REVIEW ARTICLE

Phagosome maturation: aging gracefully

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Foreign particles and apoptotic bodies are eliminated from the body by phagocytic leucocytes. The initial stage of the elimination process is the internalization of the particles into a plasma membrane-derived vacuole known as the phagosome. Such nascent phagosomes, however, lack the ability to kill pathogens or to degrade the ingested targets. These properties are acquired during the course of phagosomal maturation, a complex sequence of reactions that result in drastic remodelling of the phagosomal membrane and contents. The determinants and consequences of the fusion and fission reactions that underlie phagosomal maturation are the topic of this review.

Key words: endocytosis, macrophage, neutrophil, phagocytosis, phosphoinositide, Rab, SNARE.

INTRODUCTION

Macrophages and neutrophils eliminate invading micro-organisms and other foreign particles by first ingesting them into a plasma membrane-derived intracellular vacuole, or phagosome. The ingestion, or phagocytosis, is a receptor-mediated, actin-dependent process. The resulting intracellular vacuoles, termed phagosomes, then undergo a series of fission and fusion events that modify the composition of their limiting membrane and of their contents, by a sequence that resembles the progression of the endocytic pathway. This process is referred to as phagosome maturation, and bestows the vacuole with a host of degradative properties, which are central to its microbicidal function [1,2]. The biological importance of particle engulfment and phagosome maturation is not restricted to the function of mammalian professional phagocytes. These processes developed early in evolution – they are patent in *Dictyostelium*, nematodes and insect haemocytes – and function not only in host defence, but also in nutrition, tissue remodelling and removal of apoptotic bodies [3–8]. Paradoxically, phagocytosis can also have deleterious effects for the host: certain pathogens, exemplified by *Mycobacteria*, take advantage of the phagocytic machinery to gain access to the cell interior where, by subverting the maturation process, they become intracellular pathogens [9–11]. Because of the diversity and complexity of phagocytosis and phagosome maturation, the scope of this review will be limited to phagosome maturation in 'professional phagocytes' of the mammalian immune system, i.e. granulocytes and monocyte/macrophages. For further information on phagocytosis and/or phagosome maturation in other model systems, the reader is directed to the following reviews [4–6,8].

PHAGOCYTOSIS: A PRIMER

A large number of mammalian cell types are capable of phagocytosis, but their phagocytic efficiency varies greatly. To reflect this varying ability, Rabinovitch [12] coined the terms 'non-professional phagocytes', 'paraprofessional phagocytes' and 'professional phagocytes', referring to cells with low, average and high phagocytic competence respectively. Professional phagocytes encompass mainly neutrophils and cells of the monocytic/macrophage lineage, sentinels of the immune system that hunt and destroy senescent, apoptotic or otherwise defective host cells, pollutant particles and, perhaps most importantly, foreign, potentially pathogenic organisms [12,13]. The unique ability of phagocytic leucocytes to efficiently internalize a variety of targets is attributable to the expression of an array of specialized phagocytic receptors. Supporting this notion, it has been shown that the phagocytic capacity of non-professional phagocytes, such as Chinese hamster ovary or COS cells, is greatly increased by the heterologous expression of specialized phagocytic receptors [e.g. Fcγ receptors (FcγRs)] that are normally found in neutrophils or macrophages [14–16]. We will refer to these heterologous transfectants hereafter as 'engineered phagocytes'.

Phagocytosis is initiated by the interaction of surface receptors with their cognate ligand. Ligands can be endogenous components of the particle, exemplified by lipopolysaccharides of bacteria and phosphatidylserine in apoptotic cells [13,17]. Internalization triggered by endogenous ligands of the particle is known as non-opsonic. The immune system is equipped with a variety of receptors that recognize non-opsonic ligands, including CD14 that binds to lipopolysaccharides, as well as receptors that recognize specifically phosphatidylserine, mannose or fucose residues [18]. Alternatively, phagocytic ligands can be classified as opsonins, which are host-derived proteins that coat the surface of a particle. The best characterized opsonins are the complement fragment C3bi and IgG antibodies. The former binds relatively non-specifically to the surface of foreign particles, whereas IgG molecules attach to the phagocytic target by recognizing specific surface epitopes [13]. C3bi-opsonized particles are recognized by complement receptor 3 (CR3; also known as CD11b/CD18 or Mac-1), a member of the integrin superfamily, while IgG-opsonized particles engage FcγRs. These receptors are reviewed in detail elsewhere [19–22].

Abbreviations used: ARF, ADP-ribosylation factor; $[Ca^{2+}]_i$, cytosolic free calcium concentration; CR3, complement receptor 3; EEA1, early endosome antigen 1; FcγR, Fcγ receptor; GAP, GTPase-activating protein; GFP, green fluorescent protein; LAMP, lysosomal-associated membrane protein; MVB, multivesicular body; NSF, *N*-ethylmaleimide-sensitive factor; PI 3-kinase, phosphatidylinositol 3-kinase; PI(3)*P*, phosphatidylinositol 3-phosphate; PX domain, Phox homology domain; Q-SNARE, glutamine-containing SNARE; Rab5ip, Rab5-interacting protein; RILP, Rab-interacting lysosomal protein; R-SNARE, arginine-containing SNARE; SNAP, NSF-attachment protein; SNARE, soluble NSF-attachment protein receptor; TfR, transferrin receptor; VAMP, vesicle-associated membrane protein.

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Receptor engagement leads to internalization of the particle into a phagosome by a complex sequence of events that require kinase activation, alterations in phospholipid metabolism, remodelling of the actin cytoskeleton and acceleration of membrane traffic (see [3,6,13,23] for reviews). While in all instances phagocytosis is manifested as the enclosure of a particle within an endomembrane vacuole, it is important to consider that phagosomes formed by the various receptor–ligand pairs may have divergent fates. Supporting this view, FcγR-derived, but not CR3-derived, phagosomes containing *Salmonella* fuse with secretory granules in neutrophils [24,25]. Similarly, only IgG-opsonized *Mycobacteria*, but not those entering the cells via complement receptors, can fuse with lysosomes. Thus not all phagosomes are created equal.

THE ENDOCYTIC PATHWAY: A PARADIGM FOR PHAGOSOME MATURATION

Immediately after formation, the limiting membrane of the phagosome resembles the plasma membrane and its fluid contents are a sample of the extracellular medium. As such, the phagosome does not evince bactericidal ability. However, shortly after sealing, the vacuole undergoes a complete overhaul, resulting in massive changes in its composition due to a progressive maturation process that ultimately yields a hybrid organelle, the phagolysosome [26]. Phagolysosomes possess a number of complementary degradative properties, including a very low pH, hydrolytic enzymes for particle digestion, defensins and other bactericidal peptides, and the ability to generate toxic oxidative compounds [1,27,28].

The maturation process that endows the phagosome with lytic activity depends critically on the interaction of the nascent vacuole with the endocytic pathway. The endocytic pathway is organized as a continuum of organelles ranging from early endosomes to lysosomes (Figure 1). During endocytosis, solutes, membrane-bound ligands and transmembrane proteins are trapped in small vesicles derived from the plasma membrane, primarily by clathrin- and caveolae-dependent mechanisms. Following formation, endocytic vesicles are targeted to sorting endosomes (often referred to as early endosomes; in this review, early endosomes designate the combined population of sorting and recycling endosomes). Sorting endosomes are molecularly equipped to differentiate, organize and re-route the assortment of internalized molecules. These organelles are often tubulovesicular, and can be typically recognized by the presence of Rab5 or early endosome antigen 1 (EEA1), or experimentally by applying

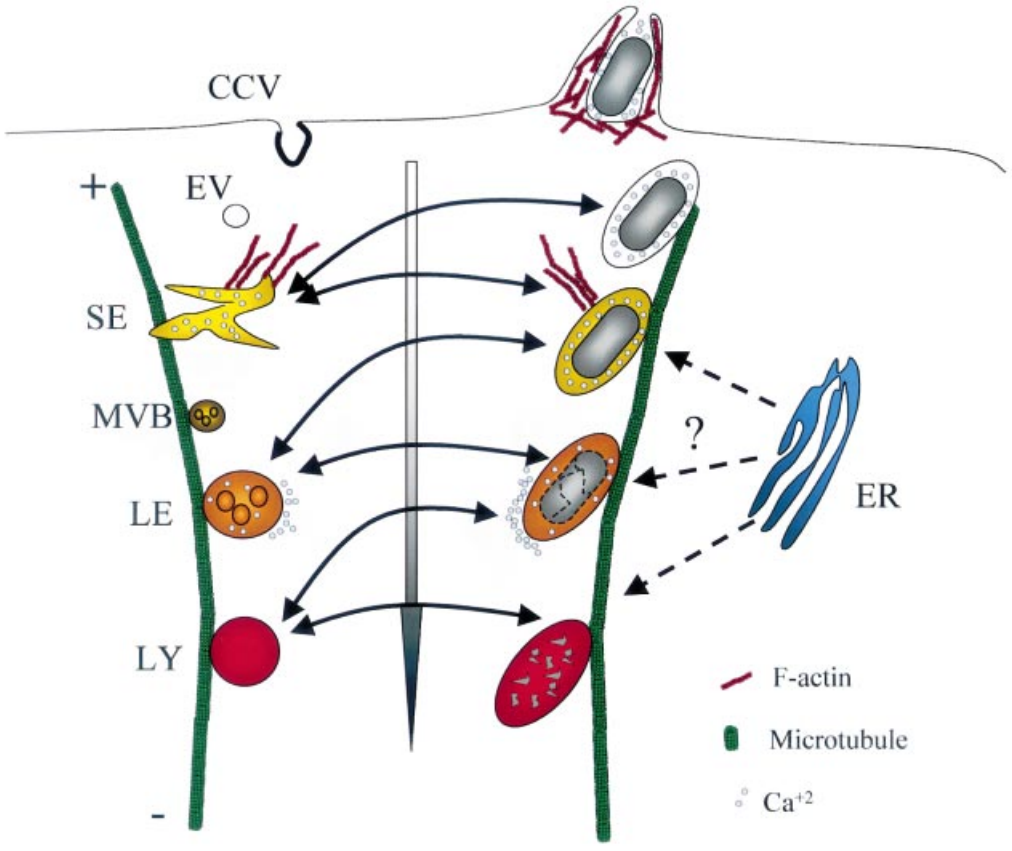

Figure 1 Endosome progression and phagosome maturation

Diagram illustrating the parallels between endosome progression to lysosome and phagosome maturation to phagolysosome. The fusion of phagosomes with compartments of the endosomal pathway is indicated by the connecting arrows. A possible interaction with the endoplasmic reticulum is also noted. Trapping of calcium during phagocytosis and the possibility of regulated organellar calcium release into the cytosol are illustrated. Note that endosomes, lysosomes and phagosomes at various stages of maturation are thought to bind to microtubules and can serve to nucleate actin. Actin is also abundant at the phagocytic cup. CCV, clathrin-coated vesicle; EV, endocytic vesicle; SE, sorting endosome; LE, late endosome; LY, lysosome; ER, endoplasmic reticulum. See the text for details.

Table 1 Molecular markers of sorting and late endosomes, lysosomes, early and late phagosomes and phagolysosomes

The pH values listed for the endocytic organelles are from Chinese hamster ovary cells [31]. The phagosomal pH acidifies to pH $\sim$ 5 within the first 5 min [213].

Organelle	Markers	pH
Sorting endosome; early phagosome	EEA1, Rab5, PI(3)*P*, syntaxin 13, transferrin, VAMP-3	$\approx$ 6.0
Late endosome; late phagosome	Rab7, Rab9, mannose 6-phosphate receptor, syntaxin 7, LAMPs, lysobisphosphatidic acid	5.5–6.0
Lysosome; phagolysosome	LAMPs, mature cathepsin D, fluid-phase markers chased for $\geqslant$ 2 h	4.5–5.5

short pulses of ligands or fluid-phase markers. The lumen of sorting endosomes is relatively poor in proteases and is mildly acidic, with a pH of $\sim$ 6.0 (Table 1). From sorting endosomes, cargo can be directed towards recycling endosomes, which are morphologically and biochemically distinct from sorting endosomes – they are often in a juxtanuclear location, near the microtubule-organizing centre, are less acidic (pH $\sim$ 6.5) than the sorting endosome, and can be identified by the presence of Rab11 [29–35]. Alternatively, molecules destined for degradation progress from sorting to late endosomes (Figure 1). Late endosomes are more acidic than sorting endosomes, reaching a pH of 5.5, and are comparatively enriched in hydrolytic enzymes. They can be identified by their multivesicular nature, i.e. they contain small intraluminal vesicles, and by the presence of Rab7, Rab9, lysobisphosphatidic acid, mannose-6-phosphate receptor and lysosomal-associated membrane proteins (LAMPs) [31,36] (see Table 1). However, there is discord as to how sorting to late endosome traffic occurs, with two predominant views: the vesicle shuttle model and the maturation model. According to the vesicle shuttle model, sorting endosomes are stable organelles from which transport intermediates, or multivesicular bodies (MVBs), are derived and subsequently targeted to late endosomes. The maturation model proposes instead that sorting endosomes are transient organelles that mature into MVBs via a series of poorly characterized fusion/fission events, ultimately generating late endosomes [37–40].

Regardless of the precise mechanism leading to the creation of late endosomes, it is agreed that lysosomes are the final step in the endocytic sequence. These organelles contain the bulk of active proteases and lipases, and are extremely acidic (pH $\leqslant$ 5.5). Lysosomes characteristically contain LAMPs and hydrolytic enzymes such as cathepsin D, but, while these proteins were thought to be unique to lysosomes, it is now apparent that they are also found in late endosomes. Therefore the best method for lysosome identification is perhaps their empirical labelling by an extended pulse followed by a long chase of fluid-phase markers, such as fluorochrome-conjugated dextrans or horseradish peroxidase (Table 1). It is noteworthy that specialized organelles resembling, but not identical to, lysosomes exist in neutrophils. These organelles, called azurophilic or primary granules, are acidic and are rich in hydrolases and cationic peptides, yet are not accessible by fluid-phase markers. Unlike the lysosomes of non-phagocytic cells, primary granules are readily secreted upon activation of surface receptors, including phagocytic receptors [28].

Interactions between phagosomes and endosomes commence soon after phagosome sealing, in a fashion that recapitulates the endocytic sequence: nascent phagosomes seemingly fuse initially with sorting endosomes, followed by late endosomes and ultimately lysosomes [41,42]. The progressive transfer of endo/lysosomal membrane and luminal constituents to phagosomes occurs during these fusion events (Figure 2). However, it is becoming clear that phagosomes are not merely a passive station for the delivery of endo/lysosomal constituents. Rather, the phagosome must express signals and beacons that trigger and guide the fusion of elements of the endocytic pathway.

There are several lines of evidence that early phagosomes interact preferentially with and effectively behave as early endosomes/endocytic vesicles. *In vitro* and *in vivo* assays clearly demonstrated that fluid-phase markers present in early endosomes – but not in lysosomes – are readily transferred to early – but not late – phagosomes, indicating that early endosomes and phagosomes coalesce [42–44]. This behaviour resembles the reported ability of early endosomes to fuse homotypically *in vitro* [45]. Secondly, early phagosomes ($<$ 30 min after formation) contain not only components characteristic of the plasma membrane, but also early-endosomal markers such as transferrin receptors (TfRs), EEA1 and Rab5, but are devoid of late-endosomal/lysosomal markers [26,46,47] (Figure 2, Table 1). Thirdly, integral and peripheral proteins such as TfR and EEA1 are eliminated from phagosomes during maturation, in a process akin to the transition from sorting to late endosomes [26,46,48]. In fact, some phagosomal proteins were shown to traffic retrogradely to the plasma membrane, suggesting that recycling from phagosomes occurs, perhaps using the same mechanisms that are employed in endosomal recycling [49,50] (see below). Together, these results suggest that phagosomes typically proceed through an early-endosome-like stage during phagolysosome biogenesis.

Phagosomes rapidly lose the characteristics of early endosomes, while attaining those of late endosomes. Although the kinetics of maturation seem to differ greatly depending on the particle and cells employed, phagosomes begin to fuse with late endosomes while becoming refractory to early endosomes about 10–30 min after formation [51–53]. As a result, as phagosomes age, they become enriched in late-endosomal components, best exemplified by Rab7, the mannose-6-phosphate receptor and lysobisphosphatidic acid [26,53,54]. While late endosomes characteristically appear as MVBs, it is not known whether phagosomes contain luminal vesicles. These could result from delivery of the contents of late endosomes or by invagination and scission of the phagosomal limiting membrane. The existence of late-endosome components on phagosomes is unquestionable, yet it is also transitory, suggesting that phagosomes are ultimately transformed from a late-endosome-like organelle into phagolysosomes, identified by the presence of hydrolytic proteases, such as processed cathepsin D, and by the acquisition of an extremely acidic pH, reported to be as low as 4.5 [44,48,54,55]. The active role of phagosomes in directing their own maturation is supported by further *in vitro* experiments showing that phagosomes isolated 1 h after formation fuse preferentially with lysosomes, but not with earlier endocytic organelles [42,44].

Together, these observations support the notion that phagosome maturation follows a hierarchy like that of the endocytic pathway, where nascent phagosomes preferentially fuse with and acquire the characteristics of early endosomes, late endosomes and lysosomes in an ordered sequential manner.

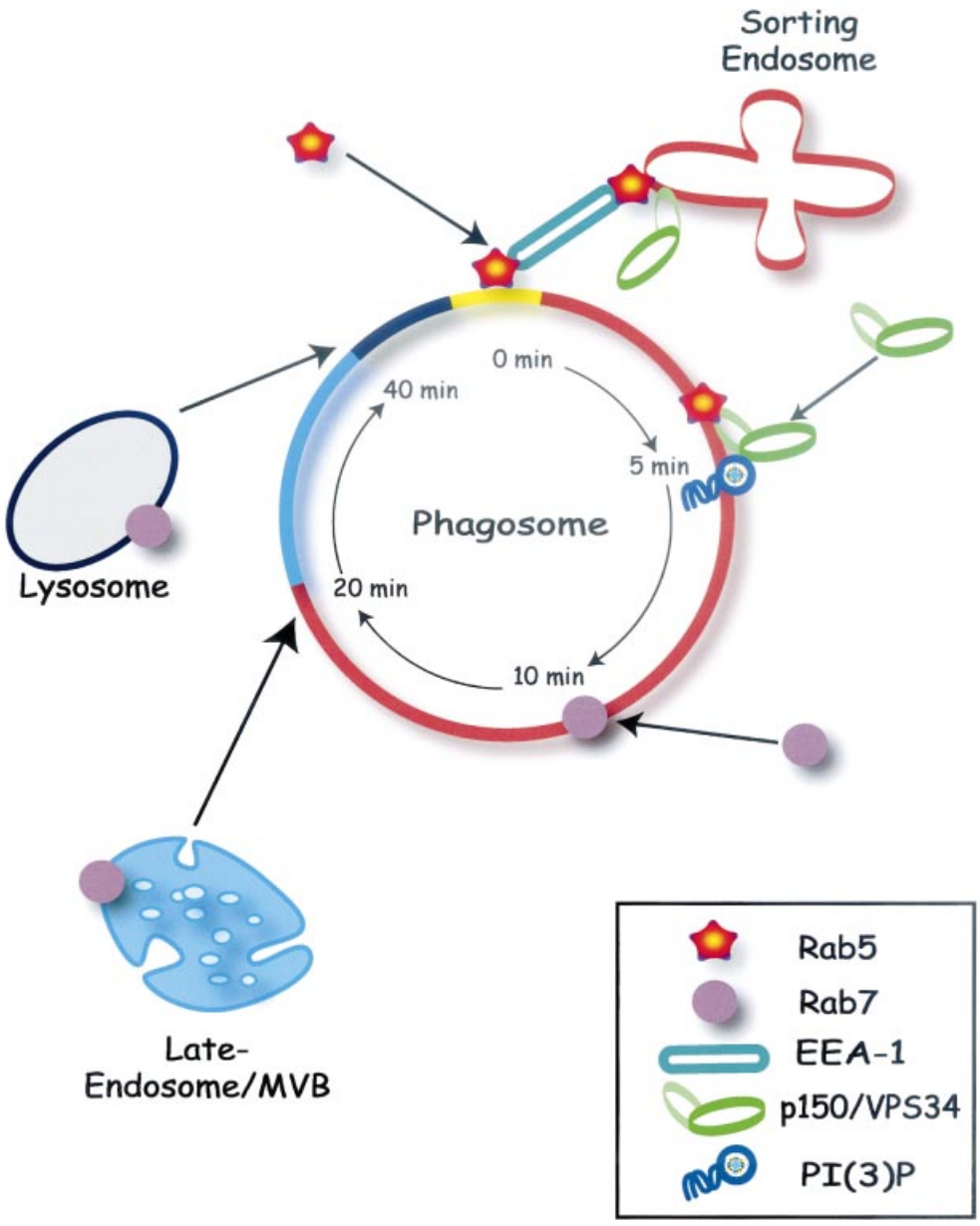

Figure 2 Changing composition of phagosomes during maturation

Diagram illustrating some of the known molecular events that dictate maturation and the approximate time course of the process. In the scheme, maturation proceeds clockwise from the top. At the outset (0 min) the nascent phagosome acquires Rab5, possibly from the plasmalemma or cytosol. Rab5 recruits p150/VPS34 to generate PI(3)*P* ($\approx$ 2–5 min) and attracts EEA1, which in turn facilitates tethering and fusion of early (sorting) endosomes. Subsequently, Rab7 is recruited ($\approx$ 5–10 min) either from the cytosol and/or along with late endosomes. Rab7 is instrumental in promoting fusion with lysosomes ($\approx$ 30–60 min). See the text for details.

An interesting paradigm suggested by Desjardins and colleagues [56] envisions that phagosomes and endosomes/lysosomes do not amalgamate into a single organelle during phagosome maturation. Rather, these investigators suggested a 'kiss-and-run' model for phagosome maturation [41,56]. According to this paradigm, phagosomes undergo only a transient and partial fusion with endocytic organelles (the 'kiss'), which allows the transfer of selected membrane and luminal contents between the phagosome and the endosome. This is promptly followed by a fission event (the 'run'), preventing the complete intermixing of the two compartments. Bolstering this model, not only were phagosomes observed to undergo transient contacts with endocytic organelles by video microscopy [41], but, of greater significance, the transfer of soluble contents and of membrane components between endosomes and phagosomes displayed distinctly different kinetics [42,57]. Moreover, smaller solutes were transferred more rapidly to phagosomes than those of greater molecular mass [42,57]. These experimental observations are not easily reconciled with a model where endosomes/lysosomes fuse completely with the phagosome, yet are consistent with momentary connection between the compartments through an opening of limited size. Independently of whether there is

complete or transient fusion between phagosomes and endocytic organelles, these findings ultimately suggest that the molecular machinery driving and regulating membrane fusion and fission along the endocytic pathway is likely to be a major contributor to phagosome maturation. The nature and function of some of these molecular complexes are described below.

INVOLVEMENT OF SNAREs AND Rabs IN PHAGOLYSOSOMAL BIOGENESIS

Vesicle- and target-SNAREs (where SNARE is defined as soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptor), a family of membrane-tethered coiled-coil proteins, *N*-ethylmaleimide-sensitive factor (NSF) and NSF-attachment proteins (SNAPs) have been identified as critical determinants of vesicular transport in a variety of systems (see [58,59] for reviews). Biochemical studies have shown that SNAREs on vesicles can selectively recognize and interact with cognate SNAREs on the target membrane, and that together they form an extremely stable complex that promotes the fusion of the interacting membranes by bringing them into close apposition. The hallmark of SNAREs is that they contain conserved heptad repeat sequences in their membrane-proximal regions that form coiled-coil structures. Based on the identity of a highly conserved residue, SNAREs have been classified into Q (glutamine-containing)-SNAREs (also known as t-SNAREs) and R (arginine-containing)-SNARES (or v-SNAREs) [60]. The fusion-competent SNARE complex consists of a four-helix bundle, where three of the helices are contributed by Q-SNAREs and one by an R-SNARE. In some instances two of the helices are provided by a single Q-SNARE, as in SNAP-25, but in others the individual helices belong to two separate proteins. This tetrahelical SNARE complex, also called a SNAREpin, is very stable, and ATP is needed to dissociate it for subsequent rounds of fusion. Disassembly is carried out by the ATPase NSF, linked through an adaptor protein α-SNAP [58,59,61–63]. To date, 30 SNARE family members have been identified in mammalian cells.

As expected, SNAREs, NSF and SNAPs have been identified as essential components of endosomal progression [64–71], and recent evidence has also implicated these proteins in phagosome maturation. Components of the fusogenic machinery have been identified on phagosomes, including target Q-SNAREs (syntaxins 2, 3, 4, 8 and 13), vesicle R-SNAREs [vesicle-associated membrane protein (VAMP)-2 and VAMP-3], as well as NSF [42,53,72–74]. This descriptive evidence has been complemented by some functional studies: *in vitro* fusion of early endosomes with phagosomes was found to require cytosol, ATP and NSF, and was inhibited by *N*-ethylmaleimide and antibodies against NSF, suggesting a functional role for SNAREs in the process [43,75]. Similarly, in permeabilized J774-E macrophages, phagosome–lysosome fusion was also reported to be sensitive to *N*-ethylmaleimide and to antibodies against NSF, suggesting that the NSF–SNAP–SNARE complex is also involved in phagosome–lysosome fusion [75].

Because there is some degree of promiscuity among SNAREs [76], docking/fusion specificity cannot be determined solely by the organellar distribution of these proteins [58]. For this reason, much effort has been devoted to identifying other determinants of selectivity in the interaction of membranes. In this context, small GTPases of the Rab family have become the focus of intense study. Rabs are considered as potential directors of membrane traffic, in part because of their restricted organellar distribution and because they can promote the selective tethering of vesicles with target organelles [77].

Several Rab GTPases localize to the endocytic pathway (Figure 2). Rab5 associates predominantly with the sorting endosome, Rab4 and Rab11 locate preferentially to recycling endosomes, while Rab7 is localized to late endosomes/MVBs and to lysosomes [36,78]. Rab5 is by far the best characterized of the endosomal Rab proteins. It has been shown to regulate a bewildering collection of functions, including the formation of clathrin-coated vesicles [79], the tethering and fusion of coated vesicles with sorting endosomes, homotypic fusion between sorting endosomes [80,81] and the motility of sorting endosomes along microtubules [82]. The functional involvement of Rab5 in endocytosis has been studied by overexpression of Rab5 mutants. Dominant-negative Rab5 mutants, with a preferential affinity for GDP, inhibit solute internalization and recycling, and block endosome fusion. On the other hand, overexpression of a constitutively activated Rab5 mutant that is deficient in GTP hydrolysis stimulated endocytosis and endosome fusion, leading to the appearance of giant endosomes [83–87].

Recruitment and/or activation of Rab5 on endosomal membranes was suggested to be modulated by Ras, perhaps through mediation of the nucleotide exchange factor RIN1 [88,89]. Rab5ip, a membrane-associated Rab5-interacting protein, may function as an anchorage site for Rab5 recruitment to endocytic vesicles and may also participate in its activation [90]. When in the active, GTP-bound state, Rab5 can interact with several effectors, such as Rabenosyn-5, Rabaptin-5 and Rabex-5. Rabex-5, which exists in association with Rabaptin-5, is another Rab5-specific guanine nucleotide exchange factor. This complex may contribute to the recruitment/retention of active Rab5 on endosomes and may cluster it in defined regions of the membrane [91,92]. Rab5–GTP also recruits EEA1 (Figure 2), a 170 kDa autoantigen associated with lupus erythematosus that associates specifically with the cytoplasmic face of the early-endosomal membranes [93]. The finding that EEA1 contains two spatially separate Rab5-binding sites, and is required prior to endosomal SNARE function, led to the suggestion that it may act by tethering two membranes containing Rab5 [94,95]. EEA1 also contains a cysteine-rich domain, termed the FYVE finger, which binds to phosphatidylinositol 3-phosphate [PI(3)*P*] with high affinity and specificity [96,97]. It has been shown that the FYVE domain is required for the endosomal targeting of EEA1 [98]. Upon recruitment, EEA1 assembles into a macromolecular complex with Rabaptin-5, Rabex-5 and NSF. This complex seemingly interacts transiently with syntaxin 13, an endosomal target SNARE, which might lead to membrane fusion [70]. Therefore EEA1 is likely to be a crucial downstream effector of Rab5, providing a functional link between Rabs and SNAREs.

The equilibrium between the active, membrane-bound Rab5–GTP and Rab5–GDP, which is mainly cytosolic, may be regulated in part by GTPase-activating proteins (GAPs), such as p120RasGAP, the tumour suppressor tuberin and RN-tre [99–101]. In favour of this notion, when GAP activity was diminished, Rab5 remained GTP-bound and resulted in enhancement of endosome fusion and endocytosis [102].

While the early steps of endocytosis have been studied extensively, comparatively little is known about the Rab proteins involved in later steps of the endocytic pathway leading to lysosomes. As stated above, Rab7 is localized to late endosomes. Data obtained with dominant-negative and constitutive active Rab7 mutants have indicated that this GTPase is important in the regulation of late-endocytic traffic [103,104]. In particular, it has been suggested that Rab7 could regulate the transition from early to late endosomes [78,103–106]. On the other hand, recent results using green fluorescent protein (GFP) fusion proteins suggested that Rab7 associates mainly with lysosomes, and

controls their aggregation and fusion with late endocytic structures [78]. The only Rab7–GTP downstream effector known to date is RILP (Rab-interacting lysosomal protein). RILP interacts specifically with Rab7–GTP and appears to target Rab7-containing organelles to microtubules via the dynein/dynactin system of motor proteins [107]. Despite these recent advances, the exact role of Rab7 and its mechanism of action in late-endocytic traffic are still incompletely understood. The isolation and characterization of Rab7-interacting proteins, possibly involved in docking and fusion of late endocytic structures, will be fundamental to understanding the molecular mechanisms of action of this GTPase.

Several Rab proteins have been found on phagosomal membranes, including Rab3, Rab4, Rab5, Rab7, Rab9, Rab10, Rab11 and Rab14 [41,74], yet little functional information is available at present. There is evidence to suggest that Rab5 plays a crucial role in phagosomal maturation. By analogy with endosomes, the earliest identified stages of phagosomal maturation are thought to be controlled by Rab5 [47,83]. Using time-lapse microscopy, Roberts and collaborators [83] showed the occurrence of a transient (1–2 min) interaction of Rab5 (conjugated to GFP) with newly formed phagosomes in intact macrophages. In these studies, Rab5–GFP appeared to have been recruited from the cytosol, since no fusion with labelled donor vesicles was detected. Moreover, studies in cell-free systems demonstrated an essential role for Rab5 in the fusion of isolated sorting endosomes with purified nascent phagosomes [44,75,108]. In intact cells, overexpression of dominant-negative Rab5–GFP precluded the acquisition of EEA1 and LAMP-1, suggesting a crucial role for this small G-protein in phagosomal maturation (O.V. Vieira, unpublished work).

While current evidence points to an important role for Rab5, we do not yet know how this GTPase is recruited to or is activated in the nascent phagosomes, nor do we fully understand its effector functions. As mentioned above, EEA1 is a well-established effector of Rab5; its possible role in phagosome maturation is discussed in detail below, in connection with phosphoinositide metabolism. With regards to other Rab5-associated proteins, Fratti and collaborators [53] reported that Rabaptin-5 is absent from isolated phagosomes. Moreover, microinjection of Rabaptin-5-inhibitory antibodies did not affect the acquisition of late-endosomal markers, suggesting that this protein is not essential for phagosomal maturation [53]. Little is known regarding possible roles of Rabenosyn-5, Rab5ip or other Rab5-associated proteins in phagocytosis.

As is the case for endocytosis, much less is known about the later stages of phagosomal maturation. By analogy with the endocytic pathway, it is likely that Rab7 functions in the transition between early and late phagosomes and/or phagolysosomes (Figure 2). This has not been documented in mammalian cells, but overexpression of dominant-negative Rab7 in *Dictyostelium* interfered with phagosomal maturation [109]. In permeabilized J774-E macrophages, the fusion between phagosomes and lysosomes was substantially inhibited by the addition of Rab GDP dissociation inhibitor, which promotes the removal of Rab proteins from intracellular membranes. This suggests the involvement of at least one Rab GTPase, conceivably Rab7, in phagosome–lysosome fusion [75]. To our knowledge, no information exists linking other Rabs to phagosome maturation in mammalian cells.

PHOSPHOINOSITIDES AND THE ENDOCYTIC PATHWAY

Products of phosphatidylinositol 3-kinases (PI 3-kinases) are thought to play a role in the traffic of membranes along the endocytic pathway and, as discussed below, are also critical for phagosome maturation. PI 3-kinases are a family of enzymes that phosphorylate the D-3 hydroxy group of phosphoinositides. Based on their regulation and substrate preference, three classes of PI 3-kinases have been recognized. For the sake of brevity, however, only classes I and III will be discussed in this review, since no evidence exists at present implicating class II enzymes in either phagosome formation or maturation. Class I PI 3-kinases are heterodimeric enzymes composed of catalytic and adaptor or regulatory subunits. These enzymes mainly generate phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate (for reviews, see [110–113]). The class III PI 3-kinase, the mammalian homologue of yeast Vps34p, known also as VPS34, generates only PI(3)*P*. VPS34 is associated with p150, a Vps15-like serine/threonine kinase, which contains an N-terminal myristoylation signal and is thought to regulate the membrane association and activity of VPS34 [110–115].

There is convincing evidence that PI 3-kinases regulate endocytic traffic (Figure 2). In yeast, PI(3)*P*, generated by Vps34p, is involved in the sorting and delivery of proteins to the vacuole [116]. This role for PI 3-kinase and PI(3)*P* is not unique to yeast. In mammalian cells, inhibitors of PI 3-kinase such as wortmannin and LY294002 are potent inhibitors of several membrane steps in endocytic traffic (for review, see [112]).

Some progress has been made in understanding the site of action of the phosphoinositides. In endosomes, Rab5 interacts directly with both class I and III PI 3-kinases in a GTP-dependent manner [117]. The function of the class I (p85α/p110β) kinase in endocytic traffic is still obscure. On the other hand, the association of Rab5 with p150/VPS34 explains the selective localization of PI(3)*P* in early endosomes [117]. The restricted localization of PI(3)*P* on early endosomes also dictates the distribution of proteins with PI(3)*P*-binding domains. Two such domains have been well documented: the FYVE domain, such as that in EEA1 and Rabenosyn-5, and the Phox homology domain (PX domain), like that in the soluble subunits of the NADPH oxidase [97,118–120].

There is convincing evidence that multiple ligands of PI(3)*P* participate actively in endosome sorting. As intimated above, EEA1 is an effector of Rab5 that interacts not only with this GTPase but also with PI(3)*P* [121]. Binding to PI(3)*P* through its FYVE domain is crucial for the interaction of EEA1 with the docking/fusion machinery of the endosomal membrane [45,96, 122,123]. In addition, Rabenosyn-5 (the mammalian homologue of yeast Vac1p) is required for homo- and hetero-typic early-endosome fusion, and binds Rab5–GTP, Rab4–GTP and PI(3)*P* [124]. Hrs, a mammalian homologue of Vps27p containing a FYVE domain, localizes in sorting endosomes and is required for normal endocytic traffic, at least in part by recruitment of clathrin, which in turn is involved in the sorting and retention of proteins destined for incorporation into the MVB [125–127]. A number of PX domain-containing proteins, such as the sorting nexins and the yeast SNARE Vam7p, have also been found on endomembrane vesicles. The ability to bind to PI(3)*P* via their PX domain is seemingly required for their function/location in the endocytic system (for review, see [119]). Jointly, these findings buttress the notion that PI(3)*P* is an essential player in endosome traffic.

Because PI(3)*P* is not present in late compartments of the endocytic pathway, it is likely that the phosphoinositide is either degraded or modified further by kinases. PI(3)*P* appears to be degraded, at least in part, in the intraluminal compartment, since it was still detectable in internal vesicles of MVBs [128]. Gillooly and collaborators [128] proposed a model where inward budding of the limiting membrane of the sorting endosome re-

moves the PI(3)*P* from the cytoplasmic face, while generating the MVB. The subsequent disappearance of PI(3)*P* is attributable, at least partly, to PIKfyve, a mammalian orthologue of the yeast PI(3)*P* 5-kinase Fab1p [129,130]. It is noteworthy that both the yeast and mammalian kinases contain a FYVE domain. In yeast, Fab1p regulates vesicle formation and/or cargo selection in the endocytic pathway through the formation of the second messenger phosphatidylinositol 3,5-bisphosphate, and a similar function is likely in mammalian cells, where PIKfyve may contribute to the formation of MVBs or to the disappearance of PI(3)*P* therein [130–133]. Thus, in addition to its function in early-endosomal dynamics, PI(3)*P* might also function in the biogenesis of MVBs through the formation of phosphatidylinositol 3,5-bisphosphate.

CRITICAL ROLE OF PHOSPHOINOSITIDES IN PHAGOLYSOSOME BIOGENESIS

Before it was realized that PI(3)*P* is key to endosomal progression, it was known that inhibition of PI 3-kinases ablated the phagocytosis of large (but less efficiently of small) particles, suggesting that activation of PI 3-kinases is essential for pseudopod extension [134–136]. Moreover, internalization of IgG-coated particles was found to be accompanied by a rapid accumulation of phosphatidylinositol 3,4,5-trisphosphate that was restricted precisely to the phagosomal cup [137]. It is noteworthy, however, that these actions are attributable to the class I PI 3-kinases, and not to the class III enzyme that generates PI(3)*P*. This was verified by microinjection of inhibitory anti-VPS34 antibodies, which were without effect on particle internalization, while cells isolated from mice lacking both the α and β isoforms of the class I p85 subunit had defects in large particle uptake that resembled those exerted by wortmannin [52].

By contrast, it is the class III PI 3-kinase that participates in phagosome maturation. The distribution of PI(3)*P* was monitored in living cells transiently transfected with two FYVE domains fused in tandem to GFP. This probe revealed a striking accumulation of PI(3)*P* in the phagosomal membrane (Figure 3). A high level of PI(3)*P* was apparent on the phagosomal membrane $\approx$ 1 min after sealing and persisted for nearly 10 min. The striking accumulation of the fluorescent probe was associated with a net increase in the total cellular content of PI(3)*P*, evaluated by HPLC, suggesting *de novo* synthesis of the phosphoinositide. The accumulation of PI(3)*P* in the phagosome was eliminated by treatment with wortmannin, consistent with mediation by PI 3-kinases [52]. More detailed information was obtained using antibodies and kinase-deficient cells. PI(3)*P* failed to accumulate in phagosomes of cells injected with anti-VPS34 antibodies, yet was clearly present in p85-deficient cells. These observations imply that the class III PI 3-kinase, rather than

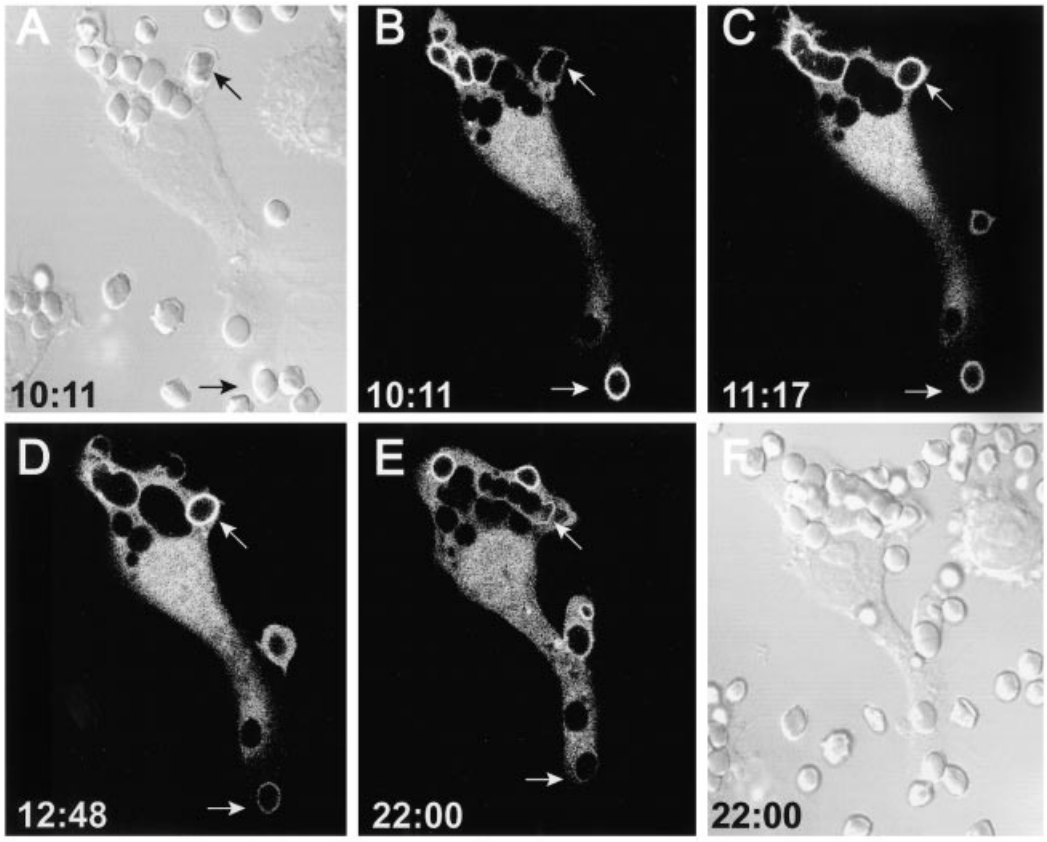

Figure 3 Accumulation of PI(3)*P* in early phagosomes

RAW264.7 macrophages were transfected with a fluorescent chimaeric construct that binds selectively to PI(3)*P*. At time 0 min, the cells were exposed to IgG-opsonized red blood cells, used as phagocytic targets. Panels (**A**) and (**F**) are differential interference contrast images. Panels (**B**)–(**E**) are fluorescence micrographs showing the distribution of the chimaeric protein. Note the transient accumulation of PI(3)*P* around forming phagosomes (e.g. arrows). The numbers at the bottom left of the panels indicate the time elapsed after addition of the red blood cells. See ref. [52] for details.

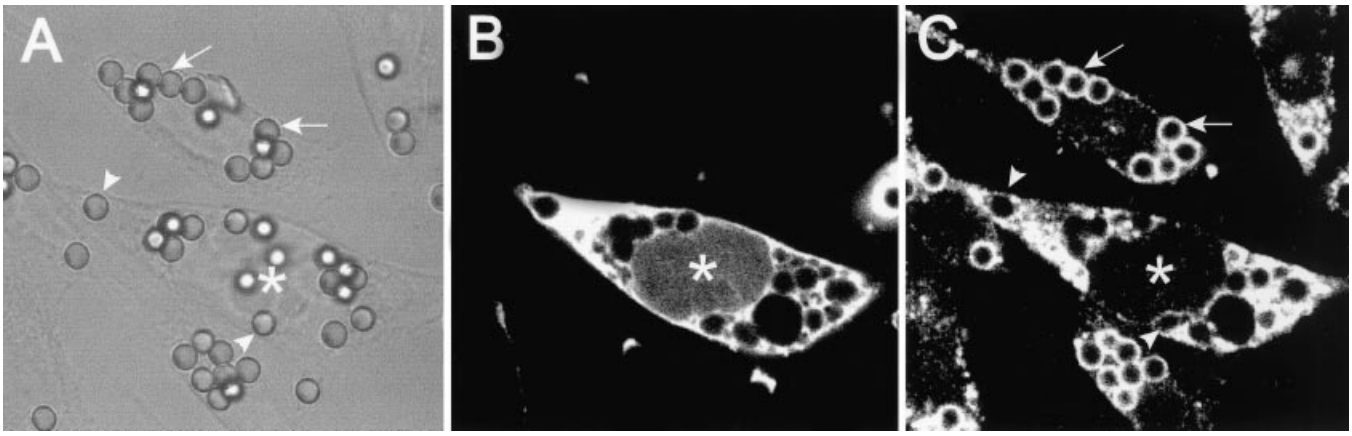

Figure 4 Inhibition of VPS34 prevents phagosomal maturation

RAW264.7 macrophages were exposed to opsonized red blood cells and the resulting phagosomes were allowed to undergo maturation for 60 min (**A**: differential interference contrast image). The cells were then fixed and permeabilized. One of the cells (indicated by the asterisk) had been injected with inhibitory anti-VPS34 antibodies (revealed in **B** by staining with labelled anti-IgG secondary antibody) prior to phagocytosis. The distribution of LAMP-1 was analysed by immunostaining (**C**). Note that LAMP-1 is incorporated into the membrane of phagosomes in uninjected cells (arrows), but not in the anti-VPS34-injected cell (arrowheads). See [52] for details.

class I enzymes, was involved. Hence, by synthesizing phosphatidylinositol 3,4,5-trisphosphate and PI(3)*P* respectively, class I and class III PI 3-kinases act consecutively in phagosome formation and maturation.

The functional role of PI(3)*P* in maturation was tested using inhibitors and also in anti-VPS34-injected cells. In macrophages treated with wortmannin, which internalize small ($\leqslant 3\,\mu$m) particles at significant rates [136], or injected with anti-VPS34 antibodies, phagosomal maturation is blocked (Figure 4). The drug attenuated recruitment of EEA1 to phagosomes and blocked acquisition of LAMP-1 [52]. This inhibitory effect cannot be attributed to the absence of SNAREs, since Fratti et al. [53] found that the phagosomal association of syntaxins 8 and 13, which co-exist with Rab5, is unaffected by PI 3-kinase antagonists.

By analogy with the endocytic pathway, PI(3)*P* production may be important for the phagosomal recruitment of EEA1 and other FYVE and PX domain-bearing proteins. In accordance with this notion, microinjection of antibodies against EEA1 depressed the phagosomal acquisition of late-endocytic markers, such as lysobisphosphatidic acid [53]. Moreover, the transient expression of dominant-negative EEA1 in engineered phagocytes abrogated LAMP-1 acquisition (O. V. Vieira, unpublished work). Together, these results suggest an important role for the transient recruitment of EEA1 in phagolysosome biogenesis. The mechanism whereby EEA1 regulates maturation remains to be characterized, but tethering of phagosomes to early endosomes is a distinct possibility.

Other FYVE and PX domain-containing proteins are equally likely to partake in the maturation sequence. Hrs and some sorting nexins also associate with nascent phagosomes (O. V. Vieira and R. J. Botelho, unpublished work), but the precise function of these molecules remains to be elucidated. Some studies have implicated the products of PI 3-kinase in stimulation of neutrophil O_2^- production, possibly by promoting the recruitment of components of the oxidase to the phagosomal membrane. Both the $p40^{phox}$ and $p47^{phox}$ subunits of the NADPH oxidase have PX domains that bind to products of PI 3-kinase [138].

The accumulation of PI(3)*P* on phagosomes is transient, suggesting that PI(3)*P* may be degraded or modified further by kinases. It has not been established yet, however, if PIKfyve associates with phagosomes or whether phosphatidylinositol 3,5-bisphosphate is synthesized and plays a role in maturation.

MEMBRANE VESICULATION FROM PHAGOSOMES

While fusion with (or 'kissing') the various endo/lysosomal compartments is a dominant force in phagosome remodelling during maturation, it is apparent that fission (or 'running') of vesicles also contributes to this process. Indeed, the size of the phagosome remains relatively constant, despite the continued fusion with endo/lysosomal organelles. Moreover, there is a progressive elimination of several transmembrane proteins from the phagosome, including SNAREs, TfRs and mannose receptors [26,48,73,139]. In addition, phagosomal constituents eventually appear in endosomes and in the plasma membrane [49,50,139–141].

Membrane traffic along the endocytic pathway relies on several membrane fission complexes, of which the best characterized are clathrin and COPI, which have been demonstrated to assemble on the cytosolic surface of endosomes and/or lysosomes and to induce the budding of vesicles therefrom [125,142,143]. COPI was originally discovered as a seven-subunit complex that mediates traffic between the endoplasmic reticulum and the Golgi complex [144,145]. Subsequently, COPI was also shown to be required for the maintenance of endosome structure and function [146–148]. This was demonstrated using brefeldin A, which prevents the activation of some members of the ADP-ribosylation factor (ARF) family of small GTPases that regulate the assembly of COPI complexes on membranes [149–151]. Confirmation of this finding was obtained using ldlF cells, a variant of the CHO line containing a temperature-sensitive mutation in the ϵ-COP subunit of COPI [152,153]. In brefeldin A-treated cells or in ϵ-COP-deficient ldlF cells, transferrin recycling is significantly retarded and early-to-late endosome traffic is disrupted [147,148].

COPI also plays a role in phagosome maturation. When COPI function was abrogated in RAW234.7 macrophages or in ldlF cells engineered to become phagocytic by transfection of FcγRs, the removal of TfRs from the phagosomal membrane was retarded, although not eliminated [48]. Similarly, protein recycling from phagosomes was also impeded in J774-E macrophages treated with brefeldin A [154]. Despite the observed defects in early phagosome processing, inactivation of COPI failed to block phagolysosome formation and acidification [48]. These findings suggest that redundant fission mechanisms may exist. Accordingly, even though phagosomal removal of TfRs is slowed by abrogation of COPI activity, the process is eventually completed [48].

Clathrin-dependent vesiculation may be one such redundant mechanism. Clathrin mediates receptor-induced endocytosis at the plasma membrane, and also the fission of vesicles from the *trans*-Golgi network. It is also known that clathrin-coated structures form on endosomes, although these are morphologically distinct from those on other organelles [125,142,155]. Clathrin assembly on to membranes requires the intermediacy of adaptor complexes such as AP-1 and AP-2, which function in the *trans*-Golgi network and plasmalemma respectively. There is currently no evidence for a role for these adaptors in phagosome maturation. Moreover, a potential function can also be contemplated for the related, but less well characterized, AP-3 and AP-4 adaptor complexes.

Scission of the budding clathrin-containing vesicles depends on dynamin, a GTPase thought to exert a 'pinching' force. Impairment of vesicle detachment using dominant-negative mutants of dynamin or of other constituents of the clathrin complex obstructs membrane traffic at multiple steps of the endocytic pathway [156,157]. Remarkably, phagolysosome biogenesis is unimpaired in cells expressing dominant-negative mutants of dynamin-1, whether in the presence or the absence of brefeldin A (S. Tse and S. Grinstein, unpublished work). However, these data do not rule out the involvement of clathrin in phagosomal maturation, since another isoform of dynamin or a dynamin-like protein may function to sever clathrin vesicles from phagosomes.

Completely unexplored is the possible function of caveolae in the fission of membranes from phagosomes. To our knowledge, the presence of caveolae or of lipid rafts, which are often associated with caveolae, has not been documented in phagosomes. However, proteins that prefer to reside within lipid rafts have been found in phagosomes [74], so that rafts and caveolae may very well exist and contribute to maturation.

A DYNAMIC ROLE FOR THE CYTOSKELETON IN PHAGOSOME MATURATION

Microtubules

The intracellular localization of many organelles is strongly linked to microtubules. Key to this function is the polarity and orientation of the tubules. Microtubules provide polarized tracks (their minus and plus ends are oriented towards the centre and periphery of the cell respectively) that guide organellar dynamics, driven by a variety of molecular motors. Most notable among these motors are members of the dynein and kinesin families, which propel organelles in a centripetal or centrifugal direction respectively. Microtubule-associated proteins serve as adaptors between microtubules, the motor proteins and their cargo [158–162].

The various components of the endocytic pathway are positioned in defined domains of the cell in a microtubule-dependent fashion, and their location influences the efficiency of endosomal progression (Figure 1). Not surprisingly, traffic between endosomes and lysosomes is severely affected when microtubules are disrupted [163,164]. Similarly, interfering with the proper function of kinesin or dynein impairs fusion between MVBs and late endosomes [165,166].

In a similar manner, phagosomal maturation is linked to microtubules (Figure 1). Newly formed phagosomes, which are by nature generated at the cell periphery, rapidly engage in centripetal motion that is arrested by microtubule disruption [167–169]. Interestingly, phagosomes were shown to possess bidirectional motility along microtubules both *in vitro* and *in vivo*, although displacement towards the minus ends predominated, as expected from the net centripetal translocation noted in cells [168]. Reconstitution assays using cell-free systems identified kinesin as the motor that powers the plus-end-directed movement of phagosomes, and kinectin, a transmembrane receptor for kinesins, as the intervening adaptor. In contrast, minus-end-oriented motility was enacted by dyneins, coupled through the dynactin complex and an uncharacterized activator X [168].

Although an early study suggested otherwise [170], it is becoming widely accepted that microtubule-based motility is essential for correct phagosomal maturation [41,75,171]. This view is supported by the observations of Desjardins et al. [41], who found that phagosomes acquired 5-fold less LAMP-2, a late-endosome/lysosome marker (Table 1), when microtubules were disrupted by nocodazole. Microtubule disassembly was also found to ablate the delivery to phagosomes of fluid-phase markers trapped in late endosomes [171], and depressed phagosome–lysosome fusion in streptolysin *O*-permeabilized cells [75].

How phagosomes become linked to microtubules remains undefined. Members of the Rab family could conceivably play a role in bridging to microtubules. Thus Rab4a was shown to bind to a cytoplasmic dynein chain [172]. Moreover, Rabphilin11, an effector of Rab11, was shown to align along peripheral microtubules, and Rab5 was demonstrated to regulate endosome motility on microtubules [82,172,173]. These Rabs are known to associate with early phagosomes and could fulfil a role in microtubule-dependent centripetal motion.

Microfilaments

Recent work suggests that organelle motility and distribution not only depend on microtubules, but also involve the actin cytoskeleton [174–176], a concept that may apply to phagosome maturation as well (Figure 1). Like microtubules, F-actin filaments are highly dynamic and polarized. F-actin is best known for its role in patterning the plasma membrane during processes such as ruffling, chemotaxis and phagocytosis. However, recent evidence suggests that F-actin can also associate with endosomes and lysosomes [177,178]. Taunton et al. [177] demonstrated that endosomes and lysosomes are propelled by the formation of actin comet tails in a manner dependent on N-WASP, Cdc42 and protein kinase C. Actin-dependent motility of endosomes and/or lysosomes may also depend on myosins, which are actin-associated motor proteins [179]. This is suggested by the attenuation of endocytic traffic induced by myosin inhibitors [176,180].

A role for actin in phagosome maturation appears to contravene the accepted notion that, once severed from the plasma membrane, phagosomes are devoid of actin. This simplistic, perhaps dogmatic, view has recently been challenged by several observations. First, mature phagosomes were reported to be often surrounded by an 'actin-rich' cytoplasm [167]. Secondly, actin was detectable as a component of fully formed phagosomes [41,74,181]. Lastly, as discussed below, in cell-free preparations actin can assemble on the surface of phagosomes [182]. Perhaps

these observations explain why numerous actin-binding proteins, including annexins, α-actinin and coronin (also called TACO), are also found to associate with phagosomes [183].

What is the relationship between actin and the phagosome after its detachment from the surface membrane? There are at least two reports that attribute to actin a role in phagosome motility [167,184]. Toyohara and Inaba [167] proposed that the size of the phagosome dictates the type of motor system utilized. In their view, small ($\leqslant 1\ \mu$m) phagosomes travel along microtubules, while larger ones are propelled by the actin cytoskeleton. Moller and colleagues [184] similarly established actin as a contributor to phagosome motility, although they also acknowledged a role for microtubules in the process. The exact molecular mechanism(s) underlying the actin-based motility of phagosomes remain obscure. The displacement force is likely to be furnished by myosins. The reported inhibition of phagosome motility by the myosin inhibitor 2,3-butanedione-2-monoxime [185] favours this view. Alternatively, *de novo* actin assembly on the phagosome surface could propel phagosomes (Figure 1), as has been suggested for endosomes, lysosomes and *Listeria* [177,178,186]. Such a mechanism is supported by the demonstration of actin assembly on the surface of phagosomes *in vitro* [182,185,187]. In these experiments, ezrin and moesin, members of the ERM family of skeletal proteins, were required for actin nucleation [182]. ERM proteins are known to act as molecular linkers between F-actin and transmembrane proteins [188]. Hence it will be important to investigate whether ERM proteins participate in phagosome maturation *in situ*.

There is now compelling evidence that actin is implicated in phagolysosome biogenesis [74,167,181,185]. Jahraus et al. [185] demonstrated that disruption of F-actin attenuated the transfer of the contents of labelled endosomes to phagosomes *in vivo*. Actin filaments formed *de novo* were proposed to extend from phagosomes and act as 'tentacles' that facilitate the capture of endosomes. Collision between the tethered organelles may then be facilitated by myosins. Indeed, actomyosin filaments extending from phagosomes have been visualized by electron microscopy. In addition, myosin inhibitors suppress phagosome–endosome fusion [185]. Paradoxically, stabilization of actin with phalloidin also abrogated phagosome–endosome fusion. In an attempt to reconcile these observations, it was suggested that overstabilization of F-actin forms a physical barrier around organelles that prevents membrane fusion, a notion supported by the inability of *Leishmania*-containing phagosomes to mature while possessing a strong periphagosomal actin ring [189].

pH AS A REGULATOR OF PHAGOSOME MATURATION

A key event in the maturation of phagosomes is the progressive acidification of their lumen, from near-neutral to pH < 5 [190]. The acidic pH directly affects the growth of pathogens, favours the dismutation of superoxide and is optimal for hydrolytic enzyme activity. Phagosomal acidification, like that of endosomes and lysosomes, is regulated by the vacuolar (V-type) ATPase complex [191,192].

However, creating an inhospitable milieu for pathogens may not be the only role of phagosomal acidification. There is ample evidence that the luminal pH of organelles has an important role in membrane traffic. Thus dissipation of the pH gradient across endomembranes by addition of V-ATPase antagonists hinders the transfer of material from early endosomes to late endosomes/lysosomes and causes endosome tubulation [193–196]. The underlying mechanism is poorly understood. Dissipation of endosomal pH is known to preclude the association of ARNO, an ARF exchange factor, with endosomes [197]. Similarly, COPI assembly on endosomes is also dependent on endosomal pH, although this may be secondary to ARF1 inhibition [143,197,198]. These observations imply that endosomes and/or lysosomes possess luminal pH sensors that convey information across the membrane. The identity of these putative sensors remains mysterious.

The above arguments suggest that phagosomal acidification may not be simply a passive event that aids in the destruction of pathogens, but also a dynamic contributor to phagosome maturation [199]. The temporal sequence of events supports this view: acidification to pH ≈ 5 precedes fusion of phagosomes with late endosomes/lysosomes [200]. More importantly, treatment of macrophages with NH_4Cl or polyanionic compounds, which alkalinize the lumen of organelles, interferes with phagosome–lysosome fusion [201–203]. Whether ARF or COPI are responsible for these effects has not yet been verified.

CALCIUM AS A REGULATOR OF PHAGOSOME MATURATION

Calcium is a key second messenger in leucocyte activation. It mediates, at least in part, activation of the respiratory burst and secretion of microbicidal granule constituents [28,204–206]. As in other systems, the resting cytosolic free calcium concentration ($[Ca^{2+}]_i$) hovers in the 100 nM range, but is acutely elevated upon the engagement of phagocytic receptors [207,208]. Release of calcium stored in the endoplasmic reticulum and opening of plasmalemmal store-operated channels are largely responsible for this elevation.

In other systems, it has been realized recently that organelles other than the endoplasmic reticulum can contribute to the elevation of $[Ca^{2+}]_i$. Calcium is now thought to be released also by early and late endosomes, lysosomes and the yeast vacuole [66,209–211]. Along the same lines, it is entirely conceivable that calcium trapped in the lumen of forming phagosomes, or accumulated afterwards by plasmalemmal calcium pumps, may be released at critical stages of the maturation sequence. Indeed, preliminary evidence to this effect has been presented [212]. Consistent with this model, a localized periphagosomal increase in $[Ca^{2+}]_i$ has been recorded [207], although this was attributed to the preferential distribution of endoplasmic reticulum in the immediate vicinity of phagosomes.

Release of intraorganellar calcium could have significant implications for membrane fusion, in that a restricted 'cloud' of high $[Ca^{2+}]_i$ may form in the immediate vicinity of the organelle, promoting and targeting fusion with cognate vesicles. In fact, a wealth of studies implicate intraorganellar Ca^{2+} in the homotypic fusion of early endosomes and yeast vacuoles, and in late-endosome–lysosome heterotypic fusion [66,209–211,213]. In the endocytic pathway, the effects of the calcium released locally are thought to be mediated by calmodulin [209,211,213], which is proposed to act downstream of the Rab GTPases and SNARE complex by promoting bilayer coalescence [66,211,214].

While the evidence implicating calcium in the endocytic pathway is tantalizing, the role of this bivalent cation in phagosome maturation is far from clear. There is at least one convincing report that discounts a role for Ca^{2+} in phagolysosome biogenesis in macrophages [215]. By contrast, preventing changes in $[Ca^{2+}]_i$ was shown to impair phagosome–lysosome fusion in neutrophils and macrophages [208,216]. Similar observations were also obtained with engineered phagocytes expressing FcγRIIA [217]. Moreover, clamping $[Ca^{2+}]_i$ prevented efficient killing of *Staphylococcus* by neutrophils, suggesting that phagosome maturation was defective [218].

Exactly how $[Ca^{2+}]_i$ may regulate phagosome maturation is not understood. One possibility is that Ca^{2+} induces disassembly

of actin coating the surface of the phagosome, permitting access to incoming vesicles [219]. As discussed above, phagosome–endosome fusion is impaired when periphagosomal actin is stabilized [185]. Interestingly, retention of an actin coat around *Mycobacterium*-containing phagosomes by inhibition of Ca^{2+} is consistent with the presence of coronin, an actin-binding protein, in *Mycobacterium*-containing phagosomes [220]. Nonetheless, exceptions have been reported: in macrophages, actin assembly and disassembly appeared to be normal when Ca^{2+} was clamped at a very low concentration [221]. Alternatively, calcium may regulate fusion by a more direct approach, through annexins, calmodulin and/or calcium/calmodulin-dependent protein kinase II [211,213,222–224]. Calmodulin and this kinase may in turn regulate tethering or docking factors such as EEA1 and syntaxin 13, and/or regulate bilayer fusion between phagosomes and endo/lysosomes [66,211,214].

THE PROTEOME OF THE PHAGOSOME: NEW INSIGHTS

The recent publication by Desjardins and colleagues [74] describing the proteome of maturing phagosomes represents a remarkable *tour de force* and constitutes a major advance in our understanding of phagosome maturation. The protein composition of 2 h-old phagosomes containing 'naked' (unopsonized) latex beads was analysed by high-resolution two-dimensional SDS/PAGE and MS, resulting in the identification of > 140 proteins. Many proteins known previously to be involved in phagosome maturation were detected, providing confirmation of the validity of the proteomic approach. These included proteins of the endosomal system such as LAMP and V-ATPase subunits, membrane fusion promoters such as EEA1 and Rab5, and cytoskeletal proteins such as coronin and actin-related protein-3. But the true impact of the proteomic project was in the identification of proteins not previously associated with the phagocytosis sequence. These included several Rab GTPases, namely Rab3, Rab10 and Rab14, and an ARF6-like GTPase. The identification of lipid-raft protein constituents, namely flotillin-1, prohibitin and stomatin, on phagosomes suggests that these specialized membrane domains may have a role in phagosome maturation [74].

Unexpectedly, numerous endoplasmic reticulum components, such as calnexin and calreticulin, were detected in the phagosome proteome, perhaps suggesting that phagosomes fuse with the reticulum. Of note, the endoplasmic reticulum is the major source of membranes for autophagosome formation and maturation, raising the prospect that phagosome formation/maturation and autophagy may be related [225–228]. This fascinating notion is supported by the detection of a ubiquitin-like protein, Ti225 or ubiquitin C, on phagosomes [74,229]. Ubiquitin is involved in the regulation of catabolic processes, particularly protein degradation, and ubiquitin-like systems are now associated with autophagy [230–232]. This raises the possibility that a ubiquitin-like system may also regulate phagosome maturation.

This proteomic technology will prove useful in the near future for comparing phagosome composition during various stages of maturation, and could also be extended to the investigation of micro-organism-containing phagosomes, in the hunt for pathogen factors that alter maturation.

CONCLUDING REMARKS

Phagosome formation and maturation encapsulate much of cellular biology in a neat, ordered sequence of events. Receptor biology, signal transduction, cytoskeletal restructuring, membrane traffic and ion transport are all merged into a purposeful matrix of reactions with a defined end goal: the elimination of foreign organisms or apoptotic bodies. As such, phagocytosis provides an unparalleled paradigm for the study of cellular biology.

Despite much knowledge acquired since Metchnikoff's initial description of the phagocytic process, enormous gaps in our understanding remain and need to be addressed. Notable among these are the determinants of selectivity that dictate the progressive fusion with elements of the endocytic pathway, the possible involvement of the endoplasmic reticulum, the fission processes that remove unwanted material from the phagosomes, and the role of ions and cytoskeletal elements in the later stages of the maturation sequence. The advent of new chemical and biophysical methods will accelerate the solution to these puzzles. We anticipate that improved proteomics techniques will facilitate the characterization of the composition of phagosomes at various, well defined stages of maturation. Specific interactions between proteins will eventually be resolved by advanced spectroscopic methods, including fluorescence correlation spectroscopy, and by high-resolution cryoelectron microscopy.

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REFERENCES

1 Tjelle, T. E., Lovdal, T. and Berg, T. (2000) Phagosome dynamics and function. BioEssays **22**, 255–263
2 Beron, W., Alvarez-Dominguez, C., Mayorga, L. and Stahl, P. D. (1995) Membrane trafficking along the phagocytic pathway. Trends Cell Biol. **5**, 100–104
3 Aderem, A. and Underhill, D. M. (1999) Mechanisms of phagocytosis in macrophages. Annu. Rev. Immunol. **17**, 593–623
4 Bangs, P., Franc, N. and White, K. (2000) Molecular mechanisms of cell death and phagocytosis in Drosophila. Cell Death Differ. **7**, 1027–1034
5 Franc, N. C., White, K. and Ezekowitz, R. A. (1999) Phagocytosis and development: back to the future. Curr. Opin. Immunol. **11**, 47–52
6 May, R. C. (2001) Phagocytosis in C. elegans: CED-1 reveals its secrets. Trends Cell Biol. **11**, 150
7 Platt, N., da Silva, R. P. and Gordon, S. (1999) Class A scavenger receptors and the phagocytosis of apoptotic cells. Immunol. Lett. **65**, 15–19
8 Cardelli, J. (2001) Phagocytosis and macropinocytosis in Dictyostelium: phosphoinositide-based processes, biochemically distinct. Traffic **2**, 311–320
9 Duclos, S. and Desjardins, M. (2000) Subversion of a young phagosome: the survival strategies of intracellular pathogens. Cell. Microbiol. **2**, 365–377
10 Hackstadt, T. (2000) Redirection of host vesicle trafficking pathways by intracellular parasites. Traffic **1**, 93–99
11 Meresse, S., Steele-Mortimer, O., Moreno, E., Desjardins, M., Finlay, B. and Gorvel, J. P. (1999) Controlling the maturation of pathogen-containing vacuoles: a matter of life and death. Nat. Cell Biol. **1**, E183–E188
12 Rabinovitch, M. (1995) Professional and non-professional phagocytes: an introduction. Trends Cell Biol. **5**, 85–87
13 Kwiatkowska, K. and Sobota, A. (1999) Signaling pathways in phagocytosis. BioEssays **21**, 422–431
14 Caron, E. and Hall, A. (1998) Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. Science **282**, 1717–1721
15 Indik, Z. K., Park, J. G., Hunter, S. and Schreiber, A. D. (1995) The molecular dissection of Fcγ receptor mediated phagocytosis. Blood **86**, 4389–4399
16 Nagarajan, S., Chesla, S., Cobern, L., Anderson, P., Zhu, C. and Selvaraj, P. (1995) Ligand binding and phagocytosis by CD16 (Fc gamma receptor III) isoforms. Phagocytic signaling by associated zeta and gamma subunits in Chinese hamster ovary cells. J. Biol. Chem. **270**, 25762–25770
17 Ofek, I., Goldhar, J., Keisari, Y. and Sharon, N. (1995) Nonopsonic phagocytosis of microorganisms. Annu. Rev. Microbiol. **49**, 239–276
18 Schutt, C. (1999) Fighting infection: the role of lipopolysaccharide binding proteins CD14 and LBP. Pathobiology **67**, 227–229
19 Daeron, M. (1997) Fc receptor biology. Annu. Rev. Immunol. **15**, 203–234

20 Gessner, J. E., Heiken, H., Tamm, A. and Schmidt, R. E. (1998) The IgG Fc receptor family. Ann. Hematol. **76**, 231–248
21 Petty, H. R. and Todd, III, R. F. (1993) Receptor-receptor interactions of complement receptor type 3 in neutrophil membranes. J. Leukocyte Biol. **54**, 492–494
22 Yefenof, E. (2000) Complement receptor 3 (CR3): a public transducer of innate immunity signals in macrophages. Adv. Exp. Med. Biol. **479**, 15–25
23 Sansonetti, P. J. (2000) Phagocytosis, a cell biology view. J. Cell Sci. **113**, 3355–3356
24 Joiner, K. A., Ganz, T., Albert, J. and Rotrosen, D. (1989) The opsonizing ligand on Salmonella typhimurium influences incorporation of specific, but not azurophil, granule constituents into neutrophil phagosomes. J. Cell Biol. **109**, 2771–2782
25 Bouvier, G., Benoliel, A. M., Foa, C. and Bongrand, P. (1994) Relationship between phagosome acidification, phagosome-lysosome fusion, and mechanism of particle ingestion. J. Leukocyte Biol. **55**, 729–734
26 Pitt, A., Mayorga, L. S., Stahl, P. D. and Schwartz, A. L. (1992) Alterations in the protein composition of maturing phagosomes. J. Clin. Invest. **90**, 1978–1983
27 Hampton, M. B., Kettle, A. J. and Winterbourn, C. C. (1998) Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood **92**, 3007–3017
28 Tapper, H. (1996) The secretion of preformed granules by macrophages and neutrophils. J. Leukocyte Biol. **59**, 613–622
29 Lemmon, S. K. and Traub, L. M. (2000) Sorting in the endosomal system in yeast and animal cells. Curr. Opin. Cell Biol. **12**, 457–466
30 Barbieri, M. A., Roberts, R. L., Mukhopadhyay, A. and Stahl, P. D. (1996) Rab5 regulates the dynamics of early endosome fusion. Biocell **20**, 331–338
31 Mukherjee, S., Ghosh, R. N. and Maxfield, F. R. (1997) Endocytosis. Physiol. Rev. **77**, 759–803
32 McMahon, H. T. (1999) Endocytosis: an assembly protein for clathrin cages. Curr. Biol. **9**, R332–R335
33 Riezman, H., Woodman, P. G., van Meer, G. and Marsh, M. (1997) Molecular mechanisms of endocytosis. Cell **91**, 731–738
34 Woodman, P. G. (2000) Biogenesis of the sorting endosome: the role of Rab5. Traffic **1**, 695–701
35 Mohrmann, K. and van der Sluijs, P. (1999) Regulation of membrane transport through the endocytic pathway by rabGTPases. Mol. Membr. Biol. **16**, 81–87
36 Somsel Rodman, J. and Wandinger-Ness, A. (2000) Rab GTPases coordinate endocytosis. J. Cell Sci. **113**, 183–192
37 Thilo, L., Stroud, E. and Haylett, T. (1995) Maturation of early endosomes and vesicular traffic to lysosomes in relation to membrane recycling. J. Cell Sci. **108**, 1791–1803
38 Gruenberg, J. (2001) The endocytic pathway: a mosaic of domains. Nat. Rev. Mol. Cell Biol. **2**, 721–730
39 Gruenberg, J. and Maxfield, F. R. (1995) Membrane transport in the endocytic pathway. Curr. Opin. Cell Biol. **7**, 552–563
40 Gu, F. and Gruenberg, J. (1999) Biogenesis of transport intermediates in the endocytic pathway. FEBS Lett. **452**, 61–66
41 Desjardins, M., Huber, L. A., Parton, R. G. and Griffiths, G. (1994) Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. J. Cell Biol. **124**, 677–688
42 Desjardins, M., Nzala, N. N., Corsini, R. and Rondeau, C. (1997) Maturation of phagosomes is accompanied by changes in their fusion properties and size-selective acquisition of solute materials from endosomes. J. Cell Sci. **110**, 2303–2314
43 Mayorga, L. S., Bertini, F. and Stahl, P. D. (1991) Fusion of newly formed phagosomes with endosomes in intact cells and in a cell-free system. J. Biol. Chem. **266**, 6511–6517
44 Jahraus, A., Tjelle, T. E., Berg, T., Habermann, A., Storrie, B., Ullrich, O. and Griffiths, G. (1998) In vitro fusion of phagosomes with different endocytic organelles from J774 macrophages. J. Biol. Chem. **273**, 30379–30390
45 Mills, I. G., Jones, A. T. and Clague, M. J. (1998) Involvement of the endosomal autoantigen EEA1 in homotypic fusion of early endosomes. Curr. Biol. **8**, 881–884
46 Scianimanico, S., Desrosiers, M., Dermine, J. F., Meresse, S., Descoteaux, A. and Desjardins, M. (1999) Impaired recruitment of the small GTPase rab7 correlates with the inhibition of phagosome maturation by Leishmania donovani promastigotes. Cell. Microbiol. **1**, 19–32
47 Duclos, S., Diez, R., Garin, J., Papadopoulou, B., Descoteaux, A., Stenmark, H. and Desjardins, M. (2000) Rab5 regulates the kiss and run fusion between phagosomes and endosomes and the acquisition of phagosome leishmanicidal properties in RAW 264.7 macrophages. J. Cell Sci. **113**, 3531–3541
48 Botelho, R. J., Hackam, D. J., Schreiber, A. D. and Grinstein, S. (2000) Role of COPI in phagosome maturation. J. Biol. Chem. **275**, 15717–15727
49 Muller, W. A., Steinman, R. M. and Cohn, Z. A. (1980) The membrane proteins of the vacuolar system. II. Bidirectional flow between secondary lysosomes and plasma membrane. J. Cell Biol. **86**, 304–314
50 Pitt, A., Mayorga, L. S., Schwartz, A. L. and Stahl, P. D. (1992) Transport of phagosomal components to an endosomal compartment. J. Biol. Chem. **267**, 126–132
51 de Chastellier, C. and Thilo, L. (1997) Phagosome maturation and fusion with lysosomes in relation to surface property and size of the phagocytic particle. Eur. J. Cell Biol. **74**, 49–62
52 Vieira, O. V., Botelho, R. J., Rameh, L., Brachmann, S. M., Matsuo, T., Davidson, H. W., Schreiber, A., Backer, J. M., Cantley, L. C. and Grinstein, S. (2001) Distinct roles of class I and class III phosphatidylinositol 3-kinases in phagosome formation and maturation. J. Cell Biol. **155**, 19–26
53 Fratti, R. A., Backer, J. M., Gruenberg, J., Corvera, S. and Deretic, V. (2001) Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. J. Cell Biol. **154**, 631–644
54 Via, L. E., Deretic, D., Ulmer, R. J., Hibler, N. S., Huber, L. A. and Deretic, V. (1997) Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. J. Biol. Chem. **272**, 13326–13331
55 Clemens, D. L. and Horwitz, M. A. (1995) Characterization of the Mycobacterium tuberculosis phagosome and evidence that phagosomal maturation is inhibited. J. Exp. Med. **181**, 257–270
56 Desjardins, M. (1995) Biogenesis of phagolysosomes: the 'kiss-and-run' hypothesis. Trends Cell Biol. **5**, 183–186
57 Wang, Y. L. and Goren, M. B. (1987) Differential and sequential delivery of fluorescent lysosomal probes into phagosomes in mouse peritoneal macrophages. J. Cell Biol. **104**, 1749–1754
58 Gotte, M. and von Mollard, G. F. (1998) A new beat for the SNARE drum. Trends Cell Biol. **8**, 215–218
59 Chen, Y. A. and Scheller, R. H. (2001) SNARE-mediated membrane fusion. Nat. Rev. Mol. Cell Biol. **2**, 98–106
60 Fasshauer, D., Sutton, R. B., Brunger, A. T. and Jahn, R. (1998) Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. Proc. Natl. Acad. Sci. U.S.A. **95**, 15781–15786
61 Rothman, J. E. and Warren, G. (1994) Implications of the SNARE hypothesis for intracellular membrane topology and dynamics. Curr. Biol. **4**, 220–233
62 Pfeffer, S. R. (1999) Transport-vesicle targeting: tethers before SNAREs. Nat. Cell Biol. **1**, E17–E22
63 Sollner, T. H. (2002) Vesicle tethers promoting fusion machinery assembly. Dev. Cell **2**, 377–378
64 Mullock, B. M., Smith, C. W., Ihrke, G., Bright, N. A., Lindsay, M., Parkinson, E. J., Brooks, D. A., Parton, R. G., James, D. E., Luzio, J. P. and Piper, R. C. (2000) Syntaxin 7 is localized to late endosome compartments, associates with Vamp 8, and is required for late endosome-lysosome fusion. Mol. Biol. Cell **11**, 3137–3153
65 Mullock, B. M., Bright, N. A., Fearon, C. W., Gray, S. R. and Luzio, J. P. (1998) Fusion of lysosomes with late endosomes produces a hybrid organelle of intermediate density and is NSF dependent. J. Cell Biol. **140**, 591–601
66 Pryor, P. R., Mullock, B. M., Bright, N. A., Gray, S. R. and Luzio, J. P. (2000) The role of intraorganellar Ca^{2+} in late endosome-lysosome heterotypic fusion and in the reformation of lysosomes from hybrid organelles. J. Cell Biol. **149**, 1053–1062
67 Ward, D. M., Pevsner, J., Scullion, M. A., Vaughn, M. and Kaplan, J. (2000) Syntaxin 7 and VAMP-7 are soluble N-ethylmaleimide-sensitive factor attachment protein receptors required for late endosome-lysosome and homotypic lysosome fusion in alveolar macrophages. Mol. Biol. Cell **11**, 2327–2333
68 Ward, D. M., Leslie, J. D. and Kaplan, J. (1997) Homotypic lysosome fusion in macrophages: analysis using an in vitro assay. J. Cell Biol. **139**, 665–673
69 Caplan, S., Hartnell, L. M., Aguilar, R. C., Naslavsky, N. and Bonifacino, J. S. (2001) Human Vam6p promotes lysosome clustering and fusion in vivo. J. Cell Biol. **154**, 109–122
70 McBride, H. M., Rybin, V., Murphy, C., Giner, A., Teasdale, R. and Zerial, M. (1999) Oligomeric complexes link Rab5 effectors with NSF and drive membrane fusion via interactions between EEA1 and syntaxin 13. Cell **98**, 377–386
71 Antonin, W., Holroyd, C., Fasshauer, D., Pabst, S., Von Mollard, G. F. and Jahn, R. (2000) A SNARE complex mediating fusion of late endosomes defines conserved properties of SNARE structure and function. EMBO J. **19**, 6453–6464
72 Hackam, D. J., Rotstein, O. D., Bennett, M. K., Klip, A., Grinstein, S. and Manolson, M. F. (1996) Characterization and subcellular localization of target membrane soluble NSF attachment protein receptors (t-SNAREs) in macrophages. Syntaxins 2, 3, and 4 are present on phagosomal membranes. J. Immunol. **156**, 4377–4383
73 Bajno, L., Peng, X. R., Schreiber, A. D., Moore, H. P., Trimble, W. S. and Grinstein, S. (2000) Focal exocytosis of VAMP3-containing vesicles at sites of phagosome formation. J. Cell Biol. **149**, 697–706
74 Garin, J., Diez, R., Kieffer, S., Dermine, J. F., Duclos, S., Gagnon, E., Sadoul, R., Rondeau, C. and Desjardins, M. (2001) The phagosome proteome. Insight into phagosome functions. J. Cell Biol. **152**, 165–180
75 Funato, K., Beron, W., Yang, C. Z., Mukhopadhyay, A. and Stahl, P. D. (1997) Reconstitution of phagosome-lysosome fusion in streptolysin O-permeabilized cells. J. Biol. Chem. **272**, 16147–16151

76 Fasshauer, D., Antonin, W., Margittai, M., Pabst, S. and Jahn, R. (1999) Mixed and non-cognate SNARE complexes. Characterization of assembly and biophysical properties. J. Biol. Chem. **274**, 15440–15446

77 Zerial, M. and McBride, H. (2001) Rab proteins as membrane organizers. Nat. Rev. Mol. Cell Biol. **2**, 107–117

78 Bucci, C., Thomsen, P., Nicoziani, P., McCarthy, J. and van Deurs, B. (2000) Rab7: a key to lysosome biogenesis. Mol. Biol. Cell **11**, 467–480

79 McLauchlan, H., Newell, J., Morrice, N., Osborne, A., West, M. and Smythe, E. (1998) A novel role for Rab5-GDI in ligand sequestration into clathrin-coated pits. Curr. Biol. **8**, 34–45

80 Bucci, C., Parton, R. G., Mather, I. H., Stunnenberg, H., Simons, K., Hoflack, B. and Zerial, M. (1992) The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. Cell **70**, 715–728

81 Gorvel, J. P., Chavrier, P., Zerial, M. and Gruenberg, J. (1991) rab5 controls early endosome fusion in vitro. Cell **64**, 915–925

82 Nielsen, E., Severin, F., Backer, J. M., Hyman, A. A. and Zerial, M. (1999) Rab5 regulates motility of early endosomes on microtubules. Nat. Cell Biol. **1**, 376–382

83 Roberts, R. L., Barbieri, M. A., Ullrich, J. and Stahl, P. D. (2000) Dynamics of rab5 activation in endocytosis and phagocytosis. J. Leukocyte Biol. **68**, 627–632

84 Stenmark, H., Parton, R. G., Steele-Mortimer, O., Lutcke, A., Gruenberg, J. and Zerial, M. (1994) Inhibition of rab5 GTPase activity stimulates membrane fusion in endocytosis. EMBO J. **13**, 1287–1296

85 Roberts, R. L., Barbieri, M. A., Pryse, K. M., Chua, M., Morisaki, J. H. and Stahl, P. D. (1999) Endosome fusion in living cells overexpressing GFP-rab5. J. Cell Sci. **112**, 3667–3675

86 Barbieri, M. A., Roberts, R. L., Gumusboga, A., Highfield, H., Alvarez-Dominguez, C., Wells, A. and Stahl, P. D. (2000) Epidermal growth factor and membrane trafficking. EGF receptor activation of endocytosis requires Rab5a. J. Cell Biol. **151**, 539–550

87 Li, G., Barbieri, M. A., Colombo, M. I. and Stahl, P. D. (1994) Structural features of the GTP-binding defective Rab5 mutants required for their inhibitory activity on endocytosis. J. Biol. Chem. **269**, 14631–14635

88 Li, G., D'Souza-Schorey, C., Barbieri, M. A., Cooper, J. A. and Stahl, P. D. (1997) Uncoupling of membrane ruffling and pinocytosis during Ras signal transduction. J. Biol. Chem. **272**, 10337–10340

89 Barbieri, M. A., Kohn, A. D., Roth, R. A. and Stahl, P. D. (1998) Protein kinase B/akt and rab5 mediate Ras activation of endocytosis. J. Biol. Chem. **273**, 19367–19370

90 Hoffenberg, S., Liu, X., Nikolova, L., Hall, H. S., Dai, W., Baughn, R. E., Dickey, B. F., Barbieri, M. A., Aballay, A., Stahl, P. D. and Knoll, B. J. (2000) A novel membrane-anchored Rab5 interacting protein required for homotypic endosome fusion. J. Biol. Chem. **275**, 24661–24669

91 Horiuchi, H., Lippe, R., McBride, H. M., Rubino, M., Woodman, P., Stenmark, H., Rybin, V., Wilm, M., Ashman, K., Mann, M. and Zerial, M. (1997) A novel Rab5 GDP/GTP exchange factor complexed to Rabaptin-5 links nucleotide exchange to effector recruitment and function. Cell **90**, 1149–1159

92 Lippe, R., Miaczynska, M., Rybin, V., Runge, A. and Zerial, M. (2001) Functional synergy between Rab5 effector Rabaptin-5 and exchange factor Rabex-5 when physically associated in a complex. Mol. Biol. Cell **12**, 2219–2228

93 Mu, F. T., Callaghan, J. M., Steele-Mortimer, O., Stenmark, H., Parton, R. G., Campbell, P. L., McCluskey, J., Yeo, J. P., Tock, E. P. and Toh, B. H. (1995) EEA1, an early endosome-associated protein. EEA1 is a conserved alpha-helical peripheral membrane protein flanked by cysteine 'fingers' and contains a calmodulin-binding IQ motif. J. Biol. Chem. **270**, 13503–13511

94 Simonsen, A., Lippe, R., Christoforidis, S., Gaullier, J. M., Brech, A., Callaghan, J., Toh, B. H., Murphy, C., Zerial, M. and Stenmark, H. (1998) EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. Nature (London) **394**, 494–498

95 Callaghan, J., Nixon, S., Bucci, C., Toh, B. H. and Stenmark, H. (1999) Direct interaction of EEA1 with Rab5b. Eur. J. Biochem. **265**, 361–366

96 Patki, V., Virbasius, J., Lane, W. S., Toh, B. H., Shpetner, H. S. and Corvera, S. (1997) Identification of an early endosomal protein regulated by phosphatidylinositol 3-kinase. Proc. Natl. Acad. Sci. U.S.A. **94**, 7326–7330

97 Gaullier, J. M., Simonsen, A., D'Arrigo, A., Bremnes, B., Stenmark, H. and Aasland, R. (1998) FYVE fingers bind PtdIns(3)P. Nature (London) **394**, 432–433

98 Stenmark, H., Aasland, R., Toh, B. H. and D'Arrigo, A. (1996) Endosomal localization of the autoantigen EEA1 is mediated by a zinc-binding FYVE finger. J. Biol. Chem. **271**, 24048–24054

99 Liu, K. and Li, G. (1998) Catalytic domain of the p120 Ras GAP binds to Rab5 and stimulates its GTPase activity. J. Biol. Chem. **273**, 10087–10090

100 Xiao, G. H., Shoarinejad, F., Jin, F., Golemis, E. A. and Yeung, R. S. (1997) The tuberous sclerosis 2 gene product, tuberin, functions as a Rab5 GTPase activating protein (GAP) in modulating endocytosis. J. Biol. Chem. **272**, 6097–6100

101 Lanzetti, L., Rybin, V., Malabarba, M. G., Christoforidis, S., Scita, G., Zerial, M. and Di Fiore, P. P. (2000) The Eps8 protein coordinates EGF receptor signalling through Rac and trafficking through Rab5. Nature (London) **408**, 374–377

102 Chen, X. and Wang, Z. (2001) Regulation of epidermal growth factor receptor endocytosis by wortmannin through activation of Rab5 rather than inhibition of phosphatidylinositol 3-kinase. EMBO Rep. **2**, 842–849

103 Feng, Y., Press, B. and Wandinger-Ness, A. (1995) Rab 7: an important regulator of late endocytic membrane traffic. J. Cell Biol. **131**, 1435–1452

104 Vitelli, R., Santillo, M., Lattero, D., Chiariello, M., Bifulco, M., Bruni, C. B. and Bucci, C. (1997) Role of the small GTPase Rab7 in the late endocytic pathway. J. Biol. Chem. **272**, 4391–4397

105 Papini, E., Satin, B., Bucci, C., de Bernard, M., Telford, J. L., Manetti, R., Rappuoli, R., Zerial, M. and Montecucco, C. (1997) The small GTP binding protein rab7 is essential for cellular vacuolation induced by Helicobacter pylori cytotoxin. EMBO J. **16**, 15–24

106 Mukhopadhyay, A., Funato, K. and Stahl, P. D. (1997) Rab7 regulates transport from early to late endocytic compartments in Xenopus oocytes. J. Biol. Chem. **272**, 13055–13059

107 Cantalupo, G., Alifano, P., Roberti, V., Bruni, C. B. and Bucci, C. (2001) Rab-interacting lysosomal protein (RILP): the Rab7 effector required for transport to lysosomes. EMBO J. **20**, 683–693

108 Alvarez-Dominguez, C., Barbieri, A. M., Beron, W., Wandinger-Ness, A. and Stahl, P. D. (1996) Phagocytosed live Listeria monocytogenes influences Rab5-regulated in vitro phagosome-endosome fusion. J. Biol. Chem. **271**, 13834–13843

109 Rupper, A., Grove, B. and Cardelli, J. (2001) Rab7 regulates phagosome maturation in Dictyostelium. J. Cell Sci. **114**, 2449–2460

110 Toker, A. and Cantley, L. C. (1997) Signalling through the lipid products of phosphoinositide-3-OH kinase. Nature (London) **387**, 673–676

111 Vanhaesebroeck, B., Leevers, S. J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P. C., Woscholski, R., Parker, P. J. and Waterfield, M. D. (2001) Synthesis and function of 3-phosphorylated inositol lipids. Annu. Rev. Biochem. **70**, 535–602

112 Backer, J. M. (2000) Phosphoinositide 3-kinases and the regulation of vesicular trafficking. Mol. Cell. Biol. Res. Commun. **3**, 193–204

113 Vanhaesebroeck, B. and Waterfield, M. D. (1999) Signaling by distinct classes of phosphoinositide 3-kinases. Exp. Cell Res. **253**, 239–254

114 Volinia, S., Dhand, R., Vanhaesebroeck, B., MacDougall, L. K., Stein, R., Zvelebil, M. J., Domin, J., Panaretou, C. and Waterfield, M. D. (1995) A human phosphatidylinositol 3-kinase complex related to the yeast Vps34p-Vps15p protein sorting system. EMBO J. **14**, 3339–3348

115 Panaretou, C., Domin, J., Cockcroft, S. and Waterfield, M. D. (1997) Characterization of p150, an adaptor protein for the human phosphatidylinositol (PtdIns) 3-kinase. Substrate presentation by phosphatidylinositol transfer protein to the p150.Ptdins 3-kinase complex. J. Biol. Chem. **272**, 2477–2485

116 Wurmser, A. E., Gary, J. D. and Emr, S. D. (1999) Phosphoinositide 3-kinases and their FYVE domain-containing effectors as regulators of vacuolar/lysosomal membrane trafficking pathways. J. Biol. Chem. **274**, 9129–9132

117 Christoforidis, S., Miaczynska, M., Ashman, K., Wilm, M., Zhao, L., Yip, S. C., Waterfield, M. D., Backer, J. M. and Zerial, M. (1999) Phosphatidylinositol-3-OH kinases are Rab5 effectors. Nat. Cell Biol. **1**, 249–252

118 Patki, V., Lawe, D. C., Corvera, S., Virbasius, J. V. and Chawla, A. (1998) A functional PtdIns(3)P-binding motif. Nature (London) **394**, 433–434

119 Wishart, M. J., Taylor, G. S. and Dixon, J. E. (2001) Phoxy lipids: revealing PX domains as phosphoinositide binding modules. Cell **105**, 817–820

120 Ellson, C. D., Andrews, S., Stephens, L. R. and Hawkins, P. T. (2002) The PX domain: a new phosphoinositide-binding module. J. Cell Sci. **115**, 1099–1105

121 Lawe, D. C., Patki, V., Heller-Harrison, R., Lambright, D. and Corvera, S. (2000) The FYVE domain of early endosome antigen 1 is required for both phosphatidylinositol 3-phosphate and Rab5 binding. Critical role of this dual interaction for endosomal localization. J. Biol. Chem. **275**, 3699–3705

122 Li, G., D'Souza-Schorey, C., Barbieri, M. A., Roberts, R. L., Klippel, A., Williams, L. T. and Stahl, P. D. (1995) Evidence for phosphatidylinositol 3-kinase as a regulator of endocytosis via activation of Rab5. Proc. Natl. Acad. Sci. U.S.A. **92**, 10207–10211

123 Jones, A. T., Mills, I. G., Scheidig, A. J., Alexandrov, K. and Clague, M. J. (1998) Inhibition of endosome fusion by wortmannin persists in the presence of activated Rab5. Mol. Biol. Cell **9**, 323–332

124 Nielsen, E., Christoforidis, S., Uttenweiler-Joseph, S., Miaczynska, M., Dewitte, F., Wilm, M., Hoflack, B. and Zerial, M. (2000) Rabenosyn-5, a novel Rab5 effector, is complexed with hVPS45 and recruited to endosomes through a FYVE finger domain. J. Cell Biol. **151**, 601–612

125 Raiborg, C., Gronvold Bache, K., Mehlum, A., Stang, E. and Stenmark, H. (2001) Hrs recruits clathrin to early endosomes. EMBO J. **20**, 5008–5021

126 Raiborg, C., Bache, K. G., Gillooly, D. J., Madshus, I. H., Stang, E. and Stenmark, H. (2002) Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes. Nat. Cell Biol. **4**, 394–398

127 Sachse, M., Urbe, S., Oorschot, V., Strous, G. J. and Klumperman, J. (2002) Bilayered clathrin coats on endosomal vacuoles are involved in protein sorting toward lysosomes. Mol. Biol. Cell **13**, 1313–1328

128 Gillooly, D. J., Morrow, I. C., Lindsay, M., Gould, R., Bryant, N. J., Gaullier, J. M., Parton, R. G. and Stenmark, H. (2000) Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. EMBO J. **19**, 4577–4588

129 Shisheva, A., Sbrissa, D. and Ikonomov, O. (1999) Cloning, characterization, and expression of a novel Zn^{2+}-binding FYVE finger-containing phosphoinositide kinase in insulin-sensitive cells. Mol. Cell. Biol. **19**, 623–634

130 Sbrissa, D., Ikonomov, O. C. and Shisheva, A. (1999) PIKfyve, a mammalian ortholog of yeast Fab1p lipid kinase, synthesizes 5-phosphoinositides. Effect of insulin. J. Biol. Chem. **274**, 21589–21597

131 Odorizzi, G., Babst, M. and Emr, S. D. (1998) Fab1p PtdIns(3)P 5-kinase function essential for protein sorting in the multivesicular body. Cell **95**, 847–858

132 Gary, J. D., Wurmser, A. E., Bonangelino, C. J., Weisman, L. S. and Emr, S. D. (1998) Fab1p is essential for PtdIns(3)P 5-kinase activity and the maintenance of vacuolar size and membrane homeostasis. J. Cell Biol. **143**, 65–79

133 Ikonomov, O. C., Sbrissa, D. and Shisheva, A. (2001) Mammalian cell morphology and endocytic membrane homeostasis require enzymatically active phosphoinositide 5-kinase PIKfyve. J. Biol. Chem. **276**, 26141–26147

134 Araki, N., Johnson, M. T. and Swanson, J. A. (1996) A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. J. Cell Biol. **135**, 1249–1260

135 Crowley, M. T., Costello, P. S., Fitzer-Attas, C. J., Turner, M., Meng, F., Lowell, C., Tybulewicz, V. L. and DeFranco, A. L. (1997) A critical role for Syk in signal transduction and phagocytosis mediated by Fcγ receptors on macrophages. J. Exp. Med. **186**, 1027–1039

136 Cox, D., Tseng, C. C., Bjekic, G. and Greenberg, S. (1999) A requirement for phosphatidylinositol 3-kinase in pseudopod extension. J. Biol. Chem. **274**, 1240–1247

137 Marshall, J. G., Booth, J. W., Stambolic, V., Mak, T., Balla, T., Schreiber, A. D., Meyer, T. and Grinstein, S. (2001) Restricted accumulation of phosphatidylinositol 3-kinase products in a plasmalemmal subdomain during Fcγ receptor-mediated phagocytosis. J. Cell Biol. **153**, 1369–1380

138 Kanai, F., Liu, H., Field, S. J., Akbary, H., Matsuo, T., Brown, G. E., Cantley, L. C. and Yaffe, M. B. (2001) The PX domains of p47phox and p40phox bind to lipid products of PI(3)K. Nat. Cell Biol. **3**, 675–678

139 Haurani, F. I. and Ryter, A. (1993) Tracing iron and transferrin in the macrophage by visual means. Am. J. Hematol. **44**, 179–186

140 Ryter, A. (1985) Relationship between ultrastructure and specific functions of macrophages. Comp. Immunol. Microbiol. Infect. Dis. **8**, 119–133

141 Muller, W. A., Steinman, R. M. and Cohn, Z. A. (1983) Membrane proteins of the vacuolar system. III. Further studies on the composition and recycling of endocytic vacuole membrane in cultured macrophages. J. Cell Biol. **96**, 29–36

142 Stoorvogel, W., Oorschot, V. and Geuze, H. J. (1996) A novel class of clathrin-coated vesicles budding from endosomes. J. Cell Biol. **132**, 21–33

143 Aniento, F., Gu, F., Parton, R. G. and Gruenberg, J. (1996) An endosomal β-COP is involved in the pH-dependent formation of transport vesicles destined for late endosomes. J. Cell Biol. **133**, 29–41

144 Cosson, P. and Letourneur, F. (1997) Coatomer (COPI)-coated vesicles: role in intracellular transport and protein sorting. Curr. Opin. Cell Biol. **9**, 484–487

145 Schekman, R. and Orci, L. (1996) Coat proteins and vesicle budding. Science **271**, 1526–1533

146 Lippincott-Schwartz, J., Yuan, L., Tipper, C., Amherdt, M., Orci, L. and Klausner, R. D. (1991) Brefeldin A's effects on endosomes, lysosomes, and the TGN suggest a general mechanism for regulating organelle structure and membrane traffic. Cell **67**, 601–616

147 Gu, F., Aniento, F., Parton, R. G. and Gruenberg, J. (1997) Functional dissection of COP-I subunits in the biogenesis of multivesicular endosomes. J. Cell Biol. **139**, 1183–1195

148 Daro, E., Sheff, D., Gomez, M., Kreis, T. and Mellman, I. (1997) Inhibition of endosome function in CHO cells bearing a temperature-sensitive defect in the coatomer (COPI) component ϵ-COP. J. Cell Biol. **139**, 1747–1759

149 Palmer, D. J., Helms, J. B., Beckers, C. J., Orci, L. and Rothman, J. E. (1993) Binding of coatomer to Golgi membranes requires ADP-ribosylation factor. J. Biol Chem. **268**, 12083–12089

150 Helms, J. B. and Rothman, J. E. (1992) Inhibition by brefeldin A of a Golgi membrane enzyme that catalyses exchange of guanine nucleotide bound to ARF. Nature (London) **360**, 352–354

151 Donaldson, J. G., Cassel, D., Kahn, R. A. and Klausner, R. D. (1992) ADP-ribosylation factor, a small GTP-binding protein, is required for binding of the coatomer protein β-COP to Golgi membranes. Proc. Natl. Acad. Sci. U.S.A. **89**, 6408–6412

152 Guo, Q., Vasile, E. and Krieger, M. (1994) Disruptions in Golgi structure and membrane traffic in a conditional lethal mammalian cell mutant are corrected by ϵ-COP. J. Cell Biol. **125**, 1213–1224

153 Hobbie, L., Fisher, A. S., Lee, S., Flint, A. and Krieger, M. (1994) Isolation of three classes of conditional lethal Chinese hamster ovary cell mutants with temperature-dependent defects in low density lipoprotein receptor stability and intracellular membrane transport. J. Biol. Chem. **269**, 20958–20970

154 Beron, W., Mayorga, L. S., Colombo, M. I. and Stahl, P. D. (2001) Recruitment of coat-protein-complex proteins on to phagosomal membranes is regulated by a brefeldin A-sensitive ADP-ribosylation factor. Biochem. J. **355**, 409–415

155 Prekeris, R., Klumperman, J., Chen, Y. A. and Scheller, R. H. (1998) Syntaxin 13 mediates cycling of plasma membrane proteins via tubulovesicular recycling endosomes. J. Cell Biol. **143**, 957–971

156 Newmyer, S. and Schmid, S. (2001) Dominant-interfering hsc70 mutants disrupt multiple stages of the clathrin-coated vesicle cycle in vivo. J. Cell Biol. **152**, 607–620

157 Nicoziani, P., Vilhardt, F., Llorente, A., Hilout, L., Courtoy, P. J., Sandvig, K. and van Deurs, B. (2000) Role for dynamin in late endosome dynamics and trafficking of the cation-independent mannose 6-phosphate receptor. Mol. Biol. Cell **11**, 481–495

158 Pierre, P., Scheel, J., Rickard, J. E. and Kreis, T. E. (1992) CLIP-170 links endocytic vesicles to microtubules. Cell **70**, 887–900

159 Sheetz, M. P. (1996) Microtubule motor complexes moving membranous organelles. Cell Struct. Funct. **21**, 369–373

160 Drewes, G., Ebneth, A. and Mandelkow, E. M. (1998) MAPs, MARKs and microtubule dynamics. Trends Biochem. Sci. **23**, 307–311

161 Hunter, A. W. and Wordeman, L. (2000) How motor proteins influence microtubule polymerization dynamics. J. Cell Sci. **113**, 4379–4389

162 Maccioni, R. B. and Cambiazo, V. (1995) Role of microtubule-associated proteins in the control of microtubule assembly. Physiol. Rev. **75**, 835–864

163 Matteoni, R. and Kreis, T. E. (1987) Translocation and clustering of endosomes and lysosomes depends on microtubules. J. Cell Biol. **105**, 1253–1265

164 Gruenberg, J., Griffiths, G. and Howell, K. E. (1989) Characterization of the early endosome and putative endocytic carrier vesicles in vivo and with an assay of vesicle fusion in vitro. J Cell Biol. **108**, 1301–1316

165 Burkhardt, J. K., Echeverri, C. J., Nilsson, T. and Vallee, R. B. (1997) Overexpression of the dynamitin (p50) subunit of the dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution. J. Cell Biol. **139**, 469–484

166 Aniento, F., Emans, N., Griffiths, G. and Gruenberg, J. (1993) Cytoplasmic dynein-dependent vesicular transport from early to late endosomes. J. Cell Biol. **123**, 1373–1387

167 Toyohara, A. and Inaba, K. (1989) Transport of phagosomes in mouse peritoneal macrophages. J. Cell Sci. **94**, 143–153

168 Blocker, A., Severin, F. F., Burkhardt, J. K., Bingham, J. B., Yu, H., Olivo, J. C., Schroer, T. A., Hyman, A. A. and Griffiths, G. (1997) Molecular requirements for bi-directional movement of phagosomes along microtubules. J. Cell Biol. **137**, 113–129

169 Blocker, A., Griffiths, G., Olivo, J. C., Hyman, A. A. and Severin, F. F. (1998) A role for microtubule dynamics in phagosome movement. J. Cell Sci. **111**, 303–312

170 Pesanti, E. L. and Axline, S. G. (1975) Phagolysosome formation in normal and colchicine-treated macrophages. J. Exp. Med. **142**, 903–913

171 Blocker, A., Severin, F. F., Habermann, A., Hyman, A. A., Griffiths, G. and Burkhardt, J. K. (1996) Microtubule-associated protein-dependent binding of phagosomes to microtubules. J. Biol. Chem. **271**, 3803–3811

172 Bielli, A., Thornqvist, P. O., Hendrick, A. G., Finn, R., Fitzgerald, K. and McCaffrey, M. W. (2001) The small GTPase Rab4A interacts with the central region of cytoplasmic dynein light intermediate chain-1. Biochem. Biophys. Res. Commun. **281**, 1141–1153

173 Mammoto, A., Ohtsuka, T., Hotta, I., Sasaki, T. and Takai, Y. (1999) Rab11BP/Rabphilin-11, a downstream target of rab11 small G protein implicated in vesicle recycling. J. Biol. Chem. **274**, 25517–25524

174 Gavin, R. H. (1997) Microtubule-microfilament synergy in the cytoskeleton. Int. Rev. Cytol. **173**, 207–242

175 Kelleher, J. F. and Titus, M. A. (1998) Intracellular motility: how can we all work together? Curr. Biol. **8**, R394–R397

176 DePina, A. S. and Langford, G. M. (1999) Vesicle transport: the role of actin filaments and myosin motors. Microsc. Res. Tech. **47**, 93–106

177 Taunton, J., Rowning, B. A., Coughlin, M. L., Wu, M., Moon, R. T., Mitchison, T. J. and Larabell, C. A. (2000) Actin-dependent propulsion of endosomes and lysosomes by recruitment of N-WASP. J. Cell Biol. **148**, 519–530

178 Taunton, J. (2001) Actin filament nucleation by endosomes, lysosomes and secretory vesicles. Curr. Opin. Cell Biol. **13**, 85–91

179 van Deurs, B., Holm, P. K., Kayser, L. and Sandvig, K. (1995) Delivery to lysosomes in the human carcinoma cell line HEp-2 involves an actin filament-facilitated fusion between mature endosomes and preexisting lysosomes. Eur. J. Cell Biol. **66**, 309–323

180 Raposo, G., Cordonnier, M. N., Tenza, D., Menichi, B., Durrbach, A., Louvard, D. and Coudrier, E. (1999) Association of myosin I alpha with endosomes and lysosomes in mammalian cells. Mol. Biol. Cell **10**, 1477–1494

181 Desjardins, M., Celis, J. E., van Meer, G., Dieplinger, H., Jahraus, A., Griffiths, G. and Huber, L. A. (1994) Molecular characterization of phagosomes. J. Biol. Chem. **269**, 32194–32200

182 Defacque, H., Egeberg, M., Habermann, A., Diakonova, M., Roy, C., Mangeat, P., Voelter, W., Marriott, G., Pfannstiel, J., Faulstich, H. and Griffiths, G. (2000) Involvement of ezrin/moesin in de novo actin assembly on phagosomal membranes. EMBO J. **19**, 199–212

183 Morrissette, N. S., Gold, E. S., Guo, J., Hamerman, J. A., Ozinsky, A., Bedian, V. and Aderem, A. A. (1999) Isolation and characterization of monoclonal antibodies directed against novel components of macrophage phagosomes. J. Cell Sci. **112**, 4705–4713

184 Moller, W., Nemoto, I., Matsuzaki, T., Hofer, T. and Heyder, J. (2000) Magnetic phagosome motion in J774A.1 macrophages: influence of cytoskeletal drugs. Biophys. J. **79**, 720–730

185 Jahraus, A., Egeberg, M., Hinner, B., Habermann, A., Sackman, E., Pralle, A., Faulstich, H., Rybin, V., Defacque, H. and Griffiths, G. (2001) ATP-dependent membrane assembly of F-actin facilitates membrane fusion. Mol. Biol. Cell **12**, 155–170

186 Tilney, L. G., Connelly, P. S. and Portnoy, D. A. (1990) Actin filament nucleation by the bacterial pathogen, Listeria monocytogenes. J. Cell Biol. **111**, 2979–2988

187 Defacque, H., Egeberg, M., Antzberger, A., Ansorge, W., Way, M. and Griffiths, G. (2000) Actin assembly induced by polylysine beads or purified phagosomes: quantitation by a new flow cytometry assay. Cytometry **41**, 46–54

188 Tsukita, S., Oishi, K., Sato, N., Sagara, J. and Kawai, A. (1994) ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. J. Cell Biol. **126**, 391–401

189 Holm, A., Tejle, K., Magnusson, K. E., Descoteaux, A. and Rasmusson, B. (2001) Leishmania donovani lipophosphoglycan causes periphagosomal actin accumulation: correlation with impaired translocation of PKCα and defective phagosome maturation. Cell. Microbiol. **3**, 439–447

190 Vergne, I., Constant, P. and Laneelle, G. (1998) Phagosomal pH determination by dual fluorescence flow cytometry. Anal. Biochem. **255**, 127–132

191 Lukacs, G. L., Rotstein, O. D. and Grinstein, S. (1990) Phagosomal acidification is mediated by a vacuolar-type H(+)-ATPase in murine macrophages. J. Biol. Chem. **265**, 21099–21107

192 Tapper, H. and Sundler, R. (1995) Bafilomycin A1 inhibits lysosomal, phagosomal, and plasma membrane H(+)-ATPase and induces lysosomal enzyme secretion in macrophages. J. Cell. Physiol. **163**, 137–144

193 van Deurs, B., Holm, P. K. and Sandvig, K. (1996) Inhibition of the vacuolar H(+)-ATPase with bafilomycin reduces delivery of internalized molecules from mature multivesicular endosomes to lysosomes in HEp-2 cells. Eur. J. Cell Biol. **69**, 343–350

194 Bayer, N., Schober, D., Prchla, E., Murphy, R. F., Blaas, D. and Fuchs, R. (1998) Effect of bafilomycin A1 and nocodazole on endocytic transport in HeLa cells: implications for viral uncoating and infection. J. Virol. **72**, 9645–9655

195 van Weert, A. W., Dunn, K. W., Gueze, H. J., Maxfield, F. R. and Stoorvogel, W. (1995) Transport from late endosomes to lysosomes, but not sorting of integral membrane proteins in endosomes, depends on the vacuolar proton pump. J. Cell Biol. **130**, 821–834

196 D'Arrigo, A., Bucci, C., Toh, B. H. and Stenmark, H. (1997) Microtubules are involved in bafilomycin A1-induced tubulation and Rab5-dependent vacuolation of early endosomes. Eur. J. Cell Biol. **72**, 95–103

197 Maranda, B., Brown, D., Bourgoin, S., Casanova, J. E., Vinay, P., Ausiello, D. A. and Marshansky, V. (2001) Intra-endosomal pH-sensitive recruitment of the Arf-nucleotide exchange factor ARNO and Arf6 from cytoplasm to proximal tubule endosomes. J. Biol. Chem. **276**, 18540–18550

198 Gu, F. and Gruenberg, J. (2000) ARF1 regulates pH-dependent COP functions in the early endocytic pathway. J. Biol. Chem. **275**, 8154–8160

199 Russell, D. G. (2001) Mycobacterium tuberculosis: here today, and here tomorrow. Nat. Rev. Mol. Cell Biol. **2**, 569–577

200 McNeil, P. L., Tanasugarn, L., Meigs, J. B. and Taylor, D. L. (1983) Acidification of phagosomes is initiated before lysosomal enzyme activity is detected. J. Cell Biol. **97**, 692–702

201 Hart, P. D. and Young, M. R. (1991) Ammonium chloride, an inhibitor of phagosome-lysosome fusion in macrophages, concurrently induces phagosome-endosome fusion, and opens a novel pathway: studies of a pathogenic mycobacterium and a nonpathogenic yeast. J. Exp. Med. **174**, 881–889

202 Gordon, A. H., Hart, P. D. and Young, M. R. (1980) Ammonia inhibits phagosome-lysosome fusion in macrophages. Nature (London) **286**, 79–80

203 Kielian, M. C., Steinman, R. M. and Cohn, Z. A. (1982) Intralysosomal accumulation of polyanions. I. Fusion of pinocytic and phagocytic vacuoles with secondary lysosomes. J. Cell Biol. **93**, 866–874

204 Brumell, J. H., Volchuk, A., Sengelov, H., Borregaard, N., Cieutat, A. M., Bainton, D. F., Grinstein, S. and Klip, A. (1995) Subcellular distribution of docking/fusion proteins in neutrophils, secretory cells with multiple exocytic compartments. J. Immunol. **155**, 5750–5759

205 Kim-Park, W. K., Moore, M. A., Hakki, Z. W. and Kowolik, M. J. (1997) Activation of the neutrophil respiratory burst requires both intracellular and extracellular calcium. Ann. N.Y. Acad. Sci. **832**, 394–404

206 Mandeville, J. T. and Maxfield, F. R. (1996) Calcium and signal transduction in granulocytes. Curr. Opin. Hematol. **3**, 63–70

207 Stendahl, O., Krause, K. H., Krischer, J., Jerstrom, P., Theler, J. M., Clark, R. A., Carpentier, J. L. and Lew, D. P. (1994) Redistribution of intracellular Ca^{2+} stores during phagocytosis in human neutrophils. Science **265**, 1439–1441

208 Malik, Z. A., Denning, G. M. and Kusner, D. J. (2000) Inhibition of Ca^{2+} signaling by Mycobacterium tuberculosis is associated with reduced phagosome-lysosome fusion and increased survival within human macrophages. J. Exp. Med. **191**, 287–302

209 Mills, I. G., Urbe, S. and Clague, M. J. (2001) Relationships between EEA1 binding partners and their role in endosome fusion. J. Cell Sci. **114**, 1959–1965

210 Holroyd, C., Kistner, U., Annaert, W. and Jahn, R. (1999) Fusion of endosomes involved in synaptic vesicle recycling. Mol. Biol. Cell **10**, 3035–3044

211 Peters, C. and Mayer, A. (1998) Ca^{2+}/calmodulin signals the completion of docking and triggers a late step of vacuole fusion. Nature (London) **396**, 575–580

212 Lundqvist-Gustafsson, H., Gustafsson, M. and Dahlgren, C. (2000) Dynamic Ca^{2+} changes in neutrophil phagosomes. A source for intracellular Ca^{2+} during phagolysosome formation? Cell Calcium **27**, 353–362

213 Colombo, M. I., Beron, W. and Stahl, P. D. (1997) Calmodulin regulates endosome fusion. J. Biol. Chem. **272**, 7707–7712

214 Wickner, W. and Haas, A. (2000) Yeast homotypic vacuole fusion: a window on organelle trafficking mechanisms. Annu. Rev. Biochem. **69**, 247–275

215 Zimmerli, S., Majeed, M., Gustavsson, M., Stendahl, O., Sanan, D. A. and Ernst, J. D. (1996) Phagosome-lysosome fusion is a calcium-independent event in macrophages. J. Cell Biol. **132**, 49–61

216 Jaconi, M. E., Lew, D. P., Carpentier, J. L., Magnusson, K. E., Sjogren, M. and Stendahl, O. (1990) Cytosolic free calcium elevation mediates the phagosome-lysosome fusion during phagocytosis in human neutrophils. J. Cell Biol. **110**, 1555–1564

217 Downey, G. P., Botelho, R. J., Butler, J. R., Moltyaner, Y., Chien, P., Schreiber, A. D. and Grinstein, S. (1999) Phagosomal maturation, acidification, and inhibition of bacterial growth in nonphagocytic cells transfected with FcγRIIA receptors. J. Biol. Chem. **274**, 28436–28444

218 Wilsson, A., Lundqvist, H., Gustafsson, M. and Stendahl, O. (1996) Killing of phagocytosed Staphylococcus aureus by human neutrophils requires intracellular free calcium. J. Leukocyte Biol. **59**, 902–907

219 Bengtsson, T., Jaconi, M. E., Gustafson, M., Magnusson, K. E., Theler, J. M., Lew, D. P. and Stendahl, O. (1993) Actin dynamics in human neutrophils during adhesion and phagocytosis is controlled by changes in intracellular free calcium. Eur. J. Cell Biol. **62**, 49–58

220 Ferrari, G., Langen, H., Naito, M. and Pieters, J. (1999) A coat protein on phagosomes involved in the intracellular survival of mycobacteria. Cell **97**, 435–447

221 Greenberg, S., el Khoury, J., di Virgilio, F., Kaplan, E. M. and Silverstein, S. C. (1991) Ca^{2+}-independent F-actin assembly and disassembly during Fc receptor-mediated phagocytosis in mouse macrophages. J. Cell Biol. **113**, 757–767

222 Ernst, J. D. (1991) Annexin III translocates to the periphagosomal region when neutrophils ingest opsonized yeast. J. Immunol. **146**, 3110–3114

223 Majeed, M., Perskvist, N., Ernst, J. D., Orselius, K. and Stendahl, O. (1998) Roles of calcium and annexins in phagocytosis and elimination of an attenuated strain of Mycobacterium tuberculosis in human neutrophils. Microb. Pathog. **24**, 309–320

224 Malik, Z. A., Iyer, S. S. and Kusner, D. J. (2001) Mycobacterium tuberculosis phagosomes exhibit altered calmodulin-dependent signal transduction: contribution to inhibition of phagosome-lysosome fusion and intracellular survival in human macrophages. J. Immunol. **166**, 3392–3401

225 Yi, J. and Tang, X. M. (1999) The convergent point of the endocytic and autophagic pathways in leydig cells. Cell Res. **9**, 243–253

226 Yokota, S. (1993) Formation of autophagosomes during degradation of excess peroxisomes induced by administration of dioctyl phthalate. Eur. J. Cell Biol. **61**, 67–80

227 Ueno, T., Muno, D. and Kominami, E. (1991) Membrane markers of endoplasmic reticulum preserved in autophagic vacuolar membranes isolated from leupeptin-administered rat liver. J. Biol. Chem. **266**, 18995–18999

228 Lord, J. M., Davey, J., Frigerio, L. and Roberts, L. M. (2000) Endoplasmic reticulum-associated protein degradation. Semin. Cell Dev. Biol. **11**, 159–164

229 Ishiguro, T., Nakajima, M., Naito, M., Muto, T. and Tsuruo, T. (1996) Identification of genes differentially expressed in B16 murine melanoma sublines with different metastatic potentials. Cancer Res. **56**, 875–879

230 Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M. D., Klionsky, D. J., Ohsumi, M. and Ohsumi, Y. (1998) A protein conjugation system essential for autophagy. Nature (London) **395**, 395–398

231 Mizushima, N., Yamamoto, A., Hatano, M., Kobayashi, Y., Kabeya, Y., Suzuki, K., Tokuhisa, T., Ohsumi, Y. and Yoshimori, T. (2001) Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. J. Cell Biol. **152**, 657–668

232 Ohsumi, Y. (2001) Molecular dissection of autophagy: two ubiquitin-like systems. Nat. Rev. Mol. Cell Biol. **2**, 211–216

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