A contractile activity that closes phagosomes in macrophages

Joel A. Swanson1,2,*, Melissa T. Johnson1, Karen Beningo2, Penny Post3, Mark Mooseker3
and Nobukazu Araki1,4

1Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA
2Department of Anatomy and Cell Biology, University of Michigan Medical School, Ann Arbor, MI 48109-0616, USA
3Department of Biology, Yale University, New Haven, CT, USA
4Department of Anatomy, Kagawa Medical University, Miki, Kagawa 791-0793, Japan

*Author for correspondence at the Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109-0620
(e-mail: jswan@umich.edu)

Accepted 7 November 1998; published on WWW 13 January 1999

SUMMARY

Studies of Fc-mediated phagocytosis by mouse macrophages identified a contractile activity at the distal margins of forming phagosomes. Time-lapse video microscopic analysis of macrophages containing rhodamine-labeled actin and fluorescein dextran showed that actin was concentrated at the distal margins of closing phagosomes. Phagocytosis-related contractile activities were observed when one IgG-opsonized erythrocyte was engaged by two macrophages. Both cells extended pseudopodia until they met midway around the erythrocyte. It was then constricted and pulled into two phagosomes, which remained interconnected by a string of erythrocyte membrane. Butanedione monoxime, an uncompetitive inhibitor of class II and perhaps other myosins, and wortmannin and LY294002, inhibitors of phosphoinositide 3-kinase, prevented the constrictions without inhibiting the initial pseudopod extension. Immunofluorescence microscopy showed the presence of myosins IC, II, V and IXb in phagosomes. Of these, only myosin IC was concentrated around the strings connecting shared erythrocytes, suggesting that myosin IC mediates the purse-string-like contraction that closes phagosomes. The sequential processes of pseudopod extension and contraction can explain how macropinosomes and spacious phagosomes form without guidance from a particle surface.

Key words: Phagosome, Macrophage, Myosin, Contraction

INTRODUCTION

Phagocytosis is a complicated rearrangement of the actin cytoskeleton that delivers extracellular particles into intracellular vacuoles called phagosomes. In macrophages, a particle opsonized with IgG can stimulate Fc receptor-mediated phagocytosis, in which pseudopodial extensions of the cell surface cover the particle and enclose it. Although it is established that these pseudopodia contain actin and many actin-associated proteins (Allen and Aderem, 1995), and that a functional actin cytoskeleton is necessary for phagocytosis (Allison et al., 1971), it is not yet known how the actin cytoskeleton is regulated to achieve this apparently coordinated combination of extension and closure.

No single extant model explains all kinds of phagocytosis. According to the zipper model for phagocytosis, pseudopod advance over a particle is guided by interactions between opsonins and cognate receptors in the macrophage plasma membrane. Such zipperping of a membrane along a particle surface, together with a membrane fusion process, has been considered sufficient to enclose particles completely (Greenberg and Silverstein, 1993; Swanson and Baer, 1995). However, in the analogous process of macropinocytosis, actin-rich pseudopodia extend and close into intracellular vesicles without any particle surface to guide them (Swanson and Watts, 1995). Moreover, some bacteria enter macrophages by a process resembling macropinocytosis (Alpuche-Aranda et al., 1994). Thus, without an additional mechanism for closing phagosomes, the zipper model cannot explain how macropinosomes and spacious phagosomes form.

Recent studies indicate that phagocytosis requires two component activities of the actin cytoskeleton. Inhibitors of phosphoinositide 3-kinase (PI3-kinase) allow pseudopodia to extend onto an opsonized particle, but prevent them from closing into phagosomes, indicating that PI3-kinase regulates phagosome closure (Araki et al., 1996). Phagosome closure could occur by regulated actin polymerization, reorganized actin gel networks, altered membrane curvature or a localized contractile activity. Although myosins localize to phagosomes (Allen and Aderem, 1995; Stendahl et al., 1980) and evidently participate in the process (Ostap and Pollard, 1996), their contribution to phagocytosis remains undefined. Evans et al. (1993), measured forces generated by leukocytes during phagocytosis of yeast particles, and identified a contraction of the entire cell that followed pseudopod extension over a particle. This indicated that some contractile activity accompanies phagocytosis. Here we describe a localized...
contraction that constricts the margins of forming phagosomes and allows extended pseudopodia to close into intracellular phagosomes. To begin to look for a motor responsible for this process, we probed bone marrow-derived macrophages for class I, II, V, VI, VII and IX myosins. Myosin-IC, myosin IIa, myosin Va and myosin IXb were present in these cells and all localized to phagosomes. The localization and timing of appearance of myosin IC suggest that it plays a role in the constriction.

**MATERIALS AND METHODS**

**Cells**
Bone marrow-derived macrophages were obtained from C3H-HeJ mice and cultured for 6 days as previously described (Swanson, 1989). Erythrocytes were labeled with biotin and fluorescein-streptavidin, and opsonized with IgG. 5x10^5 washed, sheep erythrocytes (ICN Biomedical, Costa Mesa, CA) were labeled with 1 mM NHS-X-biotin (Calbiochem, La Jolla, CA; prepared from a 50 mM stock in dimethyl formamide) in 150 mM carbonate-bicarbonate buffer, pH 9.5. After incubation for 20 minutes at 4°C, cells were washed with phosphate-buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 1 mM KH2PO4, 6 mM Na2HPO4, pH 7.4), opsonized for 60 minutes with rabbit anti-sheep erythrocyte IgG (ICN Biomedical), and washed again with PBS. To label erythrocyte surfaces, fluorescein-streptavidin (6 μg/ml final concentration) was added to erythrocytes when they were mixed with the macrophages; adding at this time reduced particle clumping. For some studies, as indicated, fluorescein-streptavidin was added to macrophages after first allowing 30 minutes for phagocytosis. Macrophages were chilled after phagocytosis and incubated with fluorescein-streptavidin (20 minutes at 4°C), then fixed. In other studies, erythrocytes were opsonized with IgG without additional labeling with NHS-X-biotin or fluorescein-streptavidin.

**Phagocytosis**
Macrophages were incubated in Ringer’s buffer (RB: 155 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 2 mM NaH2PO4, 10 mM Hepes, pH 7.2, 10 mM glucose) for 15 minutes, then in RB with or without inhibitors (100 nM wortmannin, 50 mM Hepes, pH 7.2, 10 mM glucose) for 15 minutes, then in RB ± inhibitors, all at 4°C. Opsonized erythrocytes were without inhibitors (100 nM wortmannin, 50 mM Hepes, pH 7.2, 10 mM glucose) for 15 minutes, then in RB with or without inhibitors, all at 4°C. Cells were washed with PBS, lysed in 1 ml SDS-PAGE sample buffer containing protease inhibitors (complete inhibitor tablets, Boehringer-Mannheim, 1 tablet/7 ml water). Immediately after resolubilization of proteins, 40 μg protein per sample was separated by 5% SDS-PAGE, transferred to PVDF membranes, then processed with affinity-purified rabbit antibodies raised against myosin IC (J. Skowron, W. Bement and M. Mooseker, unpublished), human platelet myosin II (Biomedical Technologies, Stoughton, MA), chick brain myosin V (Espesefico et al., 1992), porcine myosin VI (Hasson and Mooseker, 1994), human myosin VIIa (Hasson et al., 1995), human myosin IXb (Wirth et al., 1996) and horseradish peroxidase-labeled secondary antibodies.

**Immunofluorescence**
Macrophages on coverslips were allowed to phagocytose sheep erythrocytes opsonized with mouse anti-sheep erythrocyte IgG (Harlan Bioproducts, Indianapolis, IN). Cells were fixed 15 minutes at 37°C in buffer A (30 mM Hepes, pH 7.4, 10 mM EGTA, 0.5 mM EDTA, 5 mM MgSO4, 33 mM potassium acetate, 5% polyethylene glycol 400, 0.02% sodium azide) containing 4% paraformaldehyde; then fixed and permeabilized for 15 minutes in buffer A containing 4% paraformaldehyde and 1% Triton X-100. Cells were then washed 3x5 minutes in buffer B (buffer A plus 2% heat-inactivated goat serum), then incubated with anti-myosin antibodies in buffer B, 60 minutes at 37°C. Cells were washed again and incubated for 60 minutes at 37°C in buffer B containing 10 units/ml Texas Red-phalloidin, plus Oregon Green-labeled goat anti-rabbit IgG (1:5000) and aminomethylcoumarin-labeled goat anti-mouse IgG (dil 1:500) (all from Molecular Probes, Eugene, OR). Preparations were washed again with buffer B, fixed again with buffer A plus 4% paraformaldehyde, washed again with buffer B, and finally mounted in glycerol containing 1 mg/ml phenylenediamine. Cells were observed using a Zeiss Axiosphot 2 fluorescence microscope.

**Fluorescent analog cytochemistry**
Macrophages were labeled by scrape-loading (McNeil et al., 1984) with fluorescein dextran (f-dextran, average MW 10,000, Molecular Probes, Eugene OR) and rhodamine-labeled actin (a gift from Dr. Eugene Omann, University of Michigan). To prevent actin polymerization during the loading procedure, cells were scrape-loaded in solutions with cytochalasin B (Sigma Chemical Co., St Louis, MO). 10^5 cells on a Petri dish were rinsed with PBS at 37°C, then 40 μl of 500 μg/ml f-dextran, 50 μg/ml rhodamine-actin and 5 μM cytochalasin B in PBS were added and cells were scraped off the dish with a rubber policeman. The scraped cells were quickly combined with 2 ml DM10F and plated onto 25 mm diameter, circular coverslips. After 10 minutes, medium was replaced with DM10F to remove extracellular fluorophores, and cells were incubated for another hour (in 5% CO2, 37°C) before observation by fluorescence microscopy.

**Imaging**
For video microscopy, macrophages were plated onto 25 mm circular coverslips, which were assembled into Leiden chambers on a heated stage (Medical Systems Corp.) of a Zeiss IM35 inverted microscope. Cells in RB were viewed by phase-constrain optics using a 100x objective lens. Images were collected via a video camera, digitized using Metamorph software (Universal Imaging Co., West Chester, PA) and stored on optical discs.

For fluorescence microscopy of living cells, scrape-loaded macrophages on coverslips were prepared as described for video microscopy. Cells were observed using a 60x planapo objective lens. Metamorph software controlled a shutter and filter wheel that obtained pairs of fluorescein and rhodamine fluorescence signals, collected every 10 seconds using a digital cooled CCD camera (Quantix, Photometrics). Images were processed to obtain ratio images, dividing the rh-actin fluorescence by the f-dextran fluorescence.

For fluorescence confocal microscopy, macrophages fixed and stained with fluorescein streptavidin and rhodamine phalloidin were observed using a Zeiss LSM 410, as described by Araki et al. (1996).
RESULTS

Movements of actin during phagocytosis

During Fc receptor-mediated phagocytosis, actin-rich pseudopodia extend around a particle and enclose it. We reasoned that a contractile activity associated with phagosome closure should increase the concentration of actin at the distal margin of the closing phagosome. Actin concentrations were monitored directly by observing phagocytosis of IgG-opsonized sheep erythrocytes in macrophages scrape-loaded with rh-actin and f-dextran (Fig. 1). The f-dextran served as an indicator of the distribution of cytoplasm; it was accessible to the nucleus as well as the phagocytic cups, and reported the movements of cytoplasm during phagocytosis. Because rh-actin was larger than f-dextran, it was excluded from the nucleus. Ratiometric images were obtained by dividing the pixels for rh-actin by the corresponding pixels for f-dextran; and pseudocolor applied to the ratiometric image showed high concentrations of rh-actin at the cell margin and forming phagosomes as yellow and orange, and the low concentrations in the nucleus, relative to f-dextran, as purple. The time series indicated that actin was concentrated at the distal margins of closing phagosomes (Fig. 1, at 1.5, 3.5 and 4 minutes). In one time series, two labeled macrophages engaged a cluster of erythrocytes (Fig. 2). The cells appeared to bite individual erythrocytes from the cluster, and showed an increase in actin concentration coincident with phagosome closure (Fig. 2, at 2 and 3 minutes). Similar increases in actin concentration were observed in four separate observations; in no instance did phagocytosis occur without an increase in actin concentration. This increase in actin concentration indicated that closure required more than the continued advance of the pseudopod around the particle, and that perhaps a contractile activity was mediating the constriction that closed the phagosome.

The constriction of shared erythrocytes

Contractile activities could be observed directly when two macrophages attempted to phagocytose a single erythrocyte. Phase-contrast video microscopy showed that erythrocytes landing directly onto individual macrophages were quickly ingested by phagocytosis, as expected, but those landing between two macrophages were engaged by both cells. In such instances, pseudopodia extended around the erythrocyte from both macrophages until they met each other. Both phagocytes then constricted the middle of the erythrocyte, and pulled it into two phagosomes. These phagosomes remained connected (Fig. 3).

The geometry of the shared erythrocytes was visualized by fluorescence microscopy. Macrophages were fed IgG-opsonized erythrocytes labeled on their surfaces with fluorescein-streptavidin, then were fixed and observed by fluorescence microscopy. Although most erythrocytes were completely intracellular, some were clearly shared by two macrophages. Typically, the shared erythrocytes contained two bulbous ends, each enclosed in a macrophage vacuole and connected to the other by a long string of fluorescent membrane (Fig. 4a,b). Pseudopodia from each macrophage extended along the string, giving the appearance that nearly all of the erythrocyte was enclosed by one macrophage or the other. We expected that if the erythrocyte remained intact, and bridged phagosomes of two macrophages, then some portion...
of the erythrocyte membrane should remain extracellular. To identify extracellular portions of shared erythrocytes, fluorescent label was added after phagocytosis. Macrophages were first fed opsonized, biotinylated erythrocytes, then fluorescein-streptavidin was added to label exposed erythrocyte membrane. Most erythrocytes were intracellular, inaccessible to the probe and therefore dark; but the shared erythrocytes were labeled along a short stretch of the string connecting the two macrophages. Even when the phagosomes were separated by many \( \mu \)m, only 1-2 \( \mu \)m of erythrocyte membrane was accessible to fluorescent probe (Fig. 4c,d), indicating that macrophage pseudopodia extended along the length of the elongated erythrocytes.

**Inhibition of phagosome constriction**

Several studies have indicated a role for PI3-kinase in Fc receptor-mediated phagocytosis. Ligation of Fc receptors increased PI3-kinase activity and led to the generation of phosphatidylinositol 3,4,5-trisphosphate (Ninomiya et al., 1994). Moreover, the PI3-kinase inhibitors wortmannin and LY294002 inhibited macrophage phagocytosis late in the process (Araki et al., 1996). To examine the role of PI3-kinase in the constriction of shared erythrocytes, macrophages were incubated with the PI3-kinase inhibitors wortmannin or LY294002 before and during phagocytosis of fluorescent erythrocytes. Under such conditions, constricted erythrocytes were absent. Instead, many erythrocytes were wedged between two macrophages, without any evidence of constriction (Fig. 4e,f). Fluorescent labeling of erythrocyte surfaces after phagocytosis showed two non-fluorescent ends of the erythrocyte and an equatorial band of exposed erythrocyte membrane, indicating that pseudopodia extended from each neighboring macrophage along the particle surface (Fig. 4g,h). This was consistent with earlier observations that wortmannin did not inhibit pseudopod extension along opsonized erythrocyte surfaces (Araki et al., 1996). Thus, inhibition by wortmannin and LY294002 indicated a role for PI3-kinase in the purse-string-like constriction at the outer margin of the pseudopod.

To analyze the contribution of F-actin to this string formation in shared erythrocytes, F-actin of macrophages fixed during phagocytosis was labeled with rhodamine-phalloidin. In intermediate stages of the constriction process, visualized by confocal microscopy, the outer margins of the pseudopodia contained dense accumulations of F-actin (Fig. 5a,b). F-actin was also present in the extensions of macrophage surface that surrounded the connecting string of erythrocyte membrane.
Contractile activity in macrophage phagosomes

When phagosome constriction was blocked by wortmannin, the phagocytic cups surrounding erythrocytes contained a uniform distribution of F-actin (Fig. 5d), indicating that the PI3-kinase inhibitor had not prevented actin assembly or recruitment, but had prevented its concentration at the margins of contractile pseudopodia.

Myosins I and II have been localized to phagosomes (Allen and Aderem, 1995; Stendahl et al., 1980), and may contribute to the contractile activity described here. To examine the role of myosin, macrophages were incubated in 2,3-butanedione monoxime (BDM), an uncompetitive inhibitor of myosin II and perhaps other myosins (Herrmann et al., 1992; Cramer and Mitchison, 1995; McKillop et al., 1994). BDM inhibited phagocytosis and the constriction of shared erythrocytes. Like wortmannin and LY294002, BDM did not prevent the formation of actin-rich phagocytic cups around the erythrocytes: rhodamine-phalloidin staining of macrophages in BDM produced images of shared erythrocytes essentially identical to Fig. 3d. Thus, in wortmannin-, LY294002- and BDM-treated cells, string formation was completely inhibited; instead, many erythrocytes bound to macrophage surfaces were nested in cup-shaped phagosomes that labeled brightly with rhodamine-phalloidin.

An assay was devised to quantify the contractile activity, counting the number of fluorescent erythrocyte membrane strings per 100 macrophages on a coverslip. Dose-response curves for string formation in wortmannin and LY294002 were similar to those published for inhibition of PI3-kinase (Vlahos et al., 1994; Yano et al., 1995), and for the inhibition of phagocytosis in macrophages (Araki et al., 1996), further indicating that PI3-kinase was involved in the contractile activity (data not shown). In control preparations, the number of strings measured after 60 minutes was less than after 20 minutes, because the macrophages were apparently capable of degrading or breaking the strings and completing phagocytosis (Fig. 6). Complete inhibition of string formation was observed for wortmannin, LY294002 and BDM during 20- and 60-minute incubations, indicating that the inhibitors were not simply slowing the contractile activity. Inhibition of phagocytosis and thread formation by LY294002 and BDM were reversible, indicating that the drugs were not exerting some general cytotoxicity. The inhibition by wortmannin was not reversible.

Localization of myosins in phagosomes

Western staining of macrophage lysates showed immunoreactivity for myosins IC, II, V and IXb (Fig. 7), but not for myosins VI or VII (not shown). Immunofluorescent staining of macrophages engaged in phagocytosis yielded positive labeling of phagosomes with myosins IC, II, V and IXb (Fig. 8). Particles were recognized by phase-contrast optics and by fluorescent labeling of particle surfaces with aminomethylcoumarin-labeled secondary antibodies (not shown); phagocytic cups were recognizable by labeling F-actin with Texas Red-phalloidin. Although all four myosins could be localized in phagocytic cups, no one class of myosin labeled

---

**Fig. 4.** Phase contrast (a,c,e,g) and fluorescence (b,d,f,h) images of shared erythrocytes, showing the threads produced by the contractile activity at the distal margins of macrophages. Sheep erythrocytes were surface-labeled with biotin, then opsonized with IgG. Fluorescein-streptavidin was added either during phagocytosis, to label the entire erythrocyte surface (a,b,e,f), or after phagocytosis was completed, to label only exposed erythrocyte membrane (c,d,g,h). Macrophages were incubated in buffer alone (a-d) or with 100 nM wortmannin (e-h), which inhibited the contractile activity. White lines in a and c indicate macrophage borders; profiles are interrupted at regions containing shared erythrocytes. Bar, 10 μm.
all phagosomes. Rather, it appeared that myosins II and IXb labeled ruffles and early phagocytic cups, myosin IC labeled a later stage of phagosome closure, and myosin V labeled fully internalized phagosomes.

Myosin IC labeling of phagosomes indicated a role for that mechanoenzyme in phagosome closure. It formed a transient dense label around phagosomes coincident with closure. The label was internal to the phagosomal F-actin, such that the particle was closely wrapped with myosin Ic, with F-actin forming an outer layer (Fig. 8a,b). Erythrocytes that were shared between two macrophages contained myosin Ic around the connecting string (Fig. 9), whereas myosins II, V and IXb did not label those structures. This suggests that myosin IC participated in the contractions that formed strings and, by inference, in the contractions that closed phagosomes.

DISCUSSION

Contractile activities that close phagosomes

The experiments described here distinguish two component activities for ingestion by phagocytosis. The first is the extension of pseudopodia around a particle and the second is a purse-string-like constriction of the pseudopod margin (Fig. 10). Pseudopod extension probably occurs by localized polymerization of actin beneath the plasma membrane, as is the case for pseudopod advance during cell crawling (Mitchison and Cramer, 1996). The constrictions close the circular margin of the phagosome to a narrower aperture which, during normal phagocytosis, closes by membrane fusion into an intracellular vacuole or, when an erythrocyte is shared with another macrophage, remains plugged by a string of erythrocyte membrane. The contractile activity could be restricted to the margin of the phagosome, or it could occur throughout the phagocytic cup and culminate in the final closure. Consistent with this latter model, video microscopic studies of phagocytosis indicate that deformable particles, such as erythrocytes, are squeezed by phagosomes during the engulfment process (J. A. S., data not shown).

The contractile activity that closes phagosomes may be related to the contractions identified by Evans et al. (1993), who measured forces generated during phagocytosis in leukocytes. Although the contractions they described were not localized to the phagosomes, their timing was comparable to that observed for constriction of shared erythrocytes.

Mechanism

This contractility most likely involves actin and myosin. Actin is concentrated at the distal margins of closing phagosomes, and at the points of constriction of shared erythrocytes. Moreover, the myosin inhibitor BDM inhibits the constriction of shared phagosomes, consistent with the involvement of one activity.
or more myosins in this process; although it is important to note that there is no reliable information on how this drug impacts myosins other than myosins-II. In this study, we probed bone marrow-derived macrophages for class I, II, V, VI, VII and IX myosins and detected class I, II, V and IX myosins present (Fig. 7).

These myosins have previously been implicated in signal transduction and membrane trafficking. Myosin-V has been implicated in membrane trafficking events (reviewed by Mermall et al., 1998). Recently, mutations in human myosin-Va have been identified as the basis for Griscelli disease (Pastural et al., 1997). Griscelli disease patients have partial albinism, neurological defects (similar to dilute mice), and immunodeficiencies, principally in delayed type hypersensitivity responses (Griscelli et al., 1978; Hurvitz et al., 1993; Klein et al., 1994). Human myosin-IXb is most highly expressed in peripheral blood leukocytes. Its protein expression is upregulated during differentiation of HL60 cells into macrophage-like cells and it redistributes from the actin-based cortex to the cytoplasm in the differentiated cells (Wirth et al., 1996). Class IX myosins have a GAP domain in their tails for the small GTPase rho (Post et al., 1998; Muller et al., 1997); Rho regulates the organization of the actin cytoskeleton in many kinds of cells, including leukocytes (reviewed by Dharmawardhane and Bokoch, 1997). Thus, this myosin has the potential to regulate its transducing element. Presently, roles for myosins II, V and IXb in phagosomes are simply suggested by their presence in phagosomes.

Human myosin-IC, a homolog of the ‘long-tailed’ amoeboid myosins-I, has a tail containing a putative membrane-binding domain (TH1), a putative ATP-independent actin-binding domain (TH2), and an SH3 domain (Bement et al., 1994). SH3 domains allow protein-protein interactions (Feller et al., 1994; Feng et al., 1994) and recently, an Acanthamoeba protein was

Fig. 8. Immunofluorescence showing phagosomes labeled with anti-myosin antibodies. Macrophages were fixed during phagocytosis of sheep erythrocytes opsonized with mouse anti-sheep erythrocyte IgG. Cells were stained with Texas Red-phalloidin (a,c,e,g) and rabbit anti-myosins IC (b), II (d), V (f) and IXb (h), followed by Oregon Green-labeled secondary antibodies. Arrowheads indicate phagosomes, which were recognized by phase contrast microscopy and by staining particles with AMCA-labeled goat anti-mouse IgG (not shown). All four myosins labeled some phagosomes. Anti-myosin IC sometimes produced a prominent labeling of phagosomes.

Fig. 9. Immunofluorescence showing myosins in shared phagosomes. Panels show phase-contrast images of shared erythrocytes (a-e), Oregon Green-labeled secondary antibody labeling of anti-myosin primary antibodies IC (f,g), II (h), V (i) and IXb (j); and Texas Red-phalloidin labeling of F-actin (k-o). Only anti-myosin IC labels connecting strings of erythrocyte membrane. Arrowheads indicate concentrations of myosin IC at points of constriction. Bar, 10 μm.
found to associate with the SH3 domain of a class I myosin (Xu et al., 1995, 1997). Possibly myosin-Ic associates with membranes or a protein complex in the phagosome through its tail domain. Interestingly, the rat ortholog of human myosin-Ic (rat myr3) has been localized to elongate structures in regions of cell-cell contact in NRK cells (Stoffler et al., 1995). These structures are rich in filamentous actin and can be induced to form with the lectin Con A, suggesting a regulation by cell surface receptors (Stoffler et al., 1995). These structures in the rat kidney cells may be homologous to those seen here in the shared phagosomes.

Although the class of myosin that mediates closure remains undetermined, these studies indicate a significant role for myosin IC. Myosins IC, II, V and IXb localized to macrophage phagosomes, apparently at different times during the phagocytic process: myosins II and IXb were abundant in ruffles and unclosed phagosomes (M. Diakonova and J. Swanson, unpublished). Only myosin IC labeled the strings connecting shared erythrocytes, which indicates its presence during the constriction process. Previous studies of macrophages have shown phagosomal association of myosin I (Allen and Aderem, 1995) and myosin II (Stendahl et al., 1980).

Inhibition by wortmannin and LY294002 indicated that the contractions require PI3-kinase activity. Dose-response curves for string formation, using wortmannin and LY294002, showed inhibitions comparable to their effects on PI3-kinase (Vlahos et al., 1994; Yano et al., 1993) and on phagocytosis (Araki et al., 1996). Although wortmannin can inhibit myosin light chain kinase as well, it does so at higher concentrations than those used here (Yano et al., 1995). Similarly, 50 μM LY294002 does not significantly inhibit myosin light chain kinase (Yano et al., 1995). Therefore, although inhibition by BDM suggests a role for myosin in the phagocytic contractions, the effects of wortmannin and LY294002 are most likely not via direct inhibition of myosin light chain kinase but rather by their effects on PI3-kinase.

**Regulation**

To engulf a particle, pseudopod extension and contraction must be coordinated temporally and spatially. Pseudopod extension must be oriented such that the particle is enveloped and not simply pushed away. Contractions must be delayed long enough to allow pseudopodia to extend around the particle. To generate a purse-string-like contraction, contractions must also be oriented parallel to the pseudopod margin. Similar coordination is required for the formation of macropinosomes, which form from actin-rich cups that close into intracellular vesicles (Swanson and Watts, 1995). We propose that the signals that stimulate pseudopod extension also activate a slower or delayed contractile activity, producing a wave of constriction that follows pseudopod extension (Fig. 10). When that contraction reaches the end of the pseudopod, it constricts the distal end of the phagocytic cup to a narrow opening, that then closes by membrane fusion, creating an intracellular organelle. When two macrophages engage a single erythrocyte, neither can enclose all of it, so the contractile activity constricts the erythrocyte.

**Fig. 10.** A model for phagocytosis in macrophages. Ingestion entails two component activities, the extension of pseudopodia along the particle (arrows) and closure of the phagosome to form an intracellular vacuole (x). (a) The pseudopod advances by localized actin polymerization, and constricts by myosin-mediated contractions that follow. As the particle is completely enveloped, the contractile activity closes the phagosome. (b) Contractile activities that move along the forming phagosome should constrict or squeeze deformable particles. (c) When two macrophages engage a single erythrocyte, neither can enclose all of it, so the contractile activity constricts the erythrocyte.
activating Rho ( Ridley and Hall, 1992; Chrzanowska-Wodnicka and Burridge, 1996), or by organizing a complex of integral membrane proteins that can assemble a contractile apparatus. Alternatively, activation of the regulatory GTPases could activate p21-activated kinase-1 (PAK-1), which has been shown in other systems to localize to pinocytic vesicles (Dharmawardhane et al., 1997) and to activate myosin I (Sells and Chernoff, 1997).

Supported by an American Cancer Society Research Grant and NIH grant AI35950 to J. S., NIH grants DK25387 and DK38979 to M.S.M., and a NIH postdoctoral fellowship to P.L.P. N. A. was supported by the Ministry of Education, Science and Culture of Japan. The helpful suggestions of G. Rosania, K.-D. Lee, S. C. Baer, M. Swanson and C. Carpenter are gratefully acknowledged.

Supported by an American Cancer Society Research Grant and NIH grant AI35950 to J. S., NIH grants DK25387 and DK38979 to M.S.M., and a NIH postdoctoral fellowship to P.L.P. N. A. was supported by the Ministry of Education, Science and Culture of Japan. The helpful suggestions of G. Rosania, K.-D. Lee, S. C. Baer, M. Swanson and C. Carpenter are gratefully acknowledged.

REFERENCES


