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Role of microtubules and myosins in Fc gamma receptor-mediated phagocytosis

Nobukazu Araki

Department of Histology and Cell Biology, School of Medicine, Kagawa University, Miki, Kagawa 761-0793, Japan

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1. ABSTRACT

Microtubules serve as tracks for vesicular traffic in both phagosome formation and phagosome maturation. In the process of phagosome formation, endomembrane vesicles are delivered to the membrane of the forming phagosomes to supply membrane. This localized endomembrane delivery, referred to as focal exocytosis, facilitates pseudopod extension for the purpose of engulfing large particles. The microtubule-based transport system is the most likely candidate for such targeted vesicle trafficking to the forming phagosomes. During their maturation process, phagosomes interact with early and late endosomes and finally fuse with lysosomes. Although phagosomal membrane fusion with other membranous compartments does not require microtubules, bi-directional transport and positioning of the two organelles on microtubules are necessary for their close positioning and subsequent membrane fusion. Microtubules are also responsible for vesicle trafficking along the antigen presentation pathway for phagocytosed materials. Some classes of myosin are involved in diverse processes of Fc gamma receptor (FcgR)-mediated phagocytosis as force generators and actin-based transport motors. The role of myosin II in phagocytic cup squeezing is complementary to the classical zipper closure model. Myosin Ic and myosin X seem to be key players in extending and closing phagocytic-cup pseudopod. Other classes of myosin may also be involved in phagosomal movement. Myosin V may control short-range phagosome movement and relay phagosomes to the long-range linear transport system using microtubules.

2. INTRODUCTION

Microtubules formed by alpha- and beta-tubulin heterodimers are common cytoskeletal components of all eukaryotic cells. In some types of cells, microtubules form cilia and flagella on the cell surface and are responsible for cell movement. Apart from the special role played by microtubules in cilia/flagella, microtubules serve universally as tracks for the transport of organelles, membrane vesicles, and secretory granules (1). In interphase cells, microtubules are regularly found arranged in a star-like array radiating from the microtubule-organizing center (MTOC), also called the centrosome (Figure 1). The microtubule is a polar structure with the faster-growing plus end pointing toward the cell periphery and the minus end toward the MTOC. The function and structure of the microtubules are highly dependent on the association of microtubule-based motors and microtubule-associated proteins. Microtubule-based motor proteins include the minus end-directed (retrograde) motors such as cytoplasmic dyneins, and the plus end-directed (anterograde) motors, termed kinesins (1). These motor proteins use the energy derived from ATP hydrolysis for movement. The microtubule system is in charge of vesicular trafficking, providing tracks along which components move within the cell. In the processes of FcgR-mediated phagocytosis, membrane trafficking between the phagosomal membrane and other membrane compartments plays an important role in the formation and maturation of phagosomes.

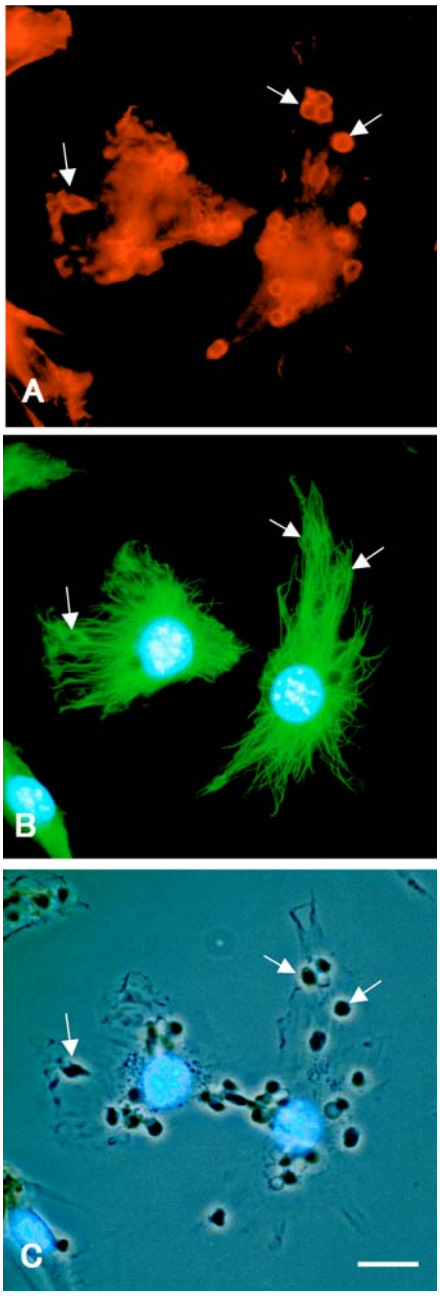

Figure 1. Fluorescence microscopy showing the distribution of microtubules in mouse macrophages during FcgR-mediated phagocytosis of IgG-opsonized erythrocytes (IgG-Es). Macrophages were fed with IgG-Es for 10 min, fixed and stained with rhodamine-phalloidin for the actin filaments, immunostained for tubulin, and stained with DAPI for the nuclei. (A) Rhodamine-phalloidin staining shows actin-filament assembly at the site of IgG-Es binding. (B) Microtubules emanate from the microtubule-organizing center located in the perinuclear region. Some microtubules extend to the vicinity of the actin-rich phagocytic cups (arrows). (C) Corresponding phase-contrast image merged with the DAPI staining image. Bar: 10 microns

The actin cytoskeleton is a key structure driving FcgR-mediated phagocytosis. In the process of phagosome formation, actin polymerization and reorganization mediated by many kinds of actin-binding proteins are particularly important to support the pseudopod extension around opsonized particles (2-6). Although actin filaments are mostly dissociated from phagosomes after internalization, some actin cytoskeleton and actin-binding proteins participate in phagosome movement and maturation. Myosins are one of the most important actin-binding proteins, and they produce the driving force for phagosome formation and transport. To date, at least eighteen classes of myosin have been identified (7). These structurally distinct classes of myosin are empirically defined on the basis of amino-acid-sequence comparisons of their conserved head or motor domains. Recent studies have implicated members of the myosin superfamily in a variety of cellular functions, such as membrane trafficking, cell motility, organelle movement and signal transduction (8). Several classes of myosin including class I, II, V, IX and X seem to participate in endocytic and phagocytic processes (3, 5, 9). Dianokova (2002) has shown that class II, and IXb myosins were concentrated at the site of phagocytic cup formation, that the concentration of myosin Ic increased at the site of phagocytic cup closure, and myosin V appeared after phagosome closure in macrophages (5). The association of each class of myosin with phagocytic cups/phagosomes at different maturation stages and locations indicates that each class of myosin plays a significant role at different stages of phagocytosis.

Thus, FcgR-mediated phagocytosis exploits both actin- and microtubule-based motors to achieve phagosome formation and intracellular trafficking between phagosomes and other endocytic compartments. In this article, the involvement of microtubules at each stage of phagosome formation, phagosome maturation and antigen presentation is reviewed. Also, the role of each myosin class in the phagocytic process will be discussed.

3. INVOLVEMENT OF MICROTUBULES IN FcgR-MEDIATED PHAGOCYTOSIS

3.1. Microtubules in phagosome formation

In FcgR-mediated phagocytosis, phagosome formation initially relies on the extension of plasmalemmal protrusion (pseudopod) to form a phagocytic cup enclosing the target particle. The pseudopod advance over the immunoglobulin G (IgG)-coated particles occurs by a zipper process, in which FcgRs in the phagocyte plasma membrane interact sequentially with Fc portions of IgG molecules (10). Signaling from the ligated FcgR leads local actin polymerization and reorganization at the site of the forming phagosomes (2, 6). Since cytochalasin treatment completely stops pseudopod extension, it is clear that actin filaments are a critical component of the phagocytic cups enclosing the particles. Unlike actin filaments, microtubules seem not to be directly required for pseudopod extension around the particles in the initial phase of phagosome formation, because phagocytic cup formation is not impaired by the disruption of the microtubules in macrophages. The subsequent

internalization of IgG-opsonized particles into phagosomes in the process of FcgR-mediated phagocytosis is also not much affected by microtubule depolymerization (11, 12), although phagocytosis of complement-opsonized particles or serum-opsonized yeast cells is significantly inhibited by microtubule-disruption (11-14). Nonetheless, recent studies have suggested the importance of microtubule-based membrane trafficking for rapid pseudopod extension which enables the efficient enclosure of large IgG-coated particles (15-19). Actually, some microtubules are found to be closely associated with forming phagosomes (Figure 1).

Before the phagocytic cups close into phagosomes, endomembrane delivery to the site of phagocytic cup formation occurs (15-19). The delivered endomembranes fuse with the plasma membrane and open at the site of particle contact. This phenomenon has been termed 'focal exocytosis' (15). Focal exocytosis, which serves as a membrane source, minimizes the use of plasma membrane and facilitates the formation and extension of phagocytic cups. In focal exocytosis, several endomembrane compartments, including recycling endosomes, late endosomes and the endoplasmic reticulum (ER), contribute to the formation of the phagosomes. The transport of VAMP3-containing vesicles, which are presumed to be compartments recycled from early (sorting) endosomes, to the site of the forming phagocytic cup is regulated by Rab11 (19) and COPI (20). It has also been shown that, during FcgR-mediated phagocytosis in macrophages, compartments of the late endocytic pathway bearing the tetanus neurotoxin-insensitive vesicle-associated membrane protein (TI-VAMP/VAMP7) are recruited upon particle binding and undergo exocytosis before phagosome sealing (17). It can be supposed that vectorial transport along the microtubules mediates focal membrane delivery to the site of the forming phagosomes. Recently, Gagnon *et al*. (2002) have revealed that the endoplasmic reticulum (ER) also contributes to the formation of phagosomes in macrophages (21, 22). The ER membrane fuses with the plasma membrane at the site of particle ingestion (23). Many studies have implicated microtubules as tracks for ER extension in cells (24-27). These studies have found by video microscopy that the tubule structures of the ER network extend along the microtubules in living cells. Interestingly, the rapid extension of the ER tubules toward the cell periphery is dependent on the microtubules, while the slower movement of the ER toward the cell center is independent of the microtubules (27). Taken together, these facts suggest that the microtubules guide the ER tubules to the bottom of the phagocytic cups, since membrane delivery should be rapid, transient and targeted. Moreover, the association of kinesin with the ER has led to the suggestion that kinesin provides the force for the centrifugal elongation of ER tubules (28).

In neutrophils, primary (azurophil) granules, which are lysosomal in nature, are exocytosed near the site of phagosome formation by focal exocytosis (29, 30). The early response of primary granule exocytosis precedes the sealing of the phagosomes, although primary granules can also fuse with internalized phagosomes. The microtubules seem to contribute to the vectorial nature of the response, since the localized delivery of primary granules can be disrupted by colchicine (30). Secondary (specific) granules are thought to be the main source of phagosomal NADPH oxidase, although only a fraction of the intrinsic oxidase component is present at the plasma membrane. Secondary granules are also capable of fusing with phagosomes (31). However, since the exocytosis of secondary granules is less polarized than that of primary granules, the transport of secondary granules may not be microtubule-dependent. It has been reported, in agreement with this, that the assembly of respiratory burst oxidase in the phagosomal membrane did not require intact microtubules (32). It has been shown that tertiary (gelatinase) granules which readily undergo exocytosis were recruited to phagosomes (33-35), although the transport mechanism is unknown.

Thus, multiple intracellular compartments and pathways contribute to phagosome formation. Some pathways may compensate for others when these are blocked. In FcgR-mediated phagocytosis, microtubule-independent pathways that can complement microtubule-dependent membrane trafficking may exist to provide sufficient membrane for the forming phagosomes, since FcgR-mediated phagocytosis is not completely blocked by microtubule-disruption.

Complement-receptor (CR)-mediated phagocytosis is more dependent on the integrity of microtubules than FcgR-mediated phagocytosis (11, 12). In CR-mediated phagocytosis, complement-opsonized particles sink directly into the cell without apparent pseudopod extension (11, 12, 36). Even though pseudopod extension is not required, membrane supply would be needed for particle internalization to prevent a net reduction in cell-surface dimensions. As in the case of FcgR-mediated phagocytosis, microtubule-dependent focal exocytosis would provide sufficient membrane to allow particle internalization. However, the sources and transport mechanism of endomembrane delivery may differ from those of FcgR-mediated phagocytosis. The presence or lack of a microtubule-independent compensation pathway for providing membrane may result in a difference in microtubule-dependence between FcgR- and CR-mediated phagocytosis. Furthermore, a recent study has shown that microtubules regulate beta-2 intergrin mobility and clustering in macrophages (37). Since beta-2 integrin functions as a complement receptor, it is consistent with the requirement for microtubules in CR-mediated phagocytosis. In addition, CR-mediated phagosome formation may occur by invagination of the plasma membrane pulled into cytoplasm along microtubules, perhaps with dynein as the microtubule-based motor.

3.2. Microtubules in phagosome maturation

Shortly after phagosomal formation, phagosomes have no microbicidal or degradative activity. For the acquisition of degradative activity, phagolysosome biogenesis was first believed to involve the complete fusion of phagosomes with lysosomes. However, recent studies have revealed that newly formed phagosomes undergo maturation by interacting with other endocytic compartments and transport vesicles. Then, the matured

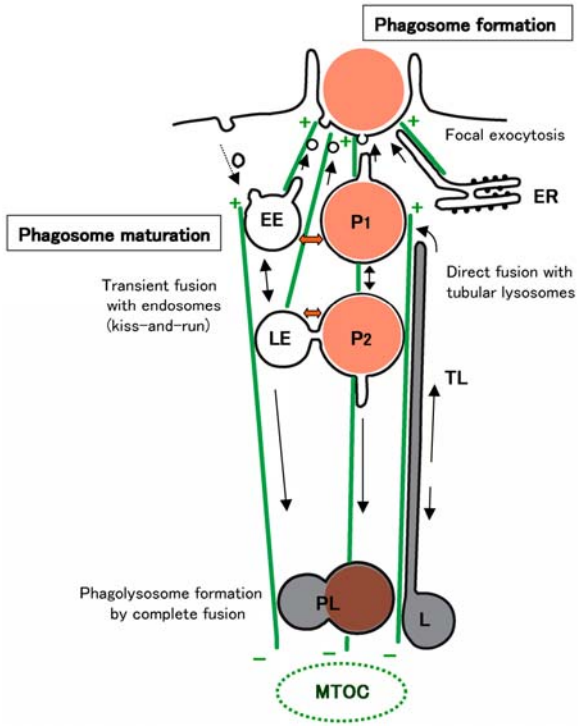

Figure 2. Scheme of microtubule-mediated transport in phagosome formation and phagosome maturation. Green lines indicate microtubules. Black arrows indicate the direction of transport. Red arrows indicate 'kiss-and-run' transient fusion. Biosynthetic transport route from Golgi apparatus is not shown. EE: early endosome; LE: late endosome; P_1: phagosome acquiring early endosomal properties; P_2: phagosome acquiring late endosomal properties; PL: phagolysosome; L: lysosome; TL: tubular lysosome; ER: endoplasmic reticulum; MTOC: microtubule-organizing center

phagosomes finally merge with preexisting lysosomes to form phagolysosomes, in which phagocytosed materials are degraded by lysosomal enzymes (38, 39). The maturation of phagosomes is characterized by the sequential alteration of both membrane composition and internal environment (40). The newly formed phagosomes, predominantly located at the cell periphery, tend to communicate with early and late endosomes through multiple fusion and fission events; this scenario is referred to as the 'kiss-and-run' hypothesis (38, 41, 42). Video microscopy has enabled us to see that transient contact (kiss) between two compartments is followed by fission and departure (run), probably along the microtubules (38, 43). Complete fusion between phagosomes and endosomes or lysosomes has also been observed in intact cells (44) and in a cell-free system (45, 46). Phagosome-endosome/lysosome fusion reconstituted in a cell-free system has indicated that direct membrane fusion between phagosomes and endosomes requires membrane fusion proteins and cofactors such as Rab GTPases, SNARE proteins, ATP and *N*-ethylmaleimide (NEM)-sensitive factor (NSF) (45, 46), but not tubulin. It is therefore unlikely that microtubules are absolutely required for the membrane fusion between phagosomes and endosomes. In intact cells, however, the two compartments must be brought close together to enable membrane fusion. Since microtubules greatly affect the intracellular location and movement of these organelles, functioning microtubules may increase the rate of phagosome-endosome fusion by promoting encounters between these two compartments. In nocodazole-treated macrophages, the recruitment of lamp2, a late endocytic marker protein, to phagosomes decreased greatly, suggesting that the interaction of phagosomes with late endocytic compartments involves microtubule-based transport of both organelles (43).

The membrane composition of phagosomes changes progressively during the process of maturation, as early endocytic markers such as Rab5 and EEA1 become scarce and late endocytic markers such as Rab7 and lamp2 are acquired. In consequence, nascent phagosomes gradually lose their ability to fuse with early endosomes, and instead gain the ability to move along the microtubules and fuse with lysosomes. Thus, the phagosome maturation pathway parallels the pathway of maturation of early endosomes into late endosomes and endolysosomes. The intracellular alignment of early endosomes, late endosomes and lysosomes from the periphery to the center of the cell, which is determined by microtubule function, would render phagosomes easy to fuse with proper counterparts in an age-dependent fashion (Figure. 2).

Maturing phagosomes acquire specific membrane components and luminal contents, not only by transient or complete fusion with endocytic compartments, but also via a biosynthetic transport pathway from the Golgi apparatus. It is conceivable that some newly synthesized proteins, including lysosomal enzymes and MHC class II proteins, are delivered to the phagosomes via microtubule-based transport from the Golgi apparatus. Dyneins and kinesins are essential for the positioning of the Golgi apparatus and vesicle transport from the Golgi apparatus, respectively (1, 47).

Despite the sequential fusion of the membrane delivered to the phagosomes, the size of phagosomes remains nearly constant. To maintain membrane dimension homeostasis in phagosomes, membrane insertion and retrieval should be balanced. This indicates that fission events and fusion events comparative in membrane amount should occur concomitantly. Plasma membrane proteins in the nascent phagosomal membrane are recycled to the plasma membrane by recycling membrane transport toward the cell surface, as in the recycling from early endosomes to the cell surface. Damiani, *et al*. (2003) have shown that tubulo-vesicular recycling from phagosomes is inhibited by the depolymerization of microtubules (48). Both Rab11and a Rab coupling protein belonging to the Rab11-FIP family associate with phagosomes and regulate the recycling from the phagosomal compartment to the cell surface (49).

Rab7, an important GTPase in the fusion events of late endocytic compartments, has been found to be associated with the phagosomal membrane after some phagosome formation. However, Rab7 recruitment to the phagosomal membrane precedes the fusion with late endosomes, suggesting that the acquisition of Rab7 is not

the result of the fusion with late endosomes (43). Although the precise pathway and regulation of Rab7 recruitment to phagosomes is still unknown, it is noteworthy that the appearance of Rab7 on the phagosomal membrane precedes the migration of the phagosome from the cell periphery to the central region of the cell. The Rab7-interacting lysosomal protein (RILP), which is known to be a Rab7 effector, localizes on late endosomes and lysosomes (50). RILP can also be observed on phagosomes. Dominant-negative Rab7 precludes RILP acquisition on phagosomes (51). Since RILP interacts with the dynein-dynactin complex, it directly promotes centripetal phagosome movement along the microtubules (52). The dominant-negative mutant of Rab7 fails to form tubular extension from phagosomes (51) and leads to a decrease in phagolysosome formation.

Phagosomes show two types of intracellular movement. The first type occurs predominantly at the cell periphery, often shortly after the phagosomes are formed, and at speeds below 0.1 micron/second. The second is faster (0.2-1.5 micron/second) and occurs mainly after the phagosomes have reached the central region of the cell (53, 54). Early-stage phagosomes have a strong preference for microtubule plus-ends, whereas late-stage phagosomes do not, and this plus-end affinity requires the presence of microtubule-associated proteins (MAPs) (54). Therefore, early phagosomes can readily ride on microtubules at the cell periphery and get off at the cell center. The majority of phagosomes show centripetal movement toward the MTOC in the perinuclear region, where phagosome-lysosome fusion frequently occurs. Both dynein and dynactin are required for this minus-end-directed movement along the microtubules (53). Although some phagosomes sometimes move to the plus-end along the microtubules using kinesin, the significance of this centrifugal movement of the phagosomes is unknown (53).

In the most cells, lysosomes usually adopt a roughly spherical shape and preferentially localize in the perinuclear region. However, in activated macrophages, lysosomes often become tubular. Tubular lysosomes are ~80 nm in diameter and up to several microns in length; they sometimes branch out and connect with each other, forming a radial network throughout the cell (55, 56). They move bi-directionally along the microtubules using dynein and kinesin (57). Thus, in such macrophages, the tubular lysosomes extending to the cell periphery can directly fuse with early phagosomes to form large spherical phagolysosomes there, prior to the transportation of the phagosomes to the perinuclear region (58). Then, the phagolysosomes move along the microtubules toward the center of the cell. The re-formation of tubular lysosomes from spherical lysosomes also requires intact microtubules (58, 59). Also, the disruption of microtubules causes the collapse of tubular lysosomes into roughly spherical ones and thus inhibits the direct fusion of lysosomes with early phagosomes (55, 56). In activated phagocytes, tubular lysosomes may play the role of a bypass route for the rapid degradation of IgG-opsonized pathogens, without awaiting full phagosome maturation. Although similar tubular lysosomes have been observed in other cell types (60-62), their function still remains to be elucidated.

3.3. Other putative roles of microtubules in phagocytosis

Phagocytosis is now considered to be one of the major entry routes of antigens for major histocompatibility complex (MHC) class I and class II antigen presentation (63-65). The internalized antigens appear to use distinct trafficking cargoes, depending on the antigen presenting pathways of the MHC class, the kind of antigens and the cell type. In any case, the processing of internalized antigens and antigen presentation are a dynamic mechanism involving several vesicular compartments and transport pathways. In the MHC class I pathway, antigen-presenting cells such as macrophages and dendritic cells concentrate and localize particulate antigens in the trans-Golgi area prior to displaying MHC class I-antigenic peptides on the cell surface. Colchicine-treated macrophages and dendritic cells internalize liposomal ovalbumin but fail to concentrate the antigens in the trans-Golgi area. This suggests that the trafficking pathway for antigen processing uses a microtubule-dependent translocation system (66). The antigen is transported to the Golgi apparatus, where it combines with MHC class I molecules, and is then transported to the cell surface for presentation of the MHC class I-antigen peptide complex to the T cells. Although the involvement of microtubules in this transport has not been established, the shuttle movement of vesicles containing the MHC class I-antigen peptide complex implies the involvement of the microtubules. Class II endosomal sorting into late multivesicular compartments seems to involve a microtubule-based system (67). A subset of lysosomal compartments (MHC class II-containing compartments) is considered a major site of class II-molecule peptide loading. The class II molecules finally present the peptides at the cell surface to the T cells. Visualization of GFP-class II molecules in living cells has revealed that the direct transport of vesicles containing class II molecules from the perinuclear regions to the plasma membrane was also carried out along the microtubules (68).

The microtubule is not only a track for vesicle/organelle trafficking, but also a scaffold for signaling molecules such as phosphoinositide 3-kinase (PI3-kinase) (69, 70). Furthermore, the Rho family of GTPases, Rac1 and Cdc42 interact with microtubules directly or indirectly, and may affect microtubule stability (71-73). Conversely, microtubule growth activates Rac1, leading to actin polymerization in the lamellipodial protrusions of fibroblasts (74). Since these signaling molecules are extensively involved in the regulation of actin remodeling during phagocytosis, the interplay between microtubules and these signaling molecules may orchestrate both microtubule- and actin-based processes during phagocytosis.

4. INVOLVEMENT OF MYOSINS IN FcgR-MEDIATED PHAGOCYTOSIS

4.1. Role of myosin II in phagocytic cup squeezing

Nonmuscle class II myosins, known as conventional myosins, have been implicated in various cellular events, including cell locomotion, cytokinesis and phagocytosis. As in the inhibition of PI3-kinase by wortmannin or LY294002, the selective inhibition of

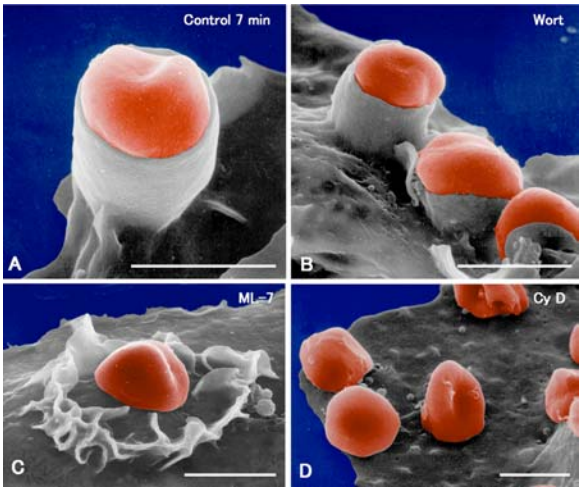

Figure 3. Scanning EM comparison of the morphology of phagocytic cups formed in the presence of various inhibitors. (A) A phagocytic cup engulfing an IgG-E in a control macrophage incubated with IgG-Es for 7 min. Note that the cylindrical phagocytic cup closely follows the surface of IgG-E. (B) Many phagocytic cups remained unclosed in wortmannin-treated cells even after a 30-min incubation with IgG-Es. The phagocytic cups are shown to be tightly fitted around the IgG-Es, just like the phagocytic cups of the control cells. (C) In ML-7 treated cells, phagocytic cup formation was not inhibited, but the morphology of the cups was remarkably different from that in the control or PI3-kinase-inhibited cells. Note that the pseudopod extends away from the surface of the IgG-E. (D) Cytochalasin D completely inhibited phagocytic cup formation, suggesting that pseudopod extension is dependent on actin polymerization. Reproduced from (4). Bars: 5 microns.

myosin II by ML-7, a myosin light-chain kinase (MLCK) inhibitor, perturbs phagocytic cup closure, but not actin filament assembly for the formation of phagocytic cups in FcgR-mediated phagocytosis by macrophages (4). However, scanning electron microscopy has revealed that the morphology of phagocytic cups in myosin II-inhibited cells apparently differs from that of PI3-kinase-inhibited ones (Figure. 3). The phagocytic cups halted during the process of engulfment of IgG-opsonized erythrocytes (IgG-Es) by PI3-kinase inhibitors have been found to be tightly fitted around the surface of IgG-Es. The shape of the phagocytic cups formed in the presence of PI3-kiase inhibitors is the same as that of normal phagocytic cups before sealing. IgG-Es engulfed by phagocytic cups are often deformed into columns, suggesting the generation of force by the contractile activity of the cup. In ML-7-treated macrophages, phagocytic cups are more open; the side wall of the phagocytic cup is not attached to the surface of the IgG-Es. These observations strongly suggest that myosin II activated by MLCK is required for the contractile activity of phagocytic cups. Furthermore, video microscopy of live macrophages expressing GFP-actin has revealed that a dense band of actin filaments moves from the bottom toward the top of the phagocytic cup during pseudopod extension. It appears that the tight ring of actin filaments squeezes the deformable particles (Figure 4A). Thus, this squeezing motion of the phagocytic cups is PI3-kinase-independent, and is a MLCK-dependent contractile activity in which actin and myosin II interact (4).

What is the significance of this squeezing motion? In the presence of ML-7, we have unexpectedly observed pseudopod extension around the particles, without apposition along the surface of the particles, although pseudopod extension is slowed down and shortened due to MLCK inhibition (Figures 3C and 4B). This finding implies that sequential IgG-FcgR binding might not occur by itself, but requires forced zipper closure. One explanation is that myosin-II contractile activity may promote the binding between the FcgR and ligands, to facilitate the efficient extension and subsequent closure of phagocytic cups. Another possibility is that the tight-fitting squeezing of the phagocytic cups pushes extra-particle fluid out of the phagosomes. This mechanism would decrease phagosomal volume and consequently increase intraphagosomal concentrations of superoxide, protons and hydrolases for bacterial killing and degradation. Interestingly, living *Salmonella typhimurium* bacteria cause pseudopods to extend and surround them, and enter into spacious phagosomes where they can live (75, 76), while opsonized pathogens are internalized into tight-fitting phagosomes where they are killed and degraded. To clarify the relationship between the myosin-mediated contractile activity of phagocytic cups and pathogen killing could be important for gaining a better understanding of innate immunity.

Olazabal *et al.* (2002) have shown that ML-7 inhibits complement receptor (CR)-mediated phagocytosis as well as actin filament assembly at the site of phagocytosis. Their study indicates that myosin II phosphorylated by Rho kinase is responsible for actin filament assembly in CR-mediated phagocytosis but not in FcgR-mediated phagocytosis (77). Thus, it is likely that myosin II is required for both types of phagocytosis, but plays distinct roles in the two modes of phagocytosis.□

4.2. Myosin I in phagosome closure

Unlike conventional myosin II, which is double-headed, myosin I is single-headed and has low molecular weight. Class I myosins have been found to localize in actin-rich cellular extensions, including the leading edges of lammelipodia and pseudopodia, macropinocytic crowns and intracellular vesicles (78). Since myosin I has a domain which binds to membranes, this class of myosins has been proposed to function at the membrane-actin interface for membrane trafficking in endocytosis and exocytosis. In mouse macrophages during FcgR-mediated phagocytosis, myosin Ic, a subclass of myosin I, is concentrated in the tip of the phagocytic cup, implicating it in the purse-string-like contraction that closes the opening of phagocytic cups (9). Myosin Ic appears a little later at the phagocytic cups than myosin II. The inhibition of PI3-kinase activity suppresses myosin Ic recruitment to the phagocytic cups, while myosin II is less affected by PI3-kinase inhibitors. In agreement with this, the inhibition of PI3-kinase activity perturbs the closure of phagocytic cups, but not of phagocytic cup squeezing by myosin II (4, 79). Taken together, these facts

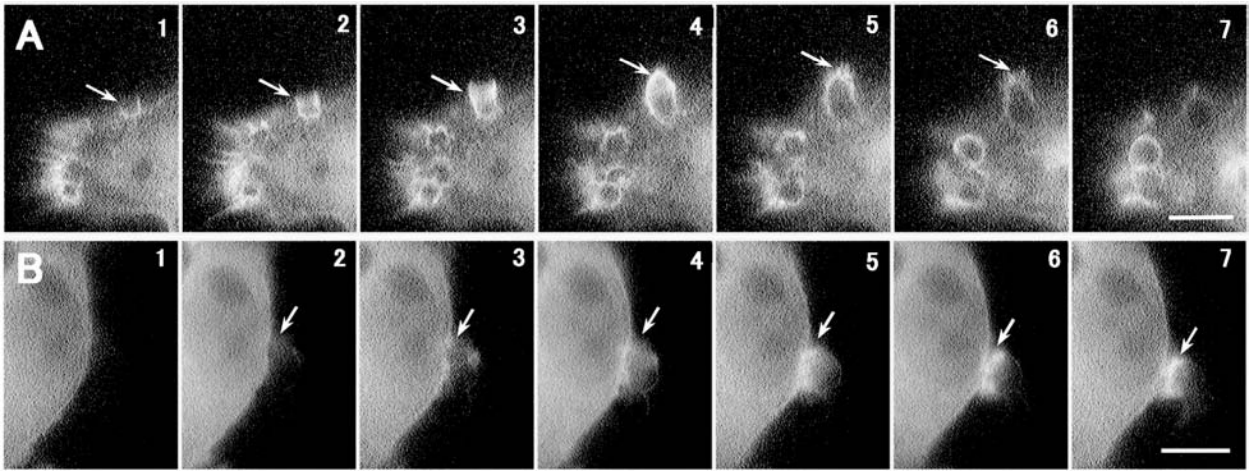

Figure 4. Time-lapse sequences of live macrophages expressing EGFP-actin, showing actin dynamics during FcgR-mediated phagocytosis of IgG-Es. (A) In a normal macrophage, actin filament assembly occurs at the site of IgG-E binding. Then, a pseudopod extends to form a phagocytic cup (arrows). It appears that a dense actin band constricts the phagocytic cup, resulting in the squeezing of the IgG-E. (B) In a ML-7-treated cell, although actin filament assembly and pseudopod extension occurred (arrows), the process was slower and the phagocytic cup was shallower than in normal. Finally, the cup collapsed without forming a phagosome. The elapsed times after the binding of the IgG-E are indicated, in minutes, in the upper right corner of each frame. Reproduced from (4). Bars: 10 microns.

indicate that myosin Ic lies downstream of PI3-kinase signaling. Class I myosins have an *src* homology 3 (SH3) domain in the tail region. In *Dictyostelium*, the SH3 domain seems to be important for myosin I function. The heavy chain of *Dictyostelium* myosin I is phosphorylated by the PAK family of serine/threonine kinases, resulting in the activation of actin-dependent ATPase (80). PAK kinases lie downstream of Cdc42 and Rac1 GTPase, which regulate actin organization. However, it is unknown how mammalian class I myosins are regulated, because the heavy chain lacks the consensus site for PAK phosphorylation. The precise role of SH3 and the signaling cascade regulating the activity and localization of myosin I during FcgR-mediated phagocytosis remain to be elucidated.

In the endocytic pathway, myosin I alpha has been shown to be associated with endosomes and lysosomes. Moreover, overexpression of the truncated nonfunctional form of myosin I alpha has been found to impair the delivery of fluid-phase marker from the endosomes to the lysosomes in a hepatoma cell line, suggesting that myosin I alpha contributes to the trafficking between late endocytic compartments (81). However, the involvement of myosin I alpha in late phagocytic events has not been reported. It is also known that the yeast myosin I acidic domain and the SH3 domain interact with the Arp2/3 complex and Bee1P, a yeast homologue of Wiskott-Aldrich syndrome protein (WASP), respectively (82, 83). It is possible that class I myosins participate in diverse actin-based movements, in cooperation with other actin-cytoskeleton-regulating molecules.

4.3. Myosin X in forming phagosomes

Recently, class X myosins have been identified as a potential downstream target of PI3-kinase, required for the pseudopod extension and closure of phagocytic cups. Myosin X has a unique tail domain structure containing a myosin-tail homology 4 (MyTH4) domain, a FERM (band 4.1/ezrin/radixin/moesin) domain and three pleckstrin homology (PH) domains. The PH domains are implicated in phosphatidylinositol phospholipid signaling. The second PH domain in the myosin X tail binds to $PI(3,4,5)P_3$, a major product of PI3-kinase. Cox, *et al*. (2002) have shown that myosin X is recruited to the phagocytic cups in a PI3-kinase-dependent manner (84). The expression of truncated myosin X tails in macrophages inhibits the phagocytosis of large IgG-coated particles (6 microns) but not of small ones (2 microns). Furthermore, myosin X tail expression inhibits pseudopod spreading, but not adhesion, on IgG-coated substrates, as a model of 'frustrated phagocytosis.' These data suggest that the role of myosin X in maximal pseudopod extension is to enclose the particle during FcgR-mediated phagocytosis. Although it is not exactly known how myosin X contributes to the mechanism of pseudopod extension, the molecular structure of myosin X suggests that the dimer of the motor head domain of myosin X becomes engaged on actin filaments and moves toward the barbed end of the actin filaments. The FERM domain in the tail, which interacts with plasma membrane proteins and $PI(4,5)P_2$, is also essential for myosin X function in phagocytosis. In other cell types, myosin X is predominantly found in actin-rich membrane protrusions, such as filopodia and lamellipodia; overexpression of myosin X increases filopodia. These findings strongly support the assumption that myosin X plays a role in extending the pseudopod during phagocytosis. However, the possibility cannot be ruled out that myosin X also participates in the closing of phagocytic cups into phagosomes, along with myosin Ic. In any case, myosin X is the only known direct PI3-kinase effector molecule required for completing phagosome formation, although

many events during phagocytosis are regulated by PI3-kinase signaling.

4.4. Myosin V and other classes of myosin in phagocytosis

Myosin V appears on fully internalized phagosomes, whereas other classes of myosin appear on forming phagosomes (5, 9). It is known that class V myosins are involved in the intracellular transport of organelles such as melanosome granules (85), brain vesicles (86) and the ER (87) in other cell types. So, it can be supposed that class V myosins are responsible for phagosomal movement rather than the formation of phagosomes. Video microscopy of phagosome dynamics has shown that newly formed phagosomes remain within the periphery of the cells for a while, and then linearly move to the center of the cells (88). The peripherally retained phagosomes exhibit short-range random movement before undergoing rapid linear transport. It is likely that myosin V mediates the short-range slow phagosomal movement at the cell periphery (88), although microtubule growth may also participate in the slow movement (54). As described above, the rapid linear movement of phagosomes from the periphery to the center of the cell is mediated by a microtubule-based transport system using the minus-end directed motor dynein (53). However, myosin Va bound to phagosomes binds to F-actin and restricts the microtubule-based centripetal movement of phagosomes (88). Therefore, it can be said that the role of myosin V is to restrict phagosome movement rather than to accelerate their transport. The retention of early phagosomes in the subcortical region may facilitate the interaction with endosomes located in the same region.

A *Dictyostelium* mutant lacking myosin VII has been found to exhibit an 80% decrease in the uptake of particles, although phagocytic cup formation was normally processed, suggesting that class VII myosins are required for phagosome formation (89). Myosin VII has been suggested to be involved in the phagocytosis of photoreceptor discs by retinal pigmented epithelial cells, but it shows a highly restricted tissue distribution (90), and has not been detected in the phagocytes of mice. Therefore, in FcgR-mediated phagocytosis by mammalian phagocytes, other classes of myosins compensate for the role of myosin VII in *Dictyostelium discoideum*.

Myosin VI is the only known myosin able to move toward the pointed ends of the actin filaments (91). Myosin VI has been implicated in early endosome transport (92-94) and in anchoring the bases of the stereocilia of the inner ear to the cortical actin cytoskeleton (95). Interestingly, myosin VI localizes on the cell-surface ruffles, which are the precursors of macropinosomes, in epidermal growth factor-stimulated A431 cells (96). However, this class of myosin has not been detected in mouse macrophages during phagocytosis (9).

The localization and timing of the appearance of class IX myosins in phagocytic cups are similar to those of class II myosins (5, 9). It can be postulated that myosin IX, like myosin II, is also involved in the contractile activity of phagocytic cups, although its functional role remains to be elucidated. In addition, myosin IX may serve as the signal transducer for the reorganization of the actin cytoskeleton, since class IX myosins contain a GTPase-activation-protein (GAP) domain that activates the intrinsic Rho GTPase activity (97) involved in the regulation of actin reorganization during phagocytosis.

5. PERSPECTIVE

It is now apparent that microtubules participate in vectorial vesicle/tubule transport in several distinct processes of phagocytosis. However, it is unclear how microtubules know in which direction to extend. Evidence for the role of microtubules as scaffolding for signaling complexes is accumulating. It is also possible that some of these signaling molecules regulate microtubule extension and direction. The interaction among signaling molecules, microtubules and microtubule-associated proteins during phagocytosis should be explored in future studies. Another fact which needs clarification is that multiple transport pathways use the same cytoskeletal component. Does one pathway use a subpopulation of microtubules exclusively? If all trafficking is commonly carried out across all microtubule tracks, it must be highly regulated to prevent traffic jams. The presence of many classes and families of motor proteins belonging to the kinesin and dynein superfamily implies that each motor protein has its own cargo, thus defining the role of each trafficking pathway in the cell (1). How a cargo binds to its correct motor is also an intriguing question. Understanding the roles of MAPs (98) and of microtubule subpopulations (99, 100) in relation to microtubule dynamics and architecture also seems to be important. With respect to myosins, it is now apparent that various classes of myosin function at distinct stages of FcgR-mediated phagocytosis, although the roles of some classes of myosin remain to be explored in more detail. Finally, the signaling cascade used for recruiting and activating distinct classes of myosin also needs to be resolved.

So far, molecular and genetic studies have identified many signaling and mechanical molecules involved in each process of phagocytosis. We are entering an exciting research stage, in which the complex web of signaling and mechanical links is being unraveled; this will bring about a better understanding of the overall system of phagocytosis, in its coordination with the immune system. The similarities and differences in molecular machinery between distinct types of phagocytosis, including complement-mediated and pathogen-induced phagocytosis, also remain to be addressed in future studies.

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Send correspondence to: Nobukazu Araki, Ph.D., Department of Histology and Cell Biology, School of Medicine, Kagawa University, Miki, Kagawa 761-0793, Japan, Tel: 81-87-891-2090, Fax: 81-87-891-2092, E-mail: naraki@med.kagawa-u.ac.jp

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