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# Distinct roles of class I and class III phosphatidylinositol 3-kinases in phagosome formation and maturation

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Phagosomes acquire their microbicidal properties by fusion with lysosomes. Products of phosphatidylinositol 3-kinase (PI 3-kinase) are required for phagosome formation, but their role in maturation is unknown. Using chimeric fluorescent proteins encoding tandem FYVE domains, we found that phosphatidylinositol 3-phosphate (PI[3]P) accumulates greatly but transiently on the phagosomal membrane. Unlike the 3′-phosphoinositides generated by class I PI 3-kinases which are evident in the nascent phagosomal cup, PI(3)P is only detectable after the phagosome has sealed. The class III PI 3-kinase VPS34 was found to be responsible for PI(3)P synthesis and essential for phagolysosome formation. In contrast, selective ablation of class I PI 3-kinase revealed that optimal phagocytosis, but not maturation, requires this type of enzyme. These results highlight the differential functional role of the two families of kinases, and raise the possibility that PI(3)P production by VPS34 may be targeted during the maturation arrest induced by some intracellular parasites.

## Introduction

Ingestion of invading microorganisms by phagocytosis is an essential component of the innate immune response that involves extensive cytoskeletal rearrangement and membrane remodelling (Aderem and Underhill, 1999; Greenberg, 2001). The resulting vacuole or phagosome then undergoes maturation, which involves sequential interactions with components of the endocytic pathway, and culminates with fusion to lysosomes (Tjelle et al., 2000). The highly acidic, oxidative, and protease-rich nature of the phagosomal lumen is instrumental in microbial elimination. However, some microorganisms have developed the ability to arrest phagosomal maturation, thereby averting killing and often becoming intracellular parasites. Despite the importance of phagosomal maturation in microbial killing, little is known about its molecular determinants.

Products of phosphatidylinositol 3-kinases (PI 3-kinases)* are thought to play a role in the traffic of membranes along the endocytic pathway. In yeast, the PI 3-kinase Vps34p, responsible for the generation of phosphatidylinositol 3-phosphate (PI[3]P), is essential for efficient sorting and delivery of proteins to the vacuole, the yeast equivalent of lysosomes (Wurmser et al., 1999). This is thought to involve recognition of the headgroups of the phosphoinositide by proteins containing FYVE domains, which are zinc finger structures recently found to bind with considerable affinity and specificity to PI(3)P (Gaullier et al., 1998; Patki et al., 1998).

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*Abbreviations used in this paper: DIC, differential interference contrast; GFP, green fluorescent protein; PI 3-kinase, phosphatidylinositol 3-kinase; PI(3)P, phosphatidylinositol 3-phosphate; RBC, red blood cells.

Like the yeast vacuole, phagosomes interact with components of the endocytic pathway during the course of their maturation; therefore, we considered the possible involvement of PI(3)P in this process. PI 3-kinase activity is known to be required for effective phagocytosis (Araki et al., 1996; Cox et al., 1999), but its role in maturation has not been investigated. Multiple isoforms of PI 3-kinase have been described in mammalian cells (Toker and Cantley, 1997), but their individual roles in the phagocytic sequence are unknown. To investigate whether the PI 3-kinase that mediates phagosomal formation is also responsible for maturation, we used the targetted ablation of genes encoding specific PI 3-kinase subunits and microinjection of isoform-specific inhibitory antibodies.

## Results and discussion

To examine the role of PI(3)P in phagosome maturation, we transfected RAW macrophages with chimeric constructs of green fluorescent protein (GFP) and the FYVE domain of EEA1. As described by others (Gillooly et al., 2000; Lawe et al., 2000), we found that the affinity of single FYVE domains is insufficient for their use as probes in vivo, but that constructs linking two or more FYVE domains in tandem bind effectively to early endosomes, which are a rich source of PI(3)P. The specificity of the interaction was verified using wortmannin, a potent inhibitor of PI 3-kinases (unpublished data). Because transferrin uptake and recycling as well as phagocytosis were unimpaired in cells expressing two tandem copies of FYVE fused to GFP (2FYVE–GFP; unpublished data), we concluded that 2FYVE–GFP was a suitable, noninvasive probe of PI(3)P distribution in macrophages.

As shown in Fig. 1, A–G, a striking accumulation of PI(3)P was observed on the membrane of phagosomes generated in RAW cells by engagement of Fcγ receptors. The phosphoinositide was only detectable after phagosomal sealing had been completed, i.e., accumulation of the 2FYVE–GFP was never detected in the cups lining nascent phagosomes. When cells were fixed and stained using phalloidin, F-actin dissociation from the phagosome was found to precede 2FYVE–GFP accumulation (unpublished data), which lasted 7–10 min. The ensuing disappearance of PI(3)P often coincided with the centripetal displacement of the phagosomes toward the nucleus.

The net accumulation of 2FYVE–GFP on the phagosomal membranes appeared to exceed the amount of the probe bound to endosomes before the cells were challenged with opsonized particles (unpublished data). This suggested that at least part of the PI(3)P appearing on the phagosome may be synthesized de novo during particle ingestion. To confirm this notion, cells were labeled metabolically using [$^3$H]-myo-inositol and their phosphatidylinositol content was compared before and after phagocytosis using HPLC. As illustrated in Fig. 1 H, the PI(3)P content of macrophages increased by 50.4 ± 6.6% 12 min after initiation of phagocytosis of IgG-opsonized beads. This increase is not only significant ($P < 0.02$), but likely underestimates the effect of phagosome formation on PI(3)P content inasmuch as phagocytosis does not occur synchronously in all the cells. To our knowledge, this is the first visualization of dynamic changes of content and/or distribution of PI(3)P, which was heretofore thought to be a constitutive and invariant component of early endosomes (Stenmark and Gillooly, 2001).

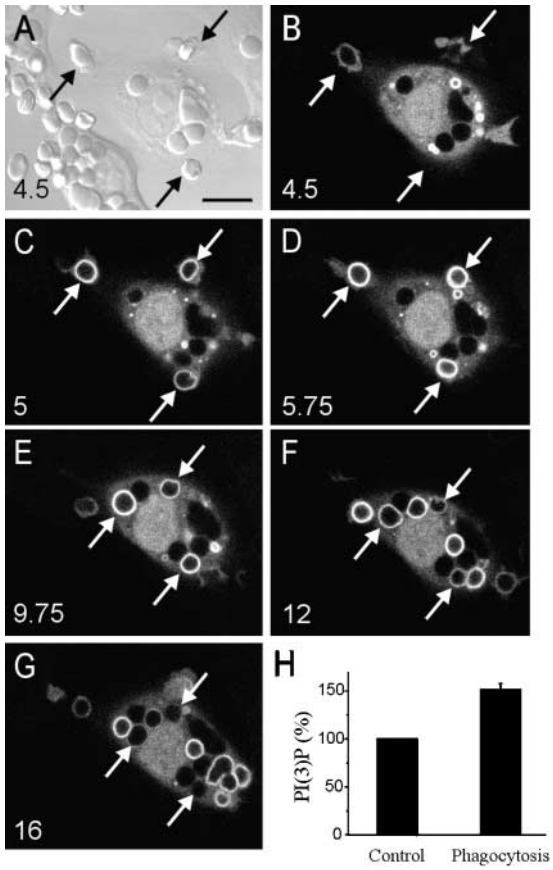


Figure 1. **Distribution and quantification of PI(3)P during phagocytosis.** (A–G) Phagocytosis of IgG-opsonized RBC by RAW macrophages expressing 2FYVE–GFP. The time elapsed after addition of the RBCs is indicated (in min). Arrows point to phagosomes formed in the 4–5-min interval. DIC image (A). Bar, 10 μm. (H) HPLC quantification of PI(3)P in RAW cells before (control) and 12 min after phagocytosis of IgG-opsonized 3-μm latex beads. The PI(3)P data were normalized to the counts of the phosphatidylinositol peak and are expressed as percent of the untreated control, to facilitate comparison between experiments. The data are means ± SE of three determinations.

In principle, the functional significance of the accumulation of PI(3)P in phagosomes could be assessed using wortmannin, which blocks the activity of most PI 3-kinases (Yano et al., 1993). However, blockade of class I PI 3-kinases was shown earlier to inhibit the phagocytosis of opsonized red blood cells (RBCs) such as those used in Fig. 1, A–G. To circumvent this problem, we took advantage of the observation made by Greenberg and colleagues (Cox et al., 1999) that the inhibitory effects of wortmannin on phagocytosis are directly proportional to the size of the target particle: although the uptake of large particles such as RBCs is virtually eliminated, particles ≤3 μm in diameter are internalized at significant rates. As shown in Fig. 2, considerable uptake of IgG-opsonized 3-μm latex beads was observed in RAW macrophages despite pretreatment with wortmannin and the somewhat diminished phagocytic efficiency (40% of control after 30 min phagocytosis). More importantly,

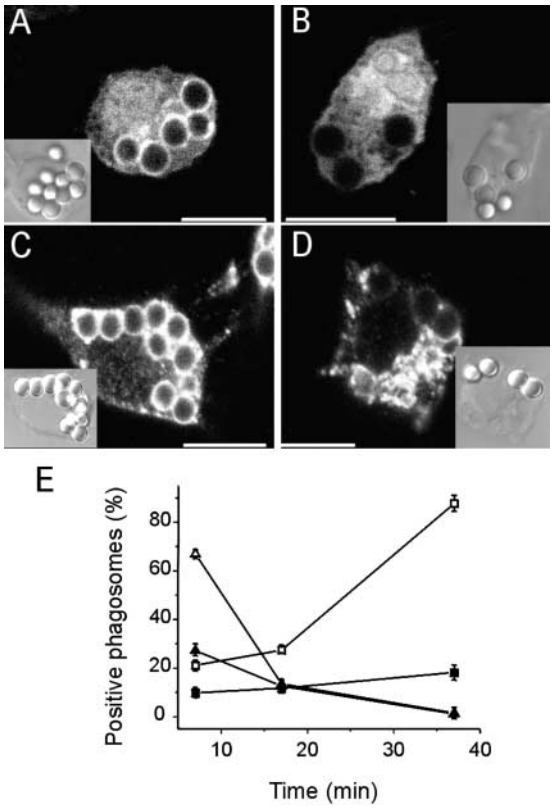


Figure 2. **Effects of wortmannin on phagosomal acquisition of PI(3)P, EEA1, and LAMP-1.** RAW cells were either left untreated (A, C, and open symbols in E) or pretreated with 100 nM wortmannin for 30 min (B, D, and solid symbols in E), before exposure to IgG-opsonized latex beads (3 μm diam). In A and B, the cells were transfected with 2FYVE–GFP and allowed to internalize beads for 7 min before fixation. In C and D, phagocytosis was allowed to occur for 7 min, followed by an additional 30 min after washing unbound beads. Subsequently, cells were immunostained for LAMP-1. Confocal fluorescence microscopy is shown in the main panels and DIC in the insets. Bars, 10 μm. (E) Quantification of the effect of wortmannin on the fraction of phagosomes containing EEA1 (triangles) or LAMP-1 (squares). Cells were pretreated with or without wortmannin and allowed to internalize beads for 7 min, as above. After washing unbound beads, incubation at 37°C proceeded for the indicated times, followed by immunostaining with antibodies to EEA1 or LAMP-1. Data are means ± SE of five experiments, each with at least 100 phagosomes counted.

whereas 2FYVE–GFP clearly accumulates around the resulting phagosomes in control cells (Fig. 2 A), no accumulation was detectable following treatment with wortmannin (Fig. 2 B). Thus, the use of the PI 3-kinase antagonist in combination with small latex beads enabled us to analyze the role of PI(3)P in phagosomal maturation.

Acquisition of the early endosomal marker EEA1 has been reported to occur during the early stages of phagosomal maturation (Duclos et al., 2000). This stage is transient and is followed by the incorporation of late endosomal and lysosomal proteins, including the lysosomal-associated membrane protein 1 (LAMP-1). A similar sequence was observed in the case of the IgG-coated 3-μm latex beads, as illustrated in Fig. 2, C and E. Pretreatment of the cells with wortmannin depressed, but did not eliminate, the ability of EEA1 to associate with phagosomes (Fig. 2 E). This suggests that although interaction with PI(3)P contributes to the association of EEA1 with the phagosomal membrane, it is not stringently required. The residual binding of EEA1 is likely mediated by attachment to Rab5, which is recruited to the phagosome despite inhibition of PI 3-kinase (unpublished data). Strikingly, the incorporation of LAMP-1 into phagosomes was affected even more profoundly by wortmannin (Fig. 2, D and E), implying that a product of PI 3-kinase is essential for progression to phagolysosomes.

Although blockade of phagosomal maturation is associated with impairment of PI(3)P formation, other 3′-phosphoinositides may be responsible for this effect. Indeed, we found that the PH domain of Akt, which binds avidly to both phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate, is rapidly recruited to the phagocytic cup and remains on the phagosomal membrane for some time after sealing (Marshall et al., 2001). The generation of these lipids, which are synthesized by class I PI 3-kinases (Toker and Cantley, 1997), is also eliminated by pretreatment of the cells with wortmannin. Thus, because of their broad spectrum of action, wortmannin and other available PI 3-kinase inhibitors are not suitable to define the type of phosphoinositide involved in maturation or the kinase responsible for its synthesis.

As an alternative approach, we used mouse embryonic fibroblasts in which both the α and β isoforms of the p85 subunit of the class I PI 3-kinase were ablated by gene targeting. The generation of the $p85\alpha^{-/-}$ and $p85\beta^{-/-}$ embryonic fibroblasts will be described in detail elsewhere. Macrophages from double knockout mice are not available and only fibroblasts can be obtained. To study phagosomal maturation in such fibroblasts, we resorted to heterologous transfection of Fcγ receptors. It was shown earlier that expression of Fcγ receptors on the surface of nonphagocytic cells confers onto them the ability to internalize IgG-coated particles (Indik et al., 1995). Moreover, the resulting phagosomes proceed to mature in a manner that is indistinguishable from that of professional phagocytes (Downey et al., 1999). Wild-type murine fibroblasts transiently transfected with FcγRIIA receptors effectively internalized opsonized latex beads as well as RBCs (Fig. 3). As reported earlier for macrophages, phagocytosis in these cells was accompanied by formation of 3′-polyphosphoinositides, which are thought to be the product of class I PI 3-kinases. This was demonstrated by the localized accumulation of a chimeric construct of the PH domain of Akt tagged with GFP (Fig. 3 A). The chimera accumulated greatly in the membrane of the nascent phagosome and the accumulation dissipated shortly after phagosome sealing. The distribution of PI(3)P in these cells was also monitored, using cotransfection of 2FYVE–GFP with FcγRIIA receptors. As illustrated in Fig. 3, C, E, and G, the maturation sequence observed in macrophages was recapitulated in the fibroblasts that expressed FcγRIIA. Not only was PI(3)P formed at the early stages of maturation, but EEA1 was recruited to the phagosomes, followed by acquisition of LAMP-1.

To evaluate the role of class I PI 3-kinases, we tested cells lacking the α and β isoforms of p85 that had been transfected with FcγRIIA receptors as above. Kinase activity determinations in immunoprecipitates of PDGF-stimulated

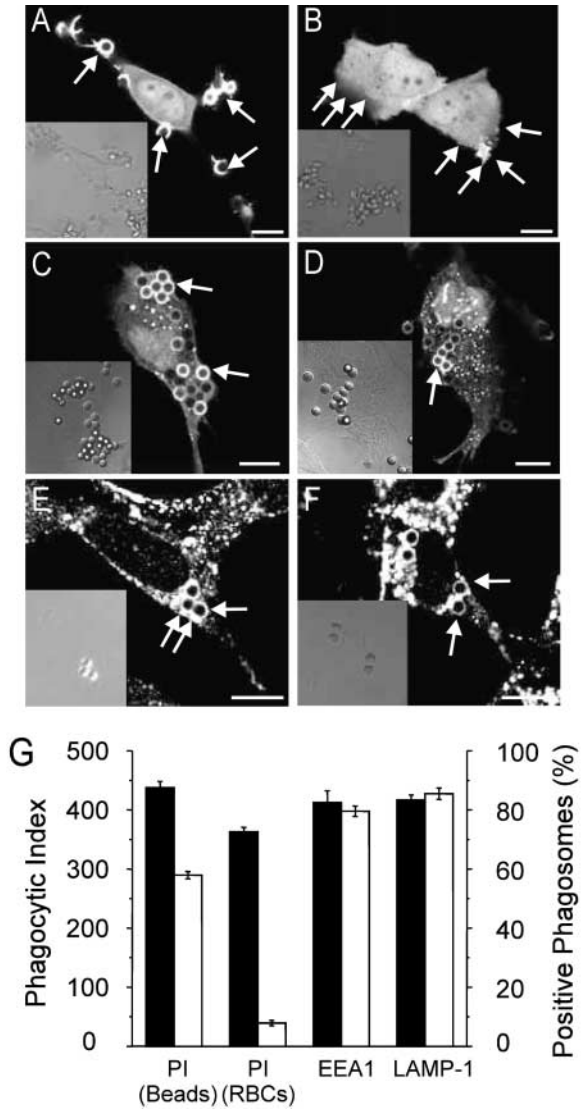


Figure 3. **Phagosomal maturation in wild-type and type I PI 3-kinase–deficient fibroblasts.** Fibroblasts were obtained from wild-type (A, C, E, and solid bars in G) or from double knockout mice lacking the α and β isoforms of the p85 subunit of type I PI 3-Kinase (B, D, F, and open bars in G). These cells were transfected with epitope-tagged FcγRIIA to confer phagocytic capacity, and then exposed to IgG-opsonized RBC (A, B, and second set of columns in G) or latex beads (C–F and leftmost bars in G). In A and B the cells were cotransfected with Akt-PH-GFP to monitor 3′-polyphosphoinositides and were allowed to internalize RBC for 6 min. Arrows indicate sites of attachment of RBCs. (C and D) Cells were cotransfected with 2FYVE–GFP to monitor PI(3)P distribution and were allowed to internalize beads for 25 min. (E and F) Cells were allowed to internalize beads for 20 min followed by a 60-min incubation after removing unbound beads. Cells were then fixed and immunostained for LAMP-1. (C–F) Arrows point to phagosomes. Bars, 10 μm. (G) Quantification of the phagocytic index using beads (PI, Beads) or RBC (PI, RBCs) (left axis, in particles/100 cells) and of the fraction of latex bead phagosomes containing EEA1 or LAMP-1 (right axis, as percent of total phagosomes). EEA1 and LAMP-1 were quantified 25 min and 80 min after phagocytosis, respectively, as described. Data are means ± SE of five experiments, each with at least 100 phagosomes counted.

cells were performed as described by Carpenter et al. (1990), and confirmed that the class Ia PI 3-kinase activity in these cells was reduced by ~90% (unpublished data). Accordingly, the rapid accumulation of 3′-polyphosphoinositides observed in wild-type cells was markedly reduced and delayed in the p85-deficient cells (Fig. 3 B). However, it is noteworthy, that the accumulation of the Akt-PH domain chimera was not entirely eliminated, and that substantial accumulation could be detected in some phagosomes after ≥20 min. This residual PI 3-kinase activity may reflect the function of the p55γ subunit, which is expressed in these cells (unpublished data).

In accordance with the results obtained in wortmannin-treated macrophages and consistent with their reduction in 3′-polyphosphoinositide synthesis, cells lacking the α and β isoforms of p85 internalized RBCs very poorly but were able to take up substantial numbers of small latex beads (Fig. 3, D and G). The inhibition of phagocytosis of beads was greater at early times (52 ± 3.7% after 20 min, $n = 4$) than at later ones (37 ± 3.2% after 60 min, $n = 4$), likely reflecting the progressive accumulation of 3′-polyphosphoinositides due to residual PI 3-kinase activity. Accordingly, 32% of the phagocytosis observed in p85-deficient cells was inhibitable by wortmannin. Jointly, these observations confirm that the effects of wortmannin on phagocytic efficiency are attributable to the inhibition of class I PI 3-kinase.

Importantly, in cells that engulfed latex beads the accumulation of 2FYVE–GFP was unabated. Similarly, EEA1 was recruited normally and LAMP-1 was acquired by >80% of the phagosomes as in wild-type cells (Fig. 3, F and G). Together, these experiments suggest that although essential for the phagocytosis of large particles, the class IA PI 3-kinases are not required for PI(3)P formation or phagosomal maturation.

VPS34, the mammalian homologue of the yeast Vps34p, is thought to be responsible for the synthesis of at least part of the endosomal PI(3)P (Siddhanta et al., 1998). We next tested if this enzyme is detectable on the membrane of phagosomes and whether it is involved in phagosomal PI(3)P synthesis. Available antibodies were unable to detect the endogenous levels of VPS34 in either professional or engineered phagocytes (unpublished data). Therefore, we increased the expression levels of the enzyme by transfection with wild-type rat VPS34 (Row et al., 2001). As shown in Fig. 4 A, VPS34 associated with phagosomes during periods of PI(3)P accumulation, revealed by cotransfection with 2FYVE–GFP.

Next, the functional involvement of VPS34 was probed using microinjection of inhibitory antibodies (Siddhanta et al., 1998). CHO cells stably transfected with FcγRIIA were used for these experiments, since native phagocytes are refractory to microinjection. Following microinjection, 2FYVE–GFP was released from endosomes into the cytosol (unpublished data), indicating disappearance of PI(3)P and verifying the effectiveness of the antibody. More importantly, the anti-VPS34 antibodies virtually eliminated the accumulation of 2FYVE–GFP on the phagosomal membranes (Fig. 4 D), whereas an equivalent amount of nonimmune antibody was ineffective (Fig. 4 C). In contrast, the anti-VPS34 antibodies had no discernible effect on the phagocytic efficiency of the cells, confirming that class I but not class III kinases are required for phagosome formation.

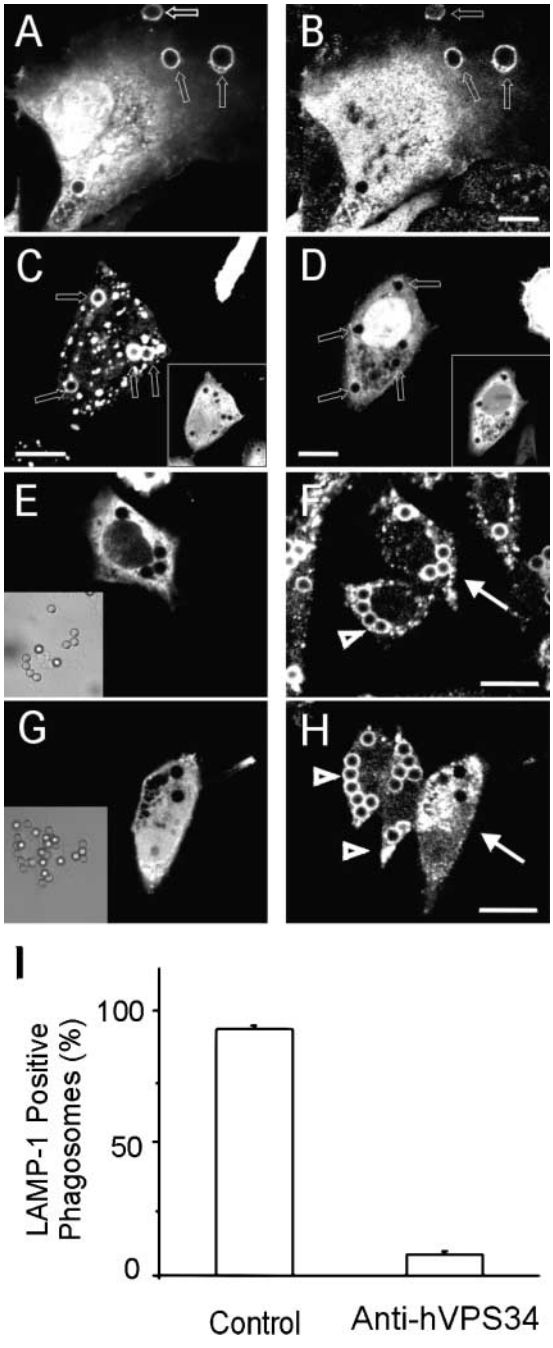


Figure 4. **Recruitment and role of VPS34 in phago–lysosome fusion.** (A and B) Distribution of 2FYVE–GFP and VPS34 during phagocytosis. COS-7 cells expressing FcγRIIA were cotransfected with 2FYVE–GFP and VPS34. After exposure to opsonized particles for 15 min, the cells were fixed and 2FYVE–GFP (A) and VPS34 (B) were visualized directly or by immunostaining, respectively. (C and D) Effect of anti-VPS34 antibodies on phagosomal distribution of 2FYVE–GFP. CHO cells transfected with 2FYVE–GFP were injected with nonimmune rabbit IgG (C) or with anti-hVPS34 antibody (D). After a 2-h period, the cells were allowed to internalize beads for 10 min. Arrows point to phagosomes. The insets identify the microinjected cells, stained with Cy3-labeled anti–rabbit IgG antibodies. (E–I) CHO cells expressing FcγRIIA were injected with either nonimmune rabbit IgG (E and F) or with anti-hVPS34 antibody (G and H). Phagocytosis of opsonized 3-μm latex beads was allowed to proceed for 20 min, followed by 50 min of maturation after removal of unbound beads, and ultimately stained for LAMP-1. (E and G) Identification of microinjected cells by staining with labeled anti-rabbit IgG. F and H: LAMP-1 staining of the cells shown in E and G, respectively. Full white arrows and open arrowheads point to injected and uninjected cells, respectively. Bars, 10 mm. (I) Quantification of phagosome acquisition of LAMP-1 in cells injected with nonimmune rabbit IgG (control) or with anti-hVPS34 antibody. Data are means ± SE of four separate experiments, each with at least 100 injected cells.

Next, we tested whether VPS34 activity is needed for phagolysosome formation. Cells were injected with anti-VPS34 or with nonimmune antibody, induced to ingest particles, and incubated for a further 50 min to allow for maturation, which was assessed by the acquisition of LAMP-1. Typical results are illustrated in Fig. 4, E–H, and the collected results are summarized in panel I. In brief, inhibition of VPS34 largely precluded LAMP-1 acquisition, whereas the control antibody had no effect. Although this manuscript was under review, a separate report appeared analyzing the role of VPS34 in phagosomal maturation. Fratti and colleagues (2001) found that inhibition of the class III PI 3-kinase markedly inhibited the acquisition of the late endosomal marker lysobisphosphatidic acid by phagosomes. Their data, as well as ours, are consistent with the notion that synthesis of PI(3)P by VPS34 is an essential component of phagosomal maturation.

By analogy with endosomes, the initial steps in phagosomal maturation have been proposed to require Rab5 (Duclos et al., 2000; Roberts et al., 2000). In the case of endosomes, Rab5 was reported to interact directly with p85α/p110β and with VPS34. Interaction with p85α/p110β was described to occur mainly on clathrin-coated vesicles, leading to the suggestion that the class I kinase may be a downstream effector of Rab5 in the heterotypic fusion of the vesicles with endosomes (Christoforidis et al., 1999). Our results suggest that, at least in the case of phagosomes, the fusion events that lead to maturation are independent of p85α or β. In contrast, functional VPS34 appears to be strictly required for successful progression of early phagosomes to phagolysosomes. The recruitment of VPS34 is associated with the transient accumulation of PI(3)P on the phagosomal membrane and with a net increase in total cellular PI(3)P. To date, this lipid was regarded as an invariant constitutive component of endosomes, and no evidence existed of regulation of the activity of VPS34. Our findings suggest that recruitment of VPS34, stimulation of its activity, and/or increased availability of its substrate, phosphatidylinositol, contribute to the accumulation of PI(3)P on the phagosomal membrane. Although delivery of preformed PI(3)P by fusion with endosomes cannot be excluded, this mechanism would not account for the observed net increase in the content of the phosphoinositide. VPS34 associates with p150, a Vps15-like serine/threonine kinase that is thought to regulate the membrane association and activity of the lipid kinase. Stimulation of FcγRIIA may attract p150 to the phagosomal membrane, perhaps via Rab5.

In summary, our results reveal distinct roles of class I and III PI 3-kinases in the phagocytic process. Class I kinases are required for phagocytosis of large particles, but are not involved in the maturation of phagosomes. Conversely, the class III PI 3-kinase (VPS34) is not essential for engulfment of particles, but appears to direct the fusion of formed pha-

gosomes with late endosomes/lysosomes. It is important to note that whereas the p85 subunit of class I PI 3-kinase was thought to couple the Fc receptor complex with the catalytic p110 subunit, 3′-phosphoinositide synthesis was nevertheless detectable in stimulated cells lacking both p85α and β. This implies that other PI 3-kinase subunits are active and capable of initiating phagocytosis, as a fraction of the uptake of opsonized beads in the p85 knockout cells was inhibited by wortmannin. Another member of the class Ia PI 3-kinases, p55γ, may be responsible for this effect, but class Ib or II kinases cannot be ruled out.

Like phagocytosis, macroautophagy is also a PI 3-kinase–dependent process, suggesting similarities between these two phenomena. However, unlike phagocytosis, the onset of macroautophagy depends on class III PI 3-kinase, but is in fact inhibited by class I enzymes (Petiot et al., 2000). Thus, phagocytosis and macroautophagy utilize different signaling pathways.

The mechanism whereby products of VPS34 facilitate phagolysosome formation remains to be elucidated. In the case of endosomes, docking and fusion are thought to be facilitated by recruitment of a multimeric complex that includes EEA1, Rab5, Rabaptin5/Rabex-5 and, NSF (McBride et al., 1999). Optimal association of EEA1 to the endosome requires PI(3)P, which accounts for the sensitivity of the process to inhibitors of PI 3-kinases. By analogy, EEA1 may mediate the maturation of phagosomes and it was recently reported that microinjection of antibodies to EEA1 arrested maturation (Fratti et al., 2001). However, in our hands a dominant–negative form of EEA1 failed to arrest phagosomal maturation, suggesting that other PI(3)P binding proteins, likely containing FYVE or PX domains, may be involved.

Despite internalization into phagosomes, a variety of pathogens avoid killing by thwarting the maturation process, often becoming intracellular parasites (Tjelle et al., 2000). Some of these, like *Mycobacterium* species, arrest maturation at an early stage, characterized by the abundance of Rab5 and the continued ability to exchange with recycling endosomes (Deretic and Fratti, 1999). It is conceivable that alterations in the activity of VPS34 or the availability of PI(3)P on the phagosomal surface are part of the abnormal maturation induced by the microorganisms (Fratti et al., 2001). If this proves to be the case, p150/VPS34 may be a suitable target for the elimination of such intracellular parasites.

## Materials and methods

### Reagents, antibodies, and DNA constructs

Rabbit and human IgG and wortmannin were from Sigma-Aldrich. Latex beads were from Bangs Laboratories. $^{3}$H-myo-inositol was from Amersham. Sheep RBCs and rabbit anti–sheep RBC antibody were from ICN Biomedicals. Goat anti-EEA1 antibody (N19) was from Santa Cruz Biotechnology. Antibodies to hamster and mouse LAMP-1 were from the Developmental Studies Hybridoma Bank, maintained by the University of Iowa (Iowa City, Iowa) and the Johns Hopkins University (Baltimore, MD). Fluorochrome-conjugated secondary antibodies were from Molecular Probes and Jackson ImmunoResearch Laboratories. The affinity-purified rabbit anti-hVPS34 antibody used in microinjection was described in Siddhanta et al. (1998). The preparation of rabbit anti-Vps34 serum was described previously (Row et al., 2001). Fusion proteins of single or two tandem copies of FYVE and GFP are encoded by pEGFP::FYVE–GFP and pEGFP::2FYVE–GFP, respectively. Wild-type rat VPS34 is encoded by pcDNA3::Vps34 (Row et al., 2001). pcDNA3::mycFcγRIIA and pEGFP::FcγRIIA–GFP encode myc-tagged human FcγRIIA and its GFP chimera, respectively.

### Cell culture and transient transfection

COS and CHO cells stably expressing human FcγRIIA and RAW264.7 macrophages were maintained in α-MEM (Wisent Inc.) supplemented with 10% FBS. Mouse embryonic fibroblasts were derived from embryos of p85α$^{-/-}$ p85β$^{-/-}$ and wild-type mice (129× C57BL/6). The generation of the p85β$^{-/-}$ mice and establishment of the embryonic fibroblasts from crosses with p85α$^{+/-}$ mice will be described in detail elsewhere (unpublished data). In brief, the embryos were transferred onto gelatinized tissue culture dishes and the mouse embryonic fibroblasts were immortalized after a few passages using SV40 large T antigen expressed by a retroviral vector. The genotypes of the cells were determined by polymerase chain reaction analysis. All transfections were done with FuGENE 6 according to the manufacturer's instructions (Roche).

### Phagocytic assays, immunofluorescence, confocal microscopy and microinjection

Sheep RBCs and latex beads, respectively, were opsonized with a 1:50 dilution of anti–sheep RBC antibodies and 1 mg/mL human IgG for >1 h. Phagocytosis was initiated by adding the phagocytic targets to cells and allowed to proceed for the indicated times, after which unbound particles were washed and phagosomes allowed to mature for the indicated periods. After fixation, external particles were labeled with Cy5-conjugated anti–human antibody before permeabilization. Antibodies to EEA1, LAMP-1, and Vps34 were employed at a dilution of 1:50, 1:2, and 1:50, respectively, followed by the corresponding secondary antibodies. Phagosome acquisition of EEA1 or LAMP-1 was assessed by differential interference contrast and confocal microscopy. Live phagocytosis was carried out in a Leiden chamber at 37°C and observed with an LSM 510 laser scanning confocal microscope (ZEISS) equipped with a 100× objective. The distribution of phosphatidylinositol 3,4,5 trisphosphate was monitored using the PH domain of Akt as described (Marshall et al., 2001). Microinjection of nonimmune rabbit or anti-VPS34 antibodies was accomplished as described previously (Siddhanta et al., 1998).

### [$^{3}$H]-myo-inositol labeling and HPLC

Phosphoinositides were metabolically labeled by incubation with [$^{3}$H] myo-inositol for 48 h. After phagocytosis, total cellular lipids were extracted, deacylated, and analyzed by HPLC as described (Serunian et al., 1991).

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