Mechanically Induced Actin-mediated Rocketing of Phagosomes[□]

Margaret Clarke,* Annette Müller-Taubenberger,^{†‡} Kurt I. Anderson,[§] Ulrike Engel,^{||} and Günther Gerisch[†]

*Program in Molecular, Cell, and Developmental Biology, Oklahoma Medical Research Foundation, Oklahoma City, OK 73121; [†]Max-Planck-Institut für Biochemie, 82152 Martinsried, Germany; [§]Max-Planck-Institut of Molecular Cell Biology and Genetics, 01037 Dresden, Germany; and ^{||}University of Heidelberg, Nikon Imaging Center, 69120 Heidelberg, Germany

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Actin polymerization can be induced in *Dictyostelium* by compressing the cells to bring phagosomes filled with large particles into contact with the plasma membrane. Asymmetric actin assembly results in rocketing movement of the phagosomes. We show that the compression-induced assembly of actin at the cytoplasmic face of the plasma membrane involves the Arp2/3 complex. We also identify two other proteins associated with the mechanically induced actin assembly. The class I myosin MyoB accumulates at the plasma membrane–phagosome interface early during the initiation of the response, and coronin is recruited as the actin filaments are disassembling. The forces generated by rocketing phagosomes are sufficient to push the entire microtubule apparatus forward and to dislocate the nucleus.

INTRODUCTION

During the uptake of a particle by *Dictyostelium* cells as by other phagocytes, actin is recruited to the incipient phagosome and disassembles immediately after the particle is engulfed (Maniak *et al.*, 1995). The Arp2/3 complex, responsible for nucleation and the dendritic assembly of actin filaments (Pollard and Borisy, 2003), is transiently enriched about the nascent phagosome (Insall *et al.*, 2001). Other actin-interacting proteins including coronin (Maniak *et al.*, 1995) and DAip1 (Konzok *et al.*, 1999) are similarly enriched and have been shown by mutational analysis to be involved in phagocytosis. Proper assembly of actin filaments during phagosome formation is likely a G protein-mediated process; the G protein β -subunit transiently labels nascent phagosomes, and its lack severely impairs phagocytosis (Peracino *et al.*, 1998). The motor protein myosin-IB, also called MyoB, is one of several unconventional myosins that have been shown to be involved in phagocytosis. MyoB binds to actin, to the plasma membrane (Senda *et al.*, 2001) and, through the linker protein CARMIL, to the Arp2/3 complex (Jung *et al.*, 2001). MyoB is concentrated in actin-rich cortical domains (Morita *et al.*, 1996), and its lack impairs lamellipod extension, membrane trafficking, and endocytosis of both fluid and particles (Titus, 2000). Once a phagosome has moved away from the cortex and become

acidified, it is devoid of actin and associated proteins, although actin, coronin, and Arp2/3 are recruited once again at a late stage of endocytic transit, after the phagosome has become neutralized (Rauchenberger *et al.*, 1997; Insall *et al.*, 2001).

Here we report that local pressure applied to phagosomes that have already lost their actin coat can trigger actin accumulation in the cell cortex and that asymmetric deposition of the actin is followed by rocketing of the phagosomes. This discovery was made in the course of studies of *Dictyostelium* cells that had taken up yeast particles and were subsequently overlaid with a thin layer of agar to flatten the cells slightly, a procedure that brings more of the cell into a single focal plane for microscopy (Yumura *et al.*, 1984). To identify proteins involved in phagosome rocketing and to determine the spatiotemporal orders of their recruitment before and during rocketing, we have labeled cells with GFP- and mRFP-tagged proteins including MyoB, members of the Arp2/3 complex, and coronin. As a marker of filamentous actin structures we used a truncated version of the LimE protein of *Dictyostelium* (LimE Δ coil), previously demonstrated to provide a brilliant label with low cytoplasmic background (Bretschneider *et al.*, 2004; Diez *et al.*, 2005).

Actin-mediated movement of pathogens has been shown to be powered by the assembly of actin filaments at the phagosome membrane (for review, see Stevens *et al.*, 2006). Our data suggest that in pressure-induced phagosome rocketing the propulsive force is provided by actin assembly at the plasma membrane/phagosome interface. We hypothesize that this local, mechanically induced force production enables cells that are filled with rigid particles to escape from narrow spaces when they are moving in a heterogeneous environment.

MATERIALS AND METHODS

Dictyostelium Strains, Cell Culture, and Vectors

Strains of *Dictyostelium discoideum* used in this study were AX2–214 (wild type) and 2A1 (*clcA*[−], a mutant of strain NC4–A2; Wang *et al.*, 2003). Cells of the wild-type strain AX2–214 were transformed by electroporation with

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[‡] Present address: Institute for Cell Biology (ABI), Ludwig Maximilians University Munich, 80336 München, Germany.

Address correspondence to: Margaret Clarke (clarkem@omrf.ouhsc.edu).

vectors expressing one or more of the following fusion proteins: GFP-Arp3 (Insall *et al.*, 2001), mRFPmars-p41-Arc (below), mRFPmars-LimEΔcoil (Fischer *et al.*, 2004; shortened to “mRFP-LimEΔ” here), LimEΔ-GFP (Bretschneider *et al.*, 2004), VatM-GFP (Clarke *et al.*, 2002a), coronin-GFP (Maniak *et al.*, 1995), GFP- α -tubulin (Neujahr *et al.*, 1998), and GFP-MyoB (a gift from Margaret Titus). The vector encoding mRFPmars-p41-Arc was constructed by cloning the sequence encoding amino acid residues 2–369 of p41-Arc (ArpC1/ArpD; DDB0214932), which was amplified by PCR using cDNA as a template, into the EcoRI-site of a pBsrH-based expression vector encoding mRFPmars (Fischer *et al.*, 2004). A linker encoding GGS-GGS was introduced between the mRFPmars and the p41-Arc sequence. Cells of the mutant 2A1 were transformed with the plasmid directing expression of GFP-Arp3.

D. discoideum strains were cultivated at 22°C in nutrient medium containing appropriate selective agents (G418 and/or blasticidin) for maintenance of the plasmids. For the rocketing experiments either living or heat-killed *Saccharomyces cerevisiae* cells were used. Living yeast cells were *S. cerevisiae* TH2–1B (Clarke *et al.*, 2002a) and 5288C (*whi5Δ::kan^R*; Jorgensen *et al.*, 2002). The yeast cells were washed in 17 mM K/Na-PO₄ buffer, pH 6.0 (PB), before addition to the *D. discoideum* cells. Heat-killed yeast were prepared from a stock purchased from Sigma (St. Louis, MO; YSC-2) by boiling for 30 min and then labeled with tetramethyl rhodamine isothiocyanate (TRITC) according to Maniak *et al.* (1995) and stored frozen.

Confocal and Total Internal Reflection Fluorescence Microscopy. For microscopic observation, *D. discoideum* cells in the exponential phase of growth were transferred to a chamber consisting of a plastic ring of 19-mm inner diameter and 4-mm height that had been attached to a coverglass with paraffin. Once the cells had settled, the nutrient medium was replaced with PB. After 30–60 min, yeast cells were added. After a period of 30 min to several hours, excess yeast were removed, and the cells were overlaid with a thin layer of agarose (Yumura *et al.*, 1984). The chamber was covered with a second coverglass held in place by silicone grease. Confocal images were collected at 2–4-s intervals using a Zeiss LSM510 laser scanning confocal microscope equipped with a Plan-Apochromat 63 $\times$, 1.4 NA DIC objective (Thornwood, NY). S65T-GFP was excited with the 488-nm laser line of an argon laser with a 505–530-nm filter for emission, and mRFP was excited with the 543-nm line of a HeNe laser, with a 560-nm long pass filter for emission. An HFT UV/488/543/633 beam splitter was used.

Three-dimensional confocal time lapse sequences were taken using an Ultra View ERS-FRET system (Perkin Elmer-Cetus LAS; Norwalk, CT) on a TE-2000 microscope equipped with a Plan-Apochromat VC 100 $\times$, 1.4 NA objective (Nikon Instruments, Melville, NY). Stacks with 0.3- μ m Z-spacing were continuously acquired with the fast sequential mode in which GFP and mRFP were excited sequentially with the 488- and 568-nm lines, respectively. The emission was detected through a triple dichroic and a double bandpass emission filter onto an EM-CCD camera.

Dual-emission total internal reflection fluorescence (TIRF) microscopy was performed essentially as previously described (Gerisch *et al.*, 2004) using an Olympus IX-70 inverted microscope and 100 $\times$, 1.45 NA objective (Central Valley, PA). The TIRF condenser (“Visi-Tirf”) was constructed in collaboration with Visi-Ton GmbH. GFP and mRFP were excited simultaneously using for both fluorophores the 488-nm line from a Coherent Innova 70c laser (Santa Clara, CA), which was fiber optically coupled into the microscope after passing through an Opto-Electronique AOTF for intensity modulation. The fluorescence filter cube in the microscope body contained a 488/10 laser cleanup filter, z488 RDC dichroic, and LP 500 emission filter (AHF Analysen Technik, Tübingen, Germany). The green and red emission signals were separated using an Optical Insights (Tucson, AZ) DualView emission splitter, which contained a 595-nm dichroic followed by Chroma HQ 525/50 and HQ 630/60 emission filters (Rockingham, VT). The camera was a Roper Scientific MicroMax 512 BFT (Tucson, AZ) and the entire system was controlled using Metamorph software (Molecular Devices Corporation, Downingtown, PA).

Measurement of Pressure Required to Induce Actin Accumulation. The pressure required to induce actin assembly at yeast-containing phagosomes was estimated using cells expressing LimEΔ-GFP. Agar pieces 1 cm² in area and 0.2 mm thick were covered by an O₂ permeable membrane (ibidi, Munich, Germany) of the same size in area. On top of the membrane a PE 390 HD gauze layer (Schweizerische Seidengazefabrik, CH-9425 Thal SG, Switzerland) was placed to allow oxygen to diffuse in, followed by 1-cm² stainless steel weights. Additional weights were sequentially added until the phagosome-associated accumulation of actin was observed.

RESULTS

Mechanical Pressure of a Phagosome Induces Accumulation of MyoB and Actin at the Plasma Membrane, followed by Phagosome Displacement

We performed a series of phagocytosis experiments in which *Dictyostelium* amoebae expressing GFP-tagged cy-

toskeletal proteins were fed TRITC-labeled yeast cells, and the behavior of the yeast-containing phagosomes was monitored in relation to that of the GFP-tagged cytoskeletal proteins. When cells were slightly flattened by an agar overlay, we observed a sudden onset of movement by previously ingested phagosomes, accompanied by actin assembly. Actin accumulation and phagosome movement could be elicited in cells by blotting the agar to dry it or by positioning the lid of the observation chamber slightly ajar, so that gradual evaporation could occur. After a few minutes of evaporation, phagosome movement began in several neighboring cells at about the same time. To specifically visualize those events occurring close to the plasma membrane before and during phagosome movement, TIRF microscopy proved to be the technique of choice. Using TIRF microscopy, GFP- or mRFP-labeled proteins were illuminated only within a depth of ~100 nm by an evanescent field generated at the substrate surface (Axelrod, 2001; Weisswange *et al.*, 2005).

In Figure 1A, TIRF images are shown of a cell expressing GFP-tagged LimEΔ, a fluorescent fusion protein that selectively labels filamentous actin structures (Bretschneider *et al.*, 2004; Diez *et al.*, 2005). This cell was fed heat-killed *S. cerevisiae* labeled with TRITC, and 4 h later, it was covered with a thin layer of agar that was allowed to press against the cells by slight evaporation. In this cell two phagosomes began to move in repeatedly changing directions in accord with the site of strongest actin deposition (Movie 1A). One phagosome moved in a series of runs and pauses. During each pause, an annulus of actin filaments visualized by GFP-LimEΔ accumulated about the region of contact between the phagosome and the plasma membrane. The annulus of actin filaments was initially symmetrical about the stationary phagosome, but this symmetry was broken as the phagosome began to move. Thus, during runs, the moving phagosome left a trail of labeled actin filaments. The comet-like appearance of this trail led us to classify the pressure-induced phagosome movement as “rocketing.” Although phagosome movement was commonly preceded by an annular accumulation of actin, sometimes a single focus of actin was observed (Movie 1B).

In cells expressing both mRFP-LimEΔ and GFP-MyoB, a class I myosin, dual emission TIRF microscopy allowed us to monitor the spatial and temporal relationship of actin assembly to recruitment of this motor protein close to the plasma membrane. Figure 1B shows images from Movie 2 of a phagosome that experienced multiple cycles of rocketing separated by pauses of a few seconds. When the phagosome paused, a build-up of structures containing GFP-MyoB occurred about the site where the phagosome pressed against the plasma membrane. When the phagosome resumed movement, the green signal of GFP-MyoB dominated at the rear of the phagosome. With increasing distance from the phagosome, the fluorescence became yellow, indicating merged signals, whereas the distal portion of the phagosome trail was labeled red by mRFP-LimEΔ. These data show that MyoB is enriched at the plasma membrane close to the phagosome and that this enrichment precedes the onset of rocketing.

The three-dimensional distribution of MyoB is clearly revealed in Z-scans obtained with a spinning disk microscope. The enrichment of MyoB at sites of plasma membrane/phagosome contact is observed not only for rocketing phagosomes, but also for phagosomes prevented from rocketing by strong pressure. Figure 2 shows such a phagosome on which actin extends up to the midregion, whereas MyoB is concentrated at regions of the plasma membrane attached

Figure 1. Phagosome rocketing in compressed cells viewed by TIRF microscopy to visualize protein recruitment to the cell cortex. Time in all figures is indicated in seconds after the initial frames. (A) Cell expressing LimEΔ-GFP to label filamentous actin structures (green), fed TRITC-labeled yeast particles (red), and compressed under an agarose sheet. When a phagosome contacted the cell cortex from the cytoplasmic face, actin assembled close to the plasma membrane. At one phagosome (indicated by an arrow), a ring of actin (20 s) became asymmetric as the phagosome moved first toward the bottom (72 and 98 s) and then toward the top of the images (138 and 178 s). At a second phagosome (open arrowhead), the site of actin deposition changed three times, and each time the direction of movement changed accordingly. These images are from Movie 1A; another example is shown in Movie 1B. (B) Cell expressing GFP-MyoB (green) and mRFP-LimEΔ (red), fed with unlabeled yeast (dark areas). When the agar overlay compressed the cell, this phagosome began a series of runs followed by pauses (Movie 2). Each time the phagosome paused, GFP-MyoB accumulated about the site of phagosome contact with the cortex (0 and 36 s, double arrows). Actin began assembling as a halo spread over a larger area of the cell cortex. The resumption of phagosome movement (6 and 53 s; the direction indicated by arrows) was associated with an asymmetric localization of MyoB and formation of an actin tail behind the transported particle. Bars, 5 μm.

to the glass surface at the bottom and to the agar layer at the top.

Movie 2 also illustrates the induction of phagosome movement in several adjoining cells by gradual drying of an agar overlay. A phagosome in the bottom cell began to rocket just after the movie started, and phagosomes in the other two cells followed not long after. If excess moisture was added back to a field that contained rocketing phagosomes, actin disassembled and the rocketing movement ceased. These data argue that mechanical pressure is a sufficient trigger for the actin-mediated rocketing. To estimate the pressure required, we covered the agar with an O₂-permeable membrane and placed weights on top. At pressures of 8 g/cm² or higher, actin was observed to assemble at yeast-containing phagosomes.

Dynamics of the Arp2/3 Complex Related to Phagosome Rocketing

Distribution of the Arp2/3 complex in relation to MyoB and actin at rocketing phagosomes was examined using confocal microscopy. A bright ring of GFP-MyoB appeared wherever

a yeast-containing phagosome pressed against the plasma membrane; this ring was interior to that of both mRFP-p41-Arc (a subunit of the Arp2/3 complex) and mRFP-LimEΔ (Figure 3, A and B). Several pairs of fluorescent proteins were examined: GFP-MyoB with mRFP-LimEΔ or mRFP-p41-Arc, and GFP-Arp3 with mRFP-LimEΔ. For all images, the focal plane was close to the plasma membrane attached to the substratum unless otherwise specified. For moving phagosomes, GFP-Arp3 and mRFP-LimEΔ exhibited almost complete overlap, although the mRFP-LimEΔ signal was slightly enriched close to the phagosome when a deeper focal plane was examined (Figure 3, C and D; Movie 3).

A striking view of the recruitment of the Arp2/3 complex to the plasma membrane was provided by cells of *D. discoideum* mutant 2A1 (Wang *et al.*, 2003). These cells are defective in cytokinesis because they lack the clathrin light chain, and their large size allows them to phagocytose many yeast cells. When we fed TRITC-labeled yeast to 2A1 cells expressing GFP-Arp3 and induced rocketing, the yeast-containing phagosomes began to move, as shown in Figure 4. A focal plane close to the glass substratum revealed bright rings of

Figure 2. Three-dimensional reconstruction of GFP-MyoB distribution (in green) relative to actin (mRFP-LimEΔ in red) at a phagosome arrested by pressure. The main panel shows an optical section in an X, Y plane close to the plasma membrane/phagosome interface. The left and upper panels represent cross-sections through the center of the phagosome in Z, Y and Z, X directions, respectively. Dual-color image stacks were recorded using a spinning disk confocal microscope with 0.3 μm Z-spacing. The rectangular scale bars indicate 2 μm in the Z direction and the same for the X, Y plane.

GFP-Arp3 that circumscribed areas of phagosome contact with the plasma membrane. A new ring of GFP-Arp3 was formed each time a phagosome paused. When the phagosome moved away, the earlier ring was left behind and finally disappeared (Movie 4; Figure 4A). Often, phagosome movement was back-and-forth, as seen for the cell in Movie 4 and also for another cell in Movie 5 and Figure 4B. In the latter cell, the phagosomes soon converted to a pinwheel motion, and the GFP-Arp3 formed tracks extending between the moving phagosomes (Figure 4B, second panel). Through-focus confocal microscopy revealed enrichment of

GFP-Arp3 at both the upper and lower plasma membrane, but not in midcell focal planes displaying equatorial sections of the phagosome (Figure 4B, third panel, and Movie 5B). Thus, GFP-Arp3 was more highly enriched at the plasma membrane than at the membrane of the phagosome.

To demonstrate a polarity in pressure-induced actin assembly around a phagosome, we used cells expressing mRFP-LimEΔ and coronin-GFP. Coronin has been shown in various cell types to and inactivate the Arp2/3 complex (Humphries *et al.*, 2002; Rodal *et al.*, 2005; Cai *et al.*, 2005). Therefore, coronin was expected to localize to sites where net actin polymerization has turned into depolymerization. In the time series shown in Figure 5, images were acquired every 4 s. When the phagosome containing an unlabeled yeast particle was moving in a confined area with frequent pauses and changes of direction, a red patch of mRFP-LimEΔ invariably labeled the rear of the moving phagosome. This red cluster changed to yellow-green (coronin-GFP merged with mRFP-LimEΔ) by the next frame, to pure green (coronin-GFP mostly alone) by the third frame, and disappeared by the fourth frame. In the meantime, the phagosome had developed a new tail labeled with mRFP-LimEΔ, which changed to the green coronin label while the phagosome moved off in a different direction. The conversion of red patches into green occurred repeatedly during this time series (Figure 5, panels 0, 8, and 16, and Movie 6).

Pressure-induced Rocketing Is Not Restricted to Early Endosomes and Does Not Require Microtubules

To substantiate the notion that mechanically induced rocketing occurs in processed phagosomes that had already lost the actin coat involved in particle uptake, we examined cells with phagosomes in the acidic phase of endocytic transit. Endosomes including phagosomes in *Dictyostelium* are acidified within a few minutes after uptake and remain acidic throughout the middle stages of

Figure 3. Localization of MyoB, Arp2/3, and F-actin at the plasma membrane in close contact with phagosomes as revealed by confocal microscopy. (A) A cell expressing GFP-MyoB and mRFP-LimEΔ. This cell has phagocytosed two yeast cells. It has also formed a macropinosome and is in the process of forming another (arrow). Note the similar relationships of MyoB and F-actin (LimEΔ) in the nascent macropinosome and in the areas surrounding the region of contact between the phagosomes and the plasma membrane. (B) Phagosomes in a cell expressing GFP-MyoB and mRFP-p41-Arc. At sites of phagosome-plasma membrane contact, the green ring of GFP-MyoB is surrounded by a red ring of Arp2/3. (C and D) The relative distribution of the Arp2/3 complex (GFP-Arp3) and F-actin (mRFP-LimEΔ) in two cells with rocketing phagosomes. There is almost complete overlap in the distribution of these two markers during phagosome movement, but with slightly greater enrichment of F-actin close to the phagosome membrane. As can be seen from the larger, clearer profiles of the yeast cells in C and D compared with A and B, the focal planes for C and D are slightly deeper in the cell. Movie 3 shows movement of the phagosome in C. The yeast cells used in these experiments were living cells of *S. cerevisiae* strain TH2-1B. Bars, 5 μm .

Figure 4. Persistence of the GFP-Arp3 label at the plasma membrane behind rocketing phagosomes revealed by confocal microscopy. For this experiment, the *Dictyostelium* clathrin light-chain mutant 2A1, which forms large cells, was used. Mutant cells expressing GFP-Arp3 were mixed with TRITC-labeled yeast and observed several hours later, after being covered with an agarose overlay. (A) On compressing the cells, rings of GFP-Arp3 appeared on the plasma membrane above and below the yeast-containing phagosomes. These rings were left behind as the phagosomes moved away. The images are from a confocal time series also shown in Movie 4. (B) In another cell, phagosome movement, initially back-and-forth, converted to a pinwheel trajectory. The last frame shows the pinwheeling phagosomes at another focal plane (Z) near the cell center. A field containing this cell is shown in Movie 5A; that time series continues with a higher magnification view of this cell in Movie 5B. Bars, 10 μm .

endocytic transit (Aubrey *et al.*, 1993; Maniak, 2001). The enzyme responsible for acidifying endosomes is the vacuolar H^+ -ATPase (V-ATPase), which is delivered to new phagosomes within a few minutes after uptake and is retrieved from phagosome membranes before exocytosis (Clarke *et al.*, 2002a). Using cells expressing mRFP-LimE Δ together with GFP-tagged VatM, a subunit of the V-ATPase (Clarke *et al.*, 2002a), we established that the membrane of rocketing phagosomes did contain VatM-GFP, indicating that the phagosomes were in the acidic phase of endocytic transit (Figure 6A; Movie 7). Thus, rocketing is not limited to

phagosomes in the very early or late stages of the endocytic cycle.

Phagosomes in the acidic phase of endocytic transit are capable of moving along microtubules (Clarke *et al.*, 2002b; Clarke and Maddera, 2006). To distinguish pressure-induced rocketing from microtubule-dependent phagosome movement, cells expressing GFP- α -tubulin and mRFP-LimE Δ were allowed to take up yeast and were thereafter treated with 20 μM nocodazole. After 30 min, the cytoplasmic microtubules had depolymerized, leaving only short stubs attached to the centrosome. When these cells were overlaid with agar, rocket-

Figure 5. Sequential labeling of phagosome comet tails with LimE Δ and coronin viewed by confocal microscopy. A cell expressing mRFP-LimE Δ and coronin-GFP was fed living yeast ~ 1 h before recording. The focal plane is close to the substratum, and the frames are separated by ~ 4 s. (The same time series is shown in Movie 6.) Direction of movement is indicated in each frame by an arrowhead on the black area representing the yeast-containing phagosome. Each time the phagosome changed direction, mRFP-LimE Δ accumulated at its back. Between one frame and the next, the red patch became yellow or green, as coronin-GFP replaced the mRFP-LimE Δ labeling of actin filaments. (Compare frames 0 and 4 s; 8 and 12 s; and 24 and 28 s.) The yeast cells used in this experiment were living cells of *S. cerevisiae* strain TH2-1B. Bar, 5 μm .

Figure 6. Rocketing of phagosomes is separated in time from particle uptake and is not dependent on microtubules (confocal microscopy). (A) Rocketing phagosome in a cell expressing mRFP-LimE Δ and VatM-GFP, a subunit of the V-ATPase. The V-ATPase, which is delivered to the membrane of phagosomes a few minutes after uptake, is responsible for acidifying the endosomal lumen. The V-ATPase also populates membranes of the contractile vacuole complex. Close to the plasma membrane attached to the substratum, enrichment of mRFP-LimE Δ is evident at the rear of a rocketing phagosome (first two panels; arrowheads overlay the rocketing phagosome and show its direction of movement). The rocketing is also shown in Movie 7. In this focal plane, VatM-GFP is seen in the tubular network of the contractile vacuole system, which lies mostly on the plasma membrane. At the midplane of the cell (third panel, Z), the presence of VatM-GFP is evident in the phagosome membrane (arrows). No mRFP-LimE Δ labels the phagosome in this focal plane. The presence of VatM-GFP means that the phagosome is in the acidic (middle) stage of endocytic transit. (B) Rocketing in a nocodazole-treated cell expressing GFP- α -tubulin and mRFP-LimE Δ . The cells were fed yeast for 40 min and then incubated with 20 μ M nocodazole. Two hours later, the cells were overlaid with agarose and observed. Phagosome movement still occurred, even though the microtubules had been depolymerized to short stubs. The yeast cells used in these experiments were living cells of *S. cerevisiae* strain TH2-1B. Bars, 10 μ m.

ing did occur, as seen in Figure 6B, showing a rocketing phagosome well separated from the microtubule stubs.

Rocketing Phagosomes Generate Forces that Displace the Microtubule System and the Nucleus

Although phagosome rocketing was usually intermittent or back-and-forth, some rocketing phagosomes exhibited long runs about the cell, and these runs often involved collisions with the cell nucleus. Figure 7A shows a cell expressing mRFP-LimE Δ and GFP-MyoB, which contained a phagosome that was moving with a circular trajectory. This rocketing phagosome made three circuits through the cytoplasm during the 232-s time series, traveling at an average velocity of 23 μ m/min (Movie 8). In each circuit the phagosome collided with the nucleus, distorting and displacing it. In Figure 7A, frames from one circuit are shown in a focal plane slightly above the plasma membrane. A comet-like flare of actin was evident behind the moving phagosome, whereas GFP-MyoB only rarely entered the focal plane.

The nucleus is connected through the centrosome to the entire microtubule array within the cell. Although microtubules are not essential for rocketing, as shown above, displacement of the nucleus suggested that rocketing phagosomes were interacting directly or indirectly with the microtubule system. Figure 7B and Movie 9 show a cell expressing GFP- α -tubulin and mRFP-LimE Δ in which a

rocketing phagosome was moving about in a rather small area, a frequently observed behavior, suggesting that microtubules were hindering or guiding phagosome movement. Each time the phagosome collided with the nucleus, the force of rocketing was sufficient to displace the nucleus and stretch out the microtubules (Figure 7B, panel 0). As soon as the rocketing phagosome turned away from the nucleus, the microtubules resumed their normal undulating appearance (panel 8). When moving orthogonally to the microtubules, the rocketing phagosome shoved against them, bending the microtubules laterally (panel 20). We infer that rocketing phagosomes are channeled toward the centrosome by microtubules and that rocketing phagosomes strike the centrosome-attached nucleus with considerable force.

DISCUSSION

Phagosome rocketing has been extensively studied in connection with the intracellular movement of infectious bacteria or viruses (reviewed by Stevens *et al.*, 2006). It has been proposed that these pathogens have harnessed a pathway of localized actin polymerization that is normally used for cell motility (see review by Rafelski and Theriot, 2004) and intracellular vesicle movement (Merrifield *et al.*, 1999; Taunton *et al.*, 2000; Kim *et al.*, 2006). Rocketing endosomes have been observed in lanthanum/zinc-treated macrophages (Southwick *et al.*, 2003), in rat basophilic leukemia cells treated with

Figure 7. Distortion of nucleus and microtubules by rocketing phagosomes. (A) Confocal view of a rocketing phagosome in a cell expressing GFP-MyoB and mRFP-LimEΔ. This phagosome made three circuits around a second larger phagosome (y) that was not moving and appears as a dark object near the cell center (Movie 8). One circuit is shown here. Each time the phagosome reached the last quadrant of the cell before nuclear impact, it accelerated briefly, momentarily outstripping its actin tail (panel 20). Subsequently, the phagosome rammed and displaced the nucleus (n), which appears slightly red because mRFP-LimEΔ has entered the nuclear matrix. Toward the end of Movie 8, the focus was shifted closer to the plasma membrane, revealing concentric rings of GFP-MyoB and mRFP-LimEΔ encircling the stationary phagosome. (B) Interactions between a rocketing phagosome and microtubules in a wild-type cell. AX2 cells expressing GFP-α-tubulin and mRFP-LimEΔ, were mixed with yeast about 2 h before the confocal microscopy time series shown in Movie 9 was captured. When the phagosome rammed the centrosome, it displaced the nucleus and stretched out the microtubules (panel 0), which relaxed when the phagosome turned away (panel 8). The microtubules were bent during lateral movements of the phagosome (panel 20). The taut appearance of the straightened microtubules suggests that they are anchored at the cell cortex as well as the centrosome. The yeast used for the experiment in Figure 7A were heat-killed, and those for the experiment in Figure 7B were living cells of *S. cerevisiae* strain 5288C, which are smaller than wild-type yeast cells. Bars, 10 μm.

phorbol ester or subjected to hypoosmotic shock (Hayes *et al.*, 2004), and in cells caused to overexpress type I phosphatidylinositol phosphate 5-kinase (Rozelle *et al.*, 2000). In cell-free systems, the mode of actin-driven movement depended on physical parameters such as bead size; beads of $<3\ \mu\text{m}$ diameter moved steadily, whereas larger beads displayed a hopping motion (Bernheim-Groswasser *et al.*, 2002).

In this article we have investigated a conditional type of phagosome rocketing, one that is induced in intact cells by mechanical pressure under physiological conditions in the absence of chemical or biological elicitors. In cells that had phagocytosed yeast particles ranging from 3 to 5 μm in diameter, we observed various types of rocketing: phases of straight propagation interrupted by periods of immobility,

back-and-forth movements, and persistent travel in circular tracks. Intermittent or back-and-forth rocketing occurred commonly. Long circular runs, while rare, allowed us to determine that the rate of phagosome movement corresponded to that previously reported for rocketing pathogens, which are also propelled by actin polymerization (Theriot *et al.*, 1992).

The large size of yeast-filled phagosomes enabled us to localize the proteins associated with the tail of rocketing phagosomes, including actin itself, predominantly to the plasma membrane-anchored cell cortex rather than to the membrane of the phagosome. In this respect phagosome rocketing in *Dictyostelium* is distinguished from the previously reported rocketing of pathogens or vesicles, which has always been attributed to the formation of an actin tail solely on the surface of the rocketing particle. The same is true for the cycles of actin assembly called "flashing," which occur on the surface of phagosomes containing *Listeria* or other particulate matter (Yam and Theriot, 2004).

In cells that had eaten yeast and been flattened by an agar overlay, rings or patches of GFP-MyoB enrichment formed at both the upper and lower plasma membrane where the phagosome pressed against it. MyoB is a class I myosin with a lipid-binding site and multiple protein-interaction domains; it binds to actin, to the plasma membrane (Senda *et al.*, 2001) and, through the linker protein CARMIL, to the Arp2/3 complex (Jung *et al.*, 2001). MyoB accumulated at the phagosome-plasma membrane interface before the onset of rocketing. For phagosomes that did begin to move, TIRF microscopy showed GFP-MyoB on the plasma membrane at the rear of the moving phagosome, with filamentous actin extending behind (Figure 1B). For some large yeast-containing phagosomes, the phagosome was pressed too strongly to allow movement, leading to the accumulation of very high levels of the fluorescent markers (Figure 3A; central phagosome in Movie 8). Rocketing phagosomes typically paused periodically, in which case a new buildup of MyoB occurred at the plasma membrane before another spurt of rocketing (Figure 1B). The correlation between these events suggests that MyoB and other class I myosins may act in the initiation of mechanically induced rocketing. Because there are seven class I myosins in *Dictyostelium* with considerable overlap in function (reviewed by Uyeda and Titus, 1997; de la Roche and Côté, 2001), single and double gene disruptions tend to cause reduction rather than loss of function (Novak *et al.*, 1995; Jung *et al.*, 1996; Falk *et al.*, 2003). Our preliminary observations of mutant strains conform to this pattern. Although actin-powered phagosome movement did occur in mutant cells lacking MyoB or both MyoA and MyoB, sustained movement appeared to be rare, and little force seemed to be exerted by a moving phagosome (Supplementary Movie 1). In contrast, pressure-induced rocketing in strain JH10, the parent of the *myoB*⁻ mutant, was quite robust (unpublished data). Additional work is needed to verify and extend these results, in particular to apply defined pressure, as described in this report, and to develop a means of measuring the force generated by a moving phagosome, for instance by the use of magnetic beads (Uhde *et al.*, 2005).

Confocal microscopy of phagosome rocketing in double-labeled strains showed that the Arp2/3 complex extended beyond the outer perimeter of the MyoB (Figure 3B). The greatest enrichment of the Arp2/3 complex was at the plasma membrane, such that bright Arp2/3 complex "prints" of the phagosome were left behind when the phagosome moved away (Figure 4). The third protein studied, coronin, is like Arp2/3 a tail constituent common to rocket-

ing phagosomes and intracellularly moving *Listeria* (David *et al.*, 1998), where coronin appears to have a regulatory function, one that is not essential for bead propulsion in vitro (Carrier *et al.*, 2003). Similarly, our analysis of coronin-null cells indicated that coronin is not essential for mechanically induced phagosome rocketing (unpublished data). The localization of coronin to the end of actin tails away from the rocketing phagosomes themselves in *Dictyostelium* suggests a role in actin disassembly, in line with the negative control of Arp2/3 complex activity reported for coronin in yeast and mammalian cells (Humphries *et al.*, 2002; Rodal *et al.*, 2005; Cai *et al.*, 2005).

If one considers the narrow space between phagosome and plasma membrane as an equivalent of the membrane fold at the leading edge of a lamellipod, the similarity of phagosome rocketing and global cell motility becomes obvious. All three components shown here to be associated with rocketing phagosomes, MyoB, the Arp2/3 complex, and coronin, are also enriched at the front of *Dictyostelium* cells (de Hostos *et al.*, 1991; Fukui *et al.*, 1999; Insall *et al.*, 2001). At both locations it remains open whether MyoB contributes directly through its motor activity to motility, or is only instrumental in the Arp2/3-dependent assembly of a dense network of actin, whose polymerization would then provide the force for phagosome movement. The Arp2/3 complex is an intrinsic component of the dense actin assemblies at the leading edge of chemotaxing *Dictyostelium* cells (Diez *et al.*, 2005), as it is in other cells (Pollard and Borisy, 2003). Coronin accumulates in a zone separated from the very edge of a migrating cell (K. Anderson, T. Bretschneider, and G. Gerisch, unpublished data), comparable to its lateral separation from the phagosome membrane.

A sequence of events similar to that observed in phagosome rocketing has recently been proposed for the internalization of clathrin-coated vesicles in yeast. Kaksonen *et al.* (2005) found that during clathrin-mediated endocytosis in *S. cerevisiae*, a WASP/Myo module at the plasma membrane regulates actin filament nucleation via the Arp2/3 complex and that addition of actin monomers at the plasma membrane drives the clathrin-coated vesicle inward. The behavior of MyoB and Arp2/3 in *Dictyostelium* during pressure-induced rocketing closely resembles the behavior of their yeast counterparts during clathrin-mediated endocytosis, suggestive of a similar mechanism for site-specific actin recruitment. An open question is how pressure triggers the recruitment of MyoB (or its possible upstream partners) in the signal transduction pathway mediating phagosome rocketing. One possibility is that distortion of the cortical actin network may stimulate MyoB recruitment, just as the stretching of cytoskeletons from detergent-treated cells activates a signaling cascade that results in the binding of specific proteins from the cytoplasm (Tamada *et al.*, 2004). Alternatively, simple contact may suffice. In mammalian cells, the contact of an extracellular vaccinia virus (Frischknecht and Way, 2001; Newsome *et al.*, 2006) or enteropathic *Escherichia coli* (Swimm *et al.*, 2004) with the cell surface triggers a signaling cascade that involves multiple tyrosine kinases and leads to the recruitment of N-WASP and Arp2/3 at the plasma membrane beneath the pathogen, stimulating actin polymerization there.

What are the functional implications of actin assembly that is stimulated at sites of contact between a phagosome and the plasma membrane? We envisage two functions of mechanically induced rocketing. One is to keep the cell cortex free of trafficking endosomes until exocytosis commences. Indeed, endosomes are concentrated in the inner region of *Dictyostelium* cells rather than at their periphery. In

compressed cells, large phagosomes cannot move away from the plasma membrane, so actin assembly is continuously reiterated at new sites of the plasma membrane. Second, under these conditions of compression, actin-dependent force generation provides a mechanism of propelling large phagosomes filled with rigid particles out of a narrow space, where the particles would hinder the cell's movement. This escape mechanism may be important not only for *Dictyostelium* cells, which in their natural habitat migrate between soil particles, but also for those cells of higher organisms that penetrate into embryonic or adult tissues.

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