Transient assembly of F-actin by phagosomes delays phagosome fusion with lysosomes in cargo-overloaded macrophages

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Summary
Dynamic remodelling of the cortical actin cytoskeleton is required for phagocytic uptake of pathogens and other particles by macrophages. Actin can also be nucleated de novo on membranes of nascent phagosomes, a process that can stimulate or inhibit phagosome fusion with lysosomes. Recently, phagosomes were shown to polymerize actin in transient pulses, called actin ‘flashing’, whose function remains unexplained. Here, we investigated phagosomal actin dynamics in live macrophages expressing actin tagged with green fluorescent protein (GFP). We show that only immature phagosomes can transiently induce assembly of actin coat, which forms a barrier preventing phagosome-lysosome docking and fusion. The capacity of phagosomes to assemble actin is enhanced in cells exposed to increased phagocytic load, which also exhibit a delay in phagosome maturation. Parallel analysis indicated that polymerization of actin on macropinosomes also induces compression and propulsion. We show that dynamic interactions between membrane elastic tension and compression forces of polymerizing actin can also lead to macropinosome constriction and scission – a process that is obstructed on rigid phagosomes. We hypothesize that the rate of individual phagosome maturation, as well as the biogenesis and remodelling of macropinosomes, can be regulated by the extent and manner of actin assembly on their membrane.

Evidence that F-actin could either stimulate or inhibit membrane fusion was first shown in the analysis of exocytosis using cytochalasin D (Orci et al., 1972). These results were confirmed and extended in detail by others in the field of exocytosis (Malacombe et al., 2006; Aunis and Bader, 1988; Muallem et al., 1995). Recently, it has been shown that after being internalized into the phagosome, some pathogens such as Leishmania or Salmonella induce the accumulation of peri-phagosomal actin to prevent phagosomal maturation (Lerm et al., 2006; Méresse et al., 2001). Nevertheless, the exact role of actin in the process of phagosome-lysosome fusion and phagosome maturation still remains unclear.

A dramatic demonstration of the ability of phagosomes to polymerize actin in epithelial cells was provided by Yam and Theriot (Yam and Theriot, 2004). They showed that phagosomes containing Listeria, as well as other types of phagosomes, had the capacity to assemble actin in a process they referred to as actin ‘flashing’, whereby the entire surface of the phagosome was transiently surrounded by a striking actin ‘coat’. This process occasionally initiated directed movement, and the link between flashing and actin comet rocketing was suggested. Nevertheless, with the exception of actin comets assembling on intracytoplasmic pathogens that have been extensively described (Gouin et al., 1999; Cudmore et al., 1995), it has been difficult to assign any function to actin-driven motility of the host cell endosomes (Taunton et al., 2000), pinosomes (Merrifield et al., 1999) or phagosomes (Zhang et al., 2002; Clarke et al., 2006).
Here, we analyzed phagosome actin flashing in mouse macrophages expressing actin tagged with green or red fluorescent protein (GFP or RFP) to elucidate the function or physiological relevance of this phenomenon for phagosome biogenesis and maturation. Our results argue that this phenomenon is not a stochastic process, but is intimately linked to the ability of phagosomes to fuse with lysosomes. Additionally, our parallel analysis of macropinosomes brought further insight to the mechanism and function of actin-driven forces on organelle membranes.

Results

Periodic waves of actin assembly-disassembly are induced on a subpopulation of internalized phagosomes

We prepared a stable cell line of mouse RAW macrophages expressing actin-GFP and actin-RFP to study actin dynamics in live macrophages pulse-chased with 3 μm latex beads (LBs). This phagocytic cargo, coated with different ligands (IgG, mannan or avidin) was followed from the initial steps of internalization until phagosome maturation (2-4 hours of chase) and dynamics of actin-GFP assembly-disassembly was monitored by time-lapse confocal imaging.

We observed that multiple phagosomes within a cell were associated with a transient, but periodic burst of actin-GFP signal soon after internalization (Fig. 1A). Vertical (x-z-y) confocal sections revealed that peri-phagosomal F-actin is not a projection of an open phagocytic cup but assembles like a ‘coat’ around the entire, fully internalized organelle (Fig. 1B). Actin initially assembled de novo from distinct nucleation sites on the phagosomal membrane and then the actin-GFP signal eventually spread rather homogenously around the phagosome surface (Fig. 1C). The period of F-actin nucleation and assembly on individual phagosome (~2 minutes) was usually followed by immediate disassembly (~2 minutes) in repeated cycles (Fig. 1D; supplementary material Movie 1). However, irregular cycles when phagosome exhibited a delay in the onset of F-actin disassembly were also observed. In this case, a prominent signal of actin-GFP persisted for various periods (from ~2 minutes up to 30 minutes) on the phagosome without significant density fluctuations (Fig. 1E; supplementary material Movie 1).

We also observed that this F-actin coat could assemble on the LBPs in primary mouse bone marrow macrophages (BMDMs) and microglial BV-2 cells, as well as on phagosomes containing zymosan, carbon particles, naked beads or beads conjugated to different ligands (IgG, mannan, avidin) in Raw macrophages (supplementary material Fig. S1). Our results thus extend the observations of Yam and Theriot (Yam and Theriot, 2004) who first described the actin-flashing phenomenon in epithelial MDCK cells internalizing bacteria or latex beads.

Polarization of actin coat on phagosomes can result in actin comet propulsion

After internalization, the large (3 μm) LBPs (whether actin-positive or not) displayed only sporadic passive movements driven by cytoplasmic contraction during continual remodelling of macrophage shape. However, in a small fraction (~5-10%) of phagosomes that displayed actin flashing, actin coat that was originally symmetrical spontaneously polarized at one pole of the phagosome. This led to the transient propulsion of these phagosomes by actin comet formation (Fig. 2A). Trajectories of different lengths and complexity were observed, from short back-and-forth movements to long-range transport over the whole cell. Although the trajectories appeared to be randomly directed, phagosomes more frequently moved to the cell periphery.

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Fig. 1. Dynamics of actin flashing on macrophage phagosomes. (A) Actin flashing phagosomes in Raw macrophage expressing actin-GFP, from 30 minutes after the pulse of IgG-coated LBs (3 μm); selected confocal sections from time-lapse series. (B) Vertical (x-z-y) confocal sections demonstrate that the entire surface of the internalized LBP is covered with actin coat (actin-GFP fluorescence). (C) F-actin is nucleated at distinct sites (arrows) on the LBP membrane and polymerizes into a uniform actin coat (actin-GFP fluorescence). (D) F-actin is nucleated at distinct sites (arrows) on the LBP membrane and polymerizes into a uniform actin coat (actin-GFP fluorescence). (E) Dynamic mode of actin ‘flashing’ on the LBP with typical waves of actin assembly (~2 minutes) and disassembly (~2 minutes) (D) and a stable F-actin coat on another LBP (E). Time plot of actin-GFP fluorescence intensity profile on selected phagosome (ROI, region of interest) and in the surrounding cytoplasm (Ref, reference) is shown. Selected confocal sections from supplementary material Movie 1 are shown on the right. Scale bars: 3 μm.
Pushing of the plasma membrane by rocketing phagosome often resulted in protrusion from the cell surface (up to 10 μm), which subsequently retracted back, so that neither the phagosome nor its content was extruded from the cell (Fig. 2B). The polymerizing actin coat appeared to generate compression of the phagosome membrane leading to phagosome ‘squeezing’ and short propulsion while this coat transiently detached from the phagosome (Fig. 2C). We then revealed by electron microscopy that this compression can induce an elastic stretching of phagosome membrane and its local retraction from the bead surface (supplementary material Fig. S2A). Although no obvious functional advantage could be attributed to the motility of phagosomes via actin comets we show that it is induced by spontaneous remodelling and polarization of actin coat on a subpopulation of phagosomes.

Phagosomal F-actin nucleation is downregulated during phagosome maturation

We next asked whether F-actin coat assembly is involved in phagosome-lysosome fusion – a process that is crucial for phagosome maturation. For this, we followed phagocytic uptake of the LBs in live macrophages preloaded with rhodamine-labelled bovine serum albumin coupled to colloidal gold (BSA-gold-rhodamine) by endocytosis. After passing through the earlier endocytic compartments, this fluid-phase fluorescent marker accumulates in the late endosomes and lysosomes (for simplicity referred to as ‘lysosomes’ from now on), and after phagosome-lysosome fusion, also within the lumen of phagosomes.

A striking finding was that only ‘early’ phagosomes, before phagosome-lysosome fusion, were able to induce actin nucleation and coat assembly, whereas phagosomes that already fused with lysosomes were never found to be positive for F-actin. This time-dependent ‘switch-off’ mechanism for phagosome actin assembly was similar for both LBPs (Fig. 3A) and zymosan-containing phagosomes (Fig. 3B).

Quantification of these observations was made on fixed cells. For this, we pulsed RAW macrophages with 3 μm LBs coated with mouse IgG for 1 hour (on ice) and cells were fixed at different time points (0.5-4 hours) after initiation of uptake (at 37°C). Surface-attached and incompletely internalized LBs were identified by immunolabelling of non-permeabilized cells (as described in Materials and Methods). After permeabilization, late-endosome-associated membrane protein 2 (LAMP-2) was detected in matured phagosomes with anti-LAMP-2 antibody, and F-actin
Polymerization of actin coat generates a transient block of vesicle docking to phagosome

We used live-cell imaging at the limit of resolution to follow dynamic events of phagosome-lysosome docking and fusion. Lysosomes were preloaded with BSA-gold-rhodamine by 1 hour pulse and 4 hour chase before uptake of the beads. When de novo actin-coat assembly was not induced on the nascent LBPs, they acquired rhodamine-gold from preloaded lysosomes from as early as about 10 minutes after phagosome internalization. The transport of lysosomes towards a subpopulation of the LBPs that assembled actin coat was not affected, but lysosomes were not able to cross the actin barrier and dock on the LBP membrane (Fig. 4A; supplementary material Movie 2). At a later stage of actin flashing (around 1 hour after LB internalization) the actin-GFP density began to fluctuate on phagosomes until the homogenous actin coat disintegrated. Here, actin-free docking sites seemed to ‘open up’ for accessing lysosomes, and fusion immediately followed (Fig. 4B; supplementary material Movie 2).

We also examined the dynamics of actin-coat disassembly and the rate of subsequent fusion on zymosan-containing phagosomes. These were more suitable for the quantitative analysis because BSA-gold-rhodamine penetrates easily throughout the zymosan particle. Although numerous lysosomes were present in close proximity to actin-coated phagosomes, their docking was always postponed until the onset of actin-coat disintegration (Fig. 4C; supplementary material Movie 3). Relative fluorescence intensity measurements on individual zymosan-containing phagosomes showed an inverse correlation between the course of actin-coat disassembly and the kinetics of BSA-gold-rhodamine acquisition by phagosomes. Loading of this marker into the phagosome lumen began as soon as actin-free docking sites on the phagosome membrane became accessible and continued gradually without any indication of further phagosomal actin assembly (Fig. 4D).

Together, these data indicate that even when it undergoes dynamic fluctuations on individual phagosomes the assembly of actin coat can temporarily block the docking of the lysosomes to the phagosome. Additionally, the actin-coat assembly can be triggered only on young immature phagosomes and is downregulated once they undergo fusion with lysosomes.

Phagosome actin forms an efficient barrier to phagosome-lysosome fusion

Since the results of our live-cell imaging argued that the actin assembly by phagosomes prevents their fusion with lysosomes, we next analyzed the ultrastructure of peri-phagosomal actin by electron microscopy (EM). Acceptable ultrastructural preservation of actin microfilaments on plastic-embedded samples allowed us to discern the morphology of actin attachment sites on the phagosome membrane and to visualize individual F-actin filaments that extend radially from the phagosome membrane (Fig. 5A). These F-actin filaments formed a uniform actin coat of homogenous density on the entire phagosome surface with an average thickness of 610±118 nm (n=10). Confirmation that these filaments were indeed actin was shown by immunolabelling of β-actin on thawed cryosections (Fig. 5B).

Analysis of macrophages that had been pulse-chased with BSA-gold into lysosomes before phagocytosis of the LBs showed that all actin-coat-positive phagosomes were devoid of colloidal gold in their lumen and had a tightly apposed membrane (which is characteristic of an early maturation stage) (Fig. 5A,B,D). Conversely, at later stages (1-2 hours after internalization), the
matured phagosomes had a rather loose membrane and an enlarged lumen filled with BSA-gold, but no detectable actin coat. No distinct actin structures were visible in between membranes of the phagosome and adjacent endocytic organelles during phagosome-lysosome fusion (supplementary material Fig. S2B). Thus, when phagosomes assemble F-actin, the filaments form a very efficient barrier that excludes all endocytic organelles from accessing the phagosome membrane (Fig. 5C). Even in the examples when actin-coat remodelling was followed by actin comet formation, the high density of F-actin filaments was entirely homogenous throughout the length and width of the actin comet (Fig. 5D).

Overloading of macrophage phagocytic capacity or lysosomal content increases actin flashing and slows phagosome maturation

Our observations showed that whereas only a subpopulation of internalized phagosomes (~5-10%) exhibited de novo nucleation of F-actin, all phagosomes (100%) eventually undergo fusion with lysosomes. This suggests that the assembly of actin coat is not required for later fusion to occur. We also noted heterogeneity in the total number of internalized particles by individual cells, with the fraction of actin flashing phagosomes usually higher in cells that had internalized higher amounts of phagocytic cargo. We therefore asked whether the ability of phagosomes to assemble F-actin de novo could be related to the total phagocytic load of individual cells. To address this question, we exposed macrophages to increasing concentrations of IgG-coated 3 μm LBs. The fraction of F-actin-positive phagosomes was analyzed in cells fixed at different time-points (0.5-4 hours), as explained in the Materials and Methods.

When low doses of the LBs were internalized (0.5-1 LBs per cell on average) the fraction of F-actin-positive phagosomes gradually decreased within the first 1-2 hours, when the majority of phagosomes (75-80%) undergo maturation. However, when cells were exposed to and internalized higher amounts of the LBs (4-8 LBs per cell on average), the number of F-actin-positive phagosomes increased significantly and the overall kinetics changed; instead of a continual decrease in this phagosome fraction within the first 1-2 hours, we detected a significant peak between 2 and 4 hours (Fig. 6A).

We next asked whether this increased phagocytic load also affected the overall course of phagosome maturation. At low dose of LBs, 50% of phagosomes were positive for the lysosomal marker LAMP-2 after 60 minutes. With increasing amount of internalized LBs, the kinetics displayed a shift in LAMP-2 acquisition to a half-time of 2-3 hours in proportion to the load concentration (Fig. 6B). Thus, the exposure of macrophages to a high dose of phagocytic cargo increases the extent of actin flashing by phagosomes in a manner that correlates with a significant delay in phagosome maturation.

A major function of lysosomes that receive and degrade macromolecules from different trafficking pathways, is to deliver hydrolytic enzymes into maturing phagosomes. We asked whether overloading of endogenous pool of lysosomes, which was
previously shown to reduce their fusion competence (Montgomery et al., 1991), could in turn also affect the rate of actin assembly by phagosomes. For this, we loaded cells with a high concentration of BSA-colloidal gold (as an indigestible fluorescent marker), which accumulates in terminal lysosomal compartments (Rabinowitz et al., 1992). Here, we used a BSA-gold concentration 10-20 times higher than that used for standard labelling of lysosomes. Cells were subsequently exposed to the LBs (3 μm) for phagocytic uptake.

The results showed that the fraction of internalized LBPs positive for F-actin was reduced within 2 hours at a low phagocytic load, whereas with higher loads it increased significantly even at later time points. (B) About 50% of the LBPs acquired LAMP-2 (marker of late endosomes and lysosomes) within 1 hour after bead pulse at a low dose of internalized beads, whereas at higher doses, the acquisition of LAMP-2 was significantly delayed. (Note that the delay correlates with protracted F-actin assembly on phagosomes shown in Fig. 6A.) (C-D) Raw macrophages were pulse-chased with high concentrations of BSA-gold (C) or 1 μm LBs (D) and 3 μm LBs were applied thereafter. The fraction of phagosomes containing 3 μm LBs and positive for F-actin was scored on cells fixed after 1, 2 and 4 hours of 3 μm LB chase and phalloidin-rhodamine labelling. In preloaded macrophages, the proportion of large (3 μm) phagosomes associated with F-actin increased (in a concentration-dependent manner) at all time points analyzed in comparison with the control (non-preloaded) cells. Values are mean values ± s.d. of three individual experiments done in duplicate.

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Thus, the preloading of macrophage phagocytic capacity increases actin assembly by phagosomes but slows down the rate of phagosome maturation. Actin polymerization on macropinosomes drives compression, shape remodelling and propulsion or scission of the organelle. During the above analysis on phagosomes in macrophages expressing actin-GFP or actin-RFP, we also detected actin nucleation

Fig. 5. Radial arrays of actin filaments form dense phagosomal actin-coat barrier on immature phagosomes. (A) Ultrathin section through the Raw macrophage fixed 30 minutes after the pulse of 3 μm LBs (processed for embedding in Epoxy resin); F-actin is nucleated on phagosome membrane, which is tightly adjacent to the LB (enlargement 1); F-actin microfilaments (arrowheads) are arranged in the radial arrays (enlargement 2). (B) Immunolabelling of phagosomal actin on thawed cryosections; anti-β actin antibody was followed by protein-A-gold (10 nm) (enlargements 1 and 2). (C) The dense F-actin coat on immature phagosome blocks endosomes (arrowheads) from the access to the phagosome membrane. (D) Cross-section through a phagosome associated with the actin comet (arrowheads) fixed 30 minutes after the pulse of the LBs and embedded in Epoxy resin; note the homogenous density of F-actin throughout the comet and the nucleus dislocated by motile phagosome.

Fig. 6. Overloading of macrophage phagocytic capacity increases actin assembly by phagosomes but slows down the rate of phagosome maturation. The fraction of F-actin-positive (A) or LAMP-2-positive (B) phagosomes was counted in Raw macrophages fixed at 0.5-4 hours post pulse of increasing dose of 3 μm LBs. (A) The number of internalized phagosomes positive for F-actin was reduced within 2 hours at a low phagocytic load, whereas with higher loads it increased significantly even at later time points. (B) About 50% of the LBPs acquired LAMP-2 (marker of late endosomes and lysosomes) within 1 hour after bead pulse at a low dose of internalized beads, whereas at higher doses, the acquisition of LAMP-2 was significantly delayed. (Note that the delay correlates with protracted F-actin assembly on phagosomes shown in Fig. 6A.) (C,D) Raw macrophages were pulse-chased with high concentrations of BSA-gold (C) or 1 μm LBs (D) and 3 μm LBs were applied thereafter. The fraction of phagosomes containing 3 μm LBs and positive for F-actin was scored on cells fixed after 1, 2 and 4 hours of 3 μm LB chase and phalloidin-rhodamine labelling. In preloaded macrophages, the proportion of large (3 μm) phagosomes associated with F-actin increased (in a concentration-dependent manner) at all time points analyzed in comparison with the control (non-preloaded) cells. Values are mean values ± s.d. of three individual experiments done in duplicate.
and assembly on the membrane of macropinosomes that were easily identified by phase-contrast microscopy. As expected, a strong cortical actin signal was found in lamellipodia and membrane ruffles that mediate macropinosome formation and drive its internalization. Actin surrounding nascent macropinosomes then rapidly disassembled. However, within minutes, de novo assembly of a new actin coat was observed on a subpopulation of macropinosomes. This was invariably less prominent and short-lived compared with the coat seen on phagosomes, and periodic actin flashing was also rarely observed on macropinosomes.

In contrast to phagosomes, where the membrane is tightly apposed to a rigid particle, fluid-containing macropinosomes are more compressible, which makes them more suitable for analysis of the mechanical forces induced by the actin assembly on their membranes. We observed that actin on macropinosomes polymerized from distinct, regularly distributed sites (10±1 for the perimeter of 3 μm macropinosome) (Fig. 7A; supplementary material Movie 4). These actin nucleation ‘hot spots’ grew in size, and within 1-2 minutes induced local membrane invaginations of the flexible macropinosome membrane (Fig. 7B; supplementary material Movie 4).

We then compared the average duration of an individual cycle of actin nucleation assembly-disassembly on phagosomes and macropinosomes of similar size. This complete event lasted 4-5 minutes (on average) on phagosomes containing the LBs or zymosan particles, but only 2 minutes (on average) on macropinosomes (Fig. 7C).

In contrast to phagosomes, each cycle of actin nucleation on a macropinosome was regularly followed by a breakage of actin-coat symmetry and macropinosome budding, through the actin-coat rupture. This process led to either macropinosome constriction and scission, or actin-coat polarization and organelle propulsion. In general, macropinosomes were more motile than phagosomes but similar distinct modes of actin-driven propulsion were observed:

1. continuous, long-distance movement initiated by polarization and persistence of actin nucleation machinery at the rear of the organelle, typical for actin comets (Fig. 7D), and
2. discontinuous, back-and-forth movement generated by compression and ‘squeezing’ forces of actin coat on macropinosome membrane that transiently detached from the polymerized actin (Fig. 7E). As with phagosomes, the trajectories of motile macropinosomes appeared random, but rocketing towards the cell surface prevailed. Macropinosomes pushing against the plasma membrane also transiently protruded from the cell surface before being pulled back into the cell interior by membrane retraction (supplementary material Fig. S3). Although, as for phagosomes, it is difficult to ascribe any relevant function to actin-comet-based motility, we found that the localized compression forces generated by actin polymerization can drive macropinosome scission – an important step in organelle biogenesis.

When the actin coat accumulated around the constriction on the budding macropinosome, the actin-driven scission was completed within 0.5-1 minutes and the ‘daughter’ pinosomes detached from the ‘mother’ organelle (Fig. 8A; supplementary material Movie 5). The most unexpected observation here was the sorting of solid (opaque) particles from fluid (translucent) content of macropinosome into the separate daughter pinosome (Fig. 8B; supplementary material Movie 5). This argues that in addition to driving membrane scission the actin polymerization also contributes to some sorting processes in the lumen of these vesiculating organelles.

![Fig. 7. Actin-coat assembly and remodelling on membrane of flexible macropinosomes.](image-url)
Actin coat concentrates at the constriction around irregularly shaped phagosomes

The observations that remodelling of the actin coat can result in actin-driven macropinosome scission led us to ask whether actin could provide similar forces in a suitable phagosome system in which the contractile forces could be visualized more easily than on the LBP. For this, as phagocytic cargo we used yeast cells (*Saccharomyces cerevisiae* ESM356–1), arrested at a late stage of budding. The rationale was that these asymmetric phagosomes would allow us to follow the polymerization of actin upon a preformed (high) curvature surface of membrane apposed to the groove between the mother yeast cell and the bud. Similarly to the LBs or zymosan particles, budding yeasts were efficiently internalized into phagosomes. These phagosomes subsequently fused with lysosomes, except those that assembled an actin coat, which temporarily prevented the fusion. As before, phagosomes that once fused with lysosomes (detected by BSA-gold-rhodamine acquisition) failed to assemble actin de novo.

The actin coat on asymmetrical yeast-containing phagosomes was not homogenous, and instead of typical flashing, we observed fluctuations of actin-GFP density all around the phagosome surface. Initially, the waves of actin polymerization spread laterally on the phagosome membrane, but actin-GFP signal finally accumulated invariably within the groove around the phagosome constriction (Fig. 8C; supplementary material Movie 5). This ring-like actin formation then persisted for relatively long periods (up to observed 20 minutes) without major intensity fluctuations. Remarkably, even when the rest of the phagosome surface had no visible actin, the docking of the lysosomes to all parts of these phagosomes was nevertheless substantially reduced. Similarly to actin flashing activity, this obstructed constriction of rigid phagosomes was only transient, and when the actin ‘constriction ring’ depolymerized, all the phagosomes proceeded to fuse rapidly with lysosomes.

These results suggest a mechanistic model where (1) actin polymerization can induce local curvature on organelle membrane that is limited by increasing membrane tension; (2) membrane curvature can in turn facilitate or enhance local actin polymerization; (3) both of these processes can result in actin-driven constriction and scission of macropinosome (but not phagosome); (4) the failure of the actin coat to induce curvature on membrane apposing rigid particle within phagosomes leads to the growth of prominent and homogenous actin coat and the flashing phenomenon.

**Discussion**

In this study, we have extended the work of Yam and Theriot (Yam and Theriot, 2004) who first described the actin ‘flashing’ phenomenon, which is the striking ability of phagosomes containing *Listeria* or latex beads to transiently polymerize actin in epithelial cells. Our live imaging and EM analysis of macrophages with internalized latex beads, zymosan or budding yeast, revealed that a fully assembled phagosomal actin coat efficiently prevents docking and fusion of lysosomes with phagosomes. Importantly, dynamic assembly of the actin coat was seen only with immature phagosomes but not mature phago-lysosomes, and we suggest that this process is involved in regulating the rate of maturation of individual phagosomes. Here, our results also provide a mechanistic rationale explaining previously published observations where the actin prevents clustering and fusion of late-endosomal vacuoles in *Dictyostelium* (Drengk et al., 2003) or where accumulation of periphagosomal actin delays maturation of *Leishmania*-containing phagosomes (Holm et al., 2001).

Oscillations in actin assembly were also observed in a cell-free system. Van der Gucht and colleagues (Van der Gucht et al., 2005) have shown that actin which initially polymerizes symmetrically on spherical beads in vitro can undergo a spontaneous break of the symmetry. The authors explain this periodic event by a model based on elasticity theory and fracture mechanics. We assume that a similar model can be applied to the behaviour of actin coat on rigid phagosomes, where breakage of the symmetry is involved in the dynamics of flashing.

Of the phagosomes that polymerized actin in our analyses, only a small fraction became motile. Of these, two distinct modes of actin-driven motility were seen: long-distance and rather continuous movement associated with typical actin comets; and discontinuous movement in short steps generated by compression of polymerizing actin – and its release. During the latter type of propulsion, the course of transient phagosome detachment from the polymerized actin suggested that it probably occurs when elastic stretching of the phagosome membrane exceeds the tension limit. Our observations from live cells are in agreement with in vitro studies of Bernheim-Groswasser et al. (Bernheim-Groswasser et al., 2002) and Dickinson...
and Purich (Dickinson and Purich, 2006) who described actin-driven saltatory motion of hard particles and vesicle deformations in a cell-free system. Nevertheless, the function of phagosome motility itself remains unclear. Polarized actin assembly on phagosomes could provide a substantial force to transport large phagosomes to the perinuclear region, to facilitate their contact with lysosomes in this area. However, we observed targeting of phagosomes more often to the cell periphery rather than towards the nucleus. We thus assume that the infrequent phagosome motility driven by formation of actin comets is merely a consequence of spontaneous actin-coat polarization.

Since the majority (90-95%) of phagosomes undergo rapid maturation without any detectable de novo actin polymerization on their membrane, we argue that actin-coat assembly is not always essential for the process of maturation. Here, the capacity of phagosomes to induce actin assembly was shown to be dependent on the state of the macrophage. The probability of actin-coat assembly on newly formed phagosomes increased as the number of preceding phagosomes (phagosome load) in the cell increased. Additionally, those phagosomes that entered the cell later were more likely to induce actin flashing than those that formed earlier. We conclude that overloading the phagocytic or lysosomal system leads to actin flashing that then blocks phagosome fusion with lysosomes. Nevertheless, how the cell can sense such overloading and increases F-actin nucleation on membrane of individual phagosomes remain to be clarified. Here, we assume that when cells are exposed to a high load of phagocytic cargo and internalize a bulk of plasma membrane, the recycling of protein and lipid components that normally occur on nascent phagosomes can be temporarily retarded. Such a delay would then also lead to retention of plasma membrane components involved in the actin nucleation. Interestingly, retention of the Cdc42, a GTPase regulating actin polymerization, is actively induced by *Leishmania* and prevents the maturation of the phagosome (Lodge and Descoteaux, 2005). We propose a model where the extent of de novo polymerization of actin by phagosomes is proportional to the amount of phagocytic load internalized by individual cell.

Although the precise mechanisms of how actin polymerization exerts forces on cellular membranes is still not completely understood, the number of theoretical and experimental studies of this process is increasing (Footer et al., 2007; Schwartz et al., 2004; Bernheim-Gros wasser, 2002; van Oudenaarden and Theriot, 1999). These show that polymerizing networks of actin filaments are able to exert significant mechanical forces to change shape or to move structures without myosin- or microtubule-associated molecular motors as power generators; such a system is used by eukaryotic cells and their prokaryotic pathogens (reviewed by Upadhyaya and Oudenaarden, 2004).

Our observations on macropinosomes brought further insight into the forces generated by actin polymerization on cellular membranes. Fluid-containing flexible macropinosomes undergo dynamic changes of the shape while fluctuations in the actin-coat density are observed on their membrane. As a result, the homogenous actin coat is disrupted and compression-induced budding of macropinosome is followed either by propulsion or by complete scission of the organelle.

Our live-imaging observations extend previous analyses of actin on synthetic liposomes in vitro, where actin assembly was shown to generate compression that deforms rocking vesicles and squeeze them forward (Upadhyaya et al., 2003; Giardini et al., 2003; Trichet et al., 2007). We suggest that the curvature of the macropinosome membrane induced by local actin polymerization can, in turn facilitate clustering of the actin nucleation machinery. Such a process would further increase heterogeneity in the actin-coat density, leading to rapid actin-coat remodelling. Thus, whether the macropinosome undergoes constriction and scission, compression-triggered propulsion or only dynamic changes of shape, probably depends on the balanced interactions between forces exerted by actin coat on their membrane and the elastic tension of the organelle membrane, which keeps the surface-to-volume ratio to a minimum.

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**Fig. 9.** Model of actin-coat-mediated block of fusion and actin-coat remodelling on phagosomes and macropinosomes. (A) After internalization, phagosomes rapidly follow the maturation pathway until they fuse with lysosomes. Under the stress conditions when macrophage phagocytic or endocytic capacity is overloaded, de novo F-actin nucleation is transiently induced on nascent phagosomes and repeated waves of actin-coat assembly-disassembly block phagosome-lyosome fusion. Occasionally, the break of actin-coat symmetry might lead to actin-comet propulsion of phagosomes. On asymmetrical phagosomes (containing e.g. budding yeast), F-actin is preferentially nucleated and assembled at the highly curved sites of phagosome constriction and resembles ‘obstructed’ scission (not shown). (B) Less prominent F-actin coat is assembled by macropinosomes. Here, the interaction between membrane elasticity and compression forces of actin polymerization drives dynamic organelle remodelling, which facilitates break of actin-coat symmetry. Macropinosome budding through the actin-coat rupture leads to either macropinosome constriction and scission or actin-coat polarization. Asymmetrical actin-coat assembly then results in long-distance comet propulsion or, when macropinosome detaches from the compressing actin coat, in short back-and-forth movements.
In contrast to macropinosomes, phagosomes usually contain rigid particles to which the membrane is tightly apposed. Consequently, actin-driven membrane deformation and inward protrusion is thus limited on phagosomes. It has been shown that when F-actin polymerizes on the surface of coated beads in vitro via incorporation of new actin monomers on the bead surface, the pre-existing F-actin coat is pushed outward and stretched (Paluch et al., 2006; Plastino and Sykes, 2005; Noireaux et al., 2000). When the tangential stress in a crosslinked actin coat exceeds a critical value, breakage of actin-coat symmetry is induced, which might then lead to transient comet propulsion ( Sekimoto et al., 2004; van der Gucht et al., 2005). We propose that differences in the mechanical properties (namely membrane plasticity and tension) can explain why the actin on phagosomes polymerizes longer, more uniformly and to a larger extent, than on flexible macropinosomes, before the actin coat breaks symmetry (Fig. 9).

On asymmetrical phagosomes containing budding yeast, the membrane is also tightly apposed to the cargo with a minimal flexibility, but the actin rarely assembled uniformly as observed with latex bead phagosomes. Instead, the bulk of the actin polymerization preferentially occurred at the high curvature of the budding zone, and was reminiscent of frustrated scission. This implies that the pre-existing membrane curvature induces clustering of the actin nucleation machinery to the groove around the phagosome constriction site. Such a scenario would be consistent with computational simulations of curvature-mediated aggregation of membrane proteins (Reynwar et al., 2007), and with experiments linking local membrane curvature and transient actin polymerization (Bettache et al., 2003; Hübner et al., 1998).

Materials and Methods

Cells lines and transfection

Mouse macrophages RAW 264.7 used for live imaging were transfected with pcGFP1-Actin or pDSRed-Actin vector (Clontech) using Transfectam kit (Promega). Stable cell lines were established by subcloning of GFP or RFP-positive cells on selection antibiotic G418 (Sigma). Fluorescence labelling of fixed cells was performed on mouse RAW 264.7 macrophages, mouse primary bone-marrow-derived macrophages (BMDMs) and on mouse BV-2 cells, an immortalized mouse microglial cell line that exhibits the morphological and functional characteristics of microglia (Bocchini et al., 1992).

Preparation of fluorescent fluid-phase marker and phagocytic cargo

The method for BSA coupling to colloidal gold was described earlier (Slot and Geuze, 1983). Stock of 10 nm BSA-gold (OD520=20) was labelled with NHS-rhodamine (Pierce Biotechnology) as described (Anes et al., 2006). To induce phagocytic uptake, we used 1.0 or 3.0 μm LBs coated with mouse IgG (kindly provided by Guilio Gabbiani, University of Geneva, Switzerland) was followed ultracut and sections transferred in sucrose-methylcellulose on the carbon-coated CuPd blocks with a razor blade, infiltrated with 2.3 M sucrose, mounted on aluminium a rubber policeman, embedded in low-melting 10% gelatin (Sigma), cut in small stamps, and contrasted in uranyl acetate and lead citrate. For immunolabelling on cryosections, cells grown in Petri dishes were pulsed with 3 μm 1.38r software (Wayne Rasband, NIH). All time-lapse series in supplementary material stamps, cropping and export to QuickTime Movie format were performed in ImageJ phase-contrast images were combined using Imaris 6.0.1 software (Bitplane); time stamps, cropping and export to QuickTime Movie format were performed in ImageJ 1.3k software (Wayne Rasband, NIH). All time-lapse series in supplementary material were acquired in xyt scanning mode at a rate of 10 frames per minute. Movies are displayed at a rate of 7 frames per second.

Kinetics of phagosome F-actin assembly and phagosome maturation in fixed cells

Raw macrophages were grown on coverslips in 24-well dishes. The 3 μm LBs coated with mouse IgG (kindly provided by Guilio Gabbiani, University of Geneva, Switzerland) was followed ultracut and sections transferred in sucrose-methylcellulose on the carbon-coated CuPd EM grids with parlodion film. The primary, anti-β actin rabbit polyclonal antibody (kindly provided by Guiulo Gabbiani, University of Geneva, Switzerland) was followed by protein A conjugated to 10 nm gold (CMC Utrecht). Sections on grids were contrasted and embedded in mixture of uranyl acetate and methylcellulose. All samples were observed with Biotwin CM120 Phillips electron microscope equipped with 1K CCD Camera (Keen View, SIS).

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References


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Phase contrast + Actin-GFP