cell cytoplasm bacterial effector proteins that are required for the bacteria to gain entry into non-phagocytic cells. Injection of these effectors shortly after bacterial adherence induces pronounced ruffling of the host cell membrane. Fusion of the tips of ruffling membranes generates vacuoles (SCV) that engulf bacteria by a process similar to macropinocytosis (Francis et al., 1993). Several of the effectors that promote ruffling have been characterized and include proteins that alter the cytoskeleton directly, by interaction with actin (e.g., SipA and SipC), or indirectly, by binding to actin-modulating proteins such as Rho-family GTPases and T-plastin, as in the case of SptP and SopE (Fu & Galan, 1998; Hardt et al., 1998; Hayward & Koronakis, 1999; Zhou, Mooseker & Galan, 1999). The SPI-1-encoded inositol phosphatase SigD/SopB may also contribute to cytoskeletal remodeling indirectly, by altering the phosphoinositide composition of the plasma membrane. It has been recently shown that SigD/SopB activity causes a rapid loss of plasmalemmal phosphatidylinositol-4,5-bisphosphate, a phosphoinositide known to regulate the actin cytoskeleton (Terebiznik et al., 2002). In addition to the well described function of SPI-1 in invasion of non-phagocytic cells, recent observations suggest a requirement of SPI-1 expression for intracellular proliferation, although the exact role of these effectors in bacterial survival is not clear (Brumell et al., 2002b).

A different pathogenicity island, SPI-2, encodes a second type III secretion system that allows delivery of a distinct set of bacterial proteins across the vacuolar membrane to the cytoplasm of the host cell. Unlike the SPI-1 encoded proteins, the SPI-2 effectors appear to function at later times after invasion. They are released in response to environmental cues that develop in the SCV as it matures, possibly including reduced Ca^{2+}, Mg^{2+} and/or PO_4^{3-} concentrations and low pH (Garcia Vescovi, Soncini & Groisman, 1996; Deiwick et al., 1999). The SPI-2 effectors are required for intracellular survival of *Salmonella* within macrophages, presumably by directing SCV maturation (Cirillo et al., 1998; Hensel et al., 1998; Ochman et al., 1996).

Manipulation of the traffic of the host cell endomembranes is fundamental to *Salmonella* viability and virulence. Shortly after formation, the SCV closely resembles an early phagosome. Both Rab5 and the Rab5 effector EEA1 are present on early SCV (Steele-Mortimer et al., 1999; Mukherjee et al., 2001; Scott et al., 2002). Further, PI(3)P, the major product of hVPS34 is also transiently present on the early SCV (Pattni et al., 2001; Scott et al., 2002). However, all of the early phagosome markers are quickly lost from the SCV, implying that maturation of *Salmonella* vacuoles proceeds beyond the early phagosome-like stage.

The loss of early phagosome markers from the SCV is accompanied by the appearance of specific lipids and proteins known to function at later stages of phagosome maturation. Several of these markers, including LAMP-1, Rab7, LBPA and cathepsin D, associate with the late SCV (Meresse et al., 1999a; Brumell et al., 2001b; Scott et al., 2002) (Fig. 4). The maturing SCV can also fuse with lysosomal compartments preloaded with a fluid-phase marker (Cuellar-Mata et al., 2002; Oh et al., 1996). Taken together, these data would suggest that maturation of the SCV is indistinguishable from that of an ordinary phagosome. However other markers, such as the mannonse-6-phosphate receptor, are not found in the late SCV (Oh et al., 1996; Cuellar-Mata et al., 2002), and in some cell types, not all of these markers are acquired (Garcia-Del Portillo et al., 2000). Further, the SCV formed by pathogenic *Salmonella* do not retain their lysosome character for long. Exposure to a lysosomal-like environment induces *Salmonella* to express a set of proteins that promote a remodeling of the SCV, yielding an environment that is conducive to bacterial survival and replication. Clearly, maturation of the SCV is not simply arrested at an easily defined stage. The biogenesis and fate of the SCV is a complex microorganism-directed process in which different maturation pathways are differentially modulated at distinct times after internalization.

Some time after invasion and the initial wave of fusion with endosomes and lysosomes, the SCV loses

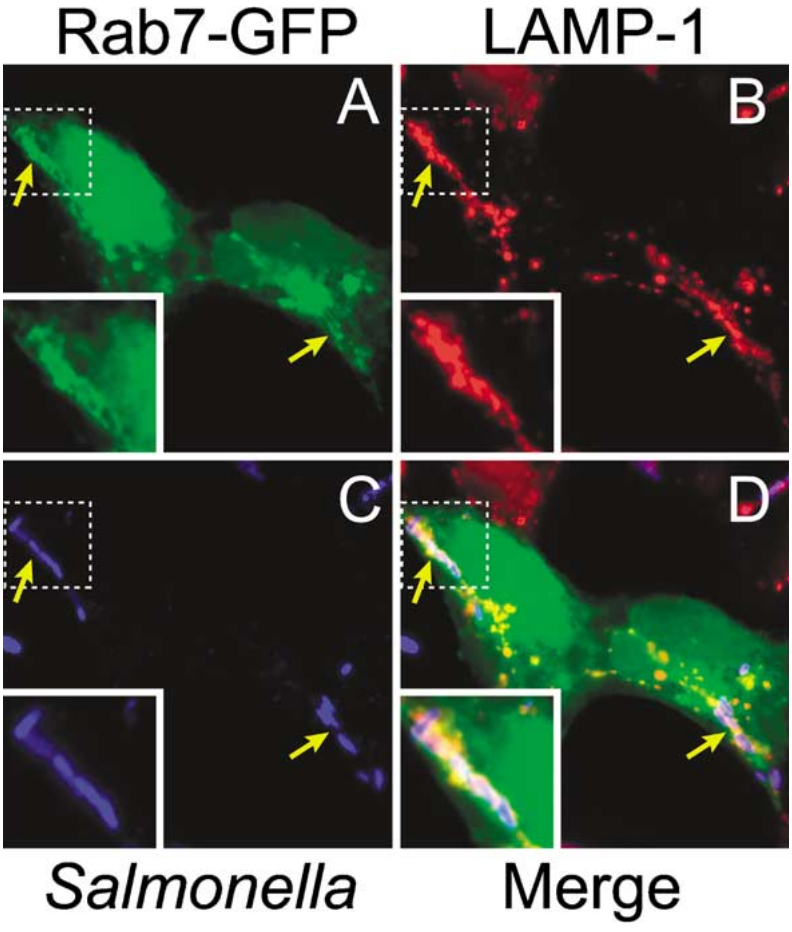

Fig. 4. Late endosomal markers associate with the *Salmonella*-containing vacuole (SCV). HeLa cells expressing a GFP-tagged Rab7 construct (*A*) were infected with *Salmonella enterica* serovar Typhimurium for 10 min and the SCV were allowed to mature for 14 h, fixed and stained with antibodies to Lysosomal Associated Membrane Protein 1 (LAMP-1) (*B*) and antibodies to *Salmonella* LPS (*C*). The arrows indicate Rab7-positive Sifs. *Inset:* Enlargement of outlined areas in each panel. Image provided by Dr. Rene Harrison.

its ability to interact with the endocytic pathway, so that material internalized by fluid phase endocytosis can no longer reach the vacuole (Garvis, Beuzon & Holden, 2001; Cuellar-Mata et al., 2002). The SPI-2 effector SpiC may be responsible for this observation. This bacterial protein has been reported to be secreted into the host cell cytoplasm where it can inhibit transferrin recycling and vacuole-lysosome fusion, as assessed by electron microscopy (Uchiya et al., 1999). The authors also demonstrated that extracts from wild-type bacteria can inhibit homotypic endosome fusion in vitro to a much greater extent than extracts from a SpiC-null strain. These results suggest that SpiC is a SPI-2 encoded effector that functions to inhibit maturation by interfering with vesicle fusion. However, two recent reports suggest that this interpretation of the data may not be entirely correct (Freeman et al., 2002; Yu et al., 2002). In both of these reports SpiC was found not to be secreted into the host cell cytoplasm. The reason for this apparent discrepancy is presently not clear. SpiC was independently shown to be required for secretion of elements of the SPI-2 type III secretion system and for assembly of this structure (Freeman et al., 2002; Yu et al., 2002). These results indicate that SpiC may act instead as a chaperone or as a regulator of the assembly of the secretion system, modulating host cell membrane traffic indirectly rather than directly.

Regardless of whether SpiC is responsible for the inhibition of fusion, the importance of SPI-2 effectors in altering membrane traffic and in promoting intracellular survival of the bacteria is well established. In HeLa cells long filamentous structures termed Sifs (*Salmonella* Induced Filaments) appear 4–6 h after invasion (Garcia-del Portillo et al., 1993) (Fig. 4). A screen for *Salmonella* mutants unable to develop Sifs yielded a single gene responsible for this phenomenon, termed *sifA* (Stein et al., 1996). It has since been shown that the product of this gene, SifA, is secreted into the host cell where it is required for maintenance and remodeling of the SCV in both epithelial cells and macrophages (Beuzon et al., 2000; Brumell et al., 2001a, 2002b). It is not clear how SifA promotes SCV integrity, although it has been demonstrated that transfection of this protein alone, in the absence of bacterial infection, is sufficient to produce Sif-like structures along the microtubule network. These observations suggest that SifA modulates microtubule-dependent membrane traffic and fusion with the SCV (Brumell, Goosney & Finlay, 2002a).

Interestingly, while it has been reported that although SifA translocation is required for SCV

integrity, it is not necessary for bacterial proliferation in all cell types. Although an intact SCV is required for bacterial proliferation in both fibroblasts and macrophages, release of *Salmonella* into the cytoplasm of epithelial cells actually increases its rate of replication (Beuzon, Salcedo & Holden, 2002; Brumell et al., 2002b). Although these surprising findings imply that Sif formation is not essential for infection of epithelial cells, it is nevertheless clear that an intact SCV is required for *Salmonella* to avoid exposure to microbicidal molecules present in the macrophage cytoplasm.

Several reports have recently demonstrated accumulation of cholesterol in the SCV, which may affect the maturation of the vacuole. Under normal conditions, internalized cholesterol is transported to late endosomes, but fails to accumulate as it is rapidly sorted to other cellular compartments (Kobayashi et al., 1999). In contrast, staining with the fluorescent sterol-binding agent filipin has revealed significant accumulation of membrane-associated cholesterol in the late SCV (Brumell et al., 2001b; Catron et al., 2002; Garner, Hayward & Koronakis, 2002). The lipid accumulation process appears to be dependent on bacterial viability and requires expression of SPI-1. The mechanism and the purpose of cholesterol accumulation are not clear (Catron et al., 2002; Garner et al., 2002). However, an analogous accumulation of cholesterol has been noted in late endosomes of cells from patients with the genetic disease Niemann-Pick type C, or in cells treated with antibody to LBPA. Such cells demonstrate defective transport between late endosomes and lysosomes (Kobayashi et al., 1999), which suggests that cholesterol retention by the SCV may be one strategy to impair its full and continued interaction with the host cell lysosomes.

In summary, the products of both pathogenicity islands cooperate to deliver and maintain *Salmonella* in a compartment where exposure to microbicidal agents is limited. While the molecular details of this preventive maneuver are incompletely defined, it is clear that sequestration in the SCV facilitates bacterial survival and is required for full *Salmonella* pathogenicity.

Mycobacteria

Bacteria of the genus *Mycobacterium* are characterized by their atypically thick and lipid-rich cell wall. Many *Mycobacterium* species are omnipresent and do not typically cause disease in humans, with the notable exception of two species, *M. leprae* and *M. tuberculosis*.

Unlike *Salmonella*, mycobacteria lack the ability to invade animal cells. However, they can gain access to intracellular vacuoles upon engulfment by professional phagocytes. However, pathogenic *Mycobacterium* species ingested by macrophages alter the intrinsic maturation program of the phagosome. As a result, they not only avoid killing, but remain sheltered from the rest of the immune system, producing a latent infection which can last for years.

Several different receptors have been implicated in the uptake of mycobacteria by phagocytes, including Fc (Armstrong & Hart, 1975; Astarie-Dequeker et al., 2002), mannose (Schlesinger, 1993; Astarie-Dequeker et al., 1999), fibronectin (Rao, Ogata & Catanzaro, 1993; Pasula, Wisniowski & Martin, 2002) and scavenger receptors (Zimmerli, Edwards & Ernst, 1996). However, complement receptors (CR3) are believed to be the primary route of entry of mycobacteria into macrophages (Schlesinger et al., 1990; El-Etr & Cirillo, 2001). Of note, opsonization by complement is not required for ingestion of mycobacteria through CR3, implying that the receptor directly recognizes ligands of the bacterial surface (Stokes et al., 1993; Cywes et al., 1997). It is also interesting that the fate of mycobacteria in macrophages depends on the type of receptor utilized for entry: maturation arrest and bacterial survival are observed when complement and/or scavenger receptors are engaged, but the mycobacteria are delivered to the lysosome and killed if opsonized by IgG for internalization via Fc receptors (Armstrong & Hart, 1975; Astarie-Dequeker et al., 2002).

Nascent phagosomes containing mycobacteria acquire many of the typical early endosomal and phagosomal markers. Biochemical purification studies found that Rab5 was rapidly acquired and retained by phagosomes containing pathogenic mycobacteria, while neither Rab7 nor LAMP-1 were detected, even days after phagosome formation (Via et al., 1997). This observation gave rise to the hypothesis that internalized *Mycobacteria* arrest maturation at a stage preceding the formation of late phagosomes. More recently, however, and in contrast to the earlier biochemical observations, an immunoelectron microscopy study of *M. tuberculosis*-infected HeLa cells found that Rab7 was in fact acquired by the vacuoles, although LAMP-1 association was still defective (Clemens, Lee & Horwitz, 2002). The source of this apparent discrepancy is unclear, but the latter observations suggest that steps other than the acquisition of Rab7 are affected by mycobacteria and can lead to maturation arrest.

Indeed, several additional differences have been noted between conventional phagosomes and those containing viable and pathogenic bacteria. The Q-SNARE syntaxin-4 has been found to be retained by phagosomes surrounding mycobacteria (Perskvist et al., 2002). This retention is likely mediated through direct interaction of the SNARE with Rab5, as these two proteins were found to co-immunoprecipitate from *M. tuberculosis*-containing phagosomes formed by human neutrophils (Perskvist et al., 2002). Interestingly, the R-SNARE VAMP-3 appears to be

specifically degraded in phagosomes enclosing *M. tuberculosis* (Fratti, Chua & Deretic, 2002). However, it is not likely that selective proteolysis of VAMP-3 is the primary mechanism of maturation arrest, as phagosomes formed by primary macrophages from a VAMP-3 null mouse were capable of acquiring LAMP-1 (Allen, Yang & Pessin, 2002), implying that this SNARE is not absolutely required for phagosome maturation.

While the aberrant behavior of SNAREs cannot readily account for the arrested maturation, changes in EEA1 binding to the phagosomal membrane may provide a likely mechanism. This tethering molecule was found not to associate with the mycobacterial phagosome (Fratti et al., 2001). Such behavior is remarkable in that EEA1 is thought to be recruited to the phagosome by the combined actions of Rab5 and PI(3)P, the main product of the class III PI3 kinase hVPS34. Both the GTPase and the inositide kinase were found on the phagosomes. To account for these observations, Fratti et al. (2001) proposed that mannose-capped lipoarabinomannan (ManLAM) released by the bacterial wall may resemble PI(3)P or may interfere with its production, and could therefore block recruitment of its physiological ligands, including EEA1. Moreover, these authors also reported that inhibition of EEA1 activity by injection of blocking antibodies prevented phagosome maturation. Combined with the preceding observations, the failure of mycobacterial phagosomes to acquire EEA1 may fully explain the maturation arrest.

It is unclear how a bacterial lipid within the phagosome could affect recruitment of EEA1 to the cytoplasmic face of the phagosome; one possibility is that ManLAM somehow interferes with PI(3)P production or accessibility on the phagosome. One possibility to resolve this topological conundrum is the observation that dextrans of up to 70 kDa injected into the cytosol were able to enter the mycobacterial phagosome, suggesting continuity between these compartments (Teitelbaum et al., 1999). However, this latter observation has been disputed recently (Clemens et al., 2002).

When stimulated with the cytokine interferon γ and lipopolysaccharide, both cultured and primary macrophages are able to overcome the maturation arrest and deliver ingested mycobacteria to the phagolysosome, where they are eliminated (Via et al., 1998). The molecular mechanism underlying this effect remains obscure, but the implications to the treatment and prevention of tuberculosis are tantalizing.

Escape from the Phagosome

An optional mechanism devised by intracellular parasites to avoid delivery to the lysosome is to escape into the comparatively comfortable milieu of the cytoplasm, by rupturing the phagosome. In the safe cytosolic environment the organism is ensured a constant near neutral pH, ample access to nutrients, and protection from many of the host cell defenses by enzymes like superoxide dismutase and catalase that eliminate reactive oxygen intermediates. To this end, many bacteria, including *Shigella*, *Listeria* and *Rickettsia*, have evolved sophisticated mechanisms to exit the phagosome. Some of these are detailed below.

Shigella

Shigella flexneri is an example of a bacterium that employs phagosomal exit as a survival strategy. Like *Salmonella*, *Shigella* uses a type III secretion system to invade epithelial cells. However, once inside the host cell, *Shigella* quickly disrupts the integrity of the invasion vacuole and escapes into the cytoplasm (Adam, 2001). Several bacterial gene products including IcsB (Allaoui et al., 1992), IpaB and IpaC (Page et al., 1999) mediate membrane destabilization, although the exact mechanism is not understood.

Once free in the cytoplasm, the bacteria induce actin polymerization, forming a 'tail' that propels the bacteria through the cytoplasm. Eventually, such swimming *Shigella* reach and exert pressure on the plasmalemma, forming protrusions that extend into neighboring cells, allowing the bacteria to spread from one cell to the next (Kadurugamuwa et al., 1991). Although the motile force generated by actin polymerization is enough to propel *Shigella* into the vicinity of an adjoining cell (Monack & Theriot, 2001), additional bacterial factors, such as IcsB, are required to lyse the double membrane resultant from this apposition, facilitating this forceful invasion (Allaoui et al., 1992).

Listeria

Another bacterium that uses a better characterized escape mechanism is *Listeria monocytogenes*. These bacteria enter a wide variety of cell types by means of surface proteins known as 'internalins', which bind to specific host cell ligands (Portnoy, Auerbuch & Glomski, 2002). Once inside a cell, *Listeria* induces rupture of the surrounding vacuole through the combined actions of two bacterial effectors: listeriolysin O (Gaillard et al., 1987), and a phosphatidylinositol-specific phospholipase or PI-PLC (Camilli, Tilney & Portnoy, 1993). After escaping from the vacuole, *Listeria* is able to induce actin polymerization, travel along the cytosol and invade surrounding cells, much like *Shigella*. Listeriolysin O is inserted into the vacuole membrane and serves as a pore of comparatively wide diameter and poor selectivity. Initially protons flow through this pore, dissipating the transmembrane pH gradient, and subsequently other bacterial effectors, such as the PI-PLC, transit to the cytoplasm (Portnoy et al., 2002). Some of the

bacterial factors delivered to the cytosol in turn activate the host cell's phospholipases, including PI-PLC and PLD. The combined actions of the bacterial PI-PLC and of host cell phospholipases degrade and eventually rupture the vacuole, liberating *Listeria* into the cytoplasm (Goldfine, Wadsworth & Johnston, 2000).

Other Hiding Places

Aside from maturation arrest and escape from the invasion vacuole, there is one other general strategy that parasites use to avoid elimination by the host. Several microorganisms have developed the ability to either hide within existing cellular organelles that are not microbicidal, or can generate a unique membrane structure to enclose and protect themselves. To this aim, the intracellular parasites have acquired means of manipulating the traffic of host cell membranes. Some of the molecular mechanisms underlying this subversion of traffic have recently been described for *Chlamydia*, *Legionella* and *Brucella*, shedding some light on how these parasites survive within cells.

Chlamydia

Chlamydia trachomatis is an obligate intracellular parasite, which invades cells using a type III secretion system similar to those of *Shigella* and *Salmonella*. Several of the chlamydial effectors secreted by this system have been characterized and include proteins designated as IncA, IncB and IncC, which are found on the inclusion vacuole (Subtil, Parsot & Dautry-Varsat, 2001). Once internalized, the chlamydial inclusion begins to acquire massive amounts of membrane, seemingly from exocytic vesicles from the host that are normally destined for the plasma membrane (Hackstadt et al., 1996), and eventually grows to occupy a significant fraction of the cell. This giant inclusion does not appear to fuse readily with any part of the endocytic pathway and is readily permeable to cytoplasmic ions, preventing the establishment of a transmembrane pH gradient (Grieshaber, Swanson & Hackstadt, 2002). Multiple inclusions formed by separate bacteria invading a single cell eventually fuse into one single inclusion. This homotypic fusion is inhibited by microinjection of an antibody raised to the IncA protein (Hackstadt et al., 1999). Remarkably, despite their striking size and rapid kinetics of formation, little is known about the composition and mechanism of formation of the inclusion vacuoles. Neither the bacterial effectors that modulate membrane fusion nor their host cell targets are well known.

Legionella

Unlike *Chlamydia*, *Legionella* uses a pre-existing compartment, the endoplasmic reticulum, to make its home within the host cell. *Legionella pneumophila*, the species responsible for the majority of cases of Legionnaires disease, is primarily a parasite of fresh water protozoa, but in certain situations can infect humans, especially the elderly and immunocompromised individuals (Steinert, Hentschel & Hacker, 2002). Once internalized, the *Legionella* vacuole immediately deviates from the endocytic pathway, never acquiring either Rab7 or LAMP-1 (Roy, Berger & Isberg, 1998). This divergence has been shown to require a functional *dotA* gene product, which is a large transmembrane protein that is part of a type IV secretion system. This secretion system is responsible for transporting across the vacuolar membrane specific factors required for modulation of intracellular membrane trafficking (Roy et al., 1998). Recently, the functional significance of one of these factors, RalF, has been described. RalF was initially identified as a protein of interest through a bioinformatic search of the *L. pneumophila* genome, which revealed that this protein contains a Sec7-homology domain, a motif known to be present in a number of ARF nucleotide exchange factors (Nagai et al., 2002). In this study, Nagai et al. (2002) also demonstrated that an ARF-1-GFP chimera was recruited to the *Legionella* phagosome by RalF. Since ARF-1 has been implicated in endoplasmic reticulum to Golgi traffic, the function of RalF is consistent with the observation that *Legionella* vacuole eventually resembles the endoplasmic reticulum, as determined by electron microscopy (Katz & Hashemi, 1982; Tilney et al., 2001). This replicative compartment also has been shown to contain the endoplasmic reticulum-resident protein BiP (Swanson & Isberg, 1995).

A thorough study of the biogenesis of the *Legionella* vacuole by Tilney et al. (2001) demonstrated that formation of this compartment occurs in at least four different stages. At very early times after phagocytosis, the *Legionella* phagosome becomes intimately associated with endoplasmic reticulum-derived vesicles and mitochondria. Eventually, these endoplasmic reticulum vesicles fuse to form a large structure that surrounds the phagosome, which eventually acquires ribosomes. Finally, the phagosome membrane is lost and the bacterium begins to replicate in this rough endoplasmic reticulum-like compartment.

Despite its resemblance to an exchange factor and its ability to recruit ARF-1, it has yet to be shown that RalF is responsible for the acquisition of endoplasmic reticulum components by the *Legionella* vacuole. Of note, RalF-deficient mutants did not exhibit any intracellular proliferation defects (Nagai et al., 2002), implying that this gene product is not essential for *Legionella* survival within macrophages. Thus, much remains to be learned about the molecular machinery that enables *Legionella* to become an intracellular parasite.

Concluding Remarks

As is illustrated by the microorganisms discussed in this review, human pathogens have evolved complex and divergent strategies for surviving intracellularly. By manipulating host cell membrane dynamics and integrity, these parasites can create niches suitable for their survival and proliferation, while evading detection by the body's immune surveillance systems. It is now clear that different microorganisms can accomplish this by targeting distinct components of the cellular membrane traffic system. Interference with specific components of the cellular machinery can effect arrest of vacuolar maturation, escape from their membrane-bound enclosure, or remodeling of the vacuole to produce an environment conducive to pathogen survival. Understanding how these amazing organisms are able to modulate their host's machinery is of paramount importance to the development of new rational treatment strategies.

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