May the force be with you: Myosin-X in phagocytosis.

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Engulfment of pathogens by phagocytosis requires the coordination of actin assembly and progressive 'zippering' of pseudopodial membranes around the particle. Recent work shows that Myosin-X, which binds phosphatidylinositol-3-OH kinase (PI(3)K) products through its pleckstrin homology (PH) domains, is required for phagocytosis, thereby providing a molecular basis for the function of PI(3)K in pseudopod extension.

In higher organisms, phagocytosis is essential for eliminating infectious agents and for the scavenging of dead cells[1], whereas in lower unicellular organisms, phagocytosis is associated with food uptake. The phagocytic process can be divided into sequential events, starting with the recognition of the particle by dedicated receptors on the phagocytic cell surface. Among the best-characterized phagocytic receptors are opsonic receptors, and particularly Fcγ receptors (FcγRs). Clustering of FcγRs triggers a local and oriented polymerization of actin filaments[2] that causes protrusion of the plasma membrane and wrapping of the particle within pseudopods. A contractile force is then generated to pull the particle into the cell, and the enclosed particle is finally degraded by lysosomal hydrolases after fusion of the phagosome with compartments of the endocytic pathway. Significant advances have recently been made in the molecular definition of the components of the phagocytic signal. Among several signalling molecules that are recruited to and are required at the site of phagocytosis[3], PI(3)K seems to be essential for pseudopod extension and phagosome closure. However, in the absence of precisely identified ligand(s) for phosphatidylinositol-3,4,5-trisphosphate ($PtdInsP_3$; the product of PI(3)K at the phagocytic site, it has been difficult to assign a definitive function to PI(3)K in phagocytosis. Work presented in this issue[4] has now identified a recently discovered myosin, Myosin-X, as a downstream effector of PI(3)K in FcγR-signalling, suggesting new roles for PI(3)K and $PtdInsP_3$ in phagocytosis.

FcγRs are characterized by the presence of pairs of tyrosine residues in the receptor (or its accessory γ chain) cytoplasmic region. After binding of a particle, receptor clustering facilitates phosphorylation of these tyrosine residues by Src family kinase(s), and brings Syk, a tyrosine kinase with two tandem src homology 2 (SH2) domains, to the phagocytic site. In turn, Syk recruits and activates PI(3)K (ref. 3). By transfecting a macrophage cell line with green fluorescent protein-tagged PH domains that bind PI(3)K products, Grinstein and coworkers have recently confirmed that $PtdInsP_3$ rapidly accumulates at sites of phagocytosis and disappears after the phagosome has been sealed off from the plasma membrane[5]. The evidence that PI(3)K activity was required for phagocytosis came from experiments using the PI(3)K inhibitors wortmannin (Wtn) and LY294002 (refs 6,7). Both drugs significantly reduced the ingestion of large particles (greater than or equal to 4 μm), whereas uptake of smaller particles was unaffected[7]. A surprising finding of these studies was that, in contrast with its role in growth factor-induced actin cytoskeleton reorganization and membrane ruffling, $PtdInsP_3$ production was not required for actin assembly during FcγR-mediated phagocytosis[6,7].

If limitation of pseudopod extension and inhibition of phagosome closure by Wtn/LY294002 was not the result of inhibition of cytoskeletal processes, it raises the question of what it could be caused by. Based on the observation that Wtn also abolished the spreading of macrophages on antibody-coated surfaces (the so-called 'frustrated phagocytosis' model), and the concomitant exocytosis of membrane from

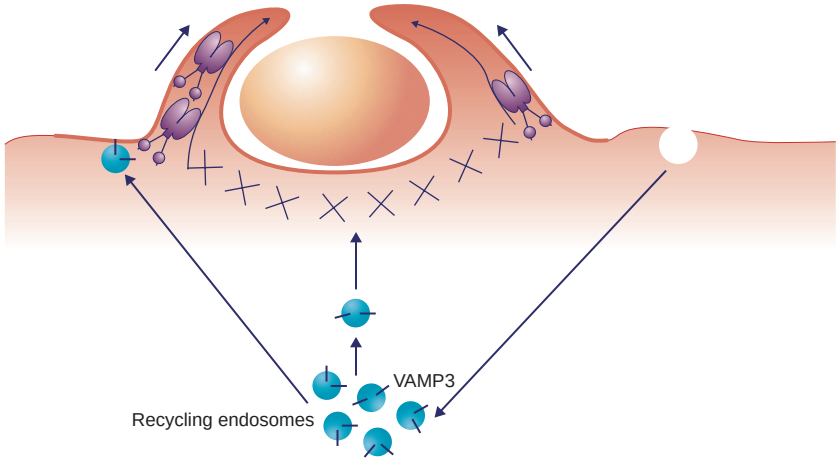

Figure 1 **A model for PI(3)K-dependent function of Myosin-X during phagocytosis. Clustering of FcγRs (not shown for clarity) at the particle attachment site triggers various signalling events that result in filament actin assembly (blue) and accumulation of $PtdInsP_3$ (red) through the activation of PI(3)K (not shown). Myosin-X (represented as dimers of heavy chains in purple) is recruited to the forming phagosome through the interaction of its PH domains (purple dots) with membrane $PtdInsP_3$. The motor head domain of Myosin-X is engaged on actin filaments and moves towards the barbed ends of filaments (indicated by arrowheads) facing the tips of the growing pseudopods. By this dual interaction, Myosin-X would be able to couple actin polymerization and pseudopod extension (black arrows). Membranes required for pseudopod extension are provided by insertion of recycling endosome membranes enriched in the SNARE VAMP3 (light blue) at the site of phagocytosis.**

intracellular pools, Greenberg and colleagues have suggested that PI(3)K could regulate the focal delivery of internal membranes to the plasma membrane, a process thought to contribute to pseudopod extension as a compensatory mechanism for the loss of membrane taken up into phagosomes[7]. Accordingly, the severity of inhibition by Wtn/LY294002 increased with particle size and hence, with the amount of membrane required to complete engulfment. This conclusion was also supported by evidence that indicated a requirement for components of the general membrane fusion machinery, such as soluble NSF attachment protein receptor (SNARE) proteins and the ATPase NSF in phagocytosis[8]. More recently, other studies[9] have examined the exocytosis of a green fluorescent protein (GFP)-tagged fusion of VAMP3, a SNARE protein that is localized to recycling early endosomes, which function as intermediates on the receptor recycling pathway. During FcγR-mediated phagocytosis, VAMP3-positive recycling endosomes fused with the plasma membrane in a polarized fashion, resulting in accumulation of GFP–VAMP3 at the phagocytic site (see Fig. 1). Wtn was found to inhibit the exocytosis of markers of recycling endosomes during phagocytosis[10]. These observations argue that recycling endosomes may function as a pool of internal membrane that can be readily mobilized to the cell surface in a PI(3)K-dependent manner as a fundamental part of the process of pseudopod extension.

Other studies have suggested the possibility that PI(3)K may be involved in the generation of a contractile activity to complete phagosome closure[6,11]. In earlier studies, the forces produced during engulfment of yeast particles by phagocytes were measured. Using a micromechanical method, an alternate phase of engulfment and membrane extension occurring without contraction was observed, before a contraction phase that started abruptly and concomitant to particle ingestion[12]. Furthermore, dumbell-shaped erythrocytes were observed in another study that examined macrophages attempting to ingest a single erythrocyte. The two bulbous ends of the erythrocyte, each enclosed in a phagosome within the two adjacent macrophages, were still connected by a thin membrane stalk. In the presence of Wtn/LY294002 or butanedione monoxime (BDM, an inhibitor of myosins), constricted erythrocytes were absent[11]. This argues that PI(3)K is involved in the generation of contractility restricted to the pseudopod margin and in the closure of the phagosome through a 'purse-string-like' mechanism. Several myosin isoforms (Myosin-Ic, -II, -Ixb and -V) that show a differential distribution at nascent phagosomes could conceivably control the generation of force during phagocytosis[11,13,14].

The missing piece of the puzzle identified by Cox and colleagues is Myosin-X (ref. 4), a newly discovered myosin characterized by a tail domain containing three PH domains[15]. Myosin-X is the founding member of a new class of unconventional myosins that have a similar motor domain to those of conventional class II myosins, but with unique structural tail domains that confer class-specific functions[16]. Human Myosin-X is a 2058-amino-acid protein with head and tail domains separated by a region predicted to form a coiled coil, suggesting that Myosin-X heavy chains exist as dimers[15]. The most unusual feature is the presence of three PH domains, the second of which interacts with PI(3)K products[17]. The carboxy-terminal end contains a MyTH4 (myosin tail homology 4) and a FERM (4.1, ezrin, radixin, moesin) domain that are conserved in the tail region of other unconventional myosins. As expected, Myosin-X binds F-actin in an ATP-sensitive manner, and is enriched in actin-rich protrusions, including lamellipodia and filopodia, in several cell types[15]. In a previous issue[18], it was reported that Myosin-X accumulates at the tips of filopodia, in agreement with the recent demonstration that Myosin-X is a motor that moves classically toward the barbed ends of actin filaments[19]. Moreover, overexpression of Myosin-X resulted in a fivefold increase in the density of filopodia at the cell perimeter[18]. These findings strongly argue for a function of Myosin-X in processes that induce the protrusion of the plasma membrane.

In this issue, Cox and colleagues show that macrophage Myosin-X accumulates at the phagocytic site with similar kinetics to F-actin, and importantly, this occurs in a PI(3)K-dependent manner (that is, recruitment is inhibited in the presence of Wtn). A series of experiments in which truncated fragments of Myosin-X were expressed in macrophages allowed the authors to establish the role of Myosin-X in phagocytosis. Expression of a Myosin-X-tail construct, comprising the three PH domains, the MyTH4 and the FERM domain, resulted in a strong (~75%) inhibition of phagocytosis. However, a similar tail construct with a point mutation in the second PH domain, which abolishes $PtdInsP_3$ binding, was not recruited to the phagocytic site, and accordingly did not inhibit phagocytosis. The requirement for Myosin-X in the phagocytic process could be further demonstrated by loading macrophages with antibodies against the Myosin-X head, resulting in a significant inhibition of particle ingestion. Together with the observation that the Myosin-X-tail did not prevent F-actin recruitment at the phagocytic site, two complementary pieces of data suggest that Myosin-X is required for membrane extension during the phagocytic process. First, the Myosin-X tail exerted its dominant inhibitory effect specifically on large particles (6 μm diameter), whereas it was unable to inhibit ingestion of small particles (2 μm) that required less membrane. Second, the tail construct inhibited spreading, but not adhesion, of macrophages on antibody-coated surfaces.

Myosin-X is a vertebrate-specific myosin[15]. However, its tail shares some conserved features with the tail of myosin VII from *Dictyostelium discoideum*. Interestingly, myosin VII-null mutants of *Dictyostelium* have a defect in phagocytosis, whereas they exhibit normal behaviour with respect to other actin-mediated processes[20]. Further evidence suggested that these mutants may have early adhesion problems, both to the particle and the substrate[21]. There are at least two different mechanisms whereby Myosin-X could exert its function during phagocytosis. First, based on compelling evidence that unconventional class V myosins function as vesicle transporters in membrane trafficking, Myosin-X could function as an actin-based motor to transport membrane cargoes to the forming pseudopods. Cargoes could include membranes from recycling endosomes that are delivered to the phagocytic site[9]. However, this is rather unlikely because biochemical evidence suggests that Myosin-X, as opposed to Myosin V, is not a processive motor[19]. Moreover, the authors have shown that expression of the inhibitory Myosin-X-tail construct does not seem to affect the recruitment of markers of the recycling pathway to the phagocytic site. Another possibility is that Myosin-X could be involved in generating the forces required for particle engulfment by pulling on the actin filament network that is present at the phagocytic site. Finally, the explanation that is most favoured by the authors is that by binding $PtdInsP_3$-enriched membrane and simultaneously moving along actin filaments, Myosin-X lifts bulk plasma membrane in the direction of the barbed ends that face the outer margin of the forming phagosome. Coupled to this process, exocytosis of recycling endosomes provides the extra membrane required for pseudopod extension (see Fig. 1).

Over the past five years, components that contribute to various aspects of the phagocytic signal have been identified. The concept that directed actin polymerization drives the protrusion of the plasma membrane has been extremely fruitful. Along these lines, proteins such as Rho family GTPases and their effectors have been found to participate actively in actin dynamics during phagocytosis[2]. However, another concept has also emerged borrowed from the related problem of cell motility. This view, initially suggested by M. Bretscher[22], proposed that the polarized insertion of membrane at the leading edge of a motile cell pushes it forward. The finding that Myosin-X functions in phagocytosis by linking PI(3)K and pseudopod extension,

and may couple the forward advance of the actin filament network to the pseudopodial movement of the plasma membrane, is important. This result brings together actin polymerization and membrane delivery to explain phagocytosis. □

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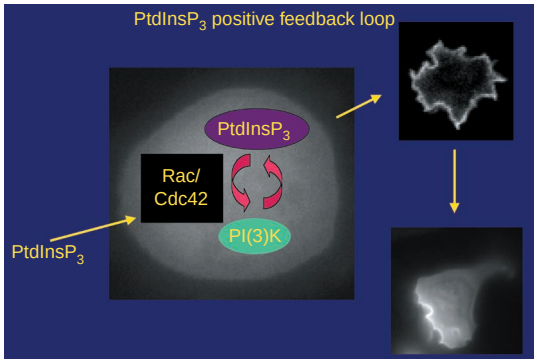
Leading the way

The ability to chemotax, that is, to sense and move in the direction of chemical signals, is a feature of a wide variety of eukaryotic cells. Chemotaxis is important for many biological responses, from the movement of leukocytes towards sites of infection or inflammation to the aggregation of *Dictyostelium discoideum* amoebae to form a multicellular organism. Recent work has firmly established the importance of the phosphatidylinositol 3-OH kinase (PI(3)K) pathway in mediating directional movement in response to chemoattractants. Insight into the mechanism that translates a shallow gradient of chemoattractant into cytoskeletal polarization and directional movement first came from work using *Dictyostelium* cells, and subsequently from studies with leukocytes and fibroblasts. These studies identified the importance of localized signalling by demonstrating that green fluorescent protein (GFP) fusions of a subfamily of pleckstrin homology (PH) domain-containing proteins, which specifically bind to the phosphoinositide products of PI(3)K, preferentially localized to the leading edge of chemotaxing cells. These findings strongly suggested that PI(3)K functions at the leading edge of the cell to mediate directional movement by using its products PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 as second messengers.

Two manuscripts in this issue of *Nature Cell Biology* (Wang, F. *et al. Nature Cell Biol* **4**, 513–518 (2002) and Weiner, R. *et al. Nature Cell Biol* **4**, 509–512 (2002)) report positive feedback loops in neutrophils that could provide the amplification mechanisms necessary for the conversion of a shallow extracellular gradient of chemoattractant into a steep intracellular second messenger gradient. In neutrophils, uniform stimulation with chemoattractant eventually results in spontaneous polarization. Wang *et al.* demonstrate that a membrane-permeable PtdIns(3,4,5)P_3 complex can elicit the same response. Using a pharmacological approach, the authors go on to show that this response is dependent on endogenous PI(3)K activity and requires a Rho family GTPase activity. Their studies suggest a model for chemotaxis in which a directional chemoattractant signal results in a small initial activation of PI(3)K, triggering a Rho GTPase-dependent feedback loop that amplifies the signal, contributing to the observed steep intracellular PtdIns(3,4,5)P_3 gradient.

Weiner *et al.* provide further evidence that inhibition of PI(3)K activity impairs the ability to maintain stable pseudopodia,



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resulting in poor chemotactic fidelity. In addition, the authors provide compelling evidence that actin polymerization at the leading edge, which drives pseudopod extension and occurs downstream of PtdIns(3,4,5)P_3 accumulation, is in turn necessary for the maintenance of the localized accumulation of PtdIns(3,4,5)P_3 at the leading edge (see figure). Actin dynamics as part of a positive feedback loop may provide neutrophils with the ability to spontaneously polarize in response to an initially diffuse stimulus and start moving, only later homing in on their target. Thus, the amplification of the response to a chemoattractant gradient by the combination of Rho GTPase and actin feedback loops provides an attractive mechanism for how an initial small response results in strong cell polarization and persistent chemoattractant movement. What is unknown in this model are the mechanisms positioning the initial response that result in the first accumulation of PtdIns(3,4,5)P_3 at the site of the cell closest to the chemoattractant source. Future studies to define the biochemistry of this step, as well as to flesh out the feedback loops outlined in these two papers, are necessary to understand how cells sense and respond to directional signals.

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