Actin and Phosphoinositide Recruitment to Fully Formed *Candida albicans* Phagosomes in Mouse Macrophages

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Key Words

Life imaging · Macrophages · Phagocytosis · *Candida albicans* · Phosphoinositides · Yeast

Abstract

Candida albicans is a dimorphic yeast that enters macrophages (Mφ) via the β-glucan receptor dectin-1. Phagocytosis of *C. albicans* is characterized by actin polymerization, Syk kinase activation and rapid acquisition of phagolysosomal markers. In mice, *C. albicans* are able to resist the harsh environment of the phagosome and form pseudohyphae inside the phagolysosomal compartment, eventually extending from the Mφ. In this study, we investigated these unique *C. albicans* phagosomes and found that actin localized dynamically around the phagosomes, before disintegrating. Membrane phosphoinositides, PI(4,5)P₂, PI(3,4,5)P₃, PI(3,4)P₂, and PI(3)P also localized to the phagosomes. Localization was not related to actin polymerization, and inhibitor studies showed that polymerization of actin on the *C. albicans* phagosome was independent of PI3K. The ability of mature *C. albicans* phagosomes to stimulate actin polymerization could facilitate the escape of the growing yeast from the Mφ.

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Introduction

Candida albicans is an opportunistic pathogen that can cause infections of mucosal tissues and is able to invade systemically, especially in immunocompromised hosts. It possesses an array of virulence factors that regulate its adaptation to the host environment, recognition of host cells, secretion of hydrolases and phenotypic switching [1]. The polymorphism of *C. albicans* has also been suggested to contribute to pathogenesis; depending on environmental factors, the yeast is able to switch between hyphal and yeast forms [2].

Dendritic cells, neutrophils and macrophages (Mφ) are able to phagocytose *C. albicans* via various receptors that have been shown to be involved in *C. albicans* recognition and regulation of subsequent immune responses [3–8]. Like phagocytosis of other particles, *C. albicans* uptake is also accompanied by actin polymerization [9]. After closure, the *C. albicans* phagosomes rapidly recruit markers of late endosomes and lysosomes [10]. In con-

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trast, lysosomal-associated membrane protein 1 (LAMP-1) marks phagosomes 30 min after entry by FcR-mediated phagocytosis [11].

Mouse M ϕ are not efficient in killing *C. albicans*. Despite the maturation of the *C. albicans* phagosomes, the yeasts are able to grow hyphal forms that eventually destroy the M ϕ [10]. Several reports have shown mechanisms used by *C. albicans* to escape phagocytosis and killing by M ϕ . *C. albicans* phospholipomannan, a surface glycolipid that is shed by *C. albicans*, was shown to mediate escape from M ϕ by inducing apoptosis [12, 13]. It has also been reported that a soluble factor from *C. albicans* suppresses nitric oxide production, but it does not stimulate the production of immunosuppressive cytokines [14]. Furthermore, *C. albicans* β -1,2-linked mannoooligosaccharides, which are part of the *C. albicans* cell wall, have been implicated in adhesion to M ϕ [15] and inhibition of tumor necrosis factor- α production [16]. Several pathogens interfere with host actin assembly, mainly by activating the Arp2/3 complex. This is used to promote uptake and to gain actin-based motility to move through the cytoplasm into neighboring cells, thereby spreading infection without activating immune responses [17]. *C. albicans* has also been shown to secrete an actin-rearranging factor that increases the transition of soluble actin to insoluble actin [18, 19]. Once inside the M ϕ , *C. albicans* adapts to oxidative stress and starvation and induces morphological changes; at a later stage when hyphal growth enables escape from the M ϕ , it activates glycolysis and downregulates stress responses [20].

Signal transitions that occur during phagosome maturation are not well characterized. Phosphoinositides (PIs) are important signaling molecules in receptor-mediated signal transduction, actin remodeling and membrane trafficking [21, 22]. PIs, the collective name of phosphorylated derivatives of phosphatidylinositol, are membrane bound and compose less than 10% of the total cellular phospholipids. A total of 7 different PIs can be produced by different combinations of phosphate groups arranged around the inositol ring. Organelle-specific PI kinases and PI phosphatases mediate rapid subcellular distribution of specific PI, leading to recruitment, binding and activation of effector proteins that mediate downstream signaling. Activation of phospholipid-modifying enzymes leads to the formation of phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P₃] from phosphatidylinositol-4,5-bisphosphate [PI(4,5)P₂] and phosphatidylinositol-3,4-bisphosphate [PI(3,4)P₂] on the phagosomal membrane [23–27]. PI(4,5)P₂ and PI(3,4,5)P₃ are involved in the regulation of actin polymerization [23,

28–33], and PI(3,4)P₂ is thought to play a role in the activation of NADPH oxidase [34]. Later stages of uptake associated with phagosome closure are the activation of Arf1 and Rac2 GTPases, formation of phosphatidylinositol-3-phosphate [PI(3)P], and the disappearance of polymerized actin from the phagosomal cup [11, 25]. PI(3)P is almost exclusively found on early endosomes and phagosomes [21, 35], where it facilitates phagosome maturation [34, 36–38].

Taken together, these observations indicate that *C. albicans* is a complex pathogen able to utilize an array of signaling mechanisms to evade the host defense response. In this study, we followed the dynamics of maturing *C. albicans* phagosomes in M ϕ using real-time microscopy. We assessed the localization of M ϕ actin and found that phagosomes containing growing *C. albicans* are accompanied by large amounts of actin. We also assessed the localization of several phosphoinositides known to be involved in phagocytosis and found that they were increased on phagosomes containing live *C. albicans*. This is suggestive of extensive signaling processes being employed around the phagosomes.

Materials and Methods

Bone Marrow M ϕ

Mice used in this study (BALB/c) were from the Sir William Dunn School of Pathology (University of Oxford) breeding colonies and between 8 and 12 weeks of age at the time of study. Animals were kept and handled in accordance with institutional guidelines. Femurs were dissected from sacrificed mice and marrow washed out of the femoral cavities. Cells were then pelleted at 500 g for 5 min prior to resuspension in fresh RPMI containing 10% heat-inactivated fetal bovine serum, 4 mM L-glutamine, 20 U/ml penicillin, 20 μ g/ml streptomycin and 20% L-cell-conditioned medium (source of M-CSF). Cells were then transferred to Petri dishes and allowed to adhere. Fresh medium was added on days 3 and 5 after isolation. Cells were plated for experiments at day 6 or 7 after isolation. Bone-marrow-derived M ϕ were used at 1×10^5 per well in 24-well plates.

RAW264.7 Cell Culture and Transfection

RAW264.7, a murine M ϕ -like cell line (RAW cells) from the American Type Culture (Manassas, Va., USA) were cultured in Advanced DMEM with 2% heat inactivated fetal bovine serum, 4 mM L-glutamine, 20 U/ml penicillin, and using Invitrogen cell culture reagents (Carlsbad, Calif., USA) at 37°C with 5% CO₂. RAW cells were prepared for ratiometric microscopy by plating $\sim 2.5 \times 10^5$ RAW cells per coverslip the day before imaging. After the cells had attached to the coverslip (3 h), they were transfected with plasmids encoding the fluorescent chimeras, using Roche FuGene-6 as described in the manufacturer's protocol (Roche Diagnostics, Indianapolis, Ind., USA). This gave a transfection efficiency of 10–20%.

Molecular Cloning and DNA Manipulation

Plasmids encoding monomeric versions (A207K) of cyan fluorescent protein (CFP) and citrine (YFP) were used where indicated [39]. β -Actin from pEYFP.actin.C1 (Clontech, Mountainview, Calif., USA) was transferred into the Clontech C1 vector expressing either monomeric CFP or monomeric Cherry (mCherry; Roger Tsien, University of California San Diego, San Diego, Calif., USA). The PH domains of human PLC δ 1 and Bruton's tyrosine kinase (Btk) were a gift from Tamas Balla [40, 41]. The PLC δ 1PH construct was PCR amplified adding *Xho*I and *Bam*HI restriction sites, then subcloned into pmCitrine-N1 (Clontech). The BtkPH construct was PCR amplified and subcloned into the pmCitrine-N1 vector between *Xho*I and *Hind*III. The AktPH construct, a gift from Tobias Meyer (Stanford, Palo Alto, Calif., USA), was subcloned into pmCitrine-C1 between *Bam*HI and *Xba*I (Clontech). Human Tapp1 constructs were obtained from the MRC Protein Phosphorylation Unit, Dundee, UK, and the C-terminal PH domain was subcloned into pmCitrine-C1 at the *Eco*RI-*Bam*HI site. GFP-2xFYVE, the tandem FYVE finger domains from hepatocyte-growth-factor-regulated tyrosine kinase substrate (Hrs), was donated by Harold Stenmark (Norwegian Radium Hospital, Oslo, Norway) and cloned into pmCitrine.C1 between *Hind*III and *Kpn*I sites (Clontech). All DNA sequences were confirmed at the University of Michigan DNA Sequencing Core. pEGFP-actin vector was from BD Biosciences (Oxford, UK).

C. albicans Infections

Stock cultures of a laboratory strain of *C. albicans* (ATCC 18804) were maintained on Sabouraud's dextrose agar (Difco Laboratories, Detroit, Mich., USA) at 4°C. For experiments, *C. albicans* was grown in 10 ml Sabouraud's dextrose broth (Difco) in a shaking incubator at 30°C for 24 h, to obtain a stationary phase culture. The yeast cultures were washed in PBS and then added to cells at a ratio of 5:1. The cells were incubated for 30 min at 4°C to allow attachment, then washed and incubated for 2 h at 37°C. Infection efficiency was around 30%. To inhibit PI3K activation, RAW cells were infected as described above, but after a 1.5-hour incubation, cells were washed and incubated with a 50- μ M solution of LY294002 for 30 min at 37°C (EMD Biosciences, San Diego, Calif., USA), to avoid inhibition of phagocytosis. Sixty phagosomes were measured for the control group and 71 phagosomes for the LY-treated population. Other particles used in this study include zymosan (Invitrogen), 6.0- μ m polystyrene polystyrene beads® (Polysciences Inc., Warrington, Pa., USA) and heat-killed *C. albicans* (killed for 1 h at 100°C). These particles were washed 3 times with medium before adding to the M ϕ at a ratio of 5:1, as described above.

For microscopy, coverslips were placed in Leiden chambers (Harvard Apparatus, Holliston, Mass., USA) at 37°C in Ringer's buffer (155 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 2 mM NaH₂PO₄, 10 mM glucose, 10 mM HEPES at pH 7.2). Transiently transfected M ϕ containing internalized *C. albicans* were imaged.

Staining of Fixed Samples

M ϕ on coverslips were cooled to 4°C and washed three times with prechilled medium. Particles were added to the M ϕ at a 5:1 ratio, incubated for 1 h at 4°C to synchronize uptake. Unbound particles were removed by washing 3 times with prechilled me-

dium. The cells were then incubated at 37°C for 2 h. Cells were fixed with 4% paraformaldehyde (Sigma) in PBS for 20 min at 4°C and permeabilized in buffer containing 0.25% saponin (Sigma), 1% BSA (Sigma), 1% heat-inactivated goat serum (Sigma) and 1% heat-inactivated rabbit serum (Sigma) in PBS (permeabilization buffer) at room temperature for 30 min. 1D4B rat anti-mouse LAMP-1 antibody (eBioscience) and FITC-phalloidin (Sigma) were added at a concentration of 10 μ g/ml in permeabilization buffer for 1 h at room temperature. Cells were washed 3 times prior to incubation with Alexa-488 goat anti-rat antibody (Invitrogen) in the same buffer. After 2 washes with buffer and 2 washes with PBS, coverslips were mounted on glass microscopy slides with Dako Cytomation Fluorescent Mounting Medium (Dako Cytomation). Slides were analyzed using a Radiance 2000 (Bio-Rad) confocal microscope as described below. To quantify actin-positive phagosomes, 100 phagosomes were scored.

Microscopy

Images were acquired on an inverted fluorescence microscope (Nikon TE300) with a 60 $\times$ 1.4 Planapo objective, a mercury arc lamp as the source of epifluorescent light and a cooled digital charge-coupled-device camera (Quantix; Photometrics). The microscope was equipped with trans- and epifluorescence shutters, a temperature-controlled stage, filter wheels for both excitation and emission filters, and dichroic mirrors that allow detection of multiple fluorophores. All images were acquired and processed using Metamorph 6.2r6 (Universal Imaging, Malvern, Pa., USA). Fluorescence excitation and emission wavelengths were selected via a JP4v2 filter set (Chroma Technology, Rockingham, Vt., USA) and Lambda 10-2 filter wheel controller (Sutter Instruments).

Phagosomes which contained growing *C. albicans* were imaged in RAW cells expressing fluorescent proteins. YFP (excitation 505 nm, emission 540 nm), CFP (excitation 435 nm, emission 490 nm) and phase-contrast images were recorded every 30 s. M ϕ expressing actin-mCherry (excitation 572 nm, emission 630 nm) were imaged using the Texas Red/FITC filter set (Omega Optical, Brattleboro, Vt., USA).

Using real-time confocal microscopy, samples were prepared as described above and images collected on a Radiance 2000 (Bio-Rad) confocal microscope, fitted with a 4-line argon laser (457, 477, 488, 514 nm), a green-HeNe laser (543 nm), a red-diode laser (638 nm) and a TiS multiphoton laser (infrared). The microscope was an inverted Nikon TE300, with a 60 $\times$ water immersion objective. The software controlling the camera was Bio-Rad LaserSharp 2000 (V. 5.0). Images were collected with the optimal iris aperture and the minimum laser power that fitted the whole gray scale. M ϕ and *C. albicans* remained viable throughout the course of these experiments.

Image Processing

To measure the amount of actin or PH domain chimera recruited to the phagosome, a region was drawn around the internalized *C. albicans* phagosome, and the average fluorescence intensity of the phagosome, I_p , was divided by the average fluorescence intensity for the cell, I_c . As a control, the average intensity of the nucleus, I_n , was divided by the I_c . Recruitment of the fluorescent chimera to the yeast phagosome was defined as I_p/I_c greater than 1.0. Images were further processed for figures using Adobe Photoshop version 6. Imaris software was used for 3-dimensional reconstruction.

Results

The early events of *C. albicans* phagocytosis have been well examined. In contrast, little is presently known about the later stages of *C. albicans* infection, when the yeast is fully internalized and forming pseudohyphae inside the Mφ. In the present study, we examined actin dynamics on mature *C. albicans* phagosomes (2+ h after infection) in Mφ. During static experiments, cells were stained for polymerized actin with FITC-phalloidin 2 h after *C. albicans* yeast phagocytosis, when the *C. albicans* had formed (pseudo) hyphae. A number of (pseudo) hyphae were marked by polymerized actin around their phagosomes. This could be seen in both RAW264.7 Mφ and primary mouse Mφ (fig. 1a, b). Furthermore, *C. albicans* (pseudo)hyphae breaking out of the Mφ also exhibited increased actin localization around the hyphae (fig. 1c and online supplementary movie 1a, b, www.karger.com/doi/10.1159/000173694). In general, more *C. albicans* (pseudo)hyphae were associated with actin compared to heat-killed *C. albicans* phagosomes (data not shown). Large concentrations of actin could be seen around *C. albicans* (pseudo)hyphae, when these (pseudo)hyphae were growing from one Mφ into adjacent Mφ (fig. 1d). A 3-dimensional reconstruction of a Z-series taken at this stage shows that a solid tube of actin was formed along the (pseudo)hyphae (fig. 1d).

While synchronized experiments give reasonable similarity in the timing and dynamics of acquired proteins at short incubation periods, certain signaling molecules

Fig. 1. Actin polymerization on *C. albicans* (pseudo)hyphae growing inside phagosomes. **a** Images of a RAW264.7 Mφ 2 h after yeast phagocytosis stained with FITC-phalloidin. The growing (pseudo)hypha was marked with increased actin. **b** FITC-phalloidin staining of primary bone marrow-derived Mφ 2 h after *C. albicans* yeast phagocytosis. Actin polymerization can be observed around the (pseudo)hypha. **c** RAW264.7 Mφ stained for LAMP-1 (green) and actin (red) 2 h after phagocytosis of *C. albicans* yeast. The two (pseudo)hyphae growing inside the Mφ are marked with LAMP-1, but only 1 phagosome exhibited actin polymerization. **d** Three-dimensional reconstruction of actin polymerization around a *C. albicans* phagosome. RAW264.7 Mφ 2 h after phagocytosis of *C. albicans* yeast. The first panel is a transmission image of a single *C. albicans* (pseudo)hypha growing from one Mφ into the next (arrow). The second panel is the equivalent fluorescent image showing actin in red and the lysosomes in green. The last two panels show three-dimensional reconstructions of a Z-series taken through the cells. Scale bar corresponds to 5 μm.

Fig. 2. Live confocal microscopy with GFP-actin-transfected RAW264.7 M ϕ . Transmission image (**a**) and fluorescent images (**b**) of *C. albicans* (pseudo)hyphae growing inside a M ϕ (arrow). Fluorescent images were taken at different time points and show actin movement around the (pseudo)hyphae. See also movie 1. Scale bar corresponds to 5 μ m.

already show differences in kinetics as soon as 10 min after synchronized uptake [11]. The dynamics of actin polymerization were assessed by real-time confocal microscopy. In these experiments, RAW264.7 M ϕ expressing GFP-actin were allowed to phagocytose *C. albicans* yeast and 2 h after phagocytosis, when *C. albicans* was growing (pseudo)hyphae inside the M ϕ , the actin dynamics were followed. Figure 2 shows a sequence of images taken from a time course experiment of a M ϕ with 3 phagosomes, 2 with *C. albicans* (pseudo)hyphae and 1 with yeast (online supplementary movie 2a, b, www.karger.com/doi/10.1159/000173694). At the start of the experiment one (pseudo)hyphal phagosome had a high concentration of surrounding actin while the other (pseudo)hyphal phagosome did not show surrounding

actin. In time, the phagosome without actin started to acquire actin. Actin moved around the phagosome and sometimes left the phagosome completely, indicating that actin localization around phagosomes is an active process.

Since actin polymerization occurs during the formation of a phagocytic cup, there may be other signals associated with the early stages of phagocytosis that are also detectable along with actin on the mature *C. albicans* phagosomes. We examined the dynamics of PI(4,5)P₂, PI(3,4,5)P₃, PI(3,4)P₂ and PI(3)P using YFP chimeras of binding domains specific for each phosphoinositide.

The PH domain of Plc δ 1 binds to PI(4,5)P₂ [42]. Plc δ 1PH-YFP and actin-CFP were expressed in M ϕ . The M ϕ were infected with *C. albicans* and after 2 h the fluo-

Fig. 3. Recruitment of PI(4,5)P₂ during *C. albicans* growth inside phagosomes. Scale bar corresponds to phase contrast, YFP and CFP images showing RAW Mφ infected with *C. albicans*. After a 2-hour incubation, Plcδ1PH-YFP increased on the *C. albicans* phagosomes, along with actin-CFP. The three rows illustrate three different cells from different experiments. Arrows indicate the localization of the fluorescent chimera to the phagosome. Plcδ1PH is a marker for PI(4,5)P₂. Scale bar corresponds to 5 μm.

rescent cells were examined for CFP and YFP chimera recruitment to the phagosomes. Detectable increases in Plcδ1PH-YFP and actin-CFP were observed on the *C. albicans* phagosomes (fig. 3). Of the 8 phagosomes measured, 7 exhibited Plcδ1PH-YFP recruitment to the phagosome, where the intensity in the phagosome I_p was greater than the intensity in the cell, I_c (I_p/I_c > 1) (fig. 4). Thus, PI(4,5)P₂ was found on mature *C. albicans* phagosomes along with actin, suggesting it may play a role in this process.

To assess the localization of PI(3,4,5)P₃ and PI(3,4)P₂, Mφ were transfected to express AktPH-YFP together with actin-CFP to measure and compare the increase in 3'-phosphoinositides on the growing yeast phagosome to the increase in actin. The PH domain of Akt binds to both PI(3,4,5)P₃ and PI(3,4)P₂ [43]. *C. albicans* phagosomes that exhibited actin polymerization also exhibited marked recruitment of AktPH-YFP (fig. 5). The localization of

Fig. 4. Recruitment of actin and phosphoinositides at the *C. albicans* phagosomes. Average of maximum I_p/I_c for actin, Plcδ1PH, AktPH and FYVE-YFP. Plot showing the increased localization of either actin-CFP or the PH domain-YFP fusion on *C. albicans* phagosomes. For each PH domain proxy, the maximum level of localization (I_p/I_c) on the phagosome was averaged for each movie. This value was then plotted. Error bars reflect standard error of the mean.

the AktPH-YFP chimera to the phagosome was observed in all 8 phagosomes measured (I_p/I_c > 1) (fig. 4). When the individual dynamics of PI(3,4,5)P₃ and PI(3,4)P₂ were measured using the BtkPH domain and the Tapp1PH domain, respectively, similar patterns were observed (data not shown). Therefore, PI(3,4,5)P₃ and PI(3,4)P₂ were present on *C. albicans* phagosomes that also contained actin.

The 2xFYVE domain from Hrs (FYVE) binds to PI(3)P [44]. The dynamics of PI(3)P on the growing *C. albicans* phagosome were traced in Mφ expressing 2xFYVE-YFP and actin-CFP. YFP fluorescence increased on those phagosomes that also had actin polymerized around them, indicating the recruitment of 2xFYVE-YFP to the phagosome (fig. 6). All 5 phagosomes measured exhibited 2XFYVE-YFP recruitment to the phagosome (I_p/I_c > 1) (fig. 4). Thus, PI(3)P was also localized to mature *C. albicans* phagosomes; however, its localization seemed to be unrelated to the dynamics of the actin polymerization.

During phagocytosis, PI3K-dependent signals facilitate actin polymerization [45]. Similar signals could drive the actin polymerization on *C. albicans* phagosomes. To examine if the actin on *C. albicans* phagosomes was dependent upon PI3K, Mφ were treated with the PI3K in-

Fig. 5. Recruitment of PI(3,4,5)P₃ and PI(3,4)P₂ to growing *C. albicans* phagosomes. Phase contrast, YFP and CFP images showing RAW Mφ transfected to express AktPH-YFP and actin-CFP, and infected with *C. albicans*. After a 2-hour incubation, AktPH-YFP increased on the *C. albicans* phagosomes, along with actin-CFP. The 3 rows illustrate 3 different cells from different experiments. Arrows indicate the localization of the fluorescent chimera to the phagosome. AktPH is a marker for PI(3,4,5)P₃ and PI(3,4)P₂. Scale bar corresponds to 5 μm.

Fig. 6. Recruitment of PI(3)P to growing *C. albicans* phagosomes. Phase contrast, YFP and CFP images showing RAW Mφ expressing 2xFYVE-YFP, a marker for PI(3)P, and actin-CFP, and infected with *C. albicans*. After a 2-hour incubation, FYVE-YFP increased on the *C. albicans* phagosomes, along with actin-CFP. The 3 rows illustrate 3 different cells from different experiments. Arrows indicate the localization of the fluorescent chimera to the phagosome. Scale bar corresponds to 5 μm.

hibitor LY294002. Since inhibition of PI3K blocks phagocytosis [46], the Mφ were only treated with LY294002 after being allowed to undergo initial phagocytosis of *C. albicans*. Thus, the actin polymerization during phagosome maturation would only be affected if dependent upon continued PI3K activation. In control and LY294002-treated Mφ transfected with actin-mCherry, the intensity of actin-mCherry on the *C. albicans* phagosome (I_P) was measured, and divided by the overall mCherry intensity in the total cell (I_C). If actin polymerized on the phagosome, I_P/I_C would be greater than the baseline level of 1.0. In LY294002-treated cells, there were comparable levels of actin polymerized on the yeast phagosomes (control = 1.18 ± 0.38 , $n = 66$, LY-treated = 1.16 ± 0.42 , $n = 71$) (fig. 7). This suggests that even though different 3'-phosphoinositides localized to the *C. albicans* phagosomes, these phosphoinositides are not necessary for actin polymerization around the *C. albicans* phagosomes.

Fig. 7. Recruitment of actin to fully formed *C. albicans* phagosomes during PI3K inhibition. Phase contrast and fluorescence images showing actin-mCherry recruited to *C. albicans* phagosomes after treatment with LY294002. Scale bar corresponds to 5 μm.

Fig. 8. Proposed model for events on growing *C. albicans* phagosomes. During phagosome formation, PI(3,4,5)P₃, PI(3,4)P₂ and PI(4,5)P₂ form at the site of phagocytosis. These PIs are converted during maturation; at later stages PI(3)P is predominantly found at the phagosome. *C. albicans* growth inside the phagosome is accompanied by large amounts of dynamic actin polymerization and association of the four PIPs.

Discussion

This is the first study to examine the dynamics of *C. albicans* phagosomes 2 h after initial infection. Our findings are summarized in figure 8. Actin was polymerized on the phagosome and, through live cell imaging, could be observed cycling on and off the phagosome. Membrane phosphoinositides that are important in FcR-mediated phagocytosis were increased on the mature *C. albicans* phagosome (fig. 8). Strikingly, inhibition of PI3K did not affect the actin polymerization observed on the yeast phagosomes. This implied that the yeast phagosome was triggering a PI3K-independent pathway leading to actin polymerization.

Actin polymerization around latex bead phagosomes has been studied extensively, showing that polymerization has a role in fusion between phagosomes and late endocytic organelles [47–49]. The increased number of actin-positive live *C. albicans* phagosomes compared to phagosomes containing heat-killed *C. albicans* may be explained by the continuous growth of the (pseudo) hyphae, which required higher levels of organelle fusion to accommodate the demand for membrane. Further analysis is needed to confirm this hypothesis. Several pathogens are known to be able to interfere with host actin assembly, mainly by activating the Arp2/3 complex.

This complex is used to promote uptake and to gain actin-based motility to move through the cytoplasm into neighboring cells, thereby spreading infection without activating humoral immune responses [17]. *C. albicans* has also been shown to secrete an actin-rearranging factor that increases the transition of soluble actin to insoluble actin [18, 19]. It is possible that actin polymerization on the yeast phagosome was mediated by *C. albicans* interference with the actin cytoskeleton. The presence of PI(4,5)P₂ could also be facilitating the actin polymerization [50]. However, in an experiment where *C. albicans* blastoconidia were phagocytosed followed by inhibition of actin polymerization with cytochalasin D, *C. albicans* was still able to grow (pseudo)hyphae and escape from the Mφ [R.P. da Silva, unpubl. data], suggesting that actin polymerization is not necessary for *C. albicans* survival inside Mφ.

It is interesting that the mature phagosomes possess membrane markers associated with the earlier stages of phagocytosis. Even PI(3)P, which marks the endocytic pathway, appears on FcR-mediated phagosomes within the first 10 min of phagocytosis [11]. The persistence of these early markers could indicate dysregulation in the maturation process of the *C. albicans* phagosome, providing a means for *C. albicans* to escape the killing mechanisms of mature phagosomes. *Mycobacterium tuberculosis* employs a similar mechanism to avoid phagocytic killing in Mφ. By altering levels of calcium inside the Mφ, the bacterium persists indefinitely in an early endosome-like compartment [51, 52]. It is possible that *C. albicans* also secretes factors that interrupt phagosome maturation, giving it additional time to develop into filaments.

PI(3)P is known to be excluded from the plasma membrane and is seen in vesicles of the endocytic pathway [11, 21]. It participates in phagosomal maturation and NADPH oxidase activation [34, 36–38]. This PI was observed around the *C. albicans* phagosomes. PI(3,4)P₂ was also present around the *C. albicans* phagosomes. The presence of PI(3)P and PI(3,4)P₂, that also activate the oxidase, suggests that the NADPH oxidase is activated on *C. albicans* phagosomes [34]. Upon phagocytosis, the NADPH oxidase is assembled and activated, leading to production of reactive oxygen intermediates that mediate killing and degradation of ingested particles. Further work is needed to assess if NADPH oxidase production takes place at these phagosomes.

Notably, not all *C. albicans* observed inside Mφ were marked by polymerized actin. This may be due to the active process of polymerization occurring at specific points during phagosomal maturation. The live *C. albicans*

phagosome is unique because of (pseudo) hyphal growth, which forces the M ϕ to accommodate this growth in order to retain the *C. albicans* inside a phagosome. The mechanism of *C. albicans* escape from M ϕ remains to be determined, but the findings described here indicate *C. albicans* evasion of normal phagosome maturation and highlights intriguing new directions for future exploration.

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