CELL SURFACE PROTEINS OF *CRYPTOCOCCUS NEOFORMANS* MEDIATE ADHERENCE AND INTERNALISATION INTO MAMMALIAN CELLS

Choo KK[1], Chin VK[1], Chong PP[1], Ho SH[1], and Yong VC[1].
[1] School of Biosciences, Faculty of Health and Medical Sciences, Taylor's University Lakeside Campus, 47500 Subang Jaya, Selangor, Malaysia

Correspondence:
Associate Professor Dr Phelim Yong Voon Chen
School of Biosciences, Faculty of Medicine and Health Sciences,
Taylor's University, Selangor, Malaysia.
Email: PhelimVoonChen.Yong@taylors.edu.my

Abstract

Cryptococcus neoformans is an encapsulated fungal pathogen that causes severe disease primarily in immunocompromised patients. Adherence and internalisation of microbial pathogens into host cells often begin with engagement of microbes to the surface receptors of host. However, the mechanisms involved remain poorly understood. In this study, we investigated the association of cell surface determinants of *C. neoformans* with mammalian cells. Our results showed that treatment with trypsin, but not paraformaldehyde or heat killing, could reduce host-cryptococci interaction, suggesting the involvement of cell surface proteins (CSPs) of *C. neoformans* in the interaction. We extended our investigations to determine the roles of CSPs during cryptococci-host cells interaction by extracting and conjugating CSPs of *C. neoformans* to latex beads. Conjugation of CSPs with both encapsulated and acapsular *C. neoformans* increased the association of latex beads with mammalian alveolar epithelial cells, alveolar macrophages and monocyte-derived macrophages. Further examination on the actin organisation of the host cells implied the involvement of actin-dependent phagocytosis in the internalisation of *C. neoformans* in CSP-conjugated latex beads. We hypothesised that CSPs present on the cell wall of *C. neoformans* mediate the adherence and actin-dependent phagocytosis of cryptococci by mammalian cells. Our results warrant further studies on the exact role of CSPs in the pathogenesis of cryptococcosis.

Keywords: *Cryptococcus neoformans*, *Cell Surface Proteins, Latex Beads, Actin*

Introduction

Cryptococcus neoformans is an encapsulated, facultative, intracellular pathogen that causes devastating cryptococcosis in immunocompromised patients and is a potential emerging pathogen in the immunocompetent population (1). Additionally, high mortality rates of cryptococcal meningitis are observed despite attentive treatment regimens, owing to factors such as increasing drug resistance, drug toxicity, cost and availability (2-7). These reflect the need for a better understanding of the pathogenesis of cryptococosis and an improved therapeutic regimen to counter this infection.

Primary cryptococcosis usually begins with inhalation of airborne infectious particles of *C. neoformans* into the lungs. An initial pulmonary infection could lead to disseminated disease, while predilection of the pathogen to the brain results in highly fatal meningoencephalitis (1, 8). To establish hematogenous dissemination, *C. neoformans* is required to cross and invade epithelial and endothelial cells of hosts. It is believed that *C. neoformans* capsular network might attribute towards disease establishment (1). Nevertheless, the mechanisms involved remain poorly understood due to the complexity of the surface architecture of *C. neoformans*.

The capsule of *C. neoformans* which consists of galactoxylomannan (GalXM) and glucuronoxylomannan (GXM), is considered an essential virulence factor involved in its pathogenesis. It is well established that capsular, wild-type *C. neoformans* strains are more virulent than acapsular mutant strain in murine infection models (9). However, acapsular *C. neoformans* are also able to produce deleterious effects, especially in severely immunocompromised hosts (10), suggesting the importance of studying both acapsular and capsular strains. Additionally, the ability of C*. neoformans* to be internalised by immune cells such as macrophages and survive in the intracellular environment of circulating host cells, could promote fungal dissemination and further establish the infection in mammalian hosts (11).

Merkel, Cunningham (12) proposed that *C. neoformans* cell surface proteins (CSPs) could possibly be involved in the adherence process to host cells, whereby the authors demonstrated that trypsin inhibited the interaction of *C. neoformans* with epithelial cells (12). Intracellular pathogenic microbes are capable of expressing cell surface proteins to target host cell receptors that function as part of the endocytosis system for their own uptake, either through an actin-dependent or actin-independent mechanism (13). Therefore, a better understanding of the role of cryptococcal surface ligand(s) that mediate interactions with mammalian cells is warranted to consolidate our knowledge on the key mechanisms involved during *C. neoformans* infection.

Thus, this study was undertaken to investigate the role of *C. neoformans* cell surface determinants that mediate interaction with mammalian cells. The study first examined the factors affecting the association of *C. neoformans* to mammalian cells. A latex-bead conjugation assay was then introduced to study the involvement of cryptococcal CSPs in *C. neoformans* interactions with the host cells.

Materials and Methods

Fungal strains and culture conditions

C. neoformans H99 (ATCC 208821) and *Cap64* (ATCC 52816, acapsular mutant) strains were purchased from the American Type Culture Collection (ATCC, USA). To obtain stationary phase culture, a seeder culture was obtained by inoculating 10 ml of Sabouraud Dextrose Broth (SDB) (BD, USA) with colonies grown on a Sabouraud Dextrose Agar (SDA) (BD, USA), followed by incubation at 37°C, with continuous agitation at 240 rpm for 24 hours. The seeder culture was then adjusted to 10^5 yeasts/ml and further cultured for 48 hours.

Mammalian cell lines and growth conditions

A549 (CCL-185, human type II alveolar epithelial-like carcinoma) and MH-S (CRL-2019, murine alveolar macrophage) were purchased from the ATCC. THP-1 (TIB-202**,** Human acute monocytic leukemia cell line) was kindly donated by Dr. SFY Yong and WC Lee. Cell lines were routinely maintained in RPMI 1640 medium (Sigma-Aldrich, USA) supplemented with 10% foetal bovine serum (JRS, USA) and penicillin (100 U/mL)/streptomycin (100 U/mL)(Gibco, USA), in a 5% CO_2 humidified incubator at 37°C. Cells were incubated in the presence of 200 nM phorbol 12-myristate 13-acetate (PMA; EMD Biosciences, USA) for 48 hours to induce differentiation into adherent, macrophage-like cells.

Chemical, heat killing and trypsin treatment of C. neoformans Cap64

C. neoformans Cap64 were killed by incubation at 100°C for 30 minutes (heat-kill) or treatment with 3.75% Perfluoroalkoxy Alkanes (PFA) for 30 minutes at room temperature (chemical-kill). Meanwhile, trypsin treatment was performed by incubating the *Cap64* with 0.25% trypsin (Gibco, USA) for 1 hour at 37°C.

Association of C. neoformans Cap64 with MH-S alveolar macrophages

MH-S cells were seeded at a density of 5×10^5 cells/well in a 24-well microplate (Orange Scientific, Belgium) and cultured for 24 hours to reach confluenceny of 70%. *C. neoformans* were then adjusted to 5×10^7 cells, pelleted, and subjected to. 1) heat, 2) PFA or 3) trypsin treatment as previously described. Cryptococci were subsequently suspended in RPMI 1640 medium and co-incubated with the mammalian cells at 5×10^6 yeasts/well. After 4 hours of infection, non-adherent yeasts were removed with three PBS washes. The cells were fixed by using 3.75% PFA for 15 minutes prior to image acquisition on an inverted epifluorescence microscope under bright field (Nikon Eclipse TI-E, Nikon Instruments). Two random fields at 200× magnifications were selected, and the number of host cells and the associated cryptococci were manually counted. The association index (yeasts/100 host cells) from three independent experiments was calculated in relative to the association index of control cells without treatment.

Extraction of C. neoformans cell surface proteins (CSPs)

The method for the extraction of CSPs from *C. neoformans* was adapted from Pitarch et al. (2000) (14). Briefly, stationary phase yeast cells were collected by centrifugation and washed twice with PBS and twice with lysis buffer (10mM Tris-HCl, pH 7.4, 1mM phenylmethylsulfonyl fluoride (PMSF)). Cells were then suspended in three volumes of ice-cold lysis buffer and four volumes of 0.5 mm acid-washed glass beads. The cell suspension was subjected to 40 cycles of 2-minutes vortexing and 1 minute resting on ice. Cell breakage was verified via microscopy examination. The glass beads were removed from the yeast solution by centrifugation at 200× g for 1 minute. Disrupted cells were separated into cell wall fraction (pellet) and cytosolic fraction (supernatant) via centrifugation at 3000×g for 10 minutes. The cell wall fraction was washed three times with ice-cold water followed washes with 5% NaCl, 2% NaCl, 1% NaCl solutions and ice-cold water. The cell wall pellet was suspended in sodium dodecyl sulphate (SDS)/ dithiothreitol (DTT) extraction buffer (50mM Tris-HCl, pH8.0, 2% SDS, 10mM DTT) and incubated at 95°C for 10 minutes. The extracted cell surface protein fraction was quantified using the RC-DC protein assay as per manufacturer's instruction (Bio-Rad Laboratories, USA) and stored at -80°C prior to use.

TCA/Acetone precipitation

TCA/Acetone precipitation was carried out as previously described (15) with slight modifications. Briefly, 100 µl of CSPs was precipitated in acetone containing 10.0% (w/v) TCA/0.3% (w/v) DTT, at -20°C for 1 hour. The supernatant was removed after centrifuged at 12,000× g, for 15 minutes at 4°C and the pellet was rinsed twice with ice-cold acetone

containing 0.3% (w/v) DTT. The sample was pelleted and air-dried for 15 minutes at room temperature before suspended in 50 µl of PBS.

Conjugation of latex-beads with C. neoformans cell surface proteins (CSPs)

Absorption reaction was initiated by mixing 4 µL of stock suspension of latex-beads (Sigma-Aldrich, USA) with 250 µl of 50 mM MES buffer (pH 6.1) containing 25 µg, 50 µg and 100 µg of *C. neoformans* CSPs, followed by incubation overnight at 4°C, with rotating agitation. The absorption reaction was terminated by centrifugation at 12,000× g and followed by three washes with PBS. For control, latex beads were conjugated with 10 mg/ml Bovine Serum Albumin (BSA) (Sigma-Aldrich, USA).

Analysis of C. neoformans CSPs-conjugated latex-beads interaction with host mammalian cells

A549 (1×10^4), MH-S and THP-1 (5×10^4) cells were seeded into a 96-well microplate (Orange Scientific, Belgium) and grown for 24 hours to reach confluence. Subsequently, the cell culture medium was replaced with fresh medium containing CSP-conjugated latex beads at a ratio of 10 beads for each host cell. After 4 hours of co-incubation, cells were washed twice with PBS, fixed with 3.75% (v/v) PFA and examined under a microscope (Nikon Eclipse TI-E, Nikon Instruments).

Actin staining

Following interactions of latex beads with mammalian cells and fixation with PFA, cells were permeabilised with 0.1% Triton X-100 for 3 minutes. The slides were subsequently incubated with 12.5µg/ml FITC-Phalloidin (Sigma-Aldrich, USA) for 45 minutes. The epifluorescence images were obtained (488-nm excitation/440-nm excitation: 520-nm emission).

Statistical analysis

Statistics was performed using a Student's t-test (GraphPad Prism, GraphPad Software Inc., USA). $p<0.05$ was considered statistically significant.

Results

Factors affecting interaction of C. neoformans Cap64 with MH-S alveolar macrophage cells

As shown in Figure 1, PFA- and heat-killed *C. neoformans Cap64* retained the ability to associate with MH-S cells at similar efficacy as the live cryptococci. In contrast, treatment with trypsin, a proteinase, significantly reduced the association to 38.5% as compared to the control cells. This indicated that adherence and internalisation of *C. neoformans* into mammalian cells could be mediated by heat-resistant but trypsin-sensitive cell surface proteins (CSPs), which were present on both viable and non-viable cryptococcal cells.

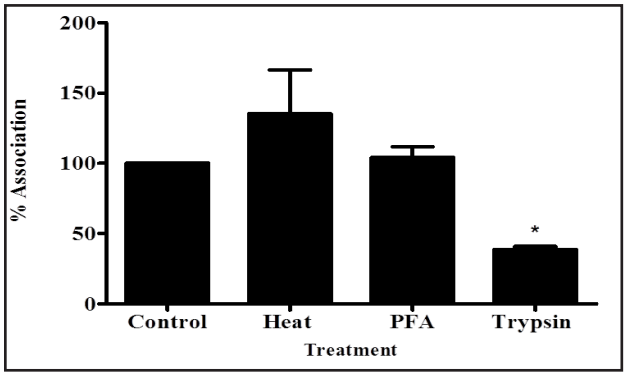

Figure 1: Influence of heat/chemical-killing and trypsin treatment towards the association of *C. neoformans Cap64* to MH-S alveolar macrophages. *Cap64* were subjected to heat-kill/PFA fixation/trypsin treatments, and subsequently co-incubated with MH-S cells for 4 hours. Association of *C. neoformans* with MH-S cells was scored and presented as means ± SEM of percentage association as compared to controls (without treatment) for 4 determinations. *p value < 0.05.

Conjugation of C. neoformans cell surface proteins (CSPs) on latex beads

As shown in Figure 2, all *C. neoformans* H99 CSP-conjugated latex beads adhered to both A549 and MH-S cells in which some were internalised into MH-S cells. The unconjugated beads bound minimally to the host cell and none of the beads was located within the mammalian cells (Figure 2).

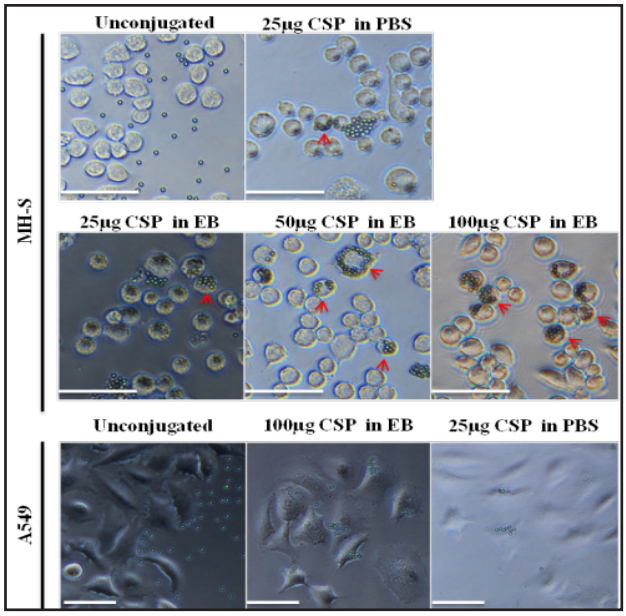

Figure 2: Association of latex beads conjugated with CSPs of *C. neoformans* H99 with mammalian cells. Control beads (unconjugated) and *C. neoformans* CSP-conjugated latex beads were co-incubated with MH-S murine alveolar macrophages and A549 alveolar epithelial cells. EB: extraction buffer. Red arrows: internalised latex beads. Bar=50 µm.

It was observed that latex beads conjugated with both CSPs in the original SDS/DTT extraction buffer (EB) or with CSPs precipitated through TCA/acetone and subsequently solubilised in PBS, could associate with host cells (Figure 2).

Interaction of C. neoformans CSP-conjugated latex beads with mammalian host cells

The association between *C. neoformans* H99 and *Cap64* CSP-conjugated latex beads with A549, MH-S and THP-1 cells, respectively, were examined and compared. Similar association efficacy was observed between mammalian cells and latex beads conjugated with CSPs of H99 and *Cap64*, respectively (Figure 3). In A549 alveolar epithelial cells, the latex beads adhered to the surface of the cells, however internalisation events were hardly observed. Meanwhile, in MH-S and THP-1 phagocytic cell lines, adherence and internalisation of latex beads within the cell cytoplasm were observed. In contrast, unconjugated- and BSA-conjugated latex beads (controls) were rarely associated with A549, MH-S or THP-1 cells, indicating the involvement of CSPs of *C. neoformans* in mediating the association of the yeasts with the host cells (Figure 3).

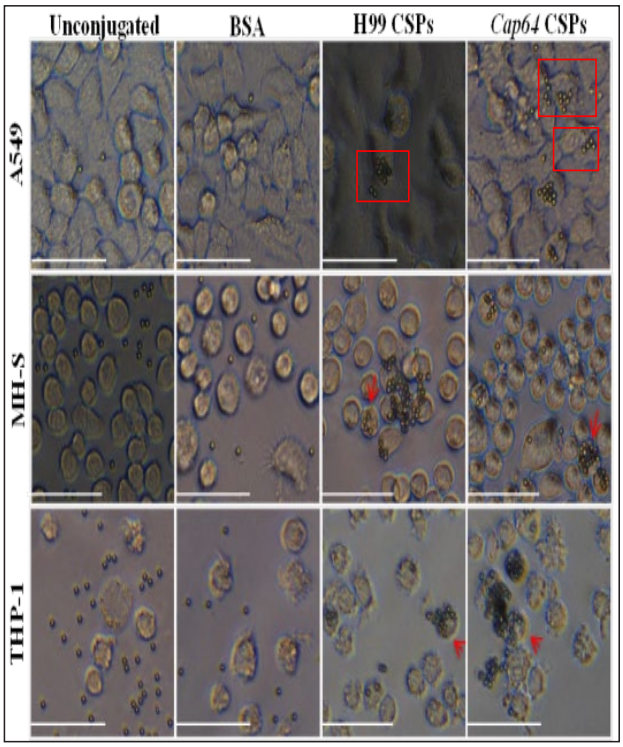

Figure 3: Adherence and internalisation of *C. neoformans* H99 and *Cap64* CSP-conjugated latex beads to A549, MH-S and THP-1 cells. Unconjugated latex beads were used as control to assess the ability of the latex beads to adhere to the mammalian cells. The mammalian cells were incubated with *C. neoformans* CSPs-conjugated latex beads for 4 hours and the associations were observed after removal of unbound latex beads. BSA serves as a non-relevant protein control. Red arrows: internalised latex beads. Red boxes: adherence of latex beads to the surface of A549 cells with internalisation events were hardly observed. Bar=50 µm.

C. neoformans CSPs mediate host cell actin cytoskeleton remodelling

For a better understanding of the mode of interaction between cryptococcal CSPs and host cells, the effects of co-incubation of *C. neoformans* CSP-conjugated latex beads on the actin organisation of A549, MH-S and THP-1 cells, respectively, were examined. Most of the associated latex beads did not cause major alterations in the actin organisation of A549, MH-S, and THP-1 cells. However, formation of phagocytic cups and actin rings were observed at some of the host-pathogen interfaces as indicated in Figure 4. This suggests that CSPs of *C. neoformans* might be involved in mediating internalisation of the pathogen into host cells through actin-dependent phagocytosis.

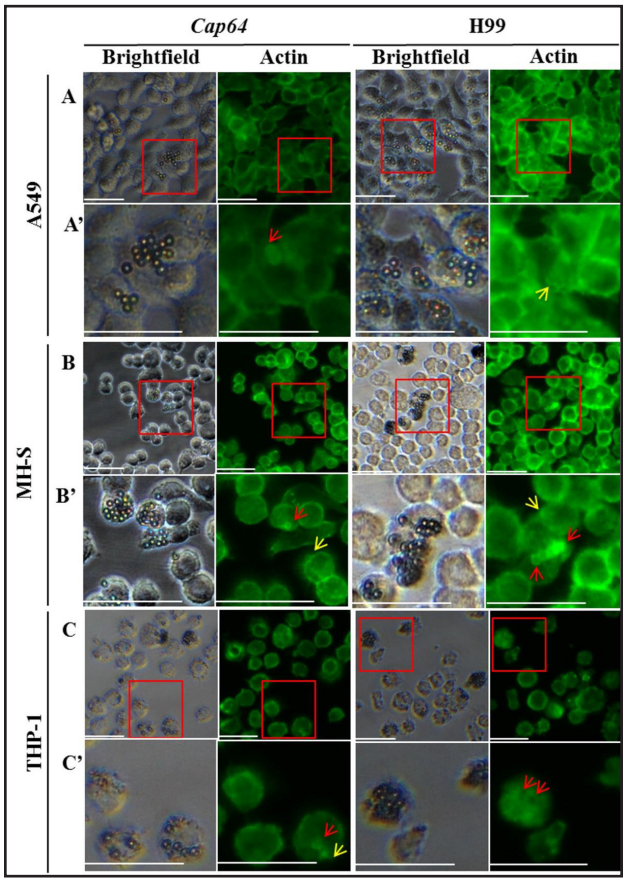

Figure 4: Effects of *C. neoformans* CSP-conjugated latex beads on actin organisation of A549, MH-S and THP-1 cells. The mammalian cells were incubated with *C. neoformans* CSP-conjugated latex beads for 4 hours and the host cells were stained with FITC-phalloidin to visualise the F-actin. A'- C' represent enlarged images of red-boxed area of images A- C. Yellow arrows: phagocytic cup; red arrows: actin ring. Bar=50 µm.

Discussion

C. neoformans has emerged as a prominent opportunistic fungal pathogen in the past two decades and is responsible for high mortality and morbidity among immunocompromised population. Adherence to host

tissue is the primary step in invasive fungal disease. Adherence and internalisation of *C. neoformans* into host cells could be mediated by interactions of the host cell membrane receptors with complementary polysaccharide/ protein complexes on the cryptococcal cell wall or capsule. The adherence of *C. neoformans* to host tissues or extracellular matrix components has been demonstrated, yet little is known about the properties of the molecules involved in this mechanism (16, 17).

The present study optimised a protocol to extract and release the surface bound proteins of *C. neoformans* and improvised an assay to study the interaction of this fraction of cell surface proteins with mammalian host cells. The study showed that upon treatment with trypsin, a protease that affects the integrity of polypeptides, the association of acapsular *C. neoformans Cap64* with MH-S cells was inhibited. This inhibition could be due to the fact that yeast cell wall ligand(s) comprise of protein moieties which play a role in mediating adherence to the host cell. Furthermore, the study demonstrated that PFA and heat killing did not inhibit the association of *C. neoformans* to MH-S cells, suggesting that heat-stable proteins could be present on the cell wall of viable and non-viable Cryptococci, and mediated the interactions. This finding is in agreement with a report by Merkel & Cunningham (1992), where trypsin treatment, but not heat or formalin-killing on cryptococci inhibited cryptococcal-epithelial cell association (12). In fact, trypsin was also shown to inhibit adherence of cell wall mannoproteins/glycoproteins of *Candida albicans* and *Sporothrix schenckii* to host cell receptors (18, 19). Taken together, the association of cryptococci to alveolar macrophages is likely mediated by a trypsin-sensitive, but heat and PFA-resistant determinant on the surface of *C. neoformans.*

It was shown that latex beads conjugated with CSPs from both acapsular and encapsulated *C. neoformans* mediated similar efficacy in association with the mammalian host cells. The ability of latex beads to conjugate with CSPs suspended in PBS after TCA/acetone precipitation and to associate with mammalian cells confirmed the role of surface protein moieties of *C. neoformans* in mediating interaction with host cells, as the process was expected to remove lipid and polysaccharide (20). In addition, non-conjugated latex beads and latex-beads that were coated with BSA did not adhere to the mammalian cells. Overall, we hypothesise that certain protein(s) in this fraction, which are likely to be present on the cell wall, are capable of mediating adherence and internalisation of cryptococci into the host cell.

When comparing the adherence and internalisation of the three separate cell lines used in this study, CSPs were shown to be able to associate with and become internalised by both the MH-S and THP-1 phagocytic cell lines, whereas the human alveolar epithelial cell lineage A549 did not display any internalisation. This is in contrast to a previous report that *C. neoformans* was able to attach to and be internalised into A549 cells via a glucuronoxylomannan (GXM)-mediated pathway. The adherence is shown to require capsule GXM, which binds to CD14 on epithelial cells which results in the internalisation of *C. neoformans* and epithelial cell lysis (21). The fact that we did not observe internalisation even for unconjugated CSPs suggests that the discrepancy in the results was not due to the latex bead conjugation in our study.

In the present study, it was found that SDS/DTT-solubilised CSPs were able to adhere to mammalian cells, coupled with earlier findings that heat and PFA treatment did not affect cryptococci association with alveolar macrophages. This indicates that the native conformation of CSPs is not essential in host-pathogen interactions. In fact, a previous study that used similar cryptococcal surface proteins extraction method as the present study, has identified plasminogen-binding proteins from the cryptococcal CSPs (17). Although uncommon, recognition of ligands in their denatured form is possible. For instance, mammalian cell integrins bind synthetic peptides containing the RGD (Arg-Gly-Asp) sequence (22) and recognise RGD motifs on pathogen surface (23, 24). A similarly prepared detergent extract from the *Histoplasma capsulatum* cell wall was successfully applied in a Far Western blot assay in identifying Heat Shock Protein 60 as a ligand of macrophage complement receptor 3 (CR3) (25). Using the same approach, another report identified proteins from SDS-released cell wall extracts of *Paracocccidiodes brasiliensis* that bind host cell extracellular matrix proteins (26). Thus, identifying host cell receptor(s) that binds the CSPs of *C. neoformans* will facilitate identification of the cryptococcal ligands from the CSP fraction.

Endocytosis pathways could be subdivided into actin-independent, clathrin- and caveolae-mediated endocytosis, and actin-dependent macropinocytosis and phagocytosis (13, 27). It was demonstrated in the current study that CSP-conjugated latex beads induced actin remodelling at the host-pathogen interface, with actin-rich phagocytic cups and rings forming around the CSP-conjugated latex beads. Assuming that the interactions of the host cells with CSP-conjugated latex beads mirror *C. neoformans* host-pathogen interactions, the result suggests that phagocytosis of *C. neoformans* is conceivably mediated by an actin-dependent process. This notion concurs with previous studies that demonstrated that *C. neoformans* were taken up by macrophages and microvascular endothelial cells through an actin-dependent process (28, 29). Additionally, Johnston & May (2010) also demonstrated that actin is repeatedly polymerised and depolymerised on phagosomes containing cryptococci (30). They suggested a potential model for the actin polymerisation and depolymerisation, or "actin flashing" in which permeabilisation of the cryptococcal phagosome induces a signal that results in localised WASp-Arp2/3 activation and subsequent actin polymerisation around the phagosome membrane that transiently inhibited cryptococcal expulsion. However, this hypothesis requires further confirmation and experiments to characterise CSPs of *C. neoformans*.

Conclusion

In conclusion, the findings from the current study demonstrated that CSPs of *C. neoformans* participates in the associations of *C. neoformans* with mammalian cells, conceivably through engagement of mammalian cell surface receptor(s) and promote actin-dependent phagocytosis. The CSPs are presumed to be localised on the cell wall of viable and non-viable cryptococci, and are sensitive to trypsin treatment. The CSPs may contain fungal adhesins pertaining to the lung colonisation, thus a future study will need to be conducted to elucidate these probable ligand candidates for the cryptococcal-host cell interaction.

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Competing interests

The authors declare that they have no competing interests.

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