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Candida albicans internalization by host cells is mediated by a clathrin-dependent mechanism

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Abstract

Candida albicans is a major cause of oropharyngeal, vulvovaginal and hematogenously disseminated candidiasis. Endocytosis of *C. albicans* hyphae by host cells is a prerequisite for tissue invasion. This internalization involves interactions between the fungal invasin Als3 and host E- or N-cadherin. Als3 shares some structural similarity with InlA, a major invasion protein of the bacterium *Listeria monocytogenes*. InlA mediates entry of *L. monocytogenes* into host cells through binding to E-cadherin. A role in internalization, for a non classical stimulation of the clathrin-dependent endocytosis machinery was recently highlighted. Based on the similarities between the *C. albicans* and *L. monocytogenes* invasion proteins, we studied the role of clathrin in the internalization of *C. albicans*. Using live-cell imaging and indirect immunofluorescence of epithelial cells infected with *C. albicans*, we observed that host E-cadherin, clathrin, dynamin and cortactin accumulated at sites of *C. albicans* internalization. Similarly, in endothelial cells, host N-cadherin, clathrin and cortactin accumulated at sites of fungal endocytosis. Furthermore, clathrin, dynamin or cortactin depletion strongly inhibited *C. albicans* internalization by epithelial cells. Finally, beads coated with Als3 were internalized in a clathrin-dependent manner. These data

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indicate that *C. albicans*, like *L. monocytogenes*, hijacks the clathrin-dependent endocytic machinery to invade host cells.

INTRODUCTION

Candida albicans is a dimorphic fungus that causes superficial oral or vaginal infections as well as life threatening disseminated candidiasis. The capacity of *C. albicans* to change from yeast to hyphae is an important virulence factor of this organism (Lo *et al.*, 1997; Saville *et al.*, 2003; Park *et al.*, 2005). *In vitro*, *C. albicans* hyphae are endocytosed by oral epithelial and endothelial cells (Rotrosen *et al.*, 1985; Drago *et al.*, 2000) and this endocytosis is a prerequisite for the organism to damage host cells (Filler *et al.*, 1995; Park *et al.*, 2005). Endocytosis is induced by the interaction of the *C. albicans* Als3 adhesin with E-cadherin on epithelial cells and N-cadherin on endothelial cells. This interaction stimulates rearrangement of host cell actin, which is necessary for *C. albicans* invasion (Rotrosen *et al.*, 1985; Filler *et al.*, 1995; Phan *et al.*, 2005; Phan *et al.*, 2007). The first steps of this process are driven by the host cell since killed hyphae are internalized as efficiently as live hyphae even though they do not cause host cell damage (Filler *et al.*, 1995; Phan *et al.*, 2000; Park *et al.*, 2005). However, the cellular machinery that mediates actin rearrangement and internalization of *C. albicans* hyphae remains unknown.

The Gram-positive bacterial pathogen *Listeria monocytogenes* is also able to invade host mammalian cells by interacting with E-cadherin (Mengaud *et al.*, 1996a; Lecuit *et al.*, 1999. Bonazzi *et al* 2009). The bacterial effector that recognizes E-cadherin is InlA (internalin), which belongs to the internalin family (Gaillard *et al.*, 1991; Mengaud *et al.*, 1996b; Mengaud *et al.*, 1996a; Lecuit *et al.*, 1997; Bierne *et al.*, 2007). Interestingly, the cleft motif found at the amino-terminus of *C. albicans* Als3 is structurally similar to bacterial leucine-rich repeat domains from internalins (Schubert *et al.*, 2002; Phan *et al.*, 2007). Both InlA and Als3 induce pathogen uptake mediated by rearrangement of the actin cytoskeleton (Hamon *et al.*, 2006). *L. monocytogenes* internalization has been extensively studied and the signal transduction pathway induced by the InlA/E-cadherin interaction is relatively well understood (Hamon *et al.*, 2006; Bonazzi *et al* 2009). This pathway, which mimics the one that induces the formation of adherens junctions, involves the recruitment of β and α catenins, ARHGAP10, Arf6 and vezatin, as well as activation of the Arp2/3 complex (Lecuit *et al.*, 2000; Sousa *et al.*, 2004; Sousa *et al.*, 2005; Hamon *et al.*, 2006; Sousa *et al.*, 2007). Recently, we have shown that *L. monocytogenes* invasion requires proteins normally involved in several endocytic pathways including clathrin, caveolin and dynamin (Veiga and Cossart, 2005; Veiga *et al.*, 2007; Bonazzi *et al.*, 2008). These results suggest that the endocytic machineries together with the actin cytoskeleton provide the force that actively engulfs invasive bacteria (Veiga and Cossart, 2005; Veiga *et al.*, 2007). Clathrin-mediated endocytosis is linked to actin rearrangement by mechanisms that, although not fully understood, are known to require the presence of cortactin, an activator of the Arp2/3 complex (Veiga and Cossart, 2005; Sousa *et al.*, 2007; Veiga *et al.*, 2007).

Clathrin and its associated proteins are in fact involved in the internalization of a large variety of pathogens, including viruses and bacteria (Conner and Schmid, 2003; Marsh and

Helenius, 2006; Veiga *et al.*, 2007). However, it was previously unknown whether similar pathways mediated the internalization of fungi, which are considerably larger than viruses and bacteria. Based on the similarities of the *C. albicans* and *L. monocytogenes* models, we investigated whether clathrin plays a role in the internalization of this fungal pathogen.

RESULTS AND DISCUSSION

Clathrin is recruited at the *C. albicans* entry site

To address whether *C. albicans* exploits a clathrin-dependent mechanism to invade non-phagocytic host cells, we analyzed the interactions of a wild-type clinical isolate of *C. albicans*, strain SC5314, with both epithelial and endothelial cells. The human epithelial HEK293 and JEG-3 cell lines were used because they are known to endocytose *L. monocytogenes* by an E-cadherin-dependent mechanism (Sousa *et al.*, 2005). Primary human umbilical-vein endothelial cells were used because they endocytose *C. albicans* via a N-cadherin-dependent mechanism (Phan *et al.*, 2005). Both cell types were infected with *C. albicans* blastospores for 90 up to 120 min in RPMI 1640 medium at 37°C to allow the *C. albicans* cells to germinate and form hyphae, which were subsequently engulfed by the host cells in an actin-dependent manner (Filler *et al.*, 1995; Park *et al.*, 2005; Phan *et al.*, 2007). Results presented in Figures 1A, 1B and Supplementary Figure S1 showed that fluorescently tagged clathrin, as well as endogenous clathrin, co-localized with endocytosed *C. albicans* hyphae in both epithelial and endothelial cells.

The time course of recruitment of clathrin around *C. albicans* hyphae was also followed by live-cell imaging. HeLa cells expressing td-Tomato-LCa (clathrin light chain) and E-cadherin-GFP were infected for 30 min at 37°C and hyphal endocytosis was monitored during the subsequent 20 min. As shown in Figure 1C and Supplementary Movie 1, clathrin was recruited to the sites at which *C. albicans* hyphae entered the epithelial cells. Similar to what has been observed during bacterial internalization, the amount of clathrin that was recruited around the internalizing hyphae fluctuated over time. This pattern contrasts with the continuous, progressive accumulation of clathrin that is observed during typical clathrin-mediated endocytosis (Ehrlich *et al.*, 2004; Boucrot *et al.*, 2006; Massol *et al.*, 2006).

The time course of E-cadherin recruitment during internalization was also determined. We found that E-cadherin co-localized with clathrin around hyphae that were internalized by epithelial cells (Figure 1C; Supplementary Movie S1). Interestingly, the amount of E-cadherin that accumulated around the hyphae also fluctuated over time, in parallel with clathrin.

Dynamin and cortactin are also recruited during *C. albicans* internalization

The GTPase dynamin is required for clathrin coated pits to pinch off the plasma membrane and form endocytic vesicles (Hinshaw, 2000; Kirchhausen, 2000; Conner and Schmid, 2003; Orth and McNiven, 2003; Ehrlich *et al.*, 2004; Merrifield *et al.*, 2005; Macia *et al.*, 2006). Furthermore, dynamin is absolutely required for bacterial internalization (Veiga and Cossart, 2005; Veiga *et al.*, 2007). During classical clathrin-dependent endocytosis and during bacterial internalization, dynamin is localized not only at the neck of vesicles pinching off

the membrane but also around the entire vesicle (Stang *et al.*, 2004; Veiga and Cossart, 2005; Veiga *et al.*, 2007).

The endocytosis of *C. albicans* is unusual because hyphae are too large to be completely internalized by a single host cell. Frequently, the blastospore attached to the hypha remains extracellular. Therefore, the endocytic tube containing the penetrating hypha cannot pinch off the membrane as observed upon endocytosis of bacteria or smaller particles. Because dynamin is required for scission of endocytic vesicles, we investigated the distribution of dynamin during the endocytosis of *C. albicans* in GFP-dynamin expressing epithelial cells. After 120 min of infection, dynamin accumulated together with actin around *C. albicans* hyphae (Fig. 2 and Supp. Fig. S2).

In addition to inducing vesicle scission, dynamin is involved in other cellular functions, which may also contribute to the endocytosis of microbial pathogens. For example, dynamin forms a complex with cortactin, which in turn activates the Arp2/3 complex (Krueger *et al.*, 2003). The Arp2/3 complex mediates the polymerization of actin, which is required for endocytosis to occur (Engqvist-Goldstein and Drubin, 2003; Kaksonen *et al.*, 2006). Consistent with this model, cortactin has been recently found to play a major role in E-cadherin dependent entry of *L. monocytogenes* (Sousa *et al.*, 2007; Veiga *et al.*, 2007). In addition, dynamin and the lymphocyte cortactin homolog Hs1 have been described to play a major role in the massive actin polymerization observed during the immune synapse formation (Billadeau *et al.*, 2007). As actin rearrangements are involved in the internalization of *C. albicans* by epithelial and endothelial cells (Rotrosen *et al.*, 1985; Filler *et al.*, 1995; Park *et al.*, 2005; Phan *et al.*, 2005; Phan *et al.*, 2007) we investigated the localization of cortactin during this process. As shown in Figure 3A and B, and Supplementary Figure S3A, cortactin accumulated with actin around *C. albicans* hyphae that were in the process of being internalized by either epithelial or endothelial cells. N-cadherin also accumulated with cortactin around organisms that were being endocytosed by endothelial cells (Figure 3C), suggesting that N-cadherin-mediated internalization of *C. albicans* occurs by the recruitment of cortactin. We also show as control a membrane protein (ICAM-I) not localizing with *C. albicans* during HEK293 infection (Supp. Fig. S3B).

Taken together, these results indicate that clathrin, dynamin and cortactin were recruited together with actin and either E-cadherin or N-cadherin at the *C. albicans* hyphae entry sites in host cells. These results suggest a clathrin-mediated-mechanism together with active remodeling of the actin cytoskeleton contributing to hyphal internalization.

Clathrin, dynamin, and cortactin are required for *C. albicans* hyphae internalization

To further examine the role of clathrin, dynamin and cortactin in the invasion of *C. albicans*, siRNA was used to knock-down (KD) these proteins in epithelial cells. Clathrin KD in endothelial cells was attempted but was unsuccessful (data not shown). We quantified the percentage of cell-associated *C. albicans* hyphae that were internalized by differential immunofluorescence labelling as described (Park *et al.*, 2005) (Fig. 4A and Supplementary Fig. S3 and Supp Movie S2). At least 200 cell-associated *C. albicans* cells were counted per condition in each experiment and a minimum of 3 independent experiments were performed with two different epithelial cell lines. As shown in Figure 4, depletion of clathrin, dynamin

or cortactin by siRNA significantly reduced the internalization of *C. albicans* (Fig. 4B). The depletion of each of these proteins was highly efficient and specific. KD of one protein did not influence the expression of any of the other proteins, and also had no effect on total actin or cadherin expression (Fig. 4C and Supp. Fig. S4A). As the observed decrease in hyphae internalization could be due to reduced levels of an Als3 receptor (i.e. E-cadherin) on the cell surface, we used flow cytometry to measure the E-cadherin surface expression in control and clathrin siRNA KD cells. As shown in Figure 5, clathrin KD cells expressed similar levels of E-cadherin on their surface as did control cells. Additionally we tested the role of AP-2 (the major clathrin adaptor at the plasma membrane). Depletion of AP-2 also significantly reduced hyphal internalization (Supp. Fig. S4B–C. These data demonstrate that clathrin, dynamin, and cortactin are required for host cell uptake of *C. albicans*.

To further study the recruitment of clathrin and its role in the *C. albicans* invasion via Als3 in the absence of any other fungal factor, we used latex beads that had been coated with the recombinant N-terminal region of Als3. As reported previously (Phan *et al*., 2007), these beads were internalized by both epithelial and endothelial cells. Moreover clathrin accumulated around the Als3-coated beads during internalization (Fig. 6A, and Supp. Fig. S5). Similar to what was observed during the internalization of hyphae, this accumulation of clathrin was not continuous and varied in amount around the beads as they entered the cells. In contrast, very few BSA-coated beads were internalized, and clathrin did not accumulate around them (data not shown). Similarly to the hypha internalization data, we observed a dramatic reduction in number of Als3-coated beads that were internalized by epithelial cells when the level of clathrin was reduced by siRNA (Fig. 6B). Therefore, clathrin plays a key role in Als3-mediated internalization. (Fig. 6B).

In conclusion, our results indicate that the internalization of *C. albicans* hyphae by host cells following Als3-cadherin interaction is mediated by the combined interactions of clathrin, dynamin and cortactin. These proteins in turn induce actin cytoskeleton remodeling, which results in endocytosis of the hyphae. Importantly, our results highlight the role of a clathrin-dependent mechanism in the entry of large pathogens. Similar to what has been observed during viral and bacterial infections, the clathrin-dependent machinery, in coordination with cytoskeleton rearrangements, seems to provide the necessary force to internalize the different pathogenic particles. Thus, clathrin appears to be a common host cell target for a variety of microbial pathogens, including fungi.

MATERIALS AND METHODS

Cells, fungi, and growth conditions

The human epithelial cell lines HeLa (ATCC number CCL-2), JEG-3 (ATCC number HTB-36), and HEK293 (ATCC number CRL-157) were grown as recommended by the ATCC. Endothelial cells were harvested from umbilical cord veins and maintained as previously described (Filler *et al.*, 1995). *C. albicans* SC5314 (Gillum *et al.*, 1984) was routinely grown at 30°C on minimal medium (0.67% yeast nitrogen base without amino acids, 0.4% glucose, pH 5.4). To induce hyphal formation, blastospores were incubated at 37°C in RPMI 1640 (Gibco) medium buffered with 50 mM Hepes pH 7.3.

Plasmids

The plasmid encoding dynamin2-GFP (isoform aa) was a gift from Prof. Mark A. McNiven (Cao *et al.*, 1998). Plasmids encoding tdTomato-LCa (Massol *et al.*, 2006) and GFP-LCa (Ehrlich *et al.*, 2004) were gifts from Prof. Tomás Kirchhausen. The plasmid encoding E-cadherin-GFP was a gift from Prof. W. James Nelson (Yamada *et al.*, 2005).

Antibodies and reagents

Alexa Fluor 488–, 546–, and 647–conjugated phalloidin, Alexa Fluor 488–, 546–, and 647–conjugated goat anti–rabbit and goat anti–mouse antibodies were purchased from Molecular Probes. Other antibodies used were: mouse monoclonal (mAb) anti-β-actin (AC15; Sigma), anti-clathrin heavy chain mAb (BD Pharmingen), rabbit polyclonal antibody (pAb) anti-dynamin2 (Calbiochem), anti-pan-cadherin pAb (Santa Cruz Biotechnologies), anti-cortactin mAb (4F11; Upstate), anti-cortactin (sc-11408, Santa Cruz Biotechnology), anti-*C. albicans* pAb (B65411R; Biodesign International), anti-tubulin mAb (Sigma Aldrich), or anti-N-cadherin mAb (clone 32, BD Biosciences), anti-CD324 (E-cadherin) Alexa Fluor 488-labeled Ab (324109, BioLegend), supernatant from the mouse myeloma P3-X63Ag8 (X63), anti-Als3 pAb (Phan *et al.*, 2007) and green fluorescent latex beads coated with either the recombinant N-terminal region of Als3 or BSA were produced as described previously (Phan et al., 2007). When needed, anti-Candida antibodies were conjugated with Alexa Fluor-647 before proceeding with the immunolabelling.

Internalization assays

Internalization of *C. albicans* hyphae and Als3-coated beads was quantified as previously described with some modifications (Park *et al.*, 2005). HEK293 or JEG-3 epithelial cells were infected with 10^5 blastospores of *C. albicans* SC5314 in RPMI 1640 for 120 min at 37°C. The samples were first incubated at 4°C with an anti-*C. albicans* pAb (1:50), washed with PBS fixed for 30 min with 3% paraformaldehyde (PFA) in PBS and incubated with Alexa Fluor 647-conjugated secondary antibodies. Cells were then permeabilized for 5 min with 0.5% Triton X-100 in PBS and incubated with anti-*C. albicans* pAb and with Alexa Fluor 488-conjugated secondary antibodies. Actin was stained using Alexa Fluor 546-conjugated phalloidin. *C. albicans* cells that stained with Alexa Fluor 647 and Alexa Fluor 488 were recorded as internalized while those staining with Alexa Fluor 647 only were counted as adherent, extracellular. Organisms that were partly engulfed were counted as internalized. To measure the internalization of Als3-coated latex beads, the epithelial cells were incubated with 3×10^5 beads ml^{-1} in RPMI 1640 for 45 min at 37°C. They were fixed in 3% PFA in PBS and then the non-internalized beads were detected with a polyclonal rabbit anti-Als3 antibody (Phan et al 2007) before permeabilization followed by an Alexa Fluor 647-conjugated secondary antibody.

Images were acquired with a fluorescence inverted microscope (Axiovert 135; Carl Zeiss MicroImaging, Inc.) equipped with a cooled charge-coupled device camera (MicroMax 5 MHz; Princetown Instruments) driven by the Metamorph Imaging System software (Universal Imaging Corp). For figure assembly, the images were treated using ImageJ 1.38x (http://rsb.info.nih.gov/ij/index.html).

Time-lapse wide-field microscopy

HeLa cells expressing E-cadherin-GFP and td-Tomato-LCa (clathrin light chain) were infected with 10^5 blastospores of *C. albicans* SC5314 in RPMI 1640 for 30 min at 37°C, then hyphal entry was monitored for 20 additional min. Images were acquired with a motorized inverted fluorescence microscope (Axiovert 200I, Carl Zeiss MicroImaging, Inc.) equipped with a temperature-controlled stage using 100x lenses (Carl Zeiss, Inc). Fluorescent illumination was driven by an ultrahigh-speed wavelength switcher Lambda DG4 (Sutter Instrument) equipped with a 175 W xenon arc lamp and excitation filters for GFP (Excitation=480 – Emission=525) and DsRed (Excitation= 565 – Emission=620) (Chroma Technology). Emission filters were selected using a high-speed Lambda 10 filter wheel (Sutter Instrument). Images were acquired with exposure times between 100 and 500 milliseconds with a cooled, digital, charge-coupled device camera (CoolSNAPHQ, Photometrics). All devices were controlled by the MetaMorph Imaging System software (Universal Imaging). Resulting images were treated using ImageJ 1.38x (http://rsb.info.nih.gov/ij/index.html).

Immunolabelling

Epithelial or endothelial cells were infected with 10^5 blastospores of *C. albicans* SC5314 suspended in RPMI 1640 medium. After 90 min or 120 min for endothelial and epithelial cells respectively, cells were rinsed and fixed in 3% PFA in PBS. Next, host cells were permeabilized for 5 min with 0.5% Triton X-100 in PBS and then incubated with an anti-*C. albicans* pAb (1:200) and with anti-clathrin, anti-cortactin and/or anti-N-cadherin antibodies. Alternatively, cells were transfected with tdTomato-LCa (fluorescent clathrin light chain), LCa-GFP or dynamin-GFP 24 h prior to infection. Actin was stained using Alexa Fluor-conjugated phalloidin. Fluorescent secondary antibodies (Alexa Fluor-conjugated) were used for differential detection of *C. albicans* and clathrin, or dynamin or cortactin.

Confocal microscopy

Confocal images of the epithelial cells were acquired using a laser scanning confocal microscope (Zeiss LSM510) using 100x lenses (Carl Zeiss, Inc.) under control of LSM (Carl Zeiss, Inc), or using a Leica TCS-SP5 confocal microscope (63x lenses) under the control of Leica LAS AF. The images were treated using ImageJ (1.38x; http://rsb.info.nih.gov/ij/index.html), Imaris (Bitplane scientific solutions), and LSM (Carl Zeiss, Inc). Confocal images of the endothelial cells were acquired using a Leica laser scanning confocal microscope (Leica Microsystems). Optical sections were collected along the z-axis and merged to produce the final image.

RNAi assays

Double stranded RNA against clathrin heavy chain (s 5′-GGC CCA GGU GGU AAU CAU Utt-3′, as 5′-AAU GAU UAC CAC CUG GGC Ctg-3′), dynamin II (s 5′ GGA GAU UGA AGC AGA GAC Ctt-3′, as 5′-GGU CUC UGC UUC AAU CUC Ctg-3′) and cortactin (s, 5′-GGG AGA AUG UCU UUC AAG ATT-3′; as, 5′-UCU UGA AAG ACA UUC UCC CTC-3′) were purchased from Ambion and Eurogentec. Control RNA (Silencer Negative

Control 1 siRNA) was purchased from Ambion and siCONTROL Non-Targeting siRNA Pool was purchased from Dharmacon. For clathrin, additional On-Target SMART pool against clathrin heavy chain (Dharmacon) was also purchased. Transfections were performed using Oligofectamine (Invitrogen) for HeLa and HEK293 cells and Dharmafect1 (Dharmacon) for JEG-3 and HEK293 cells. These reagents were used as recommended by the manufacturers. Cells were tested 72 hours after transfections.

Flow cytometry assays

E-cadherin expression was analyzed by direct immunofluorescence using an anti-human CD324 (E-cadherin) Alexa Fluor 488-labeled Ab (324109, BioLegend). Supernatant from the mouse myeloma P3-X63Ag8 (X63) was used as negative control followed by a secondary Alexa Fluor 488-labeled-Ab (Molecular Probes). All incubations were done at 4o C in PBS with 2% BSA, 1% FCS in the presence of 50 μg/ml of poly-human Ig for Fc receptor blocking. Fluorescence was analyzed in a FACSCalibur flow cytometer (BD-Biosciences); cells were gated by forward and side scatter based on wild-type cell size and shape, and mean fluorescence intensity of 1–2 × 10^4 labeled cells was calculated using CellquestPro software (BD-Biosciences).

Statistical analysis

Differences in the endocytosis of *C. albicans* by epithelial cells treated with the different siRNAs was determined by Student's T test. In other analyses, the difference between groups was analyzed by the Mann-Whitney U test. *P* values of 0.05 or less were considered significant. Unless otherwise stated, all experiments were performed atast five times, and the data are given as mean values ± SD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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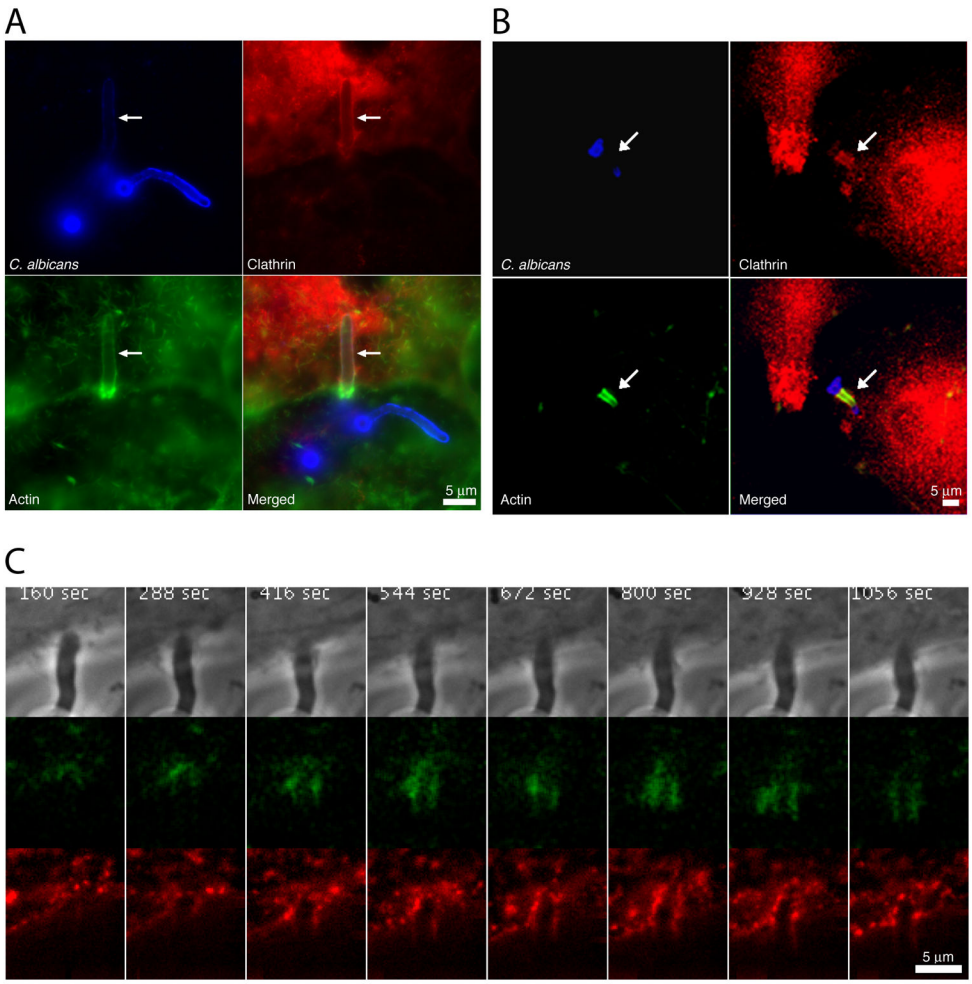

Figure 1.

Recruitment of clathrin during *C. albicans* internalization. (**A**) JEG-3 epithelial cells transformed with td-Tomato-LCa (clathrin light chain) were infected with the *C. albicans* SC5314 strain, shown in blue. Fluorescent clathrin is shown in red and actin detected with phalloidin in green. Arrows indicate internalized hyphae. Scale bar = 5µm. (**B**) Primary endothelial cells were infected with the *C. albicans* SC5314 strain, shown in blue. Clathrin, shown in red, was immunodetected using anti-clathrin heavy chain antibodies. Actin, shown in green, was detected with phalloidin. Arrows point internalized hyphae. Scale bar = 5µm. (**C**) Time series from HeLa cells transiently expressing tdTomato-LCa (red) and E-cadherin-GFP (green). Cells were infected with *C. albicans* SC5314 (phase). Recording started 32 min after infection. The figure shows one of every 16 acquisition frames from Supp. Movie 1. Scale bar = 5 µm.

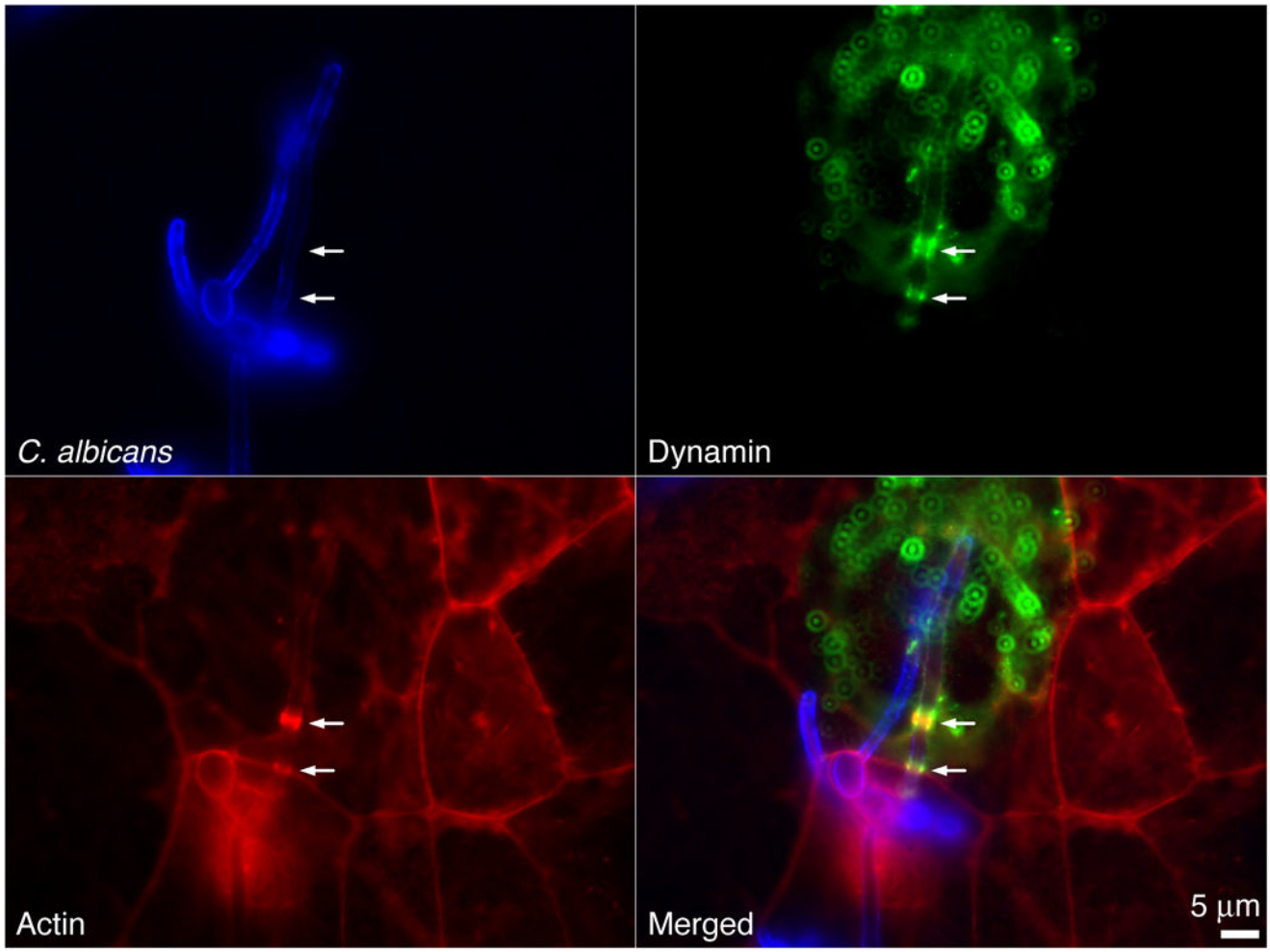

Figure 2.

Localization of dynamin during *C. albicans* internalization. JEG-3 cells transformed with dynamin-GFP were infected for 120 min with *C. albicans* SC5314, shown in blue. Dynamin is shown in green and actin in red. Arrows mark internalized hyphae and accumulation at the entry site of both dynamin and actin. Scale bar = 5μm.

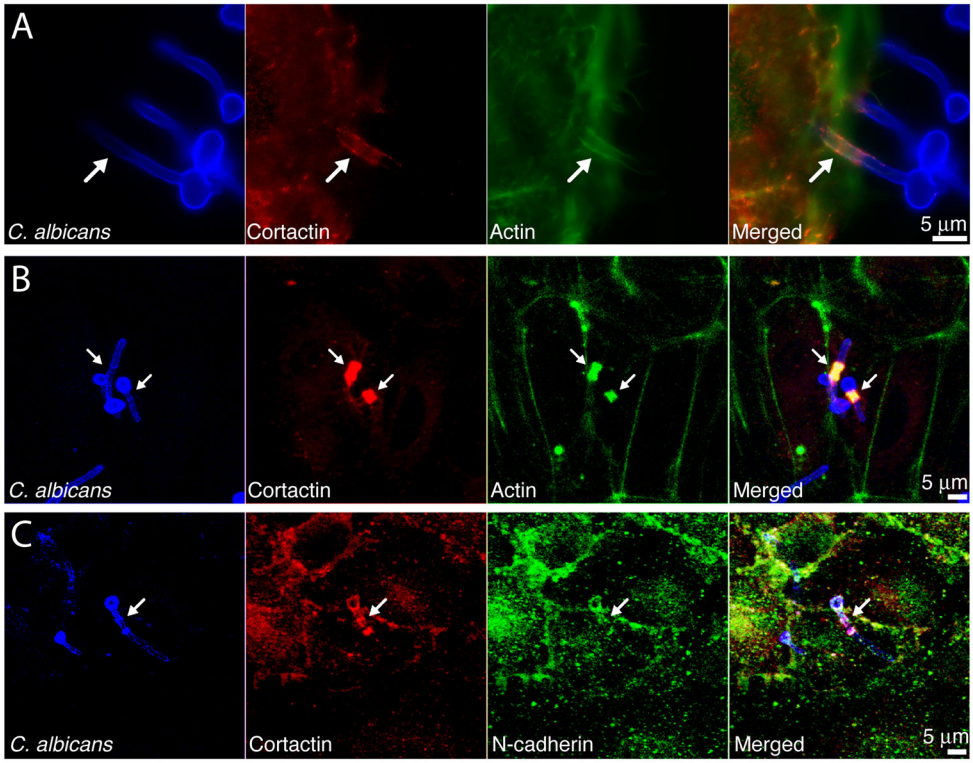

Figure 3.

Recruitment of cortactin to *C. albicans* entry site. (**A**) JEG-3 cells were infected with *C. albicans* SC5314, shown in blue. Cortactin, shown in red, was immunodetected using anti-cortactin antibodies. Actin, detected with phalloidin, is shown in green. Arrows mark internalized hyphae. Scale bar = 5µm. (**B–C**) Primary endothelial cells were infected with *C. albicans* SC5314, shown in blue. Cortactin was immunodetected with its respective antibody and is shown in red. Actin (B), detected with phalloidin, and N-cadherin (C), immunodetected with anti-N-cadherin antibody, are shown in green

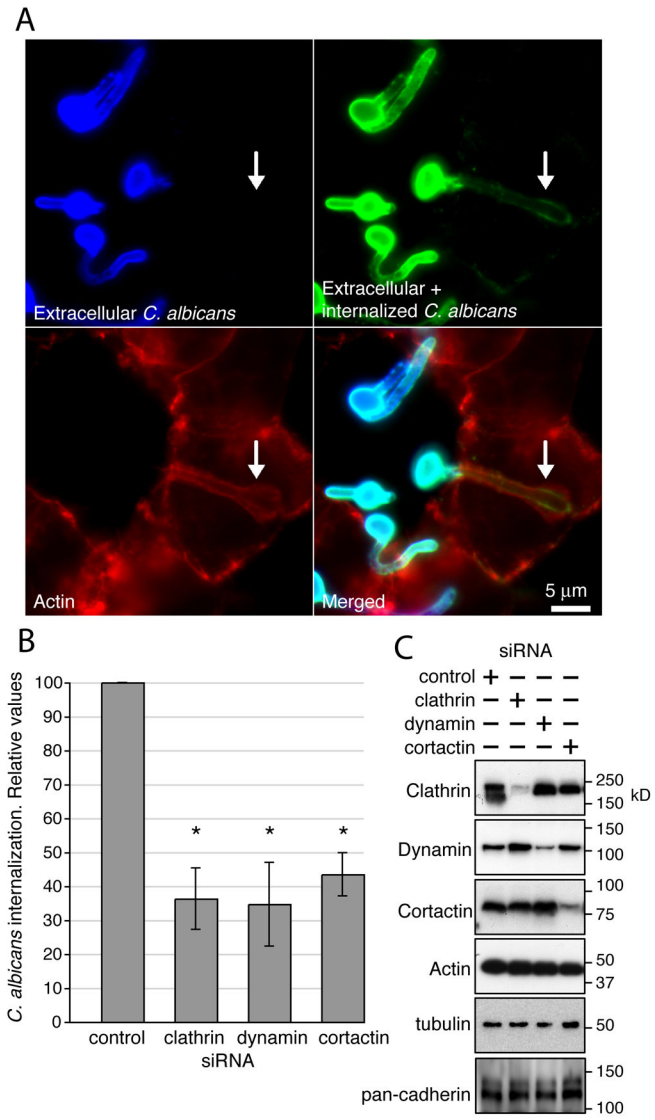

Figure 4.

Role of clathrin, dynamin, and cortactin in *C. albicans* internalization. (**A**) Extracellular *C. albicans*, immunodetected before permeabilization of epithelial cells are shown in blue. All *C. albicans* (extracellular and internalized) detected after permeablization are shown in green. Actin is shown in red. Arrows mark an internalized hypha. Scale bar = 5µm. (**B**) Clathrin, dynamin or cortactin depletion significantly decreased the endocytosis of *C. albicans*. HEK293 cells knocked-down (KD) by siRNA for the indicated proteins were infected with *C. albicans* SC5314. Fungal internalization ratio was measured by differential immunofluorescence labeling. Organisms that were at least partly internalized were counted as endocytosed. Data were normalized to control siRNA (RNA not targeting any cellular mRNA)-treated cells. Similar data were obtained in JEG-3 cells, but the siRNA treatment was more efficient using HEK293 cells (data not shown). Results are mean ± standard deviation of 3 independent experiments. * $P < 0.05$ compared to control. (**C**) Protein KD by siRNA was tested by Western-blot. + corresponds to samples treated with siRNA against the

indicated proteins. Actin and tubulin are shown as a loading control. KD of clathrin or dynamin did not affect the expression of cadherins.

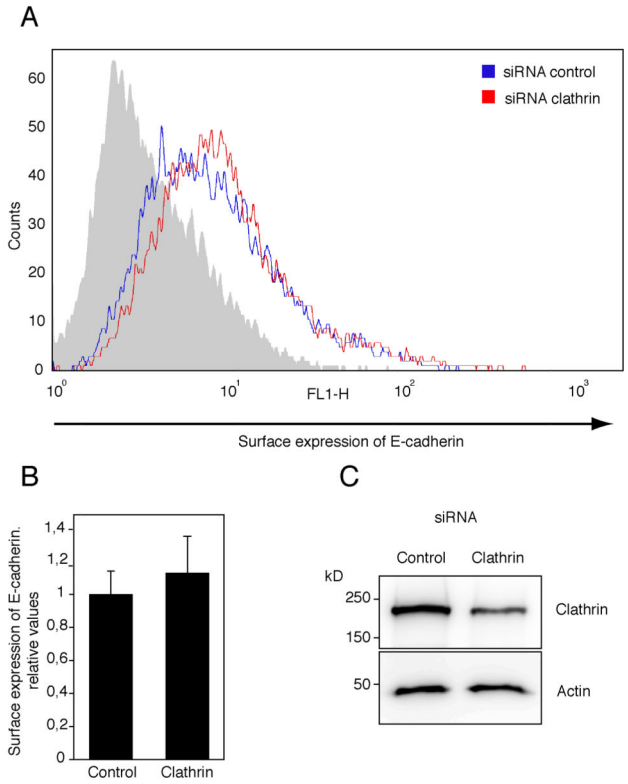

Figure 5.

Surface expression of E-cadherin. (**A**) A representative of 6 independent flow cytometry assays is shown. Surface exposed E-cadherin was immunodetected in non-permeabized epithelial cells using a Alexa Fluor 488-labelled anti E-cadherin antibody (see methods) in control (blue line) and clathrin knock-down (KD) (red line) cells. Grey histogram shows the fluorescent background observed using a non-specific antibody Alexa Fluor 488-labeled. (**B**) Combined results from flow cytometry assays (as in A). The number of events (i.e. cells) with fluorescence signal above 10^1 are represented normalized to the control. As shown, clathrin depletion did not affect E-cadherin expression on HEK293 cell surface. Results are mean ± standard deviation of 6 independent experiments. (**C**) Protein KD by siRNA in HEK293 cells was verified by immunoblotting with antibodies against clathrin and β-actin (as loading control). Clathrin siRNA KD data were normalized to control siRNA (RNA not targeting any cellular mRNA). Clathrin KD cells expressed similar levels of dynamin as did control cells (see Supp Fig. 4).

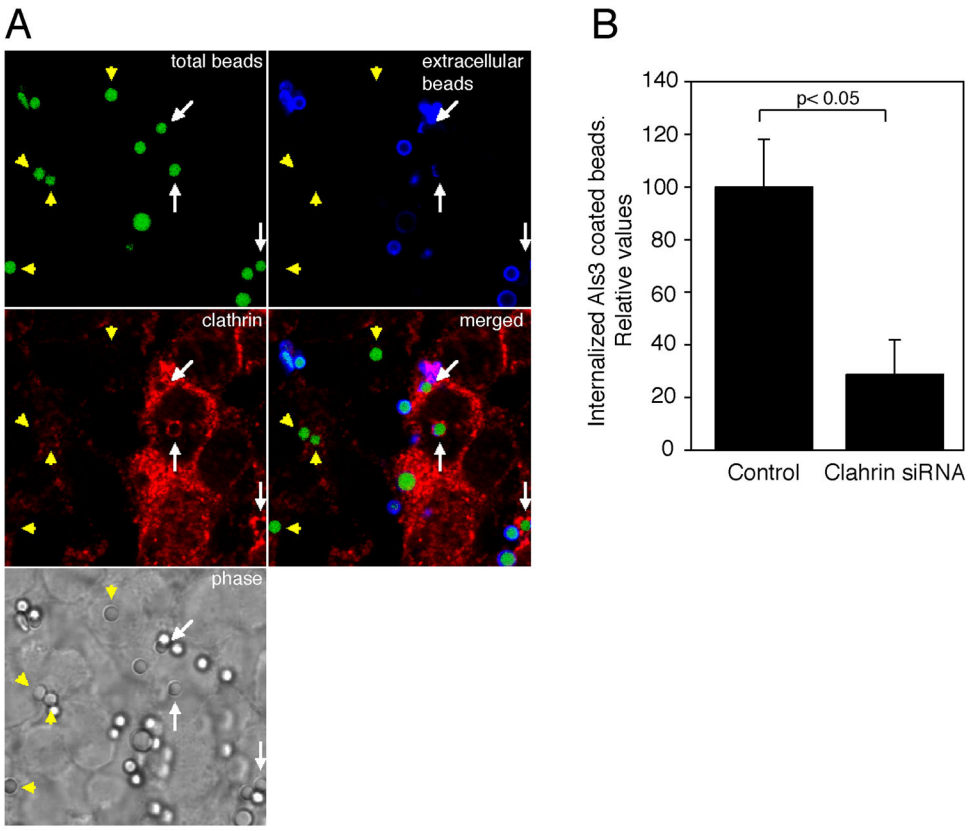

Figure 6.

Role of clathrin in the internalization of Als3-coated beads. (**A**) Als3-coated beads were incubated with HEK293 cells for 45 min. Extracellular beads were immunodetected before permeabilization (blue) using anti-Als3 antibodies. Total beads (intracellular and extracellular) are shown in green. In the merged image extracellular beads are shown in cyan and intracellular beads in green and are tagged with arrowheads. Arrows indicate beads that are in the process of being internalized (only a portion of the bead can be seen in blue). It is clear that clathrin surrounds internalizing beads. (**B**) Clathrin depletion significantly decreased the endocytosis of Als3-coated beads. HEK293 cells knocked-down (KD) by siRNA for clathrin or treated with control siRNA were incubated with Als3-coated beads. The number of internalized beads was measured by differential immunofluorescence labeling. We compared the ratio between internalized and non-internalized beads in control or clathrin KD cells. Data were normalized to control siRNA (RNA not targeting any cellular mRNA)-treated cells. Results are mean ± standard deviation of 4 independent experiments. * $P < 0.05$ compared to control. Very few beads coated with BSA were internalized and clathrin did not accumulate around them (data not shown)