# Recruitment of OCRL and Inpp5B to phagosomes by Rab5 and APPL1 depletes phosphoinositides and attenuates Akt signaling

Michal Bohdanowicz[a], Daniel M. Balkin[b], Pietro De Camilli[b], and Sergio Grinstein[a]
[a]Division of Cell Biology, Hospital for Sick Children, Toronto, ON M5G 1X8, Canada; [b]Department of Cell Biology, Howard Hughes Medical Institute, and Program in Cellular Neuroscience, Neurodegeneration and Repair, Yale University School of Medicine, New Haven, CT 06510

**ABSTRACT** **Sealing of phagosomes is accompanied by the disappearance of phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)$P_2$) from their cytoplasmic leaflet. Elimination of PtdIns(4,5)$P_2$, which is required for actin remodeling during phagosome formation, has been attributed to hydrolysis by phospholipase C and phosphorylation by phosphatidylinositol 3-kinase. We found that two inositol 5-phosphatases, OCRL and Inpp5B, become associated with nascent phagosomes. Both phosphatases, which are Rab5 effectors, associate with the adaptor protein APPL1, which is recruited to the phagosomes by active Rab5. Knockdown of APPL1 or inhibition of Rab5 impairs association of OCRL and Inpp5B with phagosomes and prolongs the presence of PtdIns(4,5)$P_2$ and actin on their membranes. Even though APPL1 can serve as an anchor for Akt, its depletion accentuated the activation of the kinase, likely by increasing the amount of PtdIns(4,5)$P_2$ available to generate phosphatidylinositol (3,4,5)-trisphosphate. Thus, inositol 5-phosphatases are important contributors to the phosphoinositide remodeling and signaling that are pivotal for phagocytosis.**

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## INTRODUCTION

Phagocytosis, a key mechanism in the clearance of apoptotic bodies and invading microorganisms, is triggered by the clustering of phagocytic receptors (Flannagan *et al.*, 2009; Poon *et al.*, 2010). Receptor clustering is induced by exposure to particles coated with multivalent ligands. In the case of Fcγ receptors (FcγRs), the ligand is the Fc portion of immunoglobulin G (IgG) antibodies that recognize epitopes on the surface of target particles. FcγR clustering induces localized actin polymerization that propels the elaboration of pseudopods, which envelop and ultimately ingest the target particle into a membrane-bound vacuole called the phagosome. Disassembly of the actin scaffold is required for completion of particle internalization (Araki *et al.*, 1996; Scott *et al.*, 2005).

The biphasic actin remodeling induced by phagocytosis requires elaborate changes in phosphoinositide metabolism (Yeung and Grinstein, 2007). Like other cells, unstimulated phagocytes constitutively produce phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)$P_2$) in the inner leaflet of their plasma membranes (Botelho *et al.*, 2000). During phagocytosis, PtdIns(4,5)$P_2$ initially accumulates in the plasma membrane of pseudopods but subsequently disappears from the sealed phagosome. Phosphorylation by type I phosphoinositide 3-kinases (PI3KI) accounts in part for the disappearance of PtdIns(4,5)$P_2$. The product of this reaction, phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)$P_3$), initially forms in nascent phagosomes but subsequently vanishes soon after sealing (Marshall *et al.*, 2001), along with its byproduct, phosphatidylinositol (3,4)-bisphosphate (PtdIns(3,4)$P_2$), which is generated when PtdIns(3,4,5)$P_3$ is dephosphorylated by SHIP1.

Both PtdIns(3,4,5)$P_3$ and PtdIns(3,4)$P_2$ are important survival signals, serving as platforms for Akt activation. Activated Akt/protein kinase B is a serine/threonine protein kinase that inhibits apoptosis (Manning and Cantley, 2007). During pathogen invasion, Akt signaling must be tightly regulated to induce an appropriate immune

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Address correspondence to: Sergio Grinstein (sergio.grinstein@sickkids.ca).

Abbreviations used: APPL1, adaptor protein containing pleckstrin-homology domain, PTB phosphotyrosine-binding domain, and leucine zipper/bin–amphiphysin–rvs domain 1; CA, constitutively active; DN, dominant negative; FcγRs, Fc gamma receptors; Gab2, Growth Factor Receptor-Bound Protein 2–associated Protein 2; GFP, green fluorescent protein; IgG, immunoglobulin G; Inpp5B, inositol polyphosphate 5-phosphatase B; OCRL, oculocerebrorenal syndrome of Lowe protein; p38 MAPK, p38 mitogen-activated protein kinase; PH, pleckstrin homology; PI3KI, type I phosphoinositide 3-kinase; PLC, phospholipase C; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(3,4)P2, phosphatidylinositol (3,4)-bisphosphate; PtdIns(3,4,5)P3, phosphatidylinositol (3,4,5)-trisphosphate; PtdIns(4,5)P2, phosphatidylinositol (4,5)-bisphosphate; RBC, red blood cells; RFP, red fluorescent protein; SHIP1, Src homology 2–containing inositol 5-phosphatase 1.

response. Insufficient signaling cripples the immune system by curtailing the survival of phagocytic cells. At the other extreme, excessive Akt signaling allows intracellular pathogens to survive within the phagocyte and eventually spread to other cells (Faherty and Maurelli, 2008). To dampen Akt activity, phagosomes recruit the phosphatase and tensin homologue, which dephosphorylates the D3 position of PtdIns(3,4,5)$P_3$ (Kamen *et al.*, 2007) and PtdIns(3,4)$P_2$.

APPL1 (adaptor protein containing pleckstrin-homology domain, PTB phosphotyrosine-binding domain, and leucine zipper/bin–amphiphysin–rvs domain 1) is an adaptor protein and Rab5 effector that interacts with Akt (Mitsuuchi *et al.*, 1999). In addition, APPL1 can bind a number of other effectors, including two inositol 5-phosphatases: the oculocerebrorenal syndrome of Lowe protein (OCRL) and the 75-kDa inositol polyphosphate 5-phosphatase B (Inpp5B; Erdmann *et al.*, 2007). These lipid phosphatases remove the D5 phosphate from both PtdIns(4,5)$P_2$ and PtdIns(3,4,5)$P_3$ (Ooms *et al.*, 2009). In theory, eliminating PtdIns(4,5)$P_2$ would reduce Akt activation by diminishing the production of PtdIns(3,4,5)$P_3$. Conversely, dephosphorylation of PtdIns(3,4,5)$P_3$ into PtdIns(3,4)$P_2$ would enhance Akt activation because Akt is activated more strongly by PtdIns(3,4)$P_2$ than by PtdIns(3,4,5)$P_3$ (Franke *et al.*, 1997). The net effect of the inositol 5-phosphatases on Akt is therefore difficult to predict a priori.

It is unclear whether APPL1, OCRL, and/or Inpp5B are present on nascent phagosomes, whether these phosphatases contribute to phosphoinositide metabolism during phagocytosis, and what their net effect is on the activation of Akt. We addressed these questions experimentally and present the results.

## RESULTS

### Rab5 recruits APPL1 to phagosomes

Earlier studies reported that APPL1 is recruited to vesicles internalized by clathrin-mediated endocytosis and macropinocytosis (Miaczynska *et al.*, 2004; Zoncu *et al.*, 2009). Because these uptake pathways share similarities with phagocytosis, we investigated whether APPL1 is also recruited to phagosomes. To this end, RAW264.7 macrophages were transiently transfected with fluorescently tagged APPL1 (green fluorescent protein [GFP]-APPL1) and analyzed by spinning disk confocal microscopy. In resting macrophages, GFP-APPL1 resided on small, motile vesicles and, to a lesser extent, in the cytosol; it was completely absent from the plasmalemma and the nucleus. Addition of IgG-coated red blood cells (RBCs; Figure 1A) or latex beads (Figure 1C)—used as targets of phagocytosis via FcγR—led to a robust but transient recruitment of APPL1 onto nascent phagosomes. Unsealed phagocytic cups were largely devoid of APPL1, which became associated with phagosomes only after they had sealed. The sealing event is denoted hereafter as 0 s and was defined as the first image in which the phagosome of interest was completely surrounded by a continuous rim of cytoplasm (Supplemental Movie S1). APPL1 recruitment peaked around 120 s after sealing and declined thereafter, becoming undetectable by ~300 s (Figure 1B). We demonstrated by immunostaining that endogenous APPL1 is also recruited to phagosomes. As shown in Figure 1D, APPL1-specific antibodies decorated phagosomes formed by macrophages challenged with IgG-coated beads (Figure 1D).

APPL1 is known to bind Akt (Mitsuuchi *et al.*, 1999), which, together with PtdIns(3,4,5)$P_3$, is involved in the early stages of phagocytosis. To investigate whether Akt helps localize APPL1 to phagosomes, RAW264.7 macrophages were transiently cotransfected with Akt1-GFP and red fluorescent protein (RFP)-APPL1. As expected, Akt was recruited to phagosomes, reaching maximal intensity after ~60 s (Figure 2, A and B). The association of APPL1 to the phagosomes lagged noticeably behind that of Akt (Figure 2, A and B). Although the behavior of the two proteins was not precisely parallel, physiologically significant but imperceptible amounts of Akt could be recruiting or maintaining APPL1 on phagosomes. To more conclusively analyze the role of Akt in APPL1 recruitment, cells were pretreated with the PI3K inhibitor LY294002, which prevents the formation of PtdIns(3,4,5)$P_3$ and the concomitant recruitment of Akt (Vlahos *et al.*, 1994). PI3K inhibition abolishes phagocytosis of large particles; however, previous studies showed that macrophages treated with PI3K inhibitors can still engulf small (≤1.5 μm) particles (Cox *et al.*, 1999; Vieira *et al.*, 2001). In untreated cells, Akt1-GFP was robustly recruited to phagosomes containing small beads (Figure 2C), but this response was obliterated in cells pretreated with LY294002 (Figure 2D, top). Despite the absence of Akt and PtdIns(3,4,5)$P_3$, RFP-APPL1 was still able to accumulate on phagosomes (Figure 2D, bottom). APPL1 was also recruited to phagosomes in cells pretreated with another general PI3K inhibitor, wortmannin, or with the class I PI3K-specific inhibitor PI-103 (Supplemental Figure S1). In fact, in cells treated with LY294002 or wortmannin, RFP-APPL1 recruitment was more pronounced and more prolonged than under control conditions, persisting on phagosomes for >300 s. Taken together, these findings indicate that Akt and PtdIns(3,4,5)$P_3$ are unnecessary for APPL1 recruitment to FcγR-induced phagosomes.

Like APPL1, the small GTPase Rab5 has been shown to persist on phagosomes when PI3K is inhibited (Vieira *et al.*, 2003). Moreover, in previous studies, Rab5 was shown to interact with APPL1 during endocytosis (Miaczynska *et al.*, 2004; Zhu *et al.*, 2007), raising the possibility that the GTPase mediates the observed recruitment of APPL1 to phagosomes. To examine this possibility, macrophages were transiently cotransfected with Rab5-RFP and GFP-APPL1. Of note, in cells expressing the exogenous tagged Rab5 the recruitment of APPL1 to phagosomal membranes was accentuated compared with cells expressing APPL1 alone (Figure 3A). The consequences of inhibiting PI3K on APPL1 recruitment were analyzed in cells allowed to ingest small beads after pretreatment with LY294002 (Supplemental Movie S2) and in cells allowed to internalize large particles (IgG-coated RBCs) that were treated with the drug 1.5 min after engulfment was completed. As described earlier (Vieira *et al.*, 2003), following treatment with PI3K inhibitors Rab5 lingered on phagosomes, replicating the behavior of APPL1 (Figure 3B and Supplemental Movie S2). Because PtdIns(3,4,5)$P_3$ and PtdIns(3,4)$P_2$ are no longer detectable on phagosomes at the stage at which APPL1 accumulates, the effect of LY294002 likely reflects its inhibition of Vps34, the type III PI3K (PI3KIII). It is noteworthy that in untreated cells, phagosomes progressively recruited 2FYVE-RFP, a probe for phosphatidylinositol 3-phosphate (PtdIns(3)P), the product of Vps34, as GFP-APPL1 dissociated (Figure 3C). This suggests that PtdIns(3)P may be the switch that releases APPL1 from phagosomal membranes, as was reported in another endosomal system (Zoncu *et al.*, 2009). Indeed, phagosomes that had acquired and then lost APPL1 rerecruited the adaptor protein when LY294002 was added at the stage when PtdIns(3)P normally accumulates (Supplemental Movie S3), that is, the 1- to 10-min interval following phagosome sealing (Vieira *et al.*, 2001).

APPL1 is known to interact only with the active GTP-bound form of Rab5 (Zhu *et al.*, 2007). To further document the role of Rab5 in the recruitment of APPL1 to phagosomes, macrophages were transfected with a constitutively active (CA) allele of Rab5. Expression of Rab5(Q79L) resulted in a delayed but sustained and accentuated recruitment of APPL1 to phagosomes, markedly exceeding the levels found in cells (over)expressing wild-type Rab5 (Figure 3, D and F). Conversely, the dominant negative (DN) Rab5(S34N) precluded

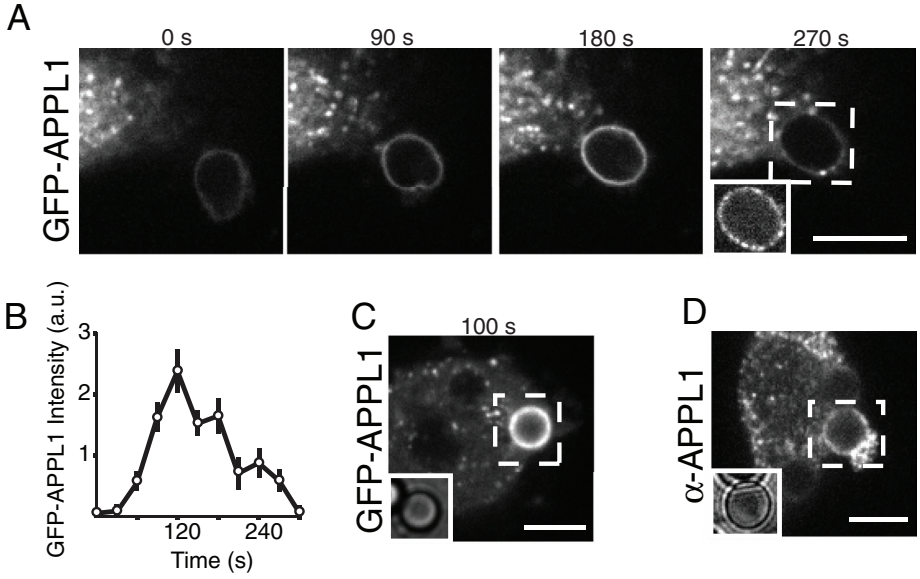


FIGURE 1: APPL1 is recruited to phagosomes. RAW264.7 macrophages transiently transfected with GFP-APPL1 (A, C) or immunolabeled with antibodies against APPL1 (D) were examined by confocal microscopy after exposure to IgG-opsonized RBCs (A) or 3.87-μm latex beads (C, D). (B) Quantification of the fluorescence intensity of GFP-APPL1 on phagosomes after phagocytosis of IgG-coated RBCs. In this and subsequent similar experiments sealing was defined as the moment when the phagosome of interest displayed a continuous rim of cytoplasm (fluorescence) surrounding it (see Supplemental Movie 1). Data are means ± SE of at least three individual experiments; a minimum of 10 phagosomes were quantified per experiment. Images in A, C, and D are representative of at least four individual experiments of each kind. In this and subsequent figures the numbers indicate time in seconds (s) after phagosome formation, and the scale bars represent 5 μm. The rightmost inset A illustrates the target RBC, which was labeled with a fluorescent anti-IgG secondary antibody. The insets in C and D display the corresponding differential interference contrast (DIC) images. The macrophage in D was fixed 5 min after addition of the beads. In fixed samples such as this, the precise time when ingestion of the illustrated bead occurred was not defined.

the association of APPL1 with vesicles and phagosomes (Figure 3, E and F). These findings imply that active Rab5 is a key determinant of APPL1 recruitment to phagosomes.

### APPL1 knockdown increases Akt activation

To examine the functional role of APPL1 during phagocytosis, gene silencing with small interfering RNA (siRNA) was used to deplete the endogenous APPL1 protein in RAW264.7 macrophages. Immunofluorescence (Figure 4A) and immunoblotting (Figure 4D) determinations using anti-APPL1 antibodies confirmed that cell cultures transfected with APPL1-directed siRNA had reduced levels of APPL1 compared with cultures treated with scrambled siRNA. The siRNA treatment reduced APPL1 protein levels by >80% (Figure 4, D and E). Depletion of APPL1 had no effect on the ability of cells to ingest IgG-coated particles (phagocytic index; Figure 4B), and similar results were obtained when the protein was overexpressed (data not shown). Likewise, APPL1 siRNA did not significantly affect phagosome maturation, as measured by Rab7-GFP accumulation 10, 20, and 30 min after phagocytosis (Figure 4C). On the basis of its aforementioned interaction with APPL1, we also measured the degree of Akt activation. The formation of the active phosphorylated form of Akt was measured by immunoblotting in macrophages treated with APPL1-directed or scrambled siRNA 20 min after challenge with IgG-coated beads. As expected, based on the accumulation of PtdIns(3,4,5)$P_3$ and PtdIns(3,4)$P_2$, FcγR-mediated phagocytosis led to phosphorylation of Akt. It is remarkable that levels of phospho-Akt were significantly higher in cells treated with APPL1 siRNA than in control cells treated only with scrambled siRNA (Figure 4D). On average, cells treated with APPL1 siRNA had 1.5 times more phospho-Akt than did control cells (Figure 4F).

To further examine the effects of APPL1 on Akt activity, RNA interference–treated macrophages were transiently transfected with Akt1-GFP and exposed to IgG-coated RBC. Akt persisted on phagosomes for longer periods of time when APPL1 was depleted (Figure 5, B and C) as compared with cells treated with scrambled siRNA (Figure 5, A and C). In contrast, overexpressing RFP-APPL1 resulted in lower levels of Akt recruitment to the phagosome (Figure 5C). The relationship between Akt and APPL1 was also studied by manipulating the association of APPL1 with phagosomes via Rab5. Because Rab5 is necessary for APPL1 recruitment (see earlier discussion), we assessed the effect of expressing DN-Rab5. As described earlier, this impaired the phagosomal localization of APPL1, and, of importance, prolonged the retention of Akt, replicating the results found with APPL1 siRNA (Figure 5C). Taken together, these results suggest that APPL1 curtails Akt phosphorylation by accelerating the dissociation of Akt from phagosomes.

Other studies reported that siRNA-induced silencing of APPL1 reduces p38 mitogen-activated protein kinase (MAPK) phosphorylation following stimulation with adiponectin (Mao *et al.*, 2006; Zoncu *et al.*, 2009; Xin *et al.*, 2011). Because p38 MAPK is also activated during phagocytosis (Figure 4D), we tested the effect of APPL1 depletion on this stress kinase. In agreement with the findings in other systems, APPL1 siRNA reduced p38 MAPK phosphorylation induced by phagocytosis in macrophages (Figure 4D). Phospho-p38 MAPK levels were 40% lower in APPL1-depleted cells than in control cells (Figure 4G), although in the limited number of experiments performed this difference did not attain statistical significance. Thus, APPL1 depletion appears to have opposite effects on Akt and p38 MAPK activation.

### APPL1 helps recruit two inositol 5-phosphatases

APPL1 interacts with a pair of inositol 5-phosphatases called OCRL and Inpp5B (Erdmann *et al.*, 2007; Mao *et al.*, 2009). These phosphatases hydrolyze the 5′ phosphate from PtdIns(4,5)$P_2$ and PtdIns(3,4,5)$P_3$. Because PtdIns(3,4,5)$P_3$ activates Akt and PtdIns(4,5)$P_2$ serves as the precursor for PtdIns(3,4,5)$P_3$ synthesis, it is conceivable that APPL1 modulates Akt signaling by recruiting the 5-phosphatases. The involvement of these 5-phosphatases in the phagocytic pathway was investigated by cotransfecting RAW264.7 macrophages with GFP-OCRL and RFP-APPL1 or RFP-Inpp5B and GFP-APPL1. As documented in Figure 6, A, B, and D, both 5-phosphatases colocalized with APPL1 on phagosomes. Expressing DN-Rab5 to eliminate APPL1 recruitment also inhibited the recruitment of OCRL and Inpp5B to the phagosome. CA-Rab5, on the other hand, accentuated OCRL and Inpp5B recruitment (Figure 6C), in accordance with its stimulatory effect on APPL1.

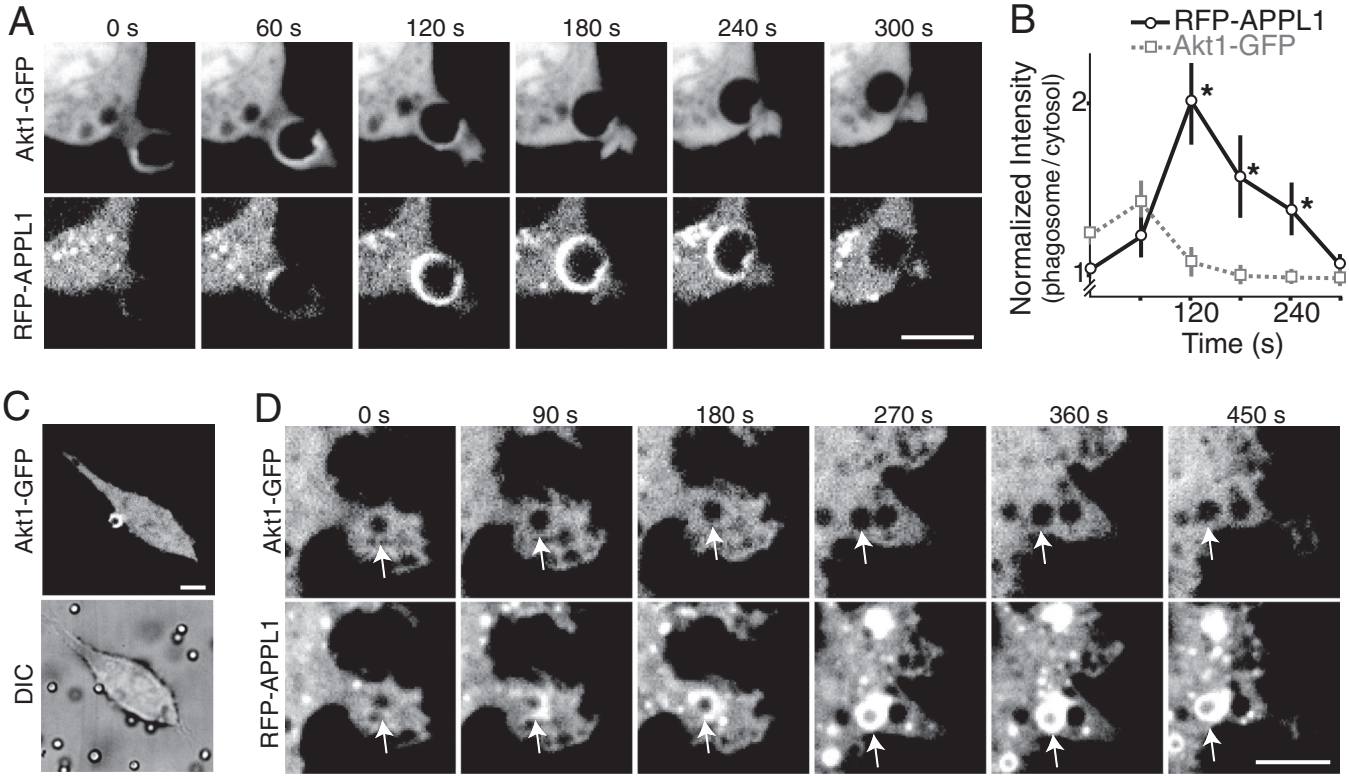


FIGURE 2: Akt1 binding to phagosomes is not required for APPL1 recruitment. RAW264.7 macrophages transiently cotransfected with Akt1-GFP and RFP-APPL1 (A, D) or Akt1-GFP alone (C) were examined by confocal microscopy after exposure to IgG-opsonized RBC (A) or 1.58 μm latex beads (C, D). (B) Quantification of the fluorescence intensity of Akt1-GFP (□) and RFP-APPL1 (○) on phagosomes as a function of time after phagocytosis of IgG-coated RBC. Data are means ± SE of three individual experiments; a total of 14 phagosomes were quantified. Asterisks indicate statistically significant differences ($p < 0.05$). Images in A, C, and D are representative of at least three individual experiments of each kind. The phagosomes in D were verified to have beads by DIC. The macrophage in D was pretreated with 100 μM LY294002 for 5 min before addition of the beads

Although these results demonstrate that Rab5 influences the behavior of OCRL and Inpp5B, they do not distinguish whether the GTPase recruits the inositol 5-phosphatases directly or indirectly through APPL1. To resolve this ambiguity, APPL1 expression was silenced using siRNA and the macrophages were transfected with CA-Rab5 together with OCRL or Inpp5B. These cells were then incubated with IgG-coated RBCs, and the association of the 5-phosphatases with phagosomes was quantified. Even though all phagosomes recruited CA-Rab5, the APPL1-depleted cells recruited considerably less OCRL and Inpp5B to their phagosomes in comparison to control cells treated with scrambled siRNA (Figure 6, E–G). Collectively, these results imply that APPL1 cooperates with Rab5 in the recruitment of OCRL and Inpp5B to phagosomes, which is consistent with findings in other endocytic systems (Erdmann *et al.*, 2007).

### APPL1 knockdown alters phosphoinositide metabolism

By degrading its precursor PtdIns(4,5)$P_2$ and dephosphorylating PtdIns(3,4,5)$P_3$ directly, OCRL and Inpp5B can in principle reduce the levels of PtdIns(3,4,5)$P_3$ on phagosomes. The ability of APPL1 to recruit these phosphatases could in turn account for the effects of the adaptor on Akt (Figure 4). To investigate whether APPL1 affects PtdIns(4,5)$P_2$ metabolism during phagosome formation, RAW264.7 macrophages were transiently transfected with 2PH-PLC-GFP, a PtdIns(4,5)$P_2$-binding probe. This high-avidity probe containing two tandem copies of the pleckstrin homology (PH) domain of phospholipase Cδ (PLCδ) was used to detect low levels of PtdIns(4,5)$P_2$. In otherwise untreated cells PtdIns(4,5)$P_2$ disappears from the phagosomal membrane soon after sealing (Figure 7A), as described earlier (Botelho *et al.*, 2000), and is never visible thereafter. It is striking that pretreatment of the cells with APPL1 siRNA or coexpression of DN-Rab5 caused phagosomes to retain 2PH-PLC-GFP for extended periods of time, often for as long as 30 min (Figure 7, B and C). To rule out that the retention of 2PH-PLC-GFP was due to incomplete scission of phagosomes from the plasmalemma, a secondary antibody to rabbit IgG was added to label external RBC. This antibody did not label RBC inside phagosomes that retained 2PH-PLC-GFP (Supplemental Figure S2 and Supplemental Movie S4), suggesting that PtdIns(4,5)$P_2$ was retained due to insufficient catabolism.

Of note, in cells depleted of APPL1, phagosomal PtdIns(4,5)$P_2$ initially decreased partially but did not disappear and often recovered to levels comparable to those in the plasmalemma (Figure 7B). The initial decrease, and the fact that only 25–30% of the phagosomes showed sustained levels of PtdIns(4,5)$P_2$ 10 min after formation, is likely due to the existence of multiple parallel pathways for PtdIns(4,5)$P_2$ metabolism. These include hydrolysis by PLC, phosphorylation by PI3KI, and dephosphorylation by additional 5-phosphatases that include not only OCRL and Inpp5B but also synaptojanin (Di Paolo and De Camilli, 2006). In this regard, it is noteworthy that whereas in normal cells phagosomal PtdIns(4,5)$P_2$ levels decreased progressively after particle engagement, complete loss of 2PH-PLC-GFP staining often coincided with the recruitment of Rab5 (Figure 7A).

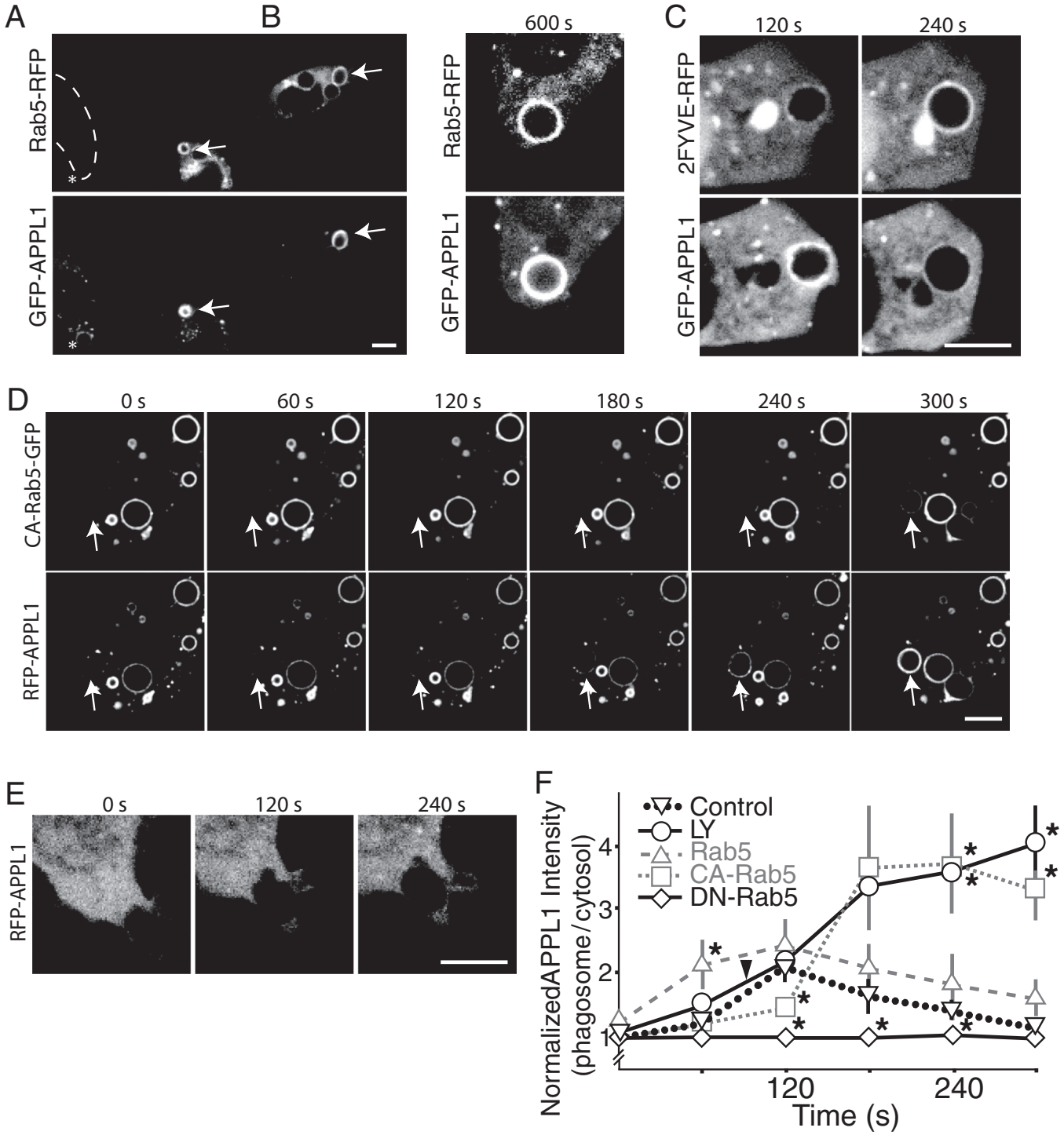


FIGURE 3: Rab5 is required for APPL1 recruitment. RAW264.7 macrophages transiently cotransfected with APPL1 and Rab5-RFP (A, B), 2FYVE-RFP (C), constitutively active Rab5 (Rab5(Q79L)-GFP) (D), or dominant-negative Rab5 (Rab5(S34N)-GFP) (E) were examined by confocal microscopy after exposure to IgG-opsonized RBC. Images in A–E are representative of at least three individual experiments of each kind. The phagosome indicated with the arrowhead in D was verified to have a RBC detecting the fluorescent anti-IgG secondary used to label the opsonin. In B, 100 μM LY294002 was added ~1.5 min after phagosome sealing. (F) Quantification of the fluorescence intensity of GFP-APPL1 on phagosomes after phagocytosis of IgG-coated RBC in macrophages transfected with GFP-APPL1 only (▽ and ○) or cotransfected with GFP-APPL1 and Rab5-RFP (△), constitutively active Rab5-mCherry (□), or dominant-negative Rab5-mCherry (◇). The arrowhead indicates when 100 μM LY294002 was added to the cells denoted by open circles (○). Data are means ± SE of at least three individual experiments; at least 10 phagosomes were quantified per experiment. Asterisks indicate statistically significant differences in comparison to control ($p < 0.01$).

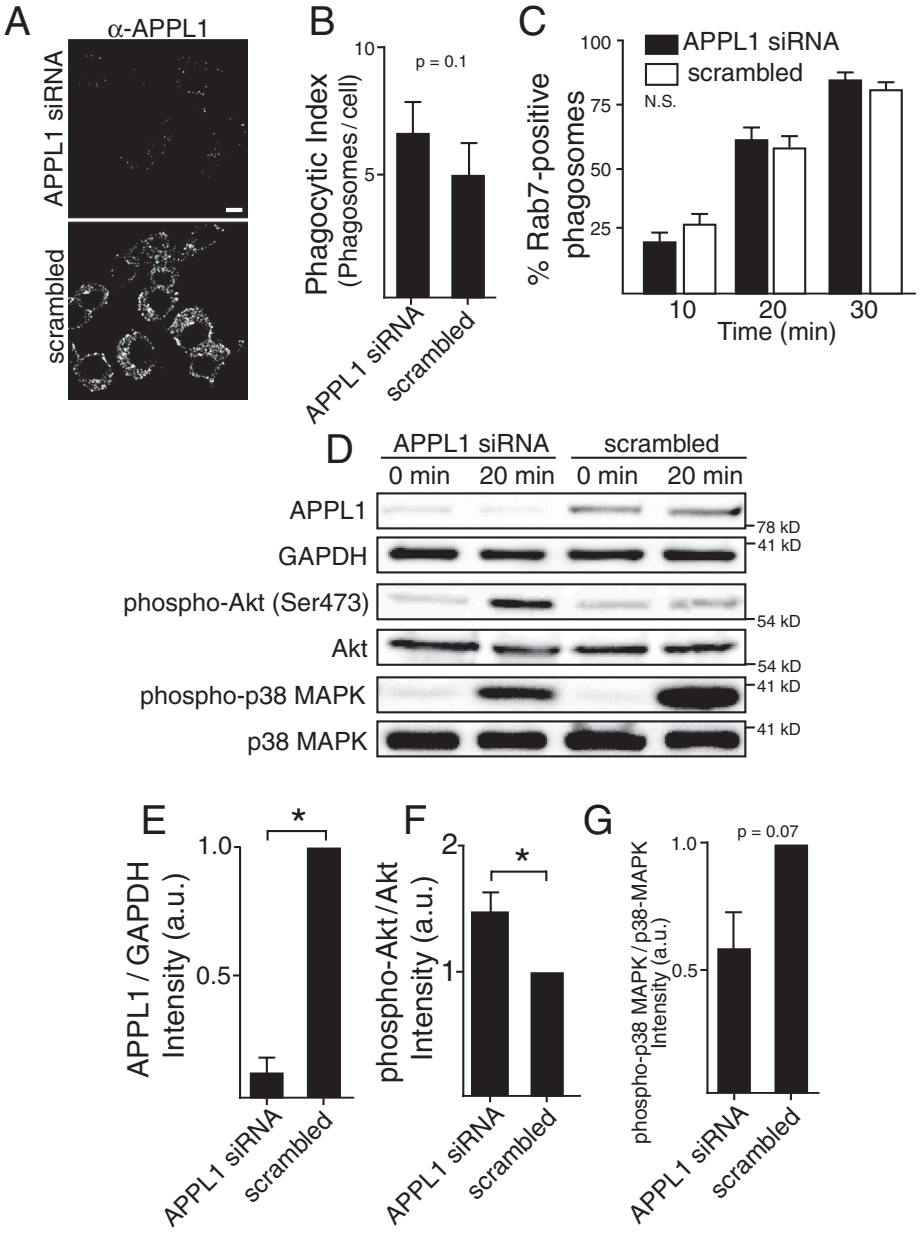


FIGURE 4: APPL1 knockdown increases Akt phosphorylation. RAW264.7 macrophages were treated with scrambled siRNA or siRNA against APPL1. (A) Macrophages were immunolabeled with antibodies against APPL1 to verify gene silencing. Images are representative of three individual experiments. (B) Quantification of the phagocytic index for macrophages challenged with IgG-coated RBC for 20 min. Data are means ± SE of three individual experiments; 20 cells were quantified per experiment. (C) Quantification of Rab7-GFP–positive phagosomes at the indicated times after sealing of phagosomes containing IgG-coated RBC. Data are means ± SE of three individual experiments; at least 100 phagosomes were quantified per time point. (D) Immunoblots of whole-cell lysates of macrophages sampled before (0 min) or 20 min after exposure to IgG-opsonized 3.87-μm latex beads. Blots are representative of at least five individual experiments of each type. Quantification of the intensities of the bands corresponding to APPL1 (E), phospho-Akt (F), and phospho-p38 MAPK (G). Data are means ± SD of at least five individual experiments of each type. *p < 0.05.

We interpret this to mean that the final stages of PtdIns(4,5)$P_2$ degradation are mediated by OCRL and Inpp5B, which are recruited by Rab5 directly and indirectly via APPL1. In support of this interpretation, we found that the 2PH-PLC-GFP probe is also retained in a fraction of the phagosomes when the cells express DN-Rab5 (Figure 7C).

If depleting APPL1 or otherwise interfering with its recruitment to phagosomes prevents PtdIns(4,5)$P_2$ degradation, it follows that the level and/or persistence of PtdIns(3,4,5)$P_3$ on phagosomes may also increase. This assumption was tested experimentally. To visualize PtdIns(3,4,5)$P_3$, RAW264.7 macrophages were transiently transfected with PH-Gab2-GFP, a probe containing the PH domain of Gab2, which preferentially binds PtdIns(3,4,5)$P_3$ (Gu *et al.*, 2003). Phagosomes lost PH-Gab2-GFP more slowly in cells transfected with DN-Rab5 or APPL1 siRNA (Figure 7E) than in those transfected with wild-type Rab5 or scrambled siRNA (Figure 7D,F). Although the resting level of plasmalemma-associated PH-Gab2-GFP varied somewhat, the basal PtdIns(3,4,5)$P_3$ did not influence the behavior of the probe during phagocytosis (compare Supplemental Figure S3 with Figure 7, D and E). It is important to note that in addition to PtdIns(4,5)$P_2$, OCRL and Inpp5B can also dephosphorylate PtdIns(3,4,5)$P_3$. A combination of these effects in all likelihood accounts for the prolonged residence of PH-Gab2-GFP on phagosomes of APPL1-depleted or DN-Rab5–expressing cells.

### OCRL and Inpp5B knockdown perturbs PtdIns(4,5)$P_2$ metabolism

The reduced PtdIns(4,5)$P_2$ degradation observed following APPL1 knockdown or inhibition of Rab5 function is most readily explained by the putative failure to recruit OCRL and Inpp5B. If correct, this assumption predicts that silencing of the OCRL and Inpp5B genes would similarly retard the hydrolysis of phagosomal PtdIns(4,5)$P_2$. To validate this prediction experimentally, we used siRNA to simultaneously deplete RAW264.7 macrophages of both phosphatases. Immunoblotting (Figure 8, A and B) confirmed that cells transfected with siRNAs directed at both phosphatases had reduced levels of OCRL compared with cells treated with scrambled siRNA, although silencing was incomplete. This partial effect was validated by RT-PCR, which also served to ascertain that the siRNAs used concomitantly reduced the level of Inpp5B transcript (Figure 8C). As earlier, the 2PH-PLC-GFP construct was used in the cells in which expression of the phosphatases was knocked down to detect possible changes in PtdIns(4,5)$P_2$ during phagocytosis. Consistent with the results obtained in cells in which APPL1 or Rab5 activity was impaired, the depletion of OCRL and Inpp5B caused phagosomes to retain 2PH-PLC-GFP for inordinately long periods of time (Figure 8, D–F). Of note, knocking down OCRL or Inpp5B individually had little effect on PtdIns(4,5)$P_2$ degradation in the murine macrophages (data not

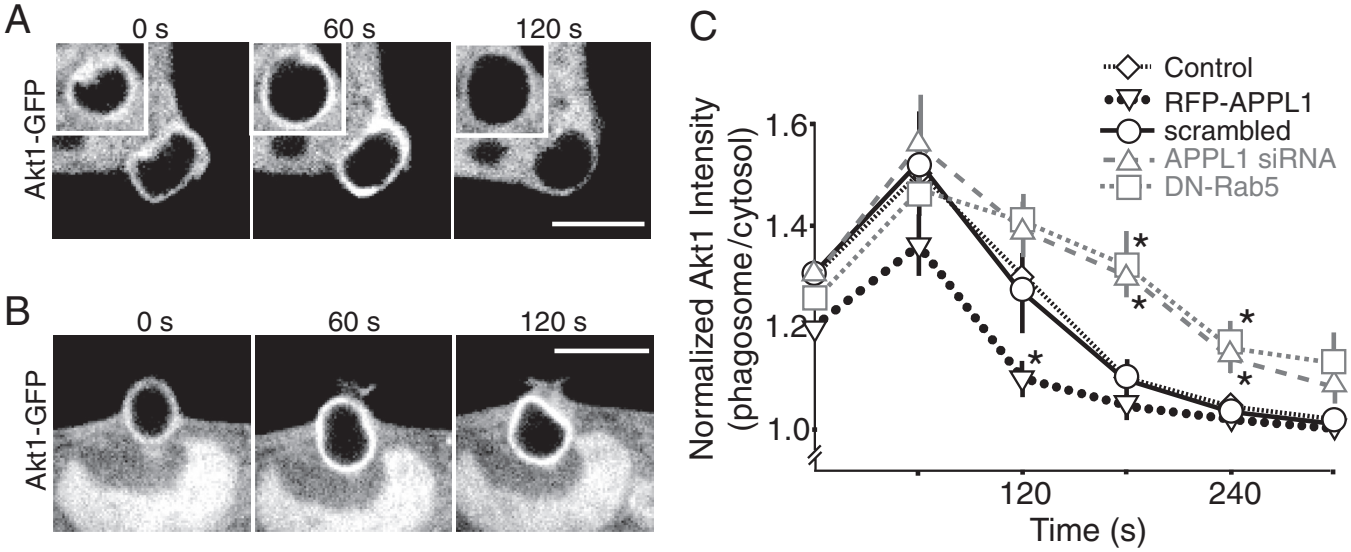


FIGURE 5: APPL1 knockdown prolongs Akt1 recruitment to phagosomes. RAW264.7 macrophages either untreated (A, inset) or treated with scrambled siRNA (A) or siRNA against APPL1 (B) were transiently transfected with Akt1-GFP, exposed to IgG-coated RBC, and examined by confocal microscopy. Images are representative of at least three individual experiments. (C) Quantification of the fluorescence intensity of Akt-GFP on phagosomes after phagocytosis of IgG-coated RBC in untreated macrophages (◇), macrophages cotransfected with RFP-APPL1 (▽) or dominant-negative Rab5-mCherry (□), or macrophages treated with scrambled siRNA (○) or siRNA against APPL1 (△). Data are means ± SE of at least three individual experiments of each type; at least 15 phagosomes were quantified per experiment. *$p < 0.01$ in comparison to the untreated control.

shown). This is in line with reported findings in knockout animals, which demonstrate that mice devoid of either OCRL or Inpp5B are phenotypically normal, whereas OCRL/Inpp5B double knockouts are not viable (Jänne *et al.*, 1998).

## DISCUSSION

Phagosomes undergo a maturation process as they progress to fusion with lysosomes. Successful completion of maturation is key to microbial destruction and to antigen presentation; indeed, a variety of pathogens survive inside host phagocytes by coopting the maturation sequence (Flannagan *et al.*, 2009). Clearly, detailed knowledge of the molecular basis of these events is essential to understand both innate immunity and its subversion by certain pathogens.

Phosphoinositides and Rab GTPases serve as important molecular switches that control the traffic and evolution of organelles, including phagosomes. They define the identity of membranous subdomains and recruit specific effector and adaptor proteins that promote fission or fusion events. APPL1 is one such adaptor (Miaczynska *et al.*, 2004; Chial *et al.*, 2008), suggesting that it may be involved in vesicular traffic. It is remarkable that APPL1 was also found to bind OCRL and Inpp5B, two inositol 5-phosphatases (Erdmann *et al.*, 2007). Despite its privileged position at the interface between Rab proteins and phosphoinositide metabolism, the participation and possible role of APPL1 in phagocytosis had not been investigated. In this study, we report that APPL1 is in fact recruited to maturing phagosomes and, more important, that it plays a role in the control of phosphoinositide metabolism, which in turn determines the activation of kinases such as Akt.

Disappearance of PtdIns(4,5)$P_2$, which is constitutively present at the plasma membrane, accompanies sealing of the phagosome and is believed to account, at least in part, for the detachment of actin required for scission (Scott *et al.*, 2005). This disappearance is the result of a complex combination of events that include termination of synthesis and accelerated degradation. The enzymes responsible for PtdIns(4,5)$P_2$ biosynthesis at the membrane, the type I phosphatidylinositol 4-phosphate 5-kinases, dissociate from the membrane as the phagosome forms (Fairn *et al.*, 2009). In parallel, hydrolysis by PLCγ and phosphorylation by PI3KI contribute to the disappearance of PtdIns(4,5)$P_2$. However, in the case of small (≤1 μm) particles, phagocytosis and PtdIns(4,5)$P_2$ disappearance proceed in the presence of PI3KI inhibitors. In addition, we find that although diacylglycerol can be visualized on a fraction of forming phagosomes, sealing and scission often proceed without any apparent diacylglycerol accumulation (unpublished observations). This could reflect active conversion to phosphatidic acid by diacylglycerol kinases, but it also raises the possibility that alternative means exist for the elimination of PtdIns(4,5)$P_2$. An obvious candidate is its conversion to PtdIns(4)P by inositol 5-phosphatases. One such phosphatase, synaptojanin, is believed to hydrolyze PtdIns(4,5)$P_2$ during the course of endocytosis (Cremona *et al.*, 1999). Although we were unable to detect synaptojanin on forming phagosomes, the observation that APPL1 associates with nascent phagocytic vacuoles raised the possibility that OCRL and/or Inpp5B might contribute to remodel the phosphoinositide makeup of the phagosome. Accordingly, both PtdIns(4,5)$P_2$ and PtdIns(3,4,5)$P_3$ persisted longer on a fraction of the phagosomes when APPL1 was silenced and also when Rab5, the GTPase that recruits both APPL1 and OCRL/INPP5B, was inhibited. Moreover, PtdIns(4,5)$P_2$ also persisted longer when OCRL and Inpp5B were simultaneously depleted below endogenous levels. It is unclear why this behavior was observed only in a subpopulation of the phagosomes; it is conceivable that under the conditions used, residual adaptors or phosphatases supported the conversion of the phosphoinositides at near-normal rates in some of the phagosomes. Of note, deletion of Dd5P4, a

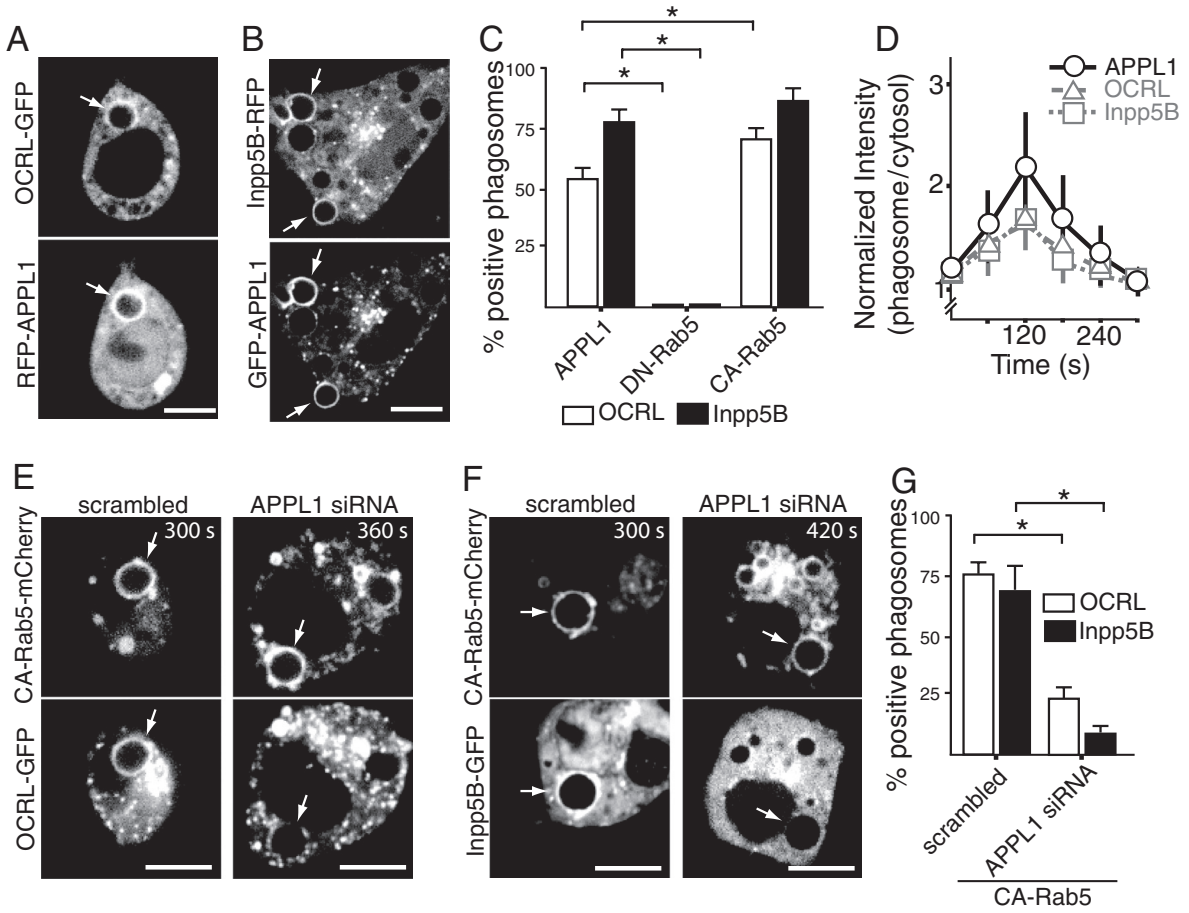


FIGURE 6: APPL1 recruits two phosphoinositide 5-phosphatases, OCRL and Inpp5B, to phagosomes. (A–C) RAW264.7 macrophages transiently cotransfected with OCRL-GFP and RFP-APPL1 (A) or Inpp5B-RFP and GFP-APPL1 (B) were examined by confocal microscopy after exposure to IgG-opsonized RBC. (C) Quantification of the percentage of OCRL-GFP–positive (□) or Inpp5B-GFP–positive (■) phagosomes 2 min after phagosome sealing. Asterisks indicate statistically significant differences ($p < 0.01$) in comparison to the control condition (APPL1). Data were acquired in macrophages cotransfected with RFP-APPL1, dominant-negative Rab5-mCherry, or constitutively active Rab5-mCherry following phagocytosis of IgG-coated RBC. Data are means ± SE of at least three individual experiments of each type; at least 50 phagosomes were quantified per experiment. (D) Quantification of the fluorescence intensity of APPL1 (○), OCRL (△), and Inpp5B (□) on phagosomes after phagocytosis of IgG-coated RBC in macrophages cotransfected with RFP-APPL1 and OCRL-GFP, or GFP-APPL1 and Inpp5B-RFP. Data are means ± SE of at least three individual experiments of each type; at least 10 phagosomes were quantified per experiment. (E–G) RAW264.7 macrophages treated with scrambled siRNA or siRNA against APPL1, transiently cotransfected with OCRL-GFP (E) or Inpp5B-GFP (F) and constitutively active Rab5-mCherry, and exposed to IgG-opsonized RBC were examined by confocal microscopy. Images in A, B, E, and F are representative of at least three individual experiments of each type. G) Quantification of OCRL-GFP–positive (□) or Inpp5B-GFP–positive (■) phagosomes 5 min after sealing. Macrophages were pretreated with scrambled siRNA or siRNA against APPL1 and transiently cotransfected with constitutively active Rab5-mCherry prior to phagocytosis of IgG-coated RBC. Data are means ± SE of three individual experiments of each kind; at least 50 phagosomes were quantified per experiment. Asterisks indicate statistically significant differences ($p < 0.01$).

homologue of OCRL, impaired phagocytosis in *Dictyostelium* (Loovers *et al.*, 2007). The authors attributed the inhibition to an inability of the cells to convert PtdIns(3,4,5)$P_3$ to PtdIns(3,4)$P_2$, but failure to eliminate PtdIns(4,5)$P_2$ could also be involved.

We propose that OCRL and Inpp5B play a role in the final stages of PtdIns(4,5)$P_2$ removal from sealing phagosomes, following the initial contribution of PLCγ and PI3KI. By completing the elimination of PtdIns(4,5)$P_2$, OCRL and Inpp5B ensure that the surface charge of the sealed phagosome drops below the levels required to retain type I phosphatidylinositol 4-phosphate 5-kinase isoforms, which associate electrostatically with the plasma membrane (Fairn *et al.*, 2009). The thorough and complete removal of PtdIns(4,5)$P_2$ ensures that actin will disassemble, that synthesis of PtdIns(3,4,5)$P_3$ will cease, and that existing PtdIns(3,4,5)$P_3$ is degraded. Two observations support this conclusion: 1) when recruitment of the 5-phosphatases was depressed by inhibition of Rab5, the nascent phagosomes retained a thin but distinct rim of actin (Supplemental Movie S4) and 2) PH-Gab2-GFP, a probe for PtdIns(3,4,5)$P_3$, was retained for inordinately long periods when Rab5 function or APPL1 recruitment was impaired (Figure 7). It is noteworthy that this paradigm applies to phagosomes formed by engagement of Fcγ receptors but cannot be generalized without testing other systems individually. In fact, after sealing, phagosomes formed via CR3 receptors undergo secondary waves of PtdIns(4,5)$P_2$ and PtdIns(3,4,5)$P_3$ accumulation, which are associated with actin reassembly and the formation of "comet tails" that propel sealed

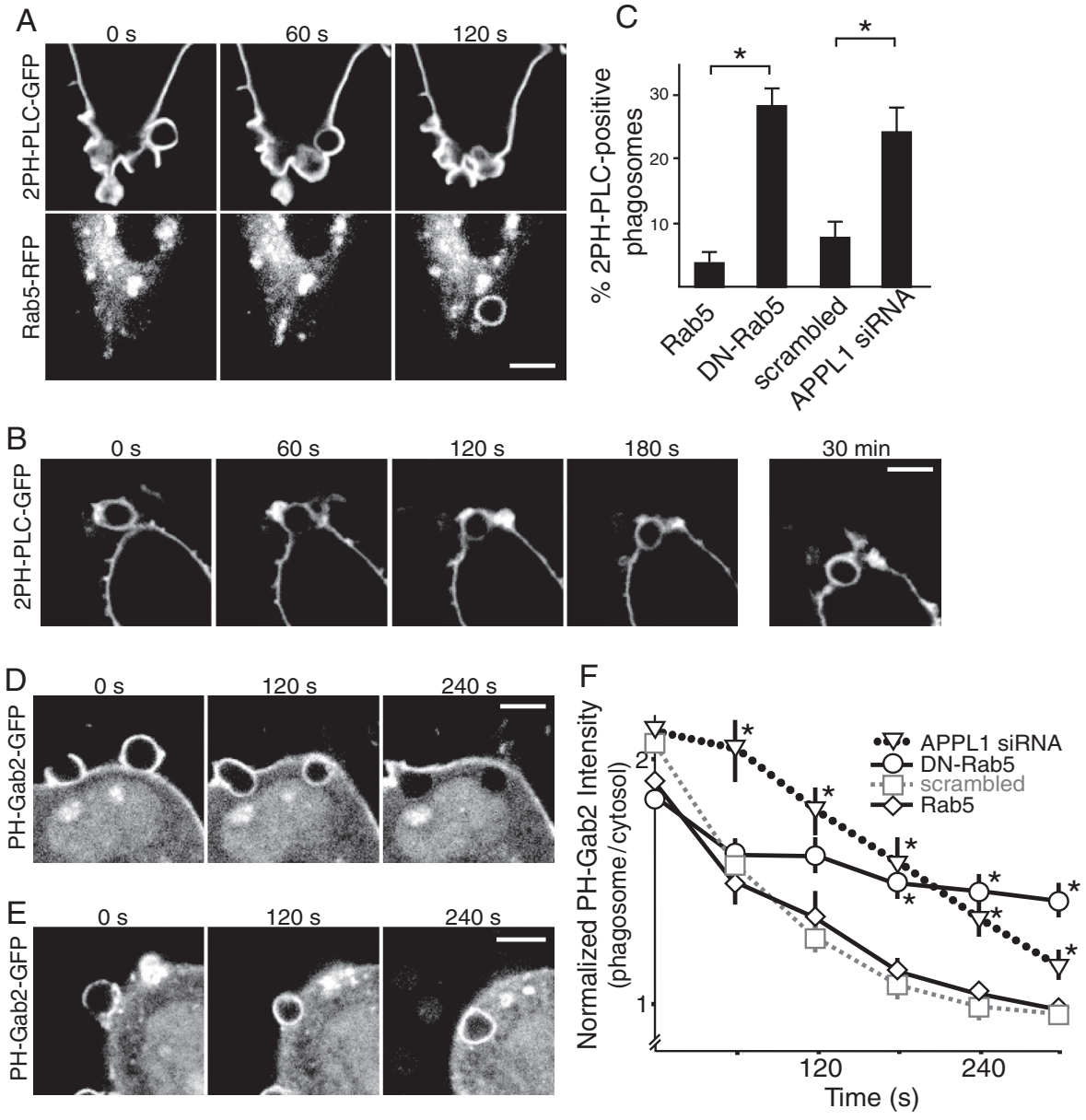


FIGURE 7: Impairing APPL1 recruitment perturbs phosphoinositide metabolism. (A, B) RAW264.7 macrophages transiently cotransfected with 2PH-PLC-GFP and either wild-type Rab5-RFP (A) or dominant-negative Rab5-mCherry (B) were examined by confocal microscopy at the indicated times after ingestion of IgG-opsonized RBC. (C) Quantification of 2PH-PLC–positive or GFP-positive phagosomes 10 min after phagosome sealing. Macrophages were cotransfected with 2PH-PLC-GFP and wild-type Rab5-RFP or dominant-negative Rab5-mCherry or were transfected with 2PH-PLC-GFP and treated with scrambled siRNA or siRNA against APPL1 before induction of phagocytosis of IgG-coated RBC. Data are means ± SE of at least three individual experiments; at least 100 phagosomes were quantified per experiment. Asterisks indicate statistically significant differences ($p < 0.05$) in comparison to the corresponding control condition. (D, E) RAW264.7 macrophages treated with scrambled siRNA (D) or siRNA against APPL1 (E), transiently transfected with PH-Gab2-GFP, and exposed to IgG-opsonized RBC were examined by confocal microscopy at the indicated times after phagosome sealing. Images in A, B, D, and E are representative of at least three individual experiments of each kind. (F) Quantification of the fluorescence intensity of PH-Gab2-GFP on phagosomes after phagocytosis of IgG-coated RBC. Macrophages were cotransfected with PH-Gab2-GFP and either Rab5-RFP (◇) or dominant-negative Rab5-mCherry (○), or were transfected with PH-Gab2-GFP and treated with scrambled siRNA (□) or siRNA against APPL1 (▽). Data are means ± SE of at least three individual experiments of each type; at least 20 phagosomes were quantified per experiment. Asterisks indicate statistically significant differences ($p < 0.01$) in comparison to the corresponding control condition.

phagosomes within the cell (Bohdanowicz *et al.*, 2010), and similar phenotypes have been described for some ingested pathogens (e.g., Gouin *et al.*, 2005).

The role of APPL1 in the regulation of Akt has been the source of controversy in other systems. Because the adaptor associates directly with Akt, it has been postulated that elimination of APPL1 reduces the activation of the kinase by certain receptors (Mao *et al.*, 2006; Schenck *et al.*, 2008; Tan *et al.*, 2010b), presumably by minimizing its recruitment to the membrane. In other instances, however, APPL1 depletion was reported to enhance Akt phosphorylation or

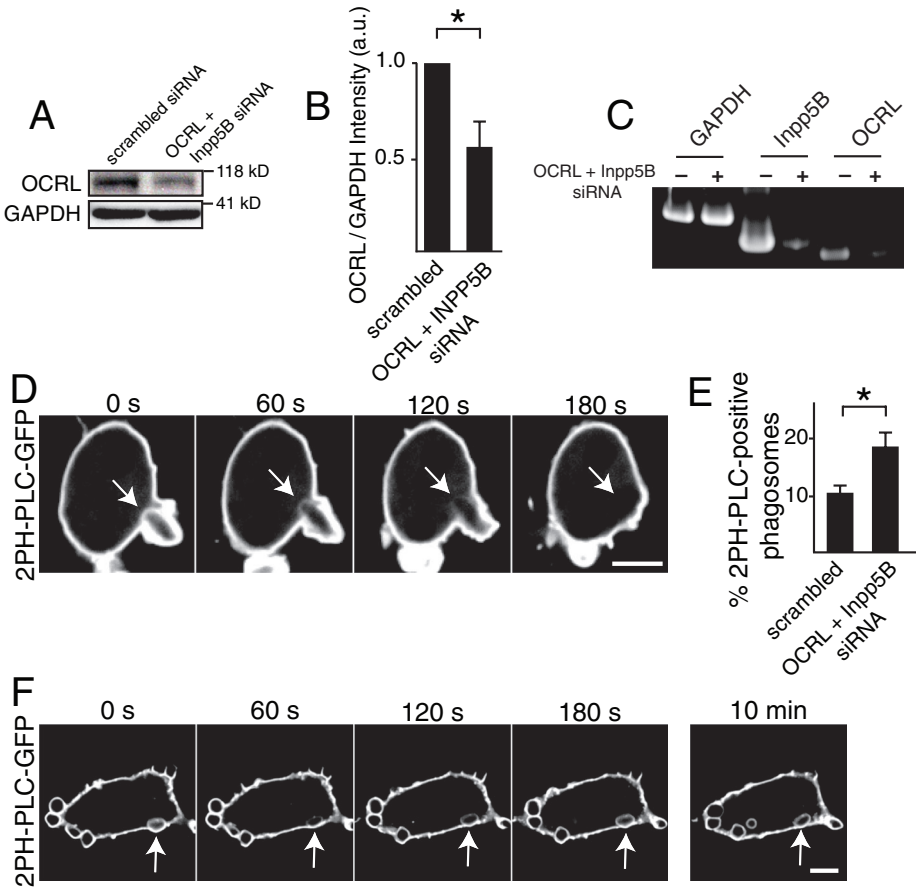


FIGURE 8: Knocking down OCRL and Inpp5B perturbs PtdIns(4,5)$P_2$ metabolism. RAW264.7 macrophages were treated with scrambled siRNA or siRNAs against OCRL and Inpp5B. Whole-cell lysates were immunoblotted with antibodies to OCRL (A) or analyzed by RT-PCR with primers for Inpp5B and OCRL (C) to verify gene silencing. Images are representative of three individual experiments. (B) Quantification of the intensity of the bands corresponding to OCRL on immunoblots like that in A. Data are means ± SE of three individual experiments. *$p < 0.05$. RAW264.7 macrophages treated with scrambled siRNA (D) or siRNAs against OCRL and Inpp5B (F) were transfected with 2PH-PLC-GFP and examined by confocal microscopy at the indicated times after ingestion of IgG-opsonized RBC. (E) Quantification of 2PH-PLC-GFP–positive phagosomes 10 min after phagosome sealing. Data are means ± SE of three individual experiments. *$p < 0.05$.

cell proliferation (Tan *et al.*, 2010a; Tu *et al.*, 2011). Our results are in line with the latter reports: we found more pronounced Akt activation in cells depleted of APPL1 (Figure 4). This cannot be simply explained by degradation of PtdIns(3,4,5)$P_3$ by OCRL/Inpp5B because the product of this reaction, PtdIns(3,4)$P_2$, is believed to be a more potent agonist of Akt than PtdIns(3,4,5)$P_3$ (Franke *et al.*, 1997). Although this possibility cannot be dismissed, we believe the predominant effect of OCRL and Inpp5B is on the depletion of PtdIns(4,5)$P_2$, leading in turn to termination of PtdIns(3,4,5)$P_3$ synthesis. As such, the net effect of APPL1, OCRL, and Inpp5B in phagosomes is the premature termination of Akt activity. Although we have no definitive evidence that this alters phagosome maturation, modulation of Akt and/or MAPK may affect the gene expression and cell survival functions of these kinases.

By converting PtdIns(3,4,5)$P_3$ to PtdIns(3,4)$P_2$, 5-phosphatases like OCRL and Inpp5B have been suggested to contribute to the formation of PtdIns(3)P in endosomes (Shin *et al.*, 2005). This requires the action of an additional 4-phosphatase that remains heretofore unidentified. Inpp4A, which binds Rab5, is a likely candidate. Nonetheless we have no evidence that this progressive pathway contributes significantly to PtdIns(3)P formation in phagosomes. In fact, virtually all of the PtdIns(3)P detected on phagosomes originates from phosphorylation of phosphatidylinositol by the class III kinase Vps34. Several lines of evidence support this contention: 1) phagosomes formed by cells in which both the α and β isoforms of the p85 subunit of the class I PI 3-kinase were ablated by gene targeting displayed normal levels of PtdIns(3)P, as assessed using GFP-tagged FYVE domain constructs (Vieira *et al.*, 2001); 2) PtdIns(3)P formation by phagosomes was seemingly normal in cells in which class I PI 3-kinase was fully inhibited using PI-103 (Bohdanowicz *et al.*, 2010); and 3) the phagosomal accumulation of PtdIns(3)P was obliterated in cells injected with inhibitory anti-Vps34 antibodies (Bohdanowicz *et al.*, 2010). In fact, instead of contributing to the formation of PtdIns(3)P, the inositol 5-phosphatases appear to be regulated by the phosphoinositide: the appearance of PtdIns(3)P coincides with and seems to dictate the dissociation of APPL1, OCRL, and Inpp5B from the phagosomal membrane (Figure 3).

In summary, our findings document a role of APPL1 during phagocytosis, which can be summarized schematically as shown in Figure 9. At the phagosomal cup, class I PI3K converts PtdIns(4,5)$P_2$ to PtdIns(3,4,5)$P_3$, favoring the activation of Akt (top). APPL1 is recruited to nascent phagosomes primarily by Rab5; the adaptor, in turn, helps Rab5 recruit two inositol 5-phosphatases that aid in elimination PtdIns(4,5)$P_2$ and possibly PtdIns(3,4,5)$P_3$ from the sealing phagosome (middle). Depletion of substrate and direct dephosphorylation forces the termination of PtdIns(3,4,5)$P_3$ synthesis, limiting the duration of the activation of Akt. In addition to recruiting APPL1, Rab5 also recruits/activates Vps34, prompting the accumulation of PtdIns(3)P. This phosphoinositide species acts to terminate the association of APPL1, which is no longer required on phagosomes that have recruited the inositol 5-phosphatases (bottom).

## MATERIALS AND METHODS

### Cell culture, transfection, and plasmids

RAW264.7 macrophages (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 medium (Wisent, St. Bruno, Canada) supplemented with 5% heat-inactivated fetal bovine serum (Wisent) at 37°C under 5% $CO_2$. For siRNA transfection, cells were trypsinized from a confluent T25 flask, spun down, and electroporated with Nucleofector Kit V (Lonza, Basel, Switzerland) according to the manufacturer's instructions. Briefly, the cell pellet was resuspended in 100 µl of Nucleofector Solution V containing 1 µM of APPL1 siRNA (L-053641; Dharmacon, Lafayette, CO), OCRL siRNA (L-064801), Inpp5B (L-057616), or scrambled siRNA (D-001810) and electroporated with program D-32 on the Nucleofector I system (Lonza). Cells were used 48 h after siRNA treatment. For cDNA transfection, cells plated on glass coverslips were transiently transfected

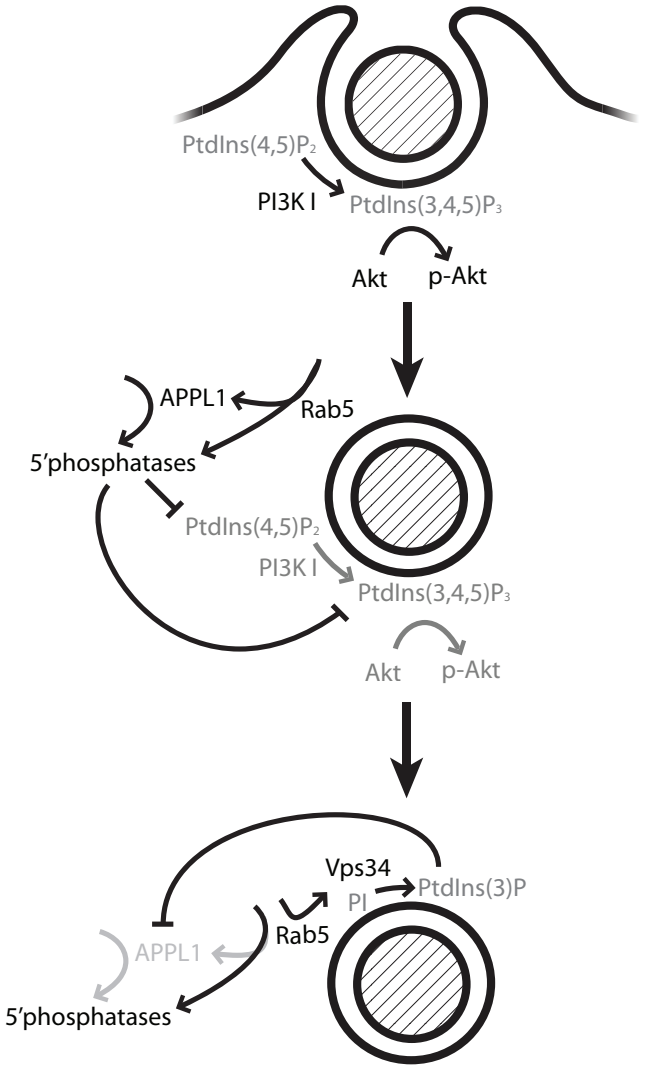


FIGURE 9: Diagrammatic representation of the role of APPL1 in phagocytosis. Top, during phagosome formation class I PI3K phosphorylates PtdIns(4,5)$P_2$ into PtdIns(3,4,5)$P_3$ and stimulates Akt phosphorylation and activation. Middle, the phagosome acquires Rab5 as it seals; Rab5 in turn recruits APPL1 and the phosphoinositide 5′-phosphatases OCRL and Inpp5B. These phosphatases dephosphorylate any remaining PtdIns(4,5)$P_2$ and PtdIns(3,4,5)$P_3$, terminating the activation signal for Akt. Bottom, Rab5 also recruits Vps34, a class III PI3K, which produces PtdIns(3)P. Accumulation of PtdIns(3)P induces the dissociation of APPL1 from maturing phagosomes.

with FuGene HD (Roche, Indianapolis, IN) according to the manufacturer's instructions. Each well of a 12-well plate was treated with 2 μg of plasmid cDNA and 6 μl of Fugene HD. Cells were generally used 12–18 h after transfection.

The plasmids encoding GFP-APPL1, RFP-APPL1, OCRL-GFP, Inpp5B-RFP, and Inpp5B-GFP were described earlier (Erdmann *et al.*, 2007; Zoncu *et al.*, 2009). The Akt1-GFP construct was generously provided by J. Brumell (Hospital for Sick Children, Toronto, Canada). The RAW264.7 macrophages stably expressing mCherry-Actin and the plasmids encoding Rab5-RFP, 2FYVE-RFP, constitutively active Rab5-GFP, dominant-negative Rab5-GFP, dominant-negative Rab5-myc, Rab7-GFP, 2PH-PLC-GFP, and PH-Gab2-GFP were described earlier (Vieira *et al.*, 2003; Bohdanowicz *et al.*, 2010). Constitutively active Rab5-mCherry and dominant-negative Rab5-mCherry were both made by subcloning the Rab5 fragment into pmCherry-C1 (Clontech, Mountain View, CA) using *Xho*I and *Bam*HI.

### Phagocytosis assays

Sheep RBCs (10% suspension; MP Biomedicals, Solon, OH) were incubated for 1 h at room temperature and constant agitation with 1:50 anti–sheep RBC IgG (MP Biomedicals). Latex beads (poly[styrene/divinylbenzene], 3.87 or 1.58 μm diameter, as indicated; Bangs Laboratories, Fishers, IN) were vigorously mixed for 1 h at 37°C with 5 mg/ml IgG from human serum (Sigma-Aldrich, St. Louis, MO). All phagocytic targets were washed with phosphate-buffered saline (Wisent) to remove excess IgG and used immediately. Where indicated, RAW264.7 macrophages were treated with 100 μM LY294002 (Enzo Life Sciences, Plymouth, PA) 5 min before phagocytosis of small beads or 1.5 min after phagocytosis of RBCs. Wortmannin and PI-103 were from Calbiochem (La Jolla, CA). When necessary, a Cy5-conjugated secondary antibody to rabbit IgG (Jackson ImmunoResearch, West Grove, PA) was used to fluorescently label the RBC. To synchronize phagocytosis, the latex beads were centrifuged onto the cells at 300 × *g* for 1 min.

### Immunoblotting and quantification

Cells were scraped into a lysis buffer (1% Nonidet P-40, 150 mM sodium chloride, 50 mM Tris, protease inhibitor cocktail [Sigma-Aldrich], 1 mM phenylmethylsulfonyl fluoride, 1 mM $Na_3VO_4$, pH 8.0) and passed through a syringe. Protein concentrations were determined using the bicinchoninic acid assay (Thermo Scientific, Waltham, MA). The samples were then mixed with a loading dye containing 5% β-mercaptoethanol, boiled for 10 min, run on a 10% SDS-polyacrylamide gel, and transferred to a nitrocellulose filter. The filter was blocked in Tris-buffered saline containing 0.1% Tween-20 and 5% bovine serum albumin. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was from Millipore (Billerica, MA; MAB374). Other antibodies were from Cell Signaling (Beverly, MA): APPL1 (3858), phospho-Ser473 Akt (4060), Akt (2938), phospho-p38 MAPK (4631), and p38 MAPK (2371). The OCRL antibody was from Sigma-Aldrich (HPA012495). After probing with horseradish peroxidase–conjugated antibody and incubating in ECL Plus (GE Healthcare, Piscataway, NJ), the immunoreactive bands were quantified using the FluorChem FC2 system and AlphaEaseFC software (version 6.0.2; Alpha Innotech, ProteinSimple, Santa Clara, CA).

### RT-PCR

RNA was extracted using the RNeasy Plus Mini-Kit (Qiagen, Valencia, CA). RT-PCR was performed using Superscript III one-step RT-PCR (Invitrogen, Carlsbad, CA) with primers for murine GAPDH (gtgcagtgccagcctcgtcc and gagagcaatgccagccccgg), OCRL (ttggagctcagcccctggc and tttgaagcttccttttcacgcgaaagc), and Inpp5B (gtaccggctggaaaatgacgcc and ctcgagctgtcgacgttagaaacgc) according to manufacturer's instructions, using 35 cycles.

### Fluorescence microscopy and quantification

Fluorescence images were captured using a spinning-disk confocal microscopy system (Quorum, Guelph, Canada) with a 63× oil immersion objective (numerical aperture 1.4) on an Axiovert 200M microscope (Carl Zeiss, Jena, Germany) equipped with diode-pumped solid-state lasers (491, 561, and 642 nm; Spectral Applied Research, Richmond Hill, Canada) and a motorized XY stage (Applied Scientific Instrumentation, Eugene, OR). Images were obtained with a back-thinned electron multiplier camera (C9100-13 ImagEM; Hamamatsu, Hamamatsu, Japan) controlled by Volocity software (version 4.1.1; PerkinElmer, Waltham, MA). Images were analyzed using the

"measure object intensity" function of Volocity (version 5.3.2) and auto-contrasted with Photoshop CS3 (Adobe, San Jose, CA). For live-cell imaging, cells were transferred to 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–buffered RPMI 1640 (Wisent). For fixed-cell imaging, cells were incubated with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 20 min at room temperature. For statistical analyses, experimental conditions were compared with control using Student's *t* tests, and multiple comparisons were accommodated with a Bonferroni correction ($p < 0.05$, two-tailed divided by the number of comparisons).


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