The coordination of signaling during Fc receptor-mediated phagocytosis

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Abstract: Phagocytosis by macrophages can be initiated by Fcγ receptors (FcR) in membranes that bind to Fc regions of immunoglobulin G (IgG). Activated FcR transduce signals to cytoplasm, which regulate the internalization of IgG-coated particles into plasma membrane-derived vacuoles, phagosomes. Particles internalized by phagocytosis are much larger than FcR, which prompts questions of if and how the receptors are coordinated with each other. FcR-mediated signal transduction entails recruitment of proteins from cytoplasm to the receptor, largely via protein phosphorylation. These FcR signaling complexes then activate proteins that regulate actin, myosin, membrane fusion, and the production of reactive oxygen intermediates. Recent fluorescence microscopic studies of phagocytosis in macrophages indicate that signaling by FcR occurs as a sequence of distinct stages, evident in the spatial and temporal patterns of phosphoinositides, protein kinase C, and Rho-family GTPase activation on forming phagosomes. The coordination of these stages may be regulated by lipids or lipid-anchored proteins, which diffuse away from FcR complexes. Lateral diffusion of FcR-derived signals could integrate FcR-dependent responses over large areas of membrane in the forming phagosome. *J. Leukoc. Biol.* 76: 1093–1103; 2004.

Key Words: *macrophage · actin · PI-3 kinase · Rac · Cdc42*

INTRODUCTION

Phagocytosis is not as simple as it appears. Time-lapse movies of macrophages internalizing erythrocytes need little explanation, even for small schoolchildren: The big cell eats the little cell in one smooth gulp. That it does so by actin-based protrusion of cell-surface membrane around the prey, followed by closure into an intracellular vacuole, is all consistent with first impressions. However, the cell biologist is hard-pressed to explain in molecular terms how signaling from a single kind of receptor produces this graceful of sequence events. Compared with the receptors, the particles internalized by phagocytosis in macrophages are immense. Here, we address the question of how molecular scale processes are integrated to coordinate micron scale events inside cells. We review how Fcγ receptors (FcR) in macrophages generate signals that regulate phagocytosis of immunoglobulin G (IgG)-coated particles. Recent microscopic studies indicate that phagocytosis is a series of transitions between distinct stages of FcR signaling. We propose that these stages are coordinated by lateral diffusion of molecules in the membrane of the forming phagosome.

WHAT HAPPENS DURING FcR-MEDIATED PHAGOCYTOSIS?

When a small particle or erythrocyte is coated (opsonized) with IgG, the Fc regions of the IgG molecules may bind to FcR in the macrophage plasma membrane and initiate a phagocytic response. Cup-shaped folds of plasma membrane extend outward from the macrophage around the particle and constrict at its distal margin, closing within 7 min into a plasma membrane-derived phagosome (see Fig. 1). During the next hour, interactions between the phagosome and other membranous organelles change its internal and surface chemistries in a maturation process that typically leads to degradation of the phagosome contents by acid hydrolases.

Movements of the underlying actin cytoskeleton drive extension of the phagocytic cup, most likely through a combination of localized actin polymerization, actin depolymerization, and myosin-based contractions [1]. In a macrophage containing, fluorescently labeled actin, such as rhodamine actin or chimeras of actin plus green or yellow fluorescent protein (actin-GFP, actin-YFP), fluorescent actin concentrates in the phagocytic cup and appears to advance over the opsonized erythrocyte as a circumferential band, which visibly squeezes the erythrocyte [2–4]. This squeezing activity reflects the interactions between actin and myosin, as inhibitors of myosin light chain kinase, which facilitates myosin contraction, inhibit squeezing [2].

Intracellular, membranous compartments also contribute to phagosome formation in essential but still poorly understood ways [5, 6]. Inhibitors of membrane fusion inhibit phagocytosis of larger particles, indicating a requirement for delivery of membrane from internal stores [7]. However, it is not known how membrane flows in the forming phagosome, where internal vesicles are delivered, or even which intracellular compart-

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ments contribute membrane. Recent work has indicated potential sources for this membrane, including recycling endosomes [6] and endoplasmic reticulum [8].

Sometime during the process, the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex is activated to deliver reactive oxygen intermediates (ROIs) into the phagosome by producing superoxide from the oxidation of NADPH and reduction of molecular oxygen. The complex consists of two membrane proteins, gp91phox and p22phox (a.k.a., cytochrome b_{558}), and four soluble proteins (p40phox, p47phox, p67phox, and Rac1 or Rac2) [9, 10]. In neutrophils, FcR signaling initiates delivery of membranes containing cytochrome b_{558} into the phagosome, recruitment of the soluble proteins from the cytoplasm, and activation of the functional complex [9, 11].

FcR AND ASSOCIATED PROTEINS

FcR are members of the immunoreceptor class of receptor tyrosine kinases. These receptors contain within their cytoplasmic tails or in associated subunits the immunoreceptor tyrosine-based activation motif (ITAM). Clustering of the FcR by IgG-opsonized particles induces phosphorylation of tyrosines within the ITAM motifs and initiates assembly of a complex of proteins around the FcR on the cytoplasmic side of the membrane [12]. Lyn and Hck, members of the Src family of tyrosine kinases, phosphorylate the ITAM motifs following clustering [13]. The phosphorylated ITAMs then recruit Syk kinase. Syk signaling is required for efficient phosphorylation of the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI-3K) [14], which activates the PI-3K catalytic subunit p110β [15], generating PI-3,4,5-trisphosphate [$PI(3,4,5)P_3$] from PI-4,5-bisphosphate [$PI(4,5)P_2$] in the membrane near the receptor complex. Other proteins recruited to the FcR complex include the protein phosphatase Src homology 2 (SH2)-containing tyrosine phosphatase-1 [16, 17], Gab2 [18], SH2 domain-containing leukocyte phosphoprotein of 76 kDa [19], Cbl, Nck [20], and the 5′ PI phosphatase, SH2 domain-containing inositol phosphatase 1 (SHIP-1) [17, 21].

FcR are ubiquitylated in response to cross-linking, and this modification is necessary for receptor-mediated endocytosis of soluble immune complexes but not phagocytosis of particles [22]. It has not been determined whether the FcR are ubiquitylated during phagocytosis.

PROTEINS DOWNSTREAM OF THE FcR COMPLEX

Most of the proteins associated with FcR-mediated phagocytosis are not bound directly to the FcR but are instead activated by recruitment to nearby membrane. Much has been learned about the biochemistry of actin polymerization, actin depolymerization, myosin regulation, vesicle fusion, and assembly of the NADPH oxidase, and many of the essential elements of these regulatory systems contribute to phagocytosis. More details about these molecules can be found in reviews about actin in phagocytosis [1, 23], membranes in phagocytosis [24], small GTPases in phagocytosis [25], and the NADPH oxidase [26]. Here, we list selected components that may mediate the major activities. Proteins implicated in the regulation of actin polymerization include Cdc42, Rac [27, 28], Wiscott-Aldrich syndrome protein (WASP) [29], Arp2/3 [30], WASP family verprolin homologous protein (WAVE) [30], ezrin [31, 32], vasodilator-stimulated phosphoprotein [19], coronin [33], phospholipase C (PLC) [34], and P21-activated kinase-1 (PAK1) [35]. Contractile proteins and their regulators include myosins IC, II, V, IXb [31, 36], and X [37], RhoA [38], and myosin light-chain kinase [2]. Proteins that regulate membrane fusion or fission include dynamin 2 [39], amphiphysin IIm [40], Arf6 [41, 42], Rab5 [43, 44], Rab11 [45], and PLA_2 [46]. Proteins that may affect the assembly and activation of the NADPH oxidase complex include Rac1, Rac2 [10], Vav1 [47], PAK1 [48], protein kinase C (PKC)α and PKCε [49, 50], myristoylated alanine-rich C kinase substrate [49], and Akt [51]. Libraries of monoclonal antibodies and proteomic analyses have indicated that many more proteins, named or unnamed, are present on phagosomes [24, 33]. Some of those proteins are likely to emerge as additional regulators of phagocytosis.

The actin cytoskeleton is regulated during FcR-mediated phagocytosis by the Rho family GTPases Cdc42 and Rac, which are recruited to phagosomes and converted from their inactive, guanosine 5′-diphosphate (GDP)-bound forms to their active, guanosine 5′-triphosphate (GTP)-bound forms [4, 25, 27, 28]. Rho family GTPases are lipid-modified on their carboxy terminus, which allows their regulated association with membranes. Guanine nucleotide disassociation inhibitors (GDIs) bind to the GTPases in the GDP-bound state and remove them from membranes by sequestering their lipid tails, a displacement that down-regulates Rho GTPase activities [52]. Guanine nucleotide exchange factors (GEFs), which facilitate the release of GDP and the loading of the more abundant GTP, are also recruited to the phagosome. The GEF Vav1 may be important for phagocytosis, as overexpression of an inhibitory Vav1 construct reduced the activation of Rac1 and inhibited phagocytosis [53]; however, another report indicates that FcR-mediated phagocytosis can occur in macrophages lacking Vav1, Vav2, and Vav3 [54]. Return to the off-state is facilitated by GTPase accelerating proteins (GAPs). Recent work has indicated that the activation of the Cdc42, Rac1, and Rac2 is tightly controlled spatially and temporally during phagocytosis [4], presumably by interactions with GAPs and GEFs.

Cdc42 and Rac contribute to phagocytosis in different ways. GTP-bound (active) Cdc42 can stimulate localized actin polymerization via interactions with WASP and the consequent activation of the Arp2/3 complex [1, 52]. Although active Cdc42 has been localized to the tips of the advancing pseudopodia, consistent with Cdc42 playing a role in guiding actin polymerization during membrane extension [4], the mechanisms regulating Cdc42 in phagocytosis are unknown. The role of Rac as a major regulator of phagocytosis is established [25]. GTP-bound Rac can activate a number of proteins important for phagocytosis, including PAK1, p67phox, WAVE [55], and PI 4-phosphate 5-kinase-α (PI4P5K) [48, 56, 57]. Artificial clustering of active Cdc42 or WASP near cell-bound particles

induced the polymerization of actin but not phagocytosis [58]. Analogous clustering of constitutively active Rac1 below particles was sufficient to induce particle uptake, although the morphology of the process was unusual [59]. Taken together, these observations suggest that Cdc42 may regulate pseudopod extension, and Rac1 may regulate phagosome closure.

NADPH oxidase complex activity depends on the interactions between GTP-Rac1 or GTP-Rac2 and p67phox and gp91phox [26]. Cdc42 does not activate the oxidase, but it can antagonize activation via Rac [60]. Additional regulatory steps include the phosphorylation of p47phox by PAK1, PKC, or Akt [26].

PAK1 is a well-characterized effector of Cdc42 and Rac and localizes to phagocytic cups [61, 62]. PAK1 could regulate phagocytosis in at least four ways (reviewed in ref. [48]). First, activation of PAK1 by Cdc42 or Rac could stimulate actin polymerization in the forming phagosome. Activated PAK1 phosphorylates LIM kinase, which phosphorylates and inactivates actin depolymerizing factor/cofilin, consequently decreasing actin filament turnover [48] (although recent work indicates that actin polymerization is stimulated by active, rather than inactive, cofilin; ref. [63]). Second, PAK1 can regulate myosin contractility in cells by phosphorylating regulatory myosin light-chain or myosin light-chain kinase [64], thereby decreasing the actin-dependent ATPase activity of myosin II. Third, PAK1 can phosphorylate RhoGDI and thereby selectively activate Rac via its release from RhoGDI [65]. Lastly, PAK1 phosphorylation of p47phox could regulate activation of the phagocyte oxidase [48].

Adenosine 5′-diphosphate-ribosylation factor 6 (ARF6) activity is necessary for phagocytosis [41]. This small GTPase is transiently activated during phagocytosis, and its activation is important for delivery of membrane to the site of the forming phagosome [42]. Although ARF6 has been implicated as a regulator of the actin cytoskeleton, it is not required for early actin polymerization during phagocytosis [42]. It may instead regulate delivery of membrane to the phagosome or activation of the NADPH oxidase.

Various PKC isoforms are recruited to forming phagosomes [49, 50, 66]. PKCε appears to regulate the rate of phagosome formation, as overexpression of PKCε increased rates of phagocytosis [50].

PHAGOSOMAL LIPIDS

Some of the many isoforms of membrane lipid-modifying enzymes contribute to FcR-mediated phagocytosis, including PI4P5K [34], PI-3K [67], PLA_2 [68], PLC [34], PLD [69], phosphatase and tensin homology deleted on chromosome 10 (PTEN) [70, 71], and SHIP-1 [17, 21]. The products of these enzymes can influence the activities of essential regulatory proteins [72]. PI4P5K can increase membrane concentrations of $PI(4,5)P_2$, which together with Cdc42, can activate WASP, leading to increased actin polymerization [73]. Although little is known about the regulation of PI4P5K activity during phagocytosis, it has been shown in separate systems that Rac can stimulate PI4P5K activity [57]. Leukotrienes have also been implicated in phagocytosis [74]; however, the molecular mechanism of their action is not well defined.

Cholesterol and ceramide contribute to the formation of detergent-resistant membranes (DRM) and so-called lipid rafts [75]. DRM have been implicated in early stages of FcR activation. In particular, ligation or cross-linking FcR facilitates its localization to DRM [76, 77], possibly through regulated generation of ceramide [78]. The consequences of FcR recruitment to DRM are not yet clear, but they appears to facilitate interaction of the receptor with early signaling kinases such as Lyn [76].

PI-3K affects many tyrosine kinase receptor and G-protein signaling systems related to cell growth and movement. In response to receptor signaling, type I PI-3K phosphorylates the 3′ position of the inositol group of $PI(4,5)P_2$ to produce $PI(3,4,5)P_3$ [12], which increases the activities of GEFs for small GTPases, including Tiam1, Vav1, and ARF nucleotide-binding site opener (ARNO) [52, 79, 80]. Rac1 and Cdc42 can bind to the p85 subunit of PI-3K [57]. It has been proposed that signaling through Rac1 and PI-3K is connected by positive-feedback interactions [81]. Conversion of $PI(3,4,5)P_3$ to $PI(4,5)P_2$ is mediated by the phosphatase PTEN [82]. Type III PI-3K phosphorylates PI to PI-3-phosphate [PI(3)P], which contributes to later stages of phagosome maturation [83, 84] and perhaps also to activation of the NADPH oxidase [85, 86]. The enzyme myotubularin, of which there are several isoforms, dephosphorylates PI(3)P [82].

PI-3K is necessary for phagocytosis of IgG-opsonized particles larger than 2 μm diameter [67]. Inhibitors of PI-3K, such as wortmannin or LY294002, do not significantly affect phagocytosis of smaller particles but arrest phagocytosis of larger particles midway through the process [87]. PI-3K-dependent contractile activities are necessary for phagosome closure [36, 67]. Myosin X, which contains a $PI(3,4,5)P_3$-binding domain, could mediate these PI-3K-dependent contractions [37]. It has been suggested that PI-3K is necessary for processes that fuse intracellular membrane compartments with the forming phagosome [42, 87], but the evidence regarding this is contradictory [5].

Several phospholipases are essential for FcR-mediated phagocytosis. PLC hydrolyzes $PI(4,5)P_2$ to diacylglycerol and inositol trisphosphate, which can activate PKC and increase cytosolic-free calcium levels, respectively. Although increases in calcium are not necessary for phagocytosis by macrophages [88, 89], local activation of PKC via diacylglycerol is important for phagosome formation [66] and activation of the NADPH oxidase complex [9]. PLA_2, $PLA\text{-}D_1$, and $PLA\text{-}D_2$ are also necessary for phagocytosis, perhaps because of their roles in membrane fusion reactions [46, 68, 69, 90].

SHIP-1, a 5′ PI phosphatase that converts $PI(3,4,5)P_3$ to $PI(3,4)P_2$, has a complex and still poorly defined role in phagocytosis. Overexpression in macrophages of wild-type SHIP-1, constitutively active, or dominant-negative inhibitory constructs of SHIP-1 inhibited phagocytosis [21]. Immunofluorescence microscopy showed that the enzyme localized to forming phagosomes [21, 91]. SHIP-1 was first shown to associate with immunoreceptor tyrosine-based inhibitory motifs of inhibitory FcRIIB, which indicated that it normally inhibits phagocytosis [92]. SHIP-1 has recently been shown to associate with FcR

containing ITAMs [17, 93]; it is therefore possible that it contributes to phagosome formation by turning off $PI(3,4,5)P_3$-dependent signals in the forming phagosome.

Membrane lipids can contribute to FcR signaling in at least three ways. First, cholesterol- or ceramide-dependent clustering could facilitate initial kinase recruitment to ligated FcR [77, 78]. Second, many cytoplasmic proteins contain structural elements—pleckstrin homology (PH); phox homology (PX); or Fab1p, YOTB, Vps27p, EEA1 (FYVE) domains—which bind to PIs and allow regulation of protein association with membranes [94–96]. Recruitment of PI-binding domain-containing enzymes to membranes brings them close to membrane-bound substrates, which then allow signal transduction by protein modification. The PI-binding domains of different signaling proteins have different specificities; the PH domain from Akt recognizes $PI(3,4,5)P_3$ and $PI(3,4)P_2$ [97], the PH domain from PLCδ1 recognizes $PI(4,5)P_2$ [97], and the PX domain of p47phox recognizes $PI(3,4)P_2$ and PI(3)P [98]. Third, the activities of enzymes recruited to membranes via protein–protein interactions may be modified by the PI microenvironment of the membrane. For example, Vav is recruited to membranes by protein–protein interactions [99]. Its GTP exchange activity is increased by $PI(3,4,5)P_3$ and decreased by $PI(4,5)P_2$ [100]. Therefore, one can envision mechanisms of coordination in which the lipid microenvironments created by FcR signaling constrain the categories of signal that follow.

SIGNAL TIMING AND LOCALIZATION

Phagocytosis has been examined by biochemical and fluorescent imaging approaches that measure the localization and activation of signaling proteins. Observations of actin cytoskeleton dynamics on forming phagosomes by fluorescence microscopy indicated a robust and intricate series of regulatory interactions governing the formation of the phagosome [2–4, 36]. Immunofluorescence microscopy of macrophages fixed at various times during synchronized phagocytosis indicates that different classes of myosin are recruited to phagosomes at different times during the process [31]. These dynamics suggest that information from the FcR orients actin polymerization and depolymerization and positions myosin-based contractions. Other proteins that associate with phagosomes at different times during or after phagosome formation include Rab5 [101, 102], Rab7 [102, 103], and Rab11 [45], small GTPases implicated in the regulation of membrane trafficking, and p67phox and p47phox, which are recruited transiently to phagosomes containing opsonized zymosan [104].

Fluorescence microscopy of living macrophages expressing GFP chimeras have shown that different proteins associate and dissociate from phagosomes at different times, with different spatial arrangements during phagocytosis (**Fig. 1**). GFP chimeras with different lipid-binding specificities allowed the distributions of membrane PIs to be followed in living cells [105]. GFP-PLCδ1PH showed that $PI(4,5)P_2$ was abundant in plasma membrane, increased slightly during the initial phases of phagocytosis, and then disappeared from the phagosomal membrane. A chimeric protein consisting of CFP and the diacylglycerol-binding region of PKCδ (C1δ) showed that di-

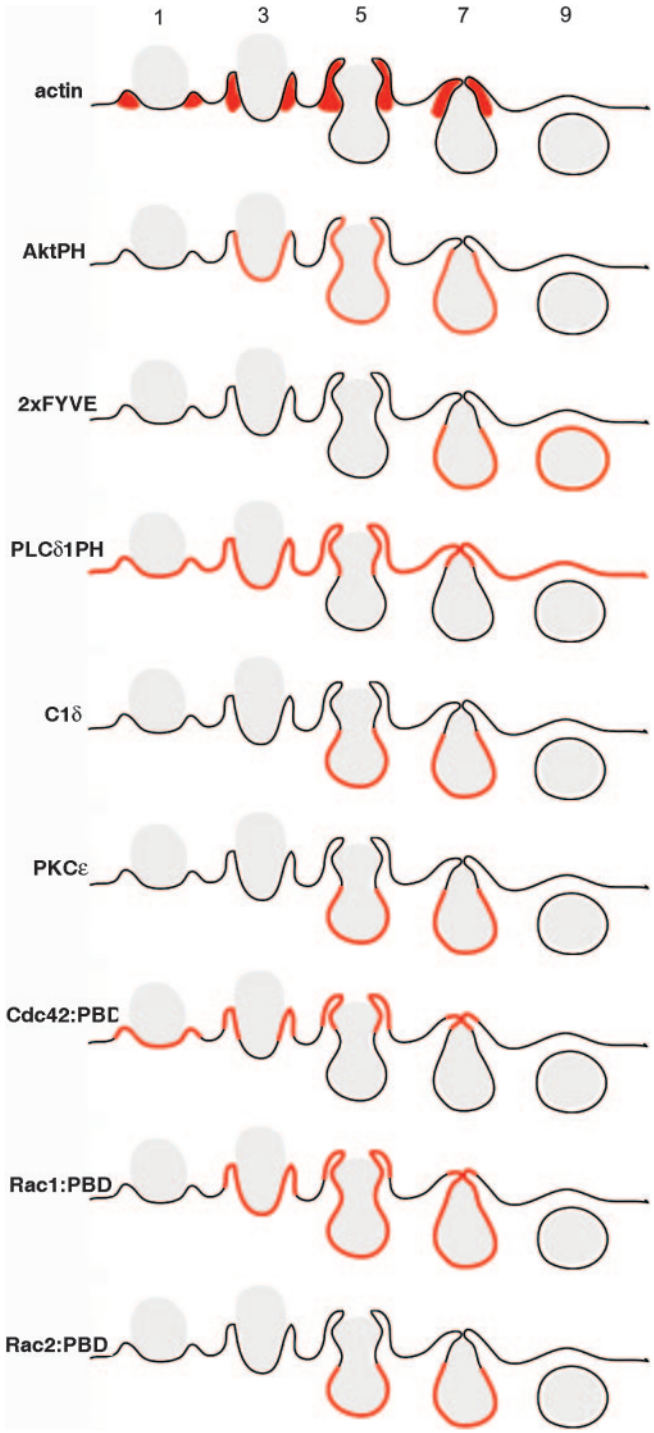

Fig. 1. Patterns of fluorescent protein chimera association with forming phagosomes. The diagram shows the movements of macrophage membrane (black and red lines) during the phagocytosis of IgG-opsonized sheep erythrocytes (gray shapes). Time (minutes) after initial binding of the erythrocyte to the macrophage is indicated in the top row. Formation of the phagocytic cup (3 min) is followed by a constriction of the outer margin (5 and 7 min) and membrane fusion to form a discrete, intracellular vacuole (9 min). The movements of phagocytosis typically distort the erythrocytes. Red lines show the approximate distributions of GFP, cyan fluorescent protein (CFP), or YFP chimeras of the indicated proteins based on different studies: actin (from refs. [3, 4, 67]; AktPH, indicating $PI(3,4,5)P_3$ plus $PI(3,4)P_2$ (from refs. [4, 91]); 2 × FYVE, indicating PI(3)P [3, 91]; PLCδ1PH, indicating $PI(4,5)P_2$ [34]; C1δ, indicating diacylglycerol [34]; PKCε [50]; Cdc42:p21-binding domain of PAK1 (PBD), indicating fluorescence resonance energy transfer (FRET)-based localization of GTP-Cdc42 [4]; Rac1:PBD, indicating FRET-based localization of GTP-Rac1; and Rac2:PBD, indicating FRET-based localization of GTP-Rac2 [4].

acylglycerol increased in the membrane as PI(4,5)P_2 levels decreased [34]. GFP-AktPH showed that PI(3,4,5)P_3 and/or PI(3,4)P_2 were largely absent from unstimulated cells but were generated transiently on the forming phagosome [4, 91]. Ratiometric fluorescence microscopy of macrophages expressing CFP plus YFP-labeled 2×FYVE, a chimera that recognizes PI(3)P, showed that PI(3)P was generated late during phagocytosis [91] and persisted for various times on the fully formed phagosome [3]. Live cell fluorescence microscopy has also been used to measure the localization dynamics of GFP or YFP chimeras of PKCε, Cdc42, Rac1, Rac2, Rab5, and Rab7 [3, 4, 50]. GFP-PKCε was recruited to phagosomes in a transient flash prior to phagosome closure [50]. YFP-Cdc42, YFP-Rac1, and YFP-Rac2 associated with forming phagosomes throughout the process. Rab5-YFP and Rab7-YFP arrived on fully formed phagosomes with a precise timing [3].

Activated, GTP-bound Cdc42, Rac1, and Rac2 bind to PBD, and this interaction allowed microscopic localization of those G-proteins in their GTP-bound state [4]. RAW264.7 macrophages expressing CFP plus a chimera of YFP and PBD (YFP-PBD) reported the distributions of the GTP-bound forms of endogenous Cdc42 and Rac during phagocytosis of IgG-opsonized sheep erythrocytes. YFP-PBD was recruited to the actin-rich region of the phagocytic cup and, transiently, to the actin-poor region of the phagosomal membrane. The pronounced recruitment of YFP-PBD to the actin-poor phagosomal membrane coincided with phagosome closure. This indicates roles for GTPases in later stages of phagocytosis.

The GTPases responsible for the striking pattern of YFP-PBD localization were identified by microscopic methods for measuring protein–protein interactions by FRET [4]. Macrophages expressing CFP-PBD plus YFP-Cdc42, YFP-Rac1, or YFP-Rac2 were imaged during phagocytosis. FRET-based stoichiometric methods were used to quantify G-protein activation relative to actin movements during phagocytosis of IgG-opsonized erythrocytes. Cdc42 activation was restricted to the leading margin of the cell, whereas Rac1 was active throughout the phagocytic cup. During phagosome closure, activation of Rac1 and Rac2 increased uniformly and transiently in the actin-poor region of the phagosomal membrane, with the same timing as the recruitment of YFP-PBD. This indicated that GTP-Cdc42 and GTP-Rac1 were prominent in the actin-rich region and that the spike of YFP-PBD recruitment seen by ratiometric fluorescence microscopy was produced by GTP-Rac1 and/or GTP-Rac2. It was speculated that the spike of Rac activation coincided with the activation of the NADPH oxidase complex.

Thus, the microscopic studies have shown that FcR signaling during phagocytosis consists of spatially and temporally distinct stages, whose sequence corresponds to the distinct, morphological stages of phagosome formation.

THE ZIPPER MODEL FOR PHAGOCYTOSIS

How are the stages of signaling organized to internalize a particle? Griffin and Silverstein [106] proposed that phagocytosis is achieved by a sequence of receptor-ligand interactions that guide the phagocytic membrane over the IgG-coated particle surface. According to their zipper model, the advance of the phagocytic cup occurs as an ordered progression of local FcR-IgG interactions, in which particle-bound Fc are continually engaged by macrophage FcR (**Fig. 2A**). Each FcR signals autonomously and governs the membrane and cytoskeletal activities in one small segment of the membrane. In support of this model, they showed that macrophage engagement of particles partially coated with IgG did not extend beyond the opsonized regions of the particle surface [107].

How can a zipper model explain the sequence of distinct activities in FcR-mediated phagocytosis? The timing of signaling from each FcR could be built into the catalytic reaction rates of the molecules in the FcR complex. Each new Fc-FcR complex could initiate a sequence of signals whose magnitude and duration depend on catalytic rates for phosphorylation and other modifications or protein–protein interactions. Cross-signaling between molecules with different catalytic rates could create a clock-like mechanism that systematically alters the state of the complex over time (Fig. 2A). The different morphological stages of phagocytosis—pseudopod extension, contraction, phagosome closure, and the generation of ROIs—would reflect the changing molecular regulatory networks associated with the FcR. At each FcR, signals for actin polymerization, ubiquitination, and Cdc42 activation would appear first; signals for activation of Rac, Arf6, membrane fusion, and PI-3K-dependent contractions would appear second; and signals for assembly and activation of the NADPH oxidase complex third. FcR activated at the distal regions of the particle surface would be initiating actin polymerization at the same time as the first activated FcR at the base of the phagosome, have begun to activate the NADPH complex. Consequently, one could have the sequence of FcR signals reflecting the continued advance of the phagocyte membrane over the particle. Much of the intracellular signaling measured by fluorescence microscopy is consistent with the zipper model, in that the rates of signal appearance and disappearance are similar to the rates of pseudopod advance over the particle [3, 4].

IS FcR SIGNALING COORDINATED DURING PHAGOCYTOSIS?

Live cell imaging of signaling during phagocytosis indicates that the process appears to be coordinated over large regions of the phagosomal membrane. Movies of the distributions of fluorescent chimeras during phagocytosis indicate that pseudopod extension, contraction at the distal margin, and phagosome closure are very similar from one phagocytic event to the next [3]. Cdc42, Rac1, and Rac2 are recruited to the phagosome in irregular patterns, but they are activated and deactivated in a very regular pattern [4]. GTP-Cdc42 is concentrated at the advancing edge of the phagocytic cup, GTP-Rac1 appears in the actin-rich part of the cup, and a spike of Rac1 and Rac2 activation appears over the actin-poor region of the cup. The zipper model envisions a smooth sequence of Fc-FcR interactions as the pseudopod advances over the particle. At the molecular level, however, the sequence of Fc-FcR engagement could be much noisier. If FcR functioned autonomously, one

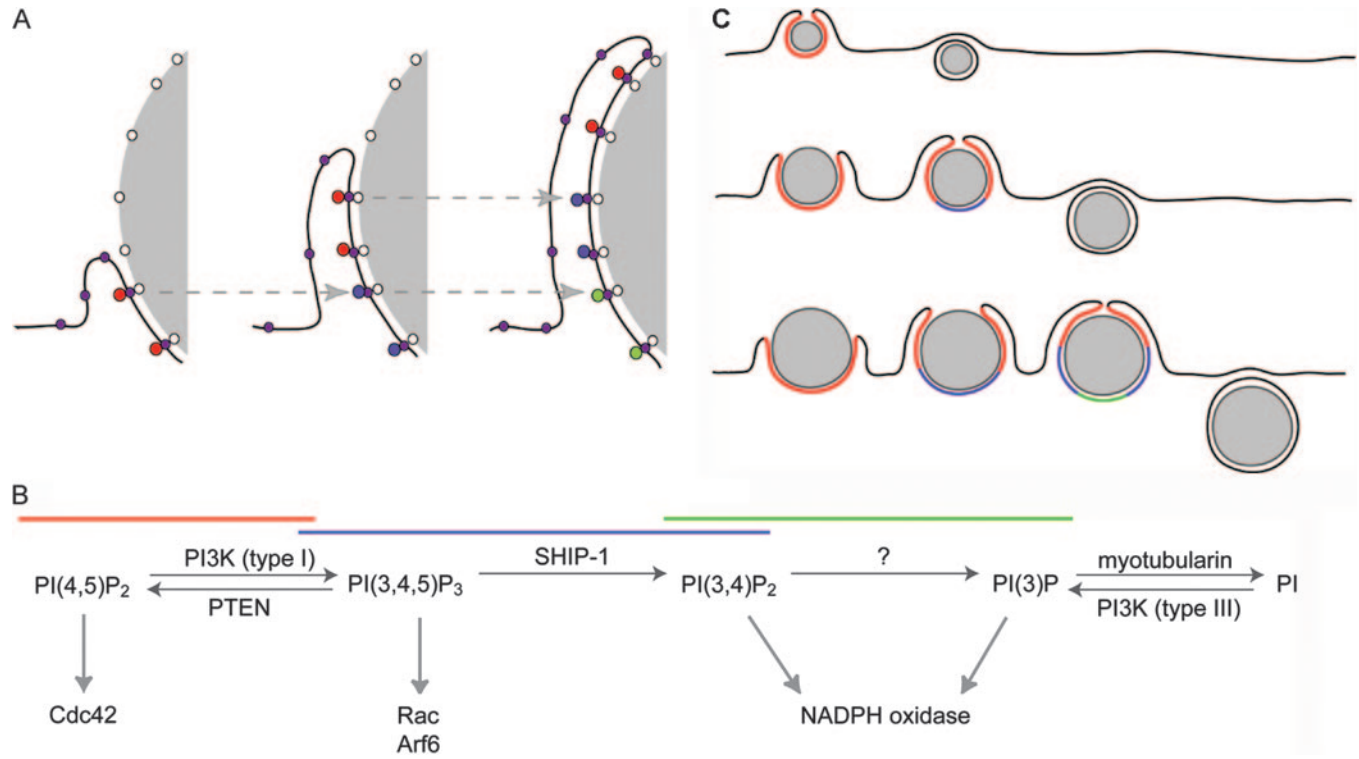

Fig. 2. Stages in FcR signaling during phagocytosis. (A) The zipper model of phagocytosis. IgG molecules (○) on the surface of a particle (gray) are engaged by FcR (●) in the macrophage plasma membrane (black lines). The three images indicate a sequence of movements and receptor signaling during phagocytosis. New interactions between IgG and FcR occur as the membrane advances over the particle. FcR recruit proteins (colored circles) to form FcR signaling complexes. Individual FcR complexes remain fixed, relative to the particle (gray arrows), but the signals they generate may change over time (red to blue to green). (B) Transitions of phosphoinositides could define or stabilize stages of FcR signaling. The general sequence of phosphoinositide transitions during phagocytosis reads left to right. Lipid-modifying enzymes associated with phagocytosis and the reactions they catalyze are indicated. Colored bars indicate possible stages in FcR signaling; gray arrows indicate corresponding molecular activities: activation of Cdc42 in the early stages of phagosome formation, 3′ PI-dependent activation of Rac and Arf6, and the assembly and activation of the NADPH oxidase. (C) Phagocytosis of large particles could use more stages of signaling than phagocytosis of small particles. Particles (gray) are progressively enclosed by macrophage membranes (black, red, blue, and green lines). Stages of FcR complex signaling are indicated by the colors: Red is stage 1, blue is stage 2, and green is stage 3. If phagosome closure could terminate signaling from FcR complexes, then phagocytosis of small particles could require only the earliest stages of FcR signaling. Accordingly, inhibitors of later stages of signaling would not affect phagocytosis of small particles as much as phagocytosis of larger particles.

might expect more irregular patterns, reflecting a stochastic pattern of Fc-FcR interactions.

Another indication that FcR signaling is coordinated laterally in the plane of the membrane can be seen in the dynamics of PI(3)P on phagosomal membranes. Using YFP-2×FYVE to monitor PI(3)P on phagosomes containing IgG-opsonized erythrocytes, Henry et al. [3] observed two patterns on phagosomes. In some phagosomes, YFP-2×FYVE labeling appeared and disappeared within 20 min, and in others, the labeling appeared gradually and persisted for a long time. These different patterns on phagosomes, whose contents contained similar amounts of surface IgG and which exhibited otherwise similar patterns of maturation, indicated that PI(3)P levels are coordinately regulated over large regions of phagosomal membrane.

Coordination is also indicated by the fact that FcR signaling varies with the size of the IgG complex or particle being ingested. FcR mediates the internalization of soluble immune complexes and IgG-coated particles, but the mechanisms of entry seem to vary with the size of the particle. Clathrin-based mechanisms have been implicated in endocytosis of soluble immune complexes and the phagocytosis of IgG particles smaller than 1 μm diameter [108, 109]. Ubiquitin-dependent, endocytic mechanisms associated with clathrin-coated vesicle formation are necessary for internalization of soluble immune complexes but not for phagocytosis of particles [22]. This indicates a size threshold of 0.5–1.0 μm for the transition between coated vesicle-mediated uptake and actin-mediated phagocytic processes. A different size threshold is indicated for actin-mediated phagocytosis. Various mechanisms for inhibiting FcR signaling to the actin cytoskeleton indicate a dependence on particle size, with a critical transition for particles larger than 2 μm diameter. Phagocytosis of large particles is inhibited by dominant-negative SHIP-1 [21] and by inhibitors of PI-3K [87], whereas the internalization of smaller particles is relatively unaffected by such treatments.

PHAGOCYTOSIS AS A SEQUENCE OF STAGES INTEGRATED BY LATERAL DIFFUSION

How might FcR signaling in one region of the phagosome be influenced by FcR signaling in another region? Imaging indicates that signals generated near FcR pass through distinct

stages. These stages may be defined and stabilized by molecules that can diffuse between FcR complexes. PIs or membrane-tethered GTPases could set the state of FcR signaling within a region of the phagosomal membrane. The sequence of transitions and spatial patterns of GTPase and PI profiles would then define the sequence of FcR signals. For example, $PI(4,5)P_2$, predominating early during phagocytosis, would restrict FcR signaling to a limited set of responses, increasing signaling to Cdc42 and WASP and inhibiting activation of later stage events (e.g., Vav). That signal would disappear from the phagosome membrane, as $PI(4,5)P_2$ is hydrolyzed to diacylglycerol or phosphorylated by PI-3K to $PI(3,4,5)P_3$. The increase in $PI(3,4,5)P_3$ could increase Rac and Arf6 GEF activities near the FcR. Subsequent hydrolysis of $PI(3,4,5)P_3$, by PTEN to $PI(4,5)P_2$ or by SHIP-1 to $PI(3,4)P_2$, could turn off the $PI(3,4,5)P_3$-dependent signals and promote later stages, such as activation of the NADPH oxidase (Fig. 2B). Thus, progressive changes in the phosphoinositide context could coordinate FcR over larger regions of membrane and possibly also dampen signals generated by FcR that are activated out of turn. Analogous patterns could be described for profiles of GTPase activities. The essential requirement of these molecules is that they must carry information laterally in the plane of the membrane between FcR complexes. Regions where the GTPase and PI profiles overlap may indicate points where signals are integrated or compared. Such integrative mechanisms could be important for coordinating the stages of phagocytosis.

There is some precedent for lateral signaling among receptors. Signals propagating away from ligated receptors were described for tyrosine kinase signaling through the epidermal growth factor receptor in response to localized stimulation [110] and in cells overexpressing growth factor receptors [111].

CHECKPOINTS AND THRESHOLDS FOR PROGRESSION THROUGH THE PHAGOCYTOSIS

The particle-size dependence of some phagocytosis inhibitors indicates that there are mechanisms for the FcR signaling apparatus to detect the dimensions of the particle. If FcR signaling progresses through a temporal series of stages, then it is possible that phagocytosis of small particles could be finished before the later stages of FcR signaling begin (Fig. 2C). Thus, if the phagosome closed into the cell as a discrete organelle at the end of stage 1, then signaling would abort, and stages 2 and 3 would not occur. Conversely, if the phagocytic cup had not closed by the end of stage 1, then later stages of FcR signaling would ensue. For small particles or soluble immune complexes, stage 1 signals would be sufficient to generate a clathrin-mediated uptake, and closure of the vesicle would terminate signaling. More time would be required for internalization of large particles, however, and the chemistries of the FcR signaling complex would be able to continue to stage 2. Absent a cancellation by phagosome closure, signaling would continue to stage 3. According to this model, inhibition of PI-3K or SHIP-1 has less effect on the phagocytosis of small particles, as they are internalized before 3′ PI-dependent processes come into play. This argues that individual FcR do not function strictly autonomously, as each receptor's progression to later stages of signaling depends on distal events in the phagosome. We imagine that FcR complexes could detect phagosome closure via changes in membrane potential or the lipid environment.

Progression from one stage to the next could also be controlled by thresholds related to particle size. Accordingly, ligated FcR on small and large particles would generate the same initial set of signals, but progression from stage 1 to 2 would require some of those signals to exceed thresholds. These size thresholds for progression could be mediated by lipids generated near FcR complexes. When only a few FcR are cross-linked, diffusion of lipids in the plane of the membrane would not allow concentrations of PIs to reach levels sufficient for progression to stage 2. If sufficient numbers of FcR were cross-linked, however, then these mediators could reach suprathreshold levels that then allow progression to later stages. For example, PI-3K-dependent receptors that recruit and activate PI-3K could be coordinated laterally in the plane of the membrane by 3′ PIs (**Fig. 3**). FcR ligation would always recruit PI-3K, but the 3′ PI-dependent signals that follow, such as Rac or Arf6 activation and recruitment of PH domain-containing proteins, would require ligation of multiple FcR. If relatively few FcR were ligated, with soluble immune complexes or small IgG-coated beads, then diffusion of 3′ PIs in the plane of the membrane, away from the FcR complexes,

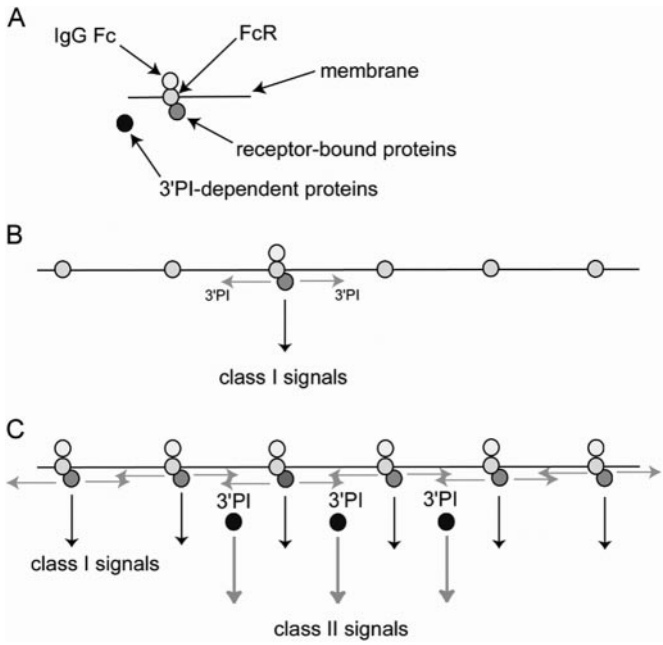

Fig. 3. Model for size-dependent transitions between stages. (A) The essential components are IgG, FcR, membrane 3′ PIs, proteins recruited to FcR, and proteins whose activation or recruitment to membranes are increased by 3′ PIs. (B) Low levels of FcR cross-linking recruit proteins, including PI-3K, which initiate intracellular signals (class I). However, because of 3′ PI diffusion in the plane of the membrane or insufficient PI-3K activities near the membrane, the 3′ PI levels remain too low to recruit or activate 3′ PI-dependent proteins. (C) Coincident activation of many receptors by larger particles or higher concentrations of soluble stimuli initiate class I signals, and 3′ PIs reach concentrations in membranes sufficient to activate 3′ PI-dependent proteins (class II).

would prevent them from reaching concentrations sufficient to recruit signaling proteins. However, if multiple receptors were ligated, such as near a large, opsonized particle, then 3′ PI levels in the membrane would reach suprathreshold levels that recruit or activate PH domain-containing proteins and initiate secondary signals, such as activation of GEFs, which stimulate formation of GTP-Rac or GTP-Arf6. It follows from this that there should be two classes of signal associated with FcR ligation: Class I signals are those associated with all ligated receptors, whereas class II signals only appear when a sufficient number or density of FcR are ligated (Fig. 3).

Other control mechanisms are possible as well. Depletion of cellular actin or other proteins in limited supply could constrain phagocytosis of larger particles such that they only enter one at a time. It is also possible that particle shape regulates progression, perhaps by translating surface curvature or contractile activities into positive or negative feedback to FcR. There is evidence that cytoskeletal tension positively regulates FcR-mediated phagocytosis [112].

GLOBAL REGULATION

There are also mechanisms that control whether phagocytosis will happen at all. Inhibitory FcR, such as FcRIIB, contain cytoplasmic domains that recruit phosphatases, which inhibit phagocytosis [113]. Overexpression of these FcR can inhibit phagocytosis. The relative expression levels of stimulatory and inhibitory FcR can simply regulate the capacity of a cell for phagocytosis. Other proteins with inhibitory motifs can modulate the ability of a phagocyte to phagocytose via FcR. The Src kinase Fgr is capable of such negative signaling [114].

Finally, the dimensions of the phagocyte may regulate progression more generally through phagocytosis. Internalization of a particle removes cell-surface membrane and displaces cell volume. Phagocytes may regulate phagosome formation based on their ability to accommodate such displacements. PI-3K has been implicated in cell-size regulation [115, 116]. The PI-3K dependence of phagocytosis could indicate a checkpoint related to cell size, which constrains the size of the particle that can be internalized.

SUMMARY AND PROSPECTS

Coordination of FcR signaling is indicated by the continuous uniformity of the process from one event to the next, by the apparent integration of 3′ PI levels in phagosomal membranes, and by the particle size-dependence of some inhibitors. The simplest model to explain this coordination requires that the FcR cannot operate strictly autonomously. Coordination could be explained as a progression of FcR signals through a series of stages, which are integrated by lipids or lipid-bound proteins that diffuse laterally in the plane of the membrane. Feedback mechanisms that define checkpoints, such as termination of signaling by phagosome closure or thresholds that reflect the overall level of FcR signaling, may regulate progression from one stage to the next.

Coordination by lateral diffusion may also apply to signaling by other kinds of phagocytic receptors and to processes that look like phagocytosis, such as bacterial entry into macrophages and otherwise nonphagocytic cells. However, some of these other kinds of phagocytosis are inconsistent with the zipper model and are better explained by an all-or-none mechanism for phagosome formation, originally referred to by Griffin et al. [107] as the trigger model. Phagosomes containing the pathogenic bacteria *Salmonella* or *Shigella* form by actin-dependent processes analogous to the movements of FcR-mediated phagocytosis but without close adherence of plasma membrane to bacterial surfaces [117]. This indicates that receptor signaling can trigger an entire phagocytic response, and more generally, locally activated receptors can sometimes govern the dynamics of a large region of cytoplasm. A mechanism that coordinates receptor signaling by lateral diffusion could apply to the zipper or the trigger model of phagocytosis. In the former, receptors govern small regions of membrane and cytoplasm but are modulated by diffusible molecules that reflect the aggregate state of the receptor population or a larger region of membrane. In the latter, signals that radiate from receptors could allow a few ligated receptors to organize large areas of cytoplasm.

These ideas prompt questions that may be answerable with new methods. Does phagocytosis of small particles initiate the same set of signals as that of large particles? Are the late-stage signals, generated during large-particle phagocytosis, missing from small-particle phagocytosis? Is the amplitude or timing of the signals generated by individual FcR affected by other ligated FcR, by the dimensions of the IgG-coated particle that binds the FcR, or by the immediate history of FcR signaling in that region of membrane? Is there measurable cooperativity between two different receptors that signal via 3′ PIs? Answers to these questions should be relevant to other receptor-mediated, large-scale cellular behaviors.

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