Microreview

Phosphoinositides and engulfment

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Summary

Cellular engulfment of particles, cells or solutes displaces large domains of plasma membrane into intracellular membranous vacuoles. This transfer of membrane is accompanied by major transitions of the phosphoinositide (PI) species that comprise the cytoplasmic face of membrane bilayers. Mapping of membrane PIs during engulfment reveals distinct patterns of protein and PI distributions associated with each stage of engulfment, which correspond with activities that regulate the actin cytoskeleton, membrane movements and vesicle secretion. Experimental manipulation of PI chemistry during engulfment indicates that PIs integrate organelle identity and orient signal transduction cascades within confined subdomains of membrane. These pathways are exploited by microbial pathogens to direct or redirect the engulfment process.

Introduction

Engulfment is the active cellular ingestion of particles, microbes or apoptotic cells by phagocytosis and of solutes by macropinocytosis. All share common component activities: distortion of plasma membrane by actin polymerization and contractility into a cup-shaped domain of plasma membrane which closes to form an intracellular vesicle or vacuole. Often, the vacuole fuses with the endolysosomal network, which includes early endosomes, late endosomes and lysosomes. Engulfment can be distinguished from other kinds of endocytic activity such as clathrin-mediated endocytosis, caveolin-mediated endocytosis and others, not only by the larger size of vesicles created by engulfment, but also by the significant involvement of the actin cytoskeleton (Kerr and Teasdale,

2009; Ravichandran, 2011; Flannagan *et al.*, 2012). Phosphoinositides (PIs) are phospholipids which localize predominantly to the cytosolic face of cellular membranes. Through reversible binding interactions with cytosolic proteins and irreversible hydrolysis reactions, PIs co-ordinate activities of the actin cytoskeleton and regulate vesicle formation and fusion between membranous compartments essential to engulfment. Many microbial pathogens manipulate the PI chemistry of engulfment to direct the formation of more hospitable vacuolar environments (Ham *et al.*, 2011), which indicates the relevance of PIs to the mechanisms of engulfment. This review examines membrane PI chemistry during the engulfment process, focusing on how plasma membrane is transformed into an intracellular vacuole.

Phosphoinositides and membrane identity

The phospholipids which form cellular membrane bilayers are mainly phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidic acid and the PIs (for a comprehensive review of phospholipids and endocytosis, see Bohdanowicz and Grinstein, 2013). The cytoplasmic leaflets of membranes involved in engulfment contain minor but significant quantities of the PI phosphatidylinositol (PtdIns), which is comprised of diacylglycerol linked to D-myo-inositol-1-phosphate by a phosphodiester linkage (Fig. 1A). The inositol hydroxyls may be reversibly altered by phosphoinositide kinases and phosphatases, generating PIs with phosphate groups in the 3, 4 or 5 positions: phosphatidylinositol 3-phosphate (PtdIns3P), PtdIns4P, PtdIns5P, phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2), PtdIns(3,4)P_2, PtdIns(3,5)P_2 and phosphatidylinositol (3,4,5)-trisphosphate (PIP$_3$). PIs can also be hydrolysed by phospholipases and the products of these irreversible reactions are important signal transduction intermediates. Phospholipase C (PLC) hydrolyses PtdIns(4,5)P_2 to form two important regulators of signal transduction: diacylglycerol (DAG), which activates protein kinase C (PKC), and InsP$_3$, which releases calcium from intracellular stores (Fig. 1B). Two hydrolases which mainly hydrolyse other phospholipids are phospholipase D (PLD) and phospholipase A2 (PLA2), which generate phosphatidic acid (PA) and arachadonic acid respectively. These
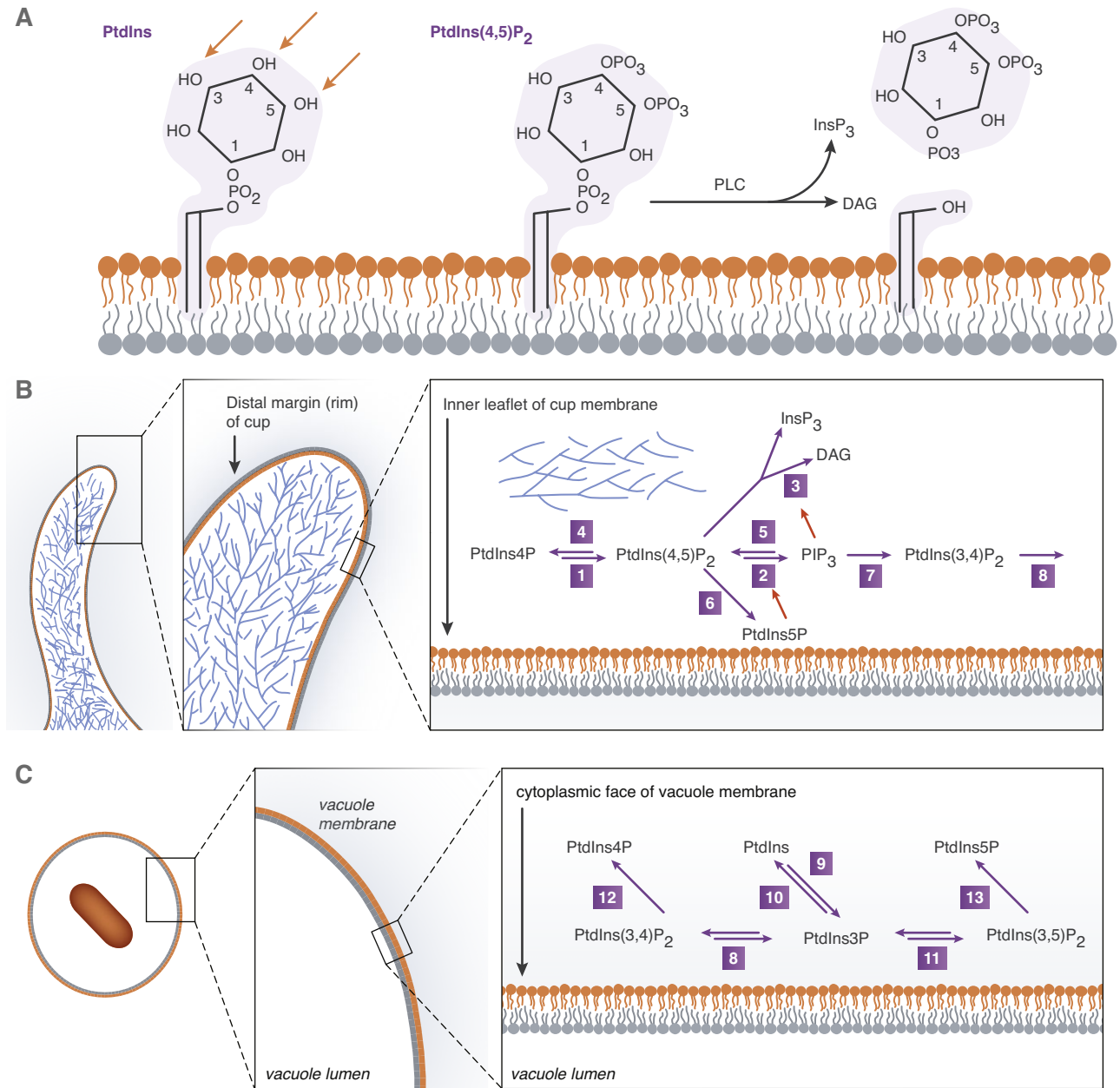
Fig. 1. Phosphoinositide structure and dynamics on membranes.

A. Schematic diagram of the structures of PtdIns, PtdIns(4,5)P_2 and the products of PLC hydrolysis. DAG is indicated by black lines, with acyl chains extending from the cytosolic surface (orange) into the bilayer. The hydroxyl groups at the 3, 4 and 5 positions of inositol (red arrows) can be phosphorylated by PI kinases and dephosphorylated by PI phosphatases.

B. Phosphoinositide dynamics in ruffles and cups. Surface extensions of plasma membrane are enriched in actin filaments (grey lines), whose polymerization and depolymerization are regulated by PIs. Significant transitions in PI profiles are indicated for the enlarged region of plasma membrane (right box). The red arrows indicate enzyme activation by PIs.

C. PI dynamics on vacuolar membranes (phagosomes and macropinosomes). The sequence of PIs appearing during macropinosome formation include PtdIns(4,5)P_2, PIP$_3$, PtdIns(3,4)P_2 and PtdIns3P. The subsequent transition to PI(3,5)P_2 on membranes has not been demonstrated directly. Microbial pathogens drive engulfment or modify vacuole maturation using PI-modifying enzymes.

(1) PtdIns4P 5-kinase, (2) PI 3-kinase type I, (3) PLCγ1, (4) OCRL and Inpp5B, (5) PTEN, (6) PtdIns-4,5-bisphosphate 4-phosphatase, *Salmonella* SopB and *Shigella flexneri* IpgD, (7) SHIP1 and SHIP2, (8) Inpp4B, (9) PI 3-kinase type III, (10) myotubularin and *Legionella* SidP, (11) PYKEfyve, (12) *Legionella* SidF, (13) *Legionella* SidP.

hydrolases can be regulated by PIs or by enzymes which modify PIs (Bohdanowicz and Grinstein, 2013).

Imaging of PIs and related protein activities during phagocytosis or macropinocytosis reveals the dynamics that maintain steady-state membrane identities. Many proteins involved in signal transduction or organelle trafficking contain structural domains which bind to PIs with high specificity. For example, the pleckstrin homology (PH) domain of the kinase Akt (AktPH) binds with high affinity to $PtdIns(3,4)P_2$ and PIP_3 (Haugh *et al.*, 2000) and the PH domain of PLC-δ binds specifically to $PtdIns(4,5)P_2$ (Rameh *et al.*, 1997; Kavran *et al.*, 1998). Fluorescent protein (FP) chimeras of PI-binding domains, such as green fluorescent protein (GFP)-AktPH, can localize the target species of PIs when expressed in living cells. When combined with other fluorescent probes for labelling compartments, they can be used to map the subcellular distributions of PIs. These and other methods have revealed that plasma membrane and the membranes of various endocytic compartments have distinct and characteristic profiles of PI species. Plasma membranes are enriched for $PtdIns(4,5)P_2$ and PtdIns4P and sometimes also the 3'phosphoinositides (3'PIs) PIP_3, PtdIns3P, $PtdIns(3,4)P_2$ or $PtdIns(3,5)P_2$ (Varnai *et al.*, 1999; Haugh *et al.*, 2000). The Golgi apparatus, some secretory vesicles and regions of endoplasmic reticulum are enriched for PtdIns4P and endocytic compartments contain 3'PIs in different proportions (Di Paolo and De Camilli, 2006). The PI profiles of different membrane surfaces within the cell restrict the subcompartments where soluble proteins can bind, or where transmembrane proteins become activated, and thereby define the chemical reactions that occur on those surfaces and the kinds of organelles that can interact with those surfaces (Bohdanowicz and Grinstein, 2013). Thus, the apparently static and uniform chemical identities of plasma membrane and endocytic organelles represent the steady-state distributions of the substrates and products of localized PI-modifying activities and the selective fission and fusion between compartments (Munro, 2004).

PI metabolism is also essential for signal transduction at the plasma membrane. Signalling by growth factor receptors and hormones initiates changes in PI chemistry which shape and propagate signals (Balla, 2013). The changes in PIs which follow receptor signalling are also evident during engulfment. The plasma membrane increases concentrations of $PtdIns(4,5)P_2$ in ruffles and cup-shaped extensions of the cell surface (Botelho *et al.*, 2000). As the cup begins to close, concentrations of $PtdIns(4,5)P_2$ in cup membranes decline and concentrations of PIP_3 and DAG increase (Botelho *et al.*, 2000) (Fig. 1B). After the cup closes into a phagosome or macropinosome, the levels of PIP_3 and DAG decrease and PtdIns3P levels increase (Vieira *et al.*, 2001; Henry

et al., 2004) (Fig. 1C). Continued maturation of the vacuole or merger of the vacuole with late endosomes replaces PtdIns3P with $PtdIns(3,5)P_2$. Thus, each morphogenetic stage of engulfment can be considered a transiently stable state, which is integrated by lateral diffusion of phospholipids over large domains of membrane and which transitions to other stable states by a limited set of vectorial reactions (Swanson, 2008; Welliver and Swanson, 2012).

Phagocytosis

Phagocytosis is usually initiated by receptors in the plasma membrane of the phagocytic cell, which bind to ligand molecules on particle surfaces and stimulate the movements of engulfment (Flannagan *et al.*, 2012). Often this begins with the formation of actin-rich, cell surface ruffles which capture the target particle (Flannagan *et al.*, 2010). Fcγ receptor (FcR)-mediated phagocytosis creates a tightly adherent phagocytic cup which extends over a particle surface coated (opsonized) with IgG, a ligand for FcR (Fig. 2A). As the cup advances over the surface, submembranous actin is organized into a tight cuff surrounding the particle, with actin filaments growing at the forward edge, contracting by the action of myosin molecules, and disassembling at the rear (Hoppe and Swanson, 2004; Scott *et al.*, 2005). At the same time, membrane from intracellular vesicles is inserted into the forming cup (Bajno *et al.*, 2000; Braun *et al.*, 2004). These very different activities of actin filaments polymerizing, then depolymerizing, and vesicle fusion suggest that FcR signal transduction during phagocytosis is organized spatially and temporally. How does activation of a single kind of receptor control such different activities?

Much of this complex behaviour is organized by membrane phospholipids. Binding to IgG leads to FcR clustering, which in turn activates receptor tyrosine phosphorylation by Src-family kinases (Fitzer-Attas *et al.*, 2000). This is followed by recruitment and activation of the kinase Syk (Crowley *et al.*, 1997), the recruitment of adapter proteins (Gu *et al.*, 2003; Lee *et al.*, 2007) and the activation of lipid-modifying enzymes, including phosphatidylinositol 3-kinase (PI 3-kinase) and PLCγ1 (Liao *et al.*, 1992). The Rho-family GTPases Cdc42, Rac1 and Rac2 are activated (Hoppe and Swanson, 2004), as well as the GTPases Arf1 (Beemiller *et al.*, 2006), Arf6 (Zhang *et al.*, 1998; Niedergang *et al.*, 2003) and PKC-ϵ (Larsen *et al.*, 2002). Other regulatory proteins are necessary for phagocytosis, including the phospholipid-modifying enzymes PtdIns4P 5-kinase-γ (PI4P5Kγ; Mao *et al.*, 2009) and PI4P5Kα (Coppolino *et al.*, 2002), PLD1 and PLD2 (Iyer *et al.*, 2004) and PLA2 (Lennartz *et al.*, 1997). The mechanisms of their activation by FcR are not fully known. PLD-generated PA activates PI4P5K, which
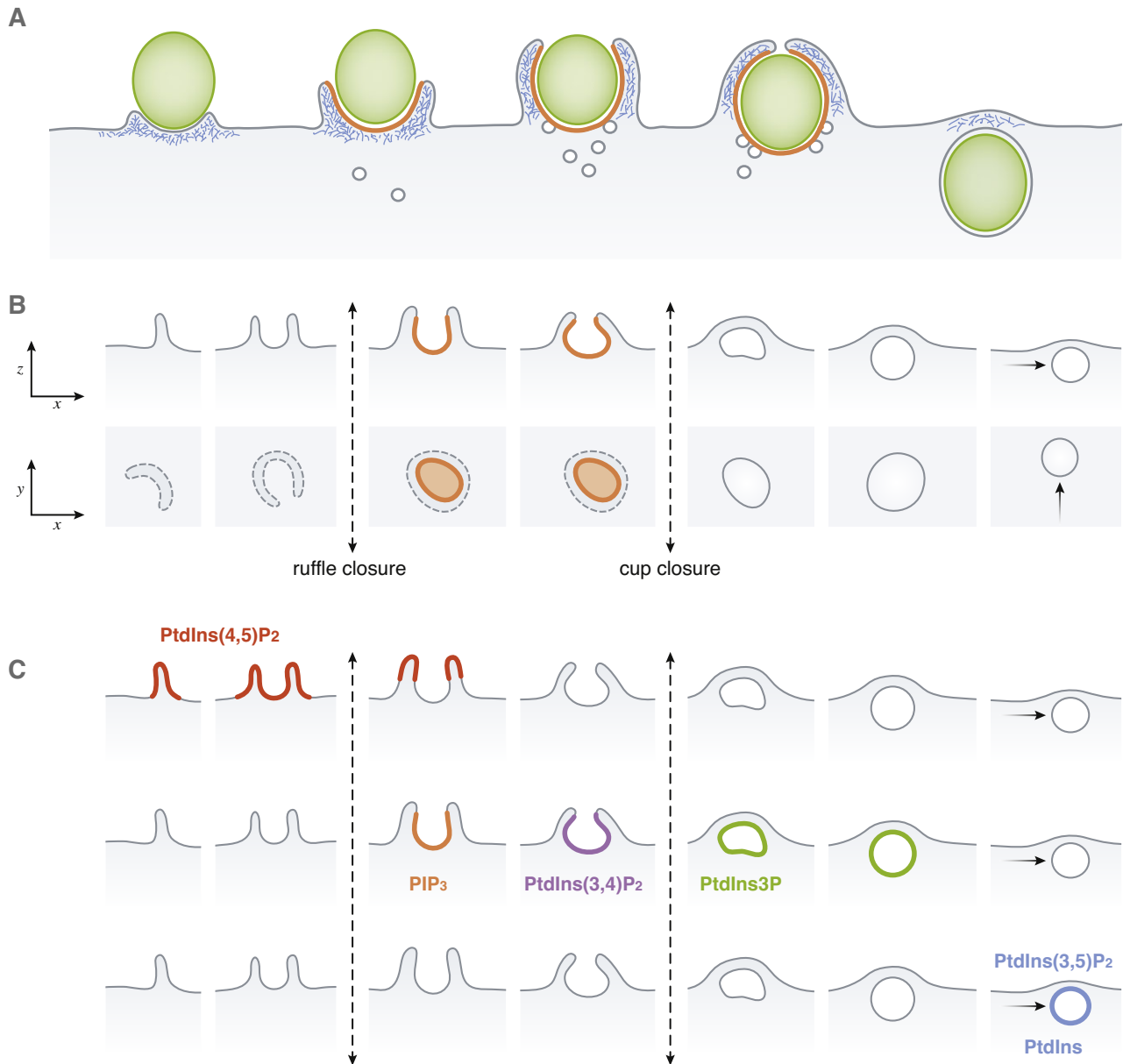
Fig. 2. Cellular movements of engulfment.

A. The cytoplasmic movements of FcR-mediated phagocytosis. The sequence left to right shows the movements of membranes during phagocytosis of a particle (green ovals). Actin filaments (grey) form a contractile cuff which advances over the particle. Membrane from intracellular organelles is inserted into the forming phagosome. Orange lines indicate plasma membrane domains with limited lateral mobility of PIs due to diffusion barriers in cup structure.

B. Stereotypical movements of macropinocytosis. The top row shows a progression, left to right, of side-view projections (*x*–*z*) of macropinocytic cup formation (ruffle closure) and membrane scission to form an intracellular macropinosome (cup closure). The second row shows the corresponding images as typically seen by light microscopy (*x*–*y* projection). Dotted lines indicate ruffles and cups in plasma membrane. Solid lines denote discrete membrane compartments. Orange lines indicate regions of plasma membrane where lateral mobility of inner leaflet molecules is constrained by diffusion barriers.

C. Dynamics of PIs during macropinosome formation. The top row shows the distributions of PtdIns(4,5)P$_2$ (red lines) which localizes to plasma membrane and is enriched in ruffles and very early cups. The middle row shows the stages of macropinosome formation which have maximal labelling with probes for PIP$_3$ (orange), PtdIns(3,4)P$_2$ (violet) and PtdIns3P (green). The bottom row indicates speculated synthesis of PtdIns or PtdIns(3,5)P$_2$, which have not been demonstrated by microscopy.

activates Arf6 (Honda *et al.*, 1999), promoting the early movements.

PtdIns(4,5)P₂ organizes the early movements of engulfment

Imaging of signalling during FcR-mediated phagocytosis revealed at least two stages of engulfment: early and late. One early event is the activation of PI4P5Kα and PI4P5Kγ, which generate PtdIns(4,5)P₂ from PtdIns4P. PtdIns(4,5)P₂ concentrations increase in cell surface ruffles and in early phagocytic cups (Botelho *et al.*, 2000). The increased concentrations of PtdIns(4,5)P₂ in plasma membrane stimulate actin polymerization by activating Cdc42 and Rac, which activate Wiscott-Aldrich Syndrome protein (WASP) and (WASP)-family verprolin homologous proteins 1/2 (WAVE1/2) respectively (Miki *et al.*, 1998; Park and Cox, 2009). WASP and WAVE1/2 bind to Arp2/3 and stimulate actin polymerization (May *et al.*, 2000). Fluorescence resonance energy transfer (FRET)-based microscopic methods for localizing GTPase activities during phagocytosis determined that Cdc42, Arf6 and Rac1 are active (i.e. in their GTP-bound configurations) in ruffles and the advancing edge of the phagocytic cup, indicating that signals which generate and respond to PtdIns(4,5)P₂ are active in the early stages of phagocytosis (Hoppe and Swanson, 2004; Beemiller *et al.*, 2006).

PtdIns(4,5)P₂ is depleted from the cup membranes even before the cup closes into the cell (Botelho *et al.*, 2002). PtdIns(4,5)P₂ is removed by three distinct enzyme activities which are necessary for phagocytosis: hydrolysis by PLCγ1 to DAG and IP₃ (Botelho *et al.*, 2000), phosphorylation by PI-3-kinase to form PIP₃ (Araki *et al.*, 1996) and, to a lesser extent, dephosphorylation by the PtdIns5P phosphatases, oculocerebrorenal syndrome of Lowe protein (OCRL) and inositol polyphosphate 5-phosphatase B (Inpp5B), which are activated by the GTPase Rab5a (Bohdanowicz *et al.*, 2012) (Fig. 1B). Loss of PtdIns(4,5)P₂ from the cup membrane facilitates removal of actin, which may help to maintain the advancing actin cuff (Scott *et al.*, 2005). Consistent with this model, imaging showed that DAG, PIP₃ and PI(3,4)P₂ concentrations increased in cup membranes as the PtdIns(4,5)P₂ was depleted (Botelho *et al.*, 2000; Vieira *et al.*, 2001; Hoppe and Swanson, 2004). Thus, as the cup advances over the particle, PtdIns(4,5)P₂ is elevated at the front of the actin cuff and depleted at the rear.

Another pathway for the activation of Rac during phagocytosis could involve the localized synthesis of PtdIns5P, which was recently shown to increase circular ruffle formation through activation of the Rac guanine nucleotide exchange factor (GEF) Tiam1 and consequent activation of Rac (Viaud *et al.*, 2014). PtdIns5P is synthesized via phosphorylation of PtdIns by the PI 5-kinase PYKfyve (Shisheva, 2012) or via dephosphorylation of PtdIns(4,5)P₂ by PtdIns-4,5-bisphosphate 4-phosphatases (Niebuhr *et al.*, 2002; Ungewickell *et al.*, 2005). A direct role for PtdIns5P in phagocytosis has not been demonstrated, although the synthesis of PtdIns5P by the *Shigella flexneri* effector protein IpgD is required for invasion of epithelial cells (Niebuhr *et al.*, 2002) (see below).

3′PIs organize late stages of engulfment

Concentrations of PIP₃ and PtdIns(3,4)P₂ increase on phagocytic cup membranes and remain elevated until after the phagosome closes into the cell (Marshall *et al.*, 2001; Vieira *et al.*, 2001; Kamen *et al.*, 2007). In macrophages, PI 3-kinase inhibition does not stop ruffling or cup formation, but prevents cup closure into phagosomes, indicating that PI 3-kinase is necessary for the contractile activities that close cups into phagosomes (Araki *et al.*, 1996). The exact functions of PI 3-kinase during FcR-mediated phagocytosis are not fully known but, overall, its activity in phagocytic cups organizes many signals associated with engulfment. PIP₃ activates PLCγ1 (Falasca *et al.*, 1998), myosin X-based contractile activities (Cox *et al.*, 2002), the GEFs which activate Rac and the GTPase activating proteins (GAPs) which inactivate Cdc42 (Beemiller *et al.*, 2010). FRET microscopy showed that, as cups develop, the GTPases Cdc42, Arf6 and Rac1, are activated early and remain active in the advancing edge, and Rac2 and Arf1 become active later, at the cup base and during phagosome closure (Hoppe and Swanson, 2004; Beemiller *et al.*, 2006). When GTPase activities were imaged during phagocytosis in the presence of PI 3-kinase inhibitors, which stop phagocytosis of large particles midway through the process, the actin-rich cups which could not close around the particles contained persistent early signals (Cdc42, Rac1 and Arf6), and the late signals (Rac2 and Arf1) were not activated. This indicated that PIP₃ generated in cup membranes is necessary for the signal transition from the early GTPases which regulate actin polymerization to the late GTPases which regulate phagosome closure. This suggests that PIP₃ or DAG in cup membranes activates the GAPs which turn off the early GTPases Arf6 and Cdc42 and the GEFs which activate the late-stage GTPases Arf1 and Rac2. Consistent with this model of sequential signalling, phagocytosis requires cyclical activation and deactivation of Cdc42. Cdc42 activates PI 3-kinase (Zheng *et al.*, 1994) and the PI 3-kinase product PIP₃ deactivates Cdc42 (Beemiller *et al.*, 2010).

The requirement of PI 3-kinase for cup closure and transitions to later stages of signalling suggests that PIP₃ in cup membranes must exceed some concentration threshold level for phagocytosis to proceed. Zhang *et al.*

(2010) examined the relationship between PIP_3 concentrations and phagosome progression by developing quantitative microscopic methods to measure the magnitudes of signals generated in phagocytic cups as a function of ligand (IgG) density on particle surfaces. When macrophages ingested microspheres opsonized with high densities of IgG, phagosomes recruited the chimeras yellow fluorescent protein (YFP)-actin, YFP-Syk (an early signal associated with ligated Fc receptors), YFP-AktPH (indicating PIP_3 and $PtdIns(3,4)P_2$) and YFP-PKCε. Recruitment of YFP chimeras occurred in a distinct sequence of early (YFP-Syk, YFP-actin), middle (YFP-AktPH) and late (YFP-PKCε) signals. Phagocytic cups containing low IgG-density microspheres often stalled without completing phagocytosis. These stalled phagocytic cups recruited early signal reporters YFP-Syk and YFP-actin and low levels of YFP-AktPH, indicating that low concentrations of PIP_3 were generated in the phagosomes. The late signal YFP-PKCε did not appear. Overall, recruitment of YFP-chimeras for early signals (Syk, actin, PIP_3) was proportional to IgG density, whereas YFP-PKCε recruitment was all-or-none, indicating that suprathreshold concentrations of PIP_3 in cup membranes (or of the $PtdIns(3,4)P_2$ or DAG which appear after PIP_3) were required for the appearance of late signals and for completion of phagocytosis. Consistent with this model, cells engaging high IgG-density microspheres in the presence of PI 3-kinase inhibitors made cups with YFP-Syk and YFP-actin, but without YFP-AktPH or YFP-PKCε.

Thus, the movements of FcR-mediated phagocytosis can be described as early, reversible exploratory behaviour, governed by $PtdIns(4,5)P_2$, and later commitment stages. What is distinct about commitment to late signals? Early signals are governed by largely reversible PI phosphorylation and dephosphorylation reactions, whereas late signals require irreversible hydrolase activities.

Macropinocytosis

Macropinosome formation is analogous to phagocytosis. Nearly all eukaryotic cells make macropinosomes, often in response to growth factors (Kerr and Teasdale, 2009). Immature dendritic cells and cells transformed by K-Ras or v-Src form macropinosomes constitutively (Bar-Sagi and Feramisco, 1986; Amyere *et al.*, 2000).

The cellular movements leading to macropinosome formation are irregular in morphology and timing (Mercer and Helenius, 2009). In a macrophage stimulated by its growth factor, construction of a 3 μm diameter macropinosome takes about 3 min (Yoshida *et al.*, 2009). The process begins with the formation of ruffles which extend and curve into circular profiles (a.k.a. ruffle closure; Fig. 2B). These 1–6 μm diameter, crater-like subdomains of plasma membrane called macropinocytic cups either recede inconsequentially or close at their distal margins to form a discrete intracellular vesicle, the macropinosome (a.k.a. cup closure) (Yoshida *et al.*, 2009). The macropinosome then either fuses with other endocytic compartments, which may include early endosomes, late endosomes, lysosomes or other macropinosomes, or recycles to the plasma membrane without further maturation. In cells transformed by v-Src, macropinosome formation requires the sequential activities of PI 3-kinase and PLC (Amyere *et al.*, 2000). In macrophages, PI 3-kinase inhibitors do not affect cell surface ruffling or circular ruffle formation, but instead inhibit cup closure into macropinosomes (Araki *et al.*, 1996).

The PI profile of membranes changes during macropinosome formation. $PtdIns(4,5)P_2$ concentrations increase early (Araki *et al.*, 2007), followed by increases in PIP_3 and PtdIns3P (Araki *et al.*, 2006). Temporal ordering of PIs relative to the stages of macropinosome formation in macrophages revealed a sequence of PI transitions in cups (Araki *et al.*, 2007; Yoshida *et al.*, 2009; Welliver and Swanson, 2012) (Fig. 2C). $PtdIns(4,5)P_2$ levels peaked transiently following ruffle closure, then decreased. PIP_3 and DAG levels then increased for about 30 s and were followed by transient increases in $PtdIns(3,4)P_2$ and PtdIns3P, similar to the sequence of PIs seen in phagocytic cups. PI-modifying phosphatases associated with these transitions have been implicated in circular ruffle formation and macropinocytosis. SHIP2, which dephosphorylates PIP_3 to $PtdIns(3,4)P_2$, is required for circular ruffle formation (Hasegawa *et al.*, 2011). A mutant screen using *Caenorhabditis elegans* cells identified the PtdIns3P phosphatase myotubularin-related protein-6 (MTMR6) and its associated protein myotubularin-related protein-9 (MTMR9) as necessary for macropinosome formation (Maekawa *et al.*, 2014). Additionally, Maekawa, *et al.* showed a requirement for inositol polyphosphate 4-phosphatase type II (Inpp4B), which dephosphorylates $PtdIns(3,4)P_2$ to PtdIns3P, supporting a model in which macropinosome formation requires a sequential progression of 3′PIs in macropinocytic cups similar to the sequence delineated by microscopy (Welliver and Swanson, 2012). These schemes may not be universal, however. In *Dictyostelium discoideum*, although PIP_3 localizes to phagocytic and macropinocytic cups (Mercanti *et al.*, 2006), the two activities are differentially regulated by PI 3-kinase and PLC (Cardelli, 2001).

Mechanisms

Physical isolation of cup domains in plasma membrane

As mentioned above, the phospholipid composition of the cup membrane differs from plasma membrane even before

the phagosome or macropinosome separates into cytoplasm (Fig. 2A and B). The lateral segregation of membrane PIs is especially pronounced during phagocytosis of large (8 μm diameter) microspheres (Botelho *et al.*, 2000). *D. discoideum* amebae have high concentrations of PIP_3 in phagocytic and macropinocytic cups, with none in the contiguous plasma membrane (Mercanti *et al.*, 2006). Sharp boundaries localized to the edges of the cups delineate the two domains. The boundaries appear to be due to structural barriers to lateral diffusion of phospholipids. Analysis of $PtdIns(4,5)P_2$ dynamics in phagocytic cups identified barriers intrinsic to the cup wall structure that limited diffusion of $PtdIns(4,5)P_2$ from the cups into the contiguous plasma membrane (Golebiewska *et al.*, 2011). Similar barriers to lateral diffusion were identified in macropinocytic cups, using membrane-tethered photoactivatable GFP probes (Welliver *et al.*, 2011). The molecular nature of these diffusion barriers remains unknown, but the fact that lateral diffusion of PIs is limited by cup structure suggests that the cup can function as a crucible for signal amplification. That is, the physical structure of a cup may provide an essential component for positive feedback amplification of PI-dependent signalling.

Size-dependent requirements for PI3K

FcR signalling can be triggered by IgG on many different kinds of surfaces, and the requirement of PI 3-kinase for cellular responses varies with the geometry of the surface. Notably, although PI 3-kinase inhibitors block phagocytosis of microspheres larger than 3 μm, ingestion of smaller microspheres is not affected. Rod-shaped particles coated with IgG are not ingested unless they contact the cell at their narrow, highly curved ends (Champion and Mitragotri, 2006). Likewise, filamentous bacteria opsonized with IgG are internalized only when the macrophage locates a free end. It then builds an actin cuff that advances along the length of the filament, pulling it into the cell and forming an elongated phagocytic cup (Prashar *et al.*, 2013). The prolonged phagocytic response needed to ingest a long filament is not inhibited by PI 3-kinase inhibitors (Prashar *et al.*, 2013).

What explains the particle size-dependence of PI 3-kinase inhibition? One possible mechanism is that small particles are ingested before late signals appear, rendering the late signals unnecessary. However, early and late GTPase signals, as well as PIP_3, also appear on phagosomes that internalize smaller microspheres (Beemiller *et al.*, 2010). Another possibility is that small particles can be ingested by a PI 3-kinase-independent mechanism that cannot work on larger particles. Clathrin-mediated endocytosis could be a candidate for such a mechanism. So far, however, no studies have identified a pathway which preferentially inhibits engulfment of small

but not large particles. Another possibility is that PI 3-kinase is required for the actin turnover necessary for the advance an actin cuff over large particles. However, PI 3-kinase inhibitors do not block engulfment of long filaments, whose ingestion is sometimes quite prolonged (Prashar *et al.*, 2013). Thus, a PI 3-kinase-independent phagocytosis internalizes small microspheres and elongated filaments, which suggests that 3′PIs are only required for the ingestion of wide loads.

How could cup width affect the cell's ability to complete phagocytosis? Particle size and shape do not measurably limit actin polymerization or early signals of phagocytosis, but they may influence the ability of the cup to generate the suprathreshold concentrations of PIP_3 necessary for transitions to late stages of signalling. Accordingly, a phagocytic cell may use actin-based motility to explore particle surfaces and only initiate a full phagocytic response (i.e. late signals) when surface dimensions or ligand densities are right. PIP_3 synthesized in cup membranes may override a brake that prevents the ingestion of large particles. The phagocytosis of large particles entails a considerable shift in organelle dimensions; cup size and 3′PI concentration thresholds may be related to mechanisms that regulate cellular dimensions. Accordingly, the concentrations of PIP_3 that can be generated in cup membranes may be influenced by a cell's size or its capacity for increasing its volume through engulfment.

Invasion

Many pathogenic microbes manipulate PI chemistry to their advantage. Some inhibit engulfment by actively inhibiting the signal transduction of phagocytosis (Ham *et al.*, 2011). Others trigger phagocytosis as a mechanism for establishing intracellular vacuolar environments. *Yersinia entercolitica* invades epithelial cells by an integrin-mediated engulfment that requires Rac and Arf6-dependent activation of PI4P5K (Wong and Isberg, 2003). $PtdIns(4,5)P_2$ accumulates in the forming phagosome and is necessary for invasion, but phagosome closure requires removal of $PtdIns(4,5)P_2$ from the cup membranes, which is mediated by the PtdIns 5-phosphatase activities of OCRL and Inpp5b (Sarantis *et al.*, 2012). *Listeria monocytogenes* invades epithelial cells by an unusual process that requires PI 3-kinase (Ireton *et al.*, 1996), PI 4-kinase (Pizarro-Cerda *et al.*, 2007) and clathrin (Veiga and Cossart, 2005), indicating yet another novel mechanism of engulfment. *Salmonella enterica* var. Typhimurium enters epithelial cells by stimulating host cell ruffling and capture by macropinosomes (Francis *et al.*, 1993). A *Salmonella* type III secretion system (TTSS) delivers effector proteins into the host cell cytoplasm, including the PtdIns-4,5-bisphosphate 4-phosphatase SopB (also known as SigD) (Mason *et al.*, 2007; Mallo *et al.*, 2008). SopB pro-

motes increased PIP$_3$ and PtdIns(3,4)P$_2$ in ruffles (Mallo *et al.*, 2008), phagosome closure by depletion of PtdIns(4,5)P$_2$ (Terebiznik *et al.*, 2002) and the synthesis of PtdIns3P on *Salmonella*-containing vacuoles. Depletion of phagosomal PtdIns(4,5)P$_2$ by SopB increases recruitment of Rab5, which activates the type III PI 3-kinase Vps34 to generate PtdIns3P (Mallo *et al.*, 2008). Like *Salmonella*, *S. flexneri* uses a TTSS to deliver a PtdIns-4,5-bisphosphate 4-phosphatase, IpgD, into host cells (Niebuhr *et al.*, 2002). The IpgD product PtdIns5P activates PI 3-kinase (Pendaries *et al.*, 2006) and stimulates Tiam-1 and Rac-dependent ruffling (Viaud *et al.*, 2014). Thus, SopB and IpgD may drive the engulfment of bacteria both through localized synthesis of PtdIns5P, activating PI 3-kinase and Rac, and localized depletion of PtdIns(4,5)P$_2$, leading to Rab5 recruitment, activation Vps34 and increased levels of PtdIns3P necessary for vacuole maturation.

Some effector enzymes modify PIs of the cytosolic surface of the vacuole after engulfment (Hilbi, 2006). *Legionella pneumophila* enters macrophages by a macropinocytosis-like mechanism (Watarai *et al.*, 2001), then uses a type four secretion system (TFSS) to deliver effector proteins which modify the intracellular vacuole (Haneburger and Hilbi, 2013). Imaging of PIs during infection of *D. discoideum* with *L. pneumophila* indicated that the *Legionella*-containing vacuole (LCV) forms and matures by mechanisms similar to those described for macropinocytosis or FcR-mediated phagocytosis (Weber *et al.*, 2013): PIP$_3$ localizes to forming phagosomes, PtdIns(4,5)P$_2$ is stripped from phagosome and PtdIns3P appears on the closed phagosome. Then, in a manner dependent on the TFSS, PtdIns4P accumulates on the LCV over the next several hours. The *Legionella* TFSS effector proteins SidF and SidP are phosphoinositide 3-phosphatases which modify the PI profile of LCV membranes and potentially inhibit LCV fusion with lysosomes (Hsu *et al.*, 2012; Toulabi *et al.*, 2013).

Perspective

Although PIs are clearly necessary for engulfment, universal organizing principles are scant. It seems certain that the different movements that underlie engulfment require different PIs and that transitions of membrane PI chemistry organize transitions in cytoskeletal activities. Actin polymerization requires generation of PtdIns(4,5)P$_2$ or PtdIns5P. PIP$_3$ and PtdIns(3,4)P$_2$ can facilitate actin dynamics and determine thresholds for commitment to late stages of engulfment, yet their requirements vary with the target particle geometry and with other conditions in the engulfing cells. Completion of engulfment requires the removal of PtdIns(4,5)P$_2$ from cup membranes, but may also require the DAG derived from PtdIns(4,5)P$_2$. Refined mechanistic understanding of how PIs organize the movements of engulfment may reveal an essential chemical cascade. Alternatively, we may eventually recognize that PIs organize engulfment by many different routes.

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