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Cryptococcus neoformans phospholipase B1 activates host cell Rac1 for traversal across the blood-brain barrier

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SUMMARY

Cryptococcus neoformans (*C. neoformans*) penetration into the central nervous system (CNS) requires traversal of the blood-brain barrier that is composed of a single layer of human brain microvascular endothelial cells (HBMEC), but the underlying mechanisms of *C. neoformans* traversal remain incompletely understood. *C. neoformans* transcytosis of HBMEC monolayer involves rearrangements of the host cell actin cytoskeleton and small GTP-binding Rho family proteins such as Rac1 are shown to regulate host cell actin cytoskeleton. We, therefore, examined whether *C. neoformans* traversal of the blood-brain barrier involves host Rac1. While the levels of activated Rac1 (GTP-Rac1) in HBMEC increased significantly upon incubation with *C. neoformans* strains, pharmacological inhibition and down-modulation of Rac1 significantly decreased *C. neoformans* transcytosis of HBMEC monolayer. Also, Rac1 inhibition was efficient in preventing *C. neoformans* penetration into the brain. In addition, *C. neoformans* phospholipase B1 (Plb1) was shown to contribute to activating host cell Rac1, and STAT3 was observed to associate with GTP-Rac1 in HBMEC that were incubated with *C. neoformans* strain but not with its *Δplb1* mutant. These findings demonstrate for the first time that *C. neoformans* Plb1 aids fungal traversal across the blood-brain barrier by activating host cell Rac1 and its association with STAT3, and suggest that pharmacological intervention of host-microbial interaction contributing to traversal of the blood-brain barrier may prevent *C. neoformans* penetration into the brain.

Introduction

C. neoformans is encapsulated yeast responsible for life-threatening CNS infections primarily in immunocompromised patients, such as those infected with HIV-1 (Chin *et al.,* 2004, Chuck and Sande 1989, Gordon *et al.,* 2000, Hakim *et al.,* 2000, Perfect and Casadevall, 2002). A 2001 case study in Zambia showed that fatality rates of *C. neoformans* meningoencephalitis are as high as 100% (despite the fact that 56% of the patients were initially treated with antifungal therapy) (Mwaba *et al.,* 2001). *C. neoformans*

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meningoencephalitis was one of the leading causes of death in HIV-1-infected adults in Uganda (French *et al.,* 2002).

Several lines of evidence from experimental mouse models of *C. neoformans* meningoencephalitis as well as human cases indicate that *C. neoformans* invasion into the brain follows fungemia with cerebral capillaries serving as portals of entry (Chang *et al.,* 2004, Charlier *et al*., 2005, Chretien *et al*., 2002, Lee *et al.,* 1996, Olszewski *et al*., 2004, Shi *et al.,* 2010). However, the mechanisms involved in *C. neoformans* traversal of the blood-brain barrier, the essential step required for the development of meningoencephalitis remain incompletely understood.

To better understand *C. neoformans* traversal of the blood-brain barrier, we developed an *in vitro* model of the blood-brain barrier with human brain microvascular endothelial cells (HBMEC). Upon cultivation on collagen-coated Transwell inserts, HBMEC exhibit morphological and functional properties of tight junction formation and polarized monolayer (Chang *et al.,* 2004, Kim *et al.,* 2004, Ruffer *et al.,* 2004, Stins *et al.,* 1997), a unique property of the brain capillary endothelial monolayer. *C. neoformans* penetration into the brain has been shown to involve the transcytosis mechanism as well as the Trojan-horse mechanism (Dromer and Levtiz, 2011), and cryptococal interaction with the HBMEC monolayer is likely to provide the information on the transcytosis mechanism. *C. neoformans* penetration into the brain was investigated in the mouse model of experimental hematogenous meningoencephalitis, which is likely to represent the blood-brain barrier penetration using the transcellular and/or Trojan-horse mechanisms (Chang *et al.,* 2004, Charlier *et al*., 2005, Chretien *et al*., 2002, Cox *et al.,* 2001, Dromer and Levitz, 2011, Kim 2008, Olszewski *et al*., 2004, Shi *et al.,* 2010).

Using the above-mentioned in vitro and in vivo models, we showed that *C. neoformans* strains exhibit the ability to traverse the blood-brain barrier and penetrate into the brain (Chang *et al.,* 2004). The traversal of *C. neoformans* across the blood-brain barrier was shown to involve host cell actin cytoskeleton rearrangements, as demonstrated by transmission and scanning electron microscopy (Chang *et al.,* 2004). Internalization of HBMEC monolayer by *C. neoformans* was associated with microvilli-like protrusions at the entry site on the surface of HBMEC and there was no change in the integrity of HBMEC monolayer following transcytosis of *C. neoformans*, as assessed by monitoring of transendothelial electrical resistance (TEER) (Chang *et al*., 2004). In addition, internalized *C. neoformans* was found to be located within membrane-bound vacuoles of HBMEC, and *C. neoformans* transmigrates HBMEC monolayer through an enclosed vacuole without intracellular multiplication (Chang *et al.,* 2004). No free yeast is found in the cytoplasm of HBMEC and also between adjacent HBMEC (Chang *et al.,* 2004). These findings suggest the involvement of host cell actin cytoskeleton rearrangements in *C. neoformans* internalization and traversal of the blood-brain barrier, but the underlying mechanisms remain incompletely understood (Chang *et al.,* 2004, Kim 2008).

Small GTP-binding Rho family proteins such as Rac1 have been shown to regulate host cell actin cytoskeleton functions (Hall 1998) and pathogenic microbes have been shown to exploit such Rho GTPases for their entry into host cells (Galan and Zhou 2000, Kim 2008, Maruvada and Kim 2012, Nhieu and Sansonetti 1999, Shin and Kim 2006). In the present study, we examined the role of host cell Rac1 in *C. neoformans* transcytosis of HBMEC monolayer and penetration into the brain, and investigated the mechanisms associated with Rac1-mediated transcytosis of *C. neoformans* across HBMEC monolayer.

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RESULTS

1. *C. neoformans* activates Rac1 in HBMEC

We have shown that *C. neoformans* traversal of HBMEC monolayer involves host cell actin cytoskeleton rearrangements (Chang *et al.,* 2004). Host cell Rac1 is an important intracellular molecule that takes part in actin cytoskeleton rearrangement, but its role in *C. neoformans* transcytosis of HBMEC monolayer has not been studied. Hence, we examined whether Rac1 activation occurs in HBMEC in response to *C. neoformans*, initially using strain B3501A (serotype D).

Briefly, HBMEC were incubated with strain B3501A for 30 and 60 min, lysed, and the lysates subjected to a pull-down assay using p21-activated kinase (PAK-1) GST beads (see *Materials and Methods*). Since the protein-binding domain (PBD) of PAK1 captures activated Rac1 (Rac1-GTP), the beads were examined for GTP-Rac1 using Western Blot assay with specific Rac1 antibody (Shin and Kim 2006).

Rac1 activation was detected at 30 min of incubation, which increased at 60 min of incubation (Fig 1A). GTP-Rac1 levels were shown to increase in a time-dependent manner as estimated by densitometric analysis of individual bands and compared them to zero time point. The total amount of Rac1 in the lysates of HBMEC incubated with *C. neoformans* remained unchanged in the samples.

To examine the specificity of Rac1 activation, the activation of Rac1 was studied in the presence of NSC23766 (a specific inhibitor of Rac1) before adding the B3501A cells. The HBMEC were treated with various concentrations of NSC23766, incubated with B3501A for 30 min and then examined for GTP-Rac1. Analysis of Rac1 activation showed that while a linear decrease occurred at 50 μM and 100 μM of NSC23766, activation was completely suppressed at 200 μM (Fig 1B).

Microscopic examination of HBMEC, approximately 18 hours after treatment with up to 200 μM NSC23766, revealed no marked changes in cellular morphology (HBMEC viability was also unaffected, as assessed by live/dead stain, Molecular Probes, Grand Island, NY). Additionally, NSC23766 at 200 μM did not affect the growth of *C. neoformans* at 37°C during a 9 hr experimental period, as determined by CFUs. We, therefore, used NSC23766 at a concentration of 100 μM for further experiments.

2. Host cell Rac1 contributes to *C. neoformans* transcytosis of the HBMEC monolayer and penetration into the brain

Since *C. neoformans* activates Rac1 in HBMEC, the role of Rac1 in *C. neoformans* transcytosis across the blood-brain barrier was examined using the well-established *in vitro* model of HBMEC. Briefly, HBMEC treated with NSC23766 or vehicle control (PBS) were incubated with yeast cells of B3501A for determination of transcytosis. As shown in Fig 2A, a significant decrease was observed in transcytosis of B-3501A across the NSC23766-treated HBMEC compared to PBS-treated HBMEC (where transcytosis frequency of strain B3501A ranged between 1.4 % and 8.9 % of total inoculum), suggesting that pharmacological inhibition of Rac1 activation decreased the *C. neoformans* transcytosis of HBMEC monolayer. It is important to note that HBMEC integrity before and after transcytosis experiments remained unchanged, as assessed by TEER measurements.

To further confirm the results of *C. neoformans* transcytosis derived from Rac1 inhibitor-treated HBMEC, the host cells were transfected with adenovirus constructs bearing the dominant-negative construct (N17Rac1) or vector alone. Cells were monitored by fluorescent microscopy for GFP expression of the adenovirus constructs. *C. neoformans*

B-3501A showed significant decrease in transcytosis of HBMEC that were transfected with the dominant-negative Rac1 construct as compared to those that were transfected with vector alone (Fig. 2B). As noted above, HBMEC monolayers were found to maintain their integrity throughout the experiments with no significant changes in TEER values.

Next, the role of Rac1 in *C. neoformans* penetration into the brain was examined in an *in vivo* model. Briefly, mice were treated with the Rac1 inhibitor (NSC27366) or vehicle (PBS) and then inoculated with B3501A cells intravenously (see *Materials and Methods*). CFUs of *C. neoformans* were significantly reduced in the brains of mice treated with the inhibitor as compared to the control group that received PBS (Fig 2C). In contrast, the CFUs recovered from non-brain organs such as spleens, kidneys and lungs did not significantly differ between the recipients of the inhibitor or PBS. These *in vitro* and *in vivo* findings demonstrate that host Rac1 is likely to be involved in the *C. neoformans* transcytosis of the blood-brain barrier and penetration into the brain.

3. Host Rac1 is involved in HBMEC transcytosis by *C. neoformans* serotype A strain H99

We next examined whether host Rac1 also plays a role in HBMEC transcytosis by *C. neoformans* serotype A strain H99 using the method described for serotype D strain. Serotype A strains are most prevalent in clinical as well as environmental isolates of *C. neoformans* and account for the majority of cryptococcosis cases in AIDS patients (Casadevall and Perfect, 1998).

To examine whether Rac1 activation occurs in HBMEC in response to strain H99, HBMEC treated with NSC23766 or PBS were incubated with H99 for various time periods. Lysates were examined for levels of GTP-Rac1 as well as total Rac1. While GTP-Rac1 levels increased in PBS-treated HBMEC over the time periods, the activation was significantly decreased in the inhibitor-treated cells, demonstrating that the strain H99 activates Rac1 of HBMEC in a time-dependent manner (Fig 3A), similar to that of strain B3501A (Fig 1A).

Next, the contribution of Rac1 to transcytosis of strain H99 across the blood-brain barrier was examined using NSC23766. As shown in Fig 3B, the transcytosis of strain H99 was significantly decreased in the inhibitor-treated HBMEC compared to the PBS-treated HBMEC (where transcytosis frequency of strain H99 ranged between 1.1 % and 5.5 % of total inoculum). Taken together, these findings demonstrate that Rac1 activation is involved in *C. neoformans* transcytosis of HBMEC monolayer by both serotypes A and D strains.

4. Host Rac1 activation and contribution to *C. neoformans* transcytosis of HBMEC monolayer occurs independent of fungal *RAC1*

C. neoformans has the *RAC1* gene encoding a Rac1 protein that is involved in fungal cytoskeletal architecture (Vallim *et al.,* 2005, Nichols *et al.,* 2007, Shen *et al.,* 2012). Hence, to examine whether cryptococcal Rac1 plays a role in transcytosis across HBMEC, *Δrac1* and its parent strain H99 were compared for their ability to transcytose the HBMEC monolayer. Both the *Δrac1* mutant and the parent strain exhibited no significant differences in transcytosis of HBMEC monolayer (Fig 4A).

To determine whether Rac1 of *Cryptococcus* was involved in the activation of host Rac1, HBMEC were incubated with either H99 or its *rac1* mutant and examined for the levels of GTP-Rac1. Both the parent strain as well as its *Δrac1* mutant were equally capable of activating host Rac1 (Fig 4B), suggesting that cryptococcal Rac1 might not be involved in host Rac1 activation as well as host Rac1-mediated cryptococcal transcytosis across HBMEC monolayer.

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To further negate the role of cryptococcal Rac1 in host Rac1-mediated transcytosis across HBMEC, both strain H99 and its *Δrac1* mutant were incubated with HBMEC that were treated either with NSC23766 or PBS. As shown with the parent strain H99, the *Δrac1* mutant showed significantly decreased transcytosis in NSC23766-treated HBMEC as compared to PBS-treated cells, suggesting that the Rac1 inhibitor prevents the HBMEC transcytosis of the *Δrac1* mutant in a manner similar to that of the parent strain (Fig 4C). These findings suggest that host Rac1 activation and host Rac1-mediated transcytosis of *C. neoformans* in HBMEC occur independently of the yeast Rac1.

5. Cryptococcal *PLB1* contributes to fungal transcytosis across the blood-brain barrier by activating host Rac1

Previous studies have shown that cryptococcal *PLB1* contributes to penetration of yeast cells into the brain (Cox *et al.,* 2001), as shown by the demonstration that the cryptococcal *Δplb1* mutant was significantly defective in penetration into the brain following either intravenous or intratracheal administrations of *C. neoformans*. We, therefore, examined whether cryptococcal *PLB1* contributes to fungal transcytosis across the HBMEC monolayer.

Briefly, HBMEC monolayers were incubated either with strain H99, *Δplb1* mutant or the mutant reconstituted with *PLB1*. While transcytosis of the *Δplb1* mutant across the HBMEC monolayer was significantly defective, the defect was restored to the level of the parent strain by reconstitution with a wild type *PLB1* gene (Fig 5A). These findings support the notion that cryptococcal Plb1 contributes to the fungal transcytosis of the blood-brain barrier.

Since Plb1 was shown to affect cryptococcal transcytosis across the HBMEC monolayer, the contribution of the molecule was examined in the activation of host cell Rac1. Briefly, HBMEC were incubated with either the wild type H99, its *Δplb1* mutant or its reconstituted *PLB1* strain for various time periods and the cell lysates examined for GTP-Rac1.

While HBMEC incubated with either H99 or the *PLB1* reconstituted strain showed a time-dependent activation of Rac1, a significant decrease was observed in the levels of GTP-Rac1 in the HBMEC incubated with the Δ*plb1* mutant (Fig 5B). These findings suggest that cryptococcal *PLB1* is likely to play a role in *C. neoformans* transcytosis of HBMEC monolayer by activating host cell Rac1.

To further confirm the role of cryptococcal *PLB1* in penetration into the brain, the *Δplb1* mutant or the parent strain H99 was injected intravenously into mice and examined for cryptococcal penetration into the brain. Compared to H99 infected mice, significantly lower CFUs were recovered from the brains of mice infected with the *Δplb1* mutant (Fig 5C). However, the CFUs recovered from non-brain organs such as kidneys, spleens or lungs were similar between the recipients of H99 and its *Δplb1*. Taken together, these findings suggest that cryptococcal *PLB1* but not *RAC1* is involved in activating host Rac1 and also for transcytosis across HBMEC monolayer.

6. *C. neoformans* Plb1 is involved in STAT3 association with GTP-Rac1 in HBMEC

To understand a mechanism by which the Plb1 contributes to the host Rac1 activation and fungal transcytosis of HBMEC, we examined the levels of STAT3 associated with GTP-Rac1. STAT3 is a well-known transcriptional activator, but recent studies have reported its possible role in actin cytoskeletal rearrangements through its association with GTP-Rac1 and in aiding the pathogens' entry into host cells (Simon *et al.,* 2000, Maruvada and Kim 2012). Since the Plb1 was shown to play a role in activating host Rac1, the levels of STAT3 associated with GTP-Rac1 in HBMEC incubated with *C. neoformans* were examined.

Briefly, the HBMEC were incubated with either the wild type H99 or its *Δplb1* mutant for various time periods. Since the *Δrac1* mutant did not activate host Rac1, the strain was included as a positive control. PBD-GST beads that were used to bind GTP-Rac1 were washed and examined for GTP-Rac1, total Rac1 and GTP-Rac1 bound STAT3. Western blot analysis showed that while significant amounts of STAT3 were associated with GTP-Rac1 in HBMEC that were incubated with H99 and *Δrac1* mutant in a time-dependent manner, the amounts of bound STAT3 were significantly less in HBMEC incubated with the *Δplb1* mutant. However, the HBMEC lysates showed similar levels of total Rac1 (Fig 6A).

To further confirm that STAT3 association with GTP-Rac1 was indeed influenced by the cryptococcal Plb1, the samples of HBMEC incubated with *Δplb1* as well as the reconstituted *PLB1* strain along with the parent stain H99 (as shown in Fig 5B) were compared for levels of STAT3 associated with GTP-Rac1. As shown above, the amounts of STAT3 associated with GTP-Rac1 was significantly less in HBMEC incubated with the *Δplb1* as compared with those incubated with strain H99. Interestingly, the HBMEC that were incubated with the reconstituted *PLB1* showed restoration of STAT3 association with GTP-Rac1 to the level of the parent strain H99 (Fig 6B). These findings led us to hypothesize that Plb1 of Cryptococcus plays a role in the transcytosis of the blood-brain barrier through a mechanism involving host cell Rac1 and STAT3.

DISCUSSION

The traversal of *C. neoformans* across the blood-brain barrier involves host cell actin cytoskeleton rearrangements, as shown by our demonstration that the internalizing yeast was surrounded by microvilli-like projections of HBMEC membranes and *C. neoformans* transcytosis occurred without any change in the integrity of HBMEC monolayer (Chang *et al.,* 2004).

In the present study, we showed that host Rac1 is involved in *C. neoformans* traversal of the blood-brain barrier, as demonstrated by (a) host Rac1 activation occurring in response to *C. neoformans* in HBMEC, (b) prevention of *C. neoformans* transcytosis of HBMEC monolayer by pharmacological inhibition of Rac1 and transfection with dominant-negative construct of Rac1, as well as (c) decreased *C. neoformans* penetration into the brain by Rac1 inhibition. Pathogenic microbes have been shown to exploit host cell Rho GTPases for their internalization into host cells (Galan and Zhou 2000, Kim 2008, Nhieu and Sansonetti 1999, Shin and Kim 2006, Maruvada and Kim 2012), but this is the first demonstration that host Rac1 contributes to *C. neoformans* traversal of HBMEC monolayer and penetration into the brain. The microbe-host interactions involved in host Rac1 contribution to *C. neoformans* traversal of the HBMEC monolayer and penetration into the brain, however, remain unclear. Although the Rac1 inhibitor (NSC23766) was effective in significant reduction in *C. neoformans* penetration into the brain, the effect of Rac1 inhibitor on the fungus cannot be unequivocally ruled out, and additional studies are needed to clarify this issue. Also, it remains unclear why blockade of host Rac1 affects *C. neoformans* penetration into the brain, but not into non-brain organs such as spleen, kidney and lung.

While specific virulence factors have been shown to involve host molecules to aid in the entry of meningitis-causing pathogens into the CNS (Kim 2008), this issue has not been fully examined in *C. neoformans* traversal of the blood-brain barrier. The genomes of *C. neoformans* strains have been sequenced, which include strains B-3501A (serotype D) and H99 (serotype A). Serotype A strains are the most prevalent clinical isolates and account for the majority of cryptococcosis cases in AIDS patients, and serotype D strains are predominant in Europe (Casadevall and Perfect, 1998). Functional genomic approaches are

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likely to identify the cryptococal factors that are involved in Rac1 activation in HBMEC and Rac1-mediated transcytosis of the blood-brain barrier.

We initially hypothesized that cryptococcal Rac1 might be involved in host Rac1 activation and the fungal transcytosis across HBMEC monolayer, but our findings with the Δ*rac1* mutant did not support the contribution of cryptococcal Rac1 to host Rac1 activation and transcytosis of HBMEC monolayer. In contrast, cryptococcal Plb1 was shown to be involved in host Rac1 activation in HBMEC, as shown by the demonstration that the Δ*plb1* mutant was defective in Rac1 activation, and this defect was restored to the level of the parent strain in the reconstituted strain with the wild type *PLB1. C. neoformans* Plb1 is shown to be involved in penetration into the brain (Cox *et al.,* 2001), which was also documented in the present study. However, the mechanisms involved in the cryptococcal Plb1-mediated activation of host Rac1 are unclear. *C. neoformans* Plb1 has been shown to trigger capsule enlargement (Chrisman *et al.*, 2011), but the time to capsule enlargement is longer (at least overnight or 24 hr incubation) than the time used for transcytosis assay (9 hr). In addition, capsule enlargement is like to impede cryptococcal transcytosis, but our studies with the Δ*plb1* mutant and the reconstituted strain showed the seemingly contrary results, i.e., Plb1 facilitates *C. neoformans* transcytosis of HBMEC monolayer and penetration into the brain. Thus, capsule enlargement is less likely to be involved in the Plb1-mediated transcytosis of the blood-brain barrier by *C. neoformans.* Our pilot data suggests that host cytoplasmic phospholipase A2 is involved in *C. neoformans* transcytosis of the blood-brain barrier and penetration into the brain. Based on cytoplasmic phospholipase A2 catalytic subunit present in the C- terminal domain of Plb1, we hypothesize that Plb1 might generate specific lipid mediators such as phosphoinositols in HBMEC that could mediate the activation of Rac1. Studies are in progress to elucidate the contribution of cryptococcal Plb1 to host Rac1 activation.

Though STAT3 is a known transcriptional factor and translocated to the nucleus upon activation, our recent data demonstrates that its cytoplasmic functions include its association with GTP-Rac1, role in actin cytoskeletal rearrangements and contribution to pathogen's entry into host cells (Maruvada and Kim, 2012). In this study, Plb1 of *C. neoformans* is shown to activate host Rac1 and affect its association with STAT3, suggesting that GTP-Rac1-STAT3 may contribute to *C. neoformans* transcytosis of the blood-brain barrier, which is highlighted in Fig 7. We hypothesize that cryptococcal Plb1 probably acts on host membrane phospholipids to release lipid mediators that activate host Rac1, which in association with STAT3, regulates *C. neoformans* transcytosis of HBMEC monolayer and penetration into the brain. Studies are currently in progress to test this hypothesis.

Taken together, our findings demonstrate that inhibition of host cell signaling molecules involved in *C. neoformans* traversal of the blood-brain barrier, as shown here with Rac1 inhibition may provide a novel approach for prevention of medically challenging *C. neoformans* meningoencephalitis. Further determination and characterization of host cell signaling molecules involved in *C. neoformans* traversal of the blood-brain barrier are likely to identify additional targets for prevention of *C. neoformans* penetration into the brain.

MATERIALS AND METHODS

Reagents and vectors

NSC23766 (an inhibitor that specifically interferes with Rac1 interaction with upstream guanine nucleoside exchange factors) was prepared as described previously (Gao *et al.,* 2004). Adenoviruses, encoding a dominant-negative Rac1 construct (N17Rac1) bearing a GFP tag were amplified and used for transfection (Shin and Kim 2006). As a vector control, GFP expressing adenovirus from pShuttle-IRES-hrGFP-1 vector (Stratagene, La Jolla, CA)

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was used as described previously (Shin and Kim 2006). Antibodies to cPLA2, phospho-cPLA2, and Rac1 were obtained from Cell Signaling Technologies (Danvers, MA). GST beads were obtained from Pierce (Rockford, IL). PBD-PAK1 plasmid was a kind gift of Dr. Keith Burridge (North Carolina).

Yeast strains

C. neoformans strains H99 and B-3501A represent serotype A and D strains, respectively, and their genomes have been sequenced (Loftus *et al.,* 2005, Idurnum *et al.,* 2005). Deletion mutants of *rac1* and *plb1* derived from the H99 strain and their reconstituted strains were previously described (Cox *et al.,* 2001, Vallim *et al.,* 2005). Yeast cells were grown on YPD agar (1% yeast extracts, 2% peptone and 2% dextrose) at 37°C overnight, harvested, washed with PBS and resuspended in Hams-F12/M199 (1: 1, v/v), 5 % heat inactivated fetal bovine serum (FBS) (experimental medium) and 1 % fresh human serum. The number of yeast cells were determined by direct counting from a hemocytometer which was verified by colony forming units (CFUs) on YPD agar, as previously described (Chang *et al.,* 2004).

Mice

9~11 week- old female BALB/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All procedures and handling techniques were approved by The Johns Hopkins University Animal Care and Use Committee.

Characterization and culture of HBMEC

HBMEC were isolated and characterized as previously described (Stins *et al.,* 1997). HBMEC were positive for factor VIII-Rag, took up fluorescently labeled acetylated low-density lipoprotein and expressed γ-glutamyl transpeptidase, demonstrating brain endothelial cell characteristics. Cultures were maintained in RPMI-based medium, including 10 % FBS and 10 % NuSerum (BD Biosciences), at 37 °C in a humid atmosphere of 5 % CO_2.

Transcytosis of *C. neoformans* across the HBMEC monolayer

HBMEC were cultured on Transwell polycarbonate tissue-culture inserts with a pore diameter of 12 μm (Corning Costar) for 5 days, as described previously (Chang *et al.,* 2004). Confluency of the HBMEC monolayer was assessed by microscopy as well as by measurement of TEER. The HBMEC monolayers were washed with experimental medium and 10^7 *Cryptococcus* cells were added to the upper chamber. Transcytosis of *C. neoformans* across the HBMEC monolayers was assessed by counting the CFUs present in the samples removed from the lower chamber after 9 hrs of incubation 37° C as previously described (Chang *et al.,* 2004). Transcytosis frequency (%) was determined [(total CFUs recovered from the lower chamber/total number of cryptococcal cells added to the upper chamber) × 100]. Transcytosis frequency in transfected HBMEC was expressed as relative transcytosis (%) compared to transcytosis frequency in control.vector-transfected HBMEC.

Preparation of cell lysates and assays for Rac1 activation

For assessment of Rac1 activation in response to *C. neoformans*, HBMEC monolayers were incubated with *Cryptococcus* for various time periods and lysed in modified radioimmuno protection assay (RIPA) buffer containing inhibitors for proteases and phosphatases at 4°C, as previously described (Shin and Kim 2006). For adenovirus infection, 50% confluent HBMEC were infected with dialyzed adenoviral particles (at a ratio of viral particles to HBMEC of 100:1 or multiplication of infection of 100) and incubated for two more days to reach confluency before adding *C. neoformans* cells. The HBMEC lysates were sonicated, centrifuged and assayed for protein concentration.

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For Rac1 activation assay, 400 μg of protein was incubated with 30 μg of PBD-PAK-1 (which captures activated Rac1 or GTP-Rac1) bound to glutathione agarose beads. Beads were washed with the lysis buffer and subjected to SDS-PAGE and Western Blot analysis using a specific Rac1 antibody. Lysates were also examined for total Rac1.

Experimental hematogenous *C. neoformans* infection in mice

Mice were anesthetized by subcutaneous administration of pentobarbital sodium (50 mg/kg) and received NSC23766 (50 μg/mouse in 100μl PBS) via intraperitoneal administration 30 min before, and 3 hr and 6 hr after tail vein injection of B-3501A (1×10^5 cells in 100μl PBS). This dose of NSC23766 was shown to inhibit Rac1 activity in mice (Akbar H *et al* 2007). Control animals were given 100 μl PBS as vehicle control. At 24 hr after B-3501A injection, mouse chest was cut open, and blood from right ventricle was collected and cultured for determination of CFUs. The animals were, then perfused with a mammalian Ringer's solution by transcardiac perfusion through a 23-gauge needle inserted into the left ventricle of the heart under the perfusion pressure of about 100 mmHg, as previously described (Zhu *et al.,* 2010). The perfusate exited through a cut in the right atrium. At 30 min after perfusion of Ringer solution, mice were decapitated. The brains were removed, weighed, homogenized in 2 ml RPMI, and cultured on YPD agar plates to determine CFUs/gm. Kidneys, lungs and spleens were also removed, homogenized, and cultured for determinations of CFUs/gm.

Statistical Analysis

Data are expressed as mean ± SEM. Differences of *C. neoformans* transcytosis across HBMEC monolayer and *C. neoformans* penetration into mouse brains, kidneys, spleens, and lungs were determined by Student's t test. $p<0.05$ was considered significant.

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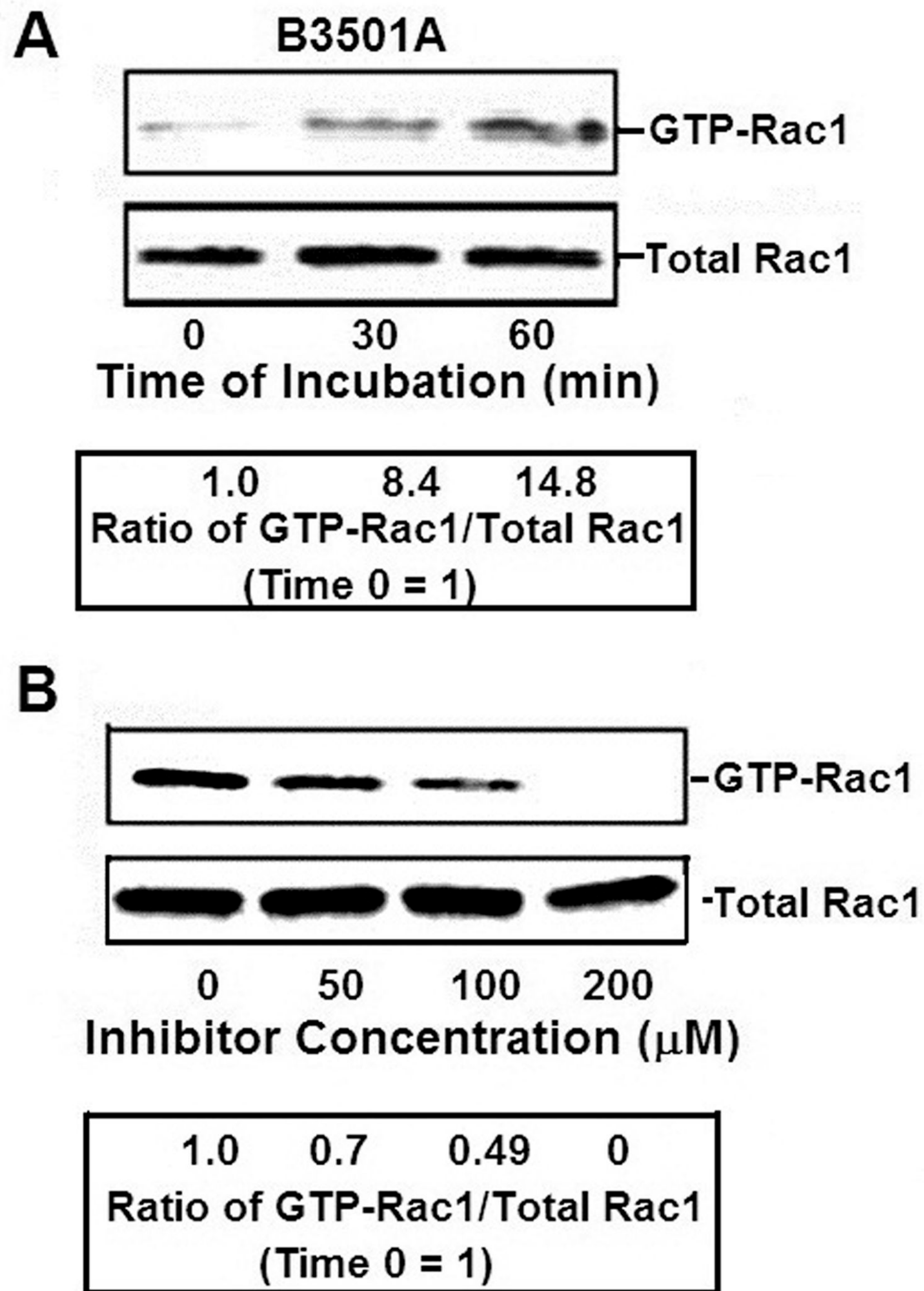

Figure 1. Rac1 activation of HBMEC by *C. neoformans*

A. To examine host cell activation of Rac1 by *C. neoformans*, HBMEC were incubated with *C. neoformans* serotype D strain B3501A for various time periods. Activated Rac1 (GTP-Rac1) were pulled down by incubation of HBMEC lysates with PBD-GST beads that bind GTP-Rac1 and beads examined for Rac1 in Western Blot assay. Total Rac1 in lysates was examined as a loading control. Band intensities was estimated densitometrically and then represented as a ratio of GTP-Rac1 to total Rac1 assuming Time 0 as 1.

B. The specificity of Rac1 activation was assessed by incubating HBMEC with various concentrations of NSC23766 (a specific inhibitor of Rac1) after which the host cells were incubated with strain B3501A for 30 min and then examined for GTP-Rac1.

Band intensities was estimated densitometrically and then represented as a ratio of GTP-Rac1 to total Rac1 assuming time 0 = 1.

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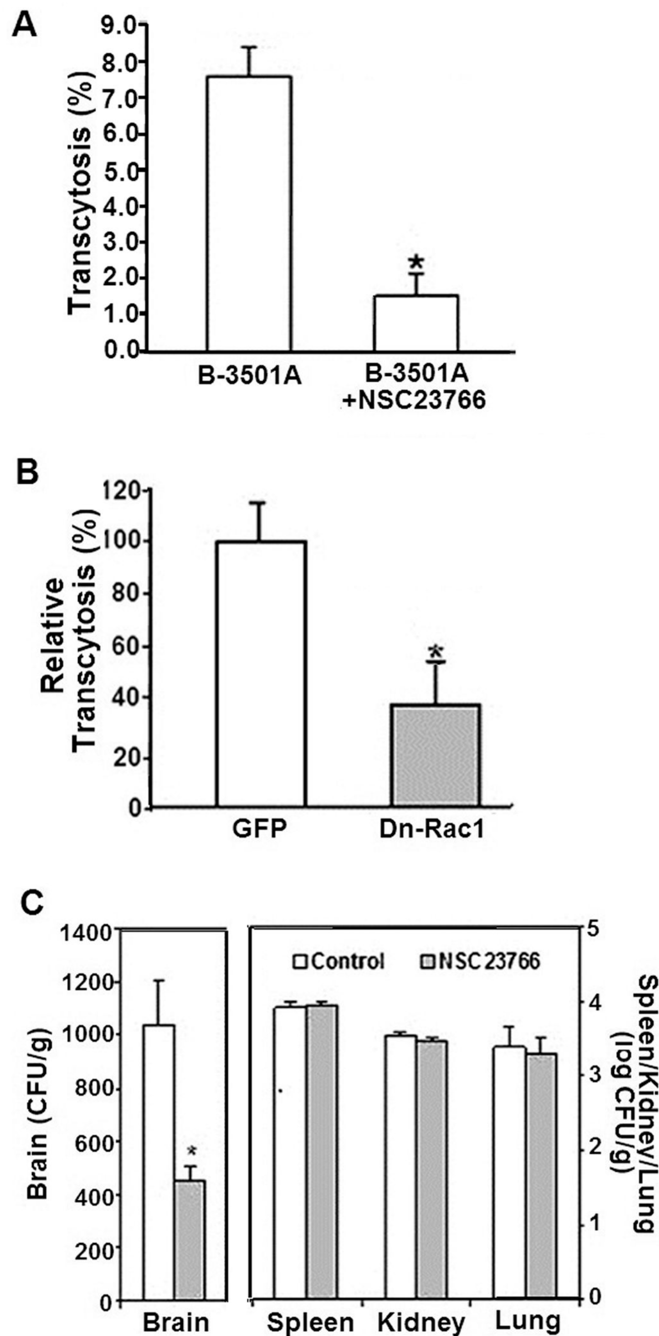

Figure 2. Role of Rac1 in *C. neoformans* transcytosis across the blood brain barrier

A. To determine the role of Rac1 in *C. neoformans* transcytosis, HBMEC monolayers grown on transwells were treated with 100 μM of NSC23766 or vehicle control and incubated with *C. neoformans* strain B3501A. After 9 hour incubation, transcytosed CFUs were determined as described in *Materials and Methods*. *$p<0.05$ compared to vehicle-treated HBMEC

B. HBMEC transfected with adenoviruses expressing dominant-negative Rac1 construct or vector control were examined for transcytosis of *C. neoformans* strain B3501A and then expressed as relative transcytosis (%) assuming transcytosed yeast numbers in control vector-transfected HBMEC as 100%. *$p<0.05$ compared to vector-transfected HBMEC

C. To analyze the role of Rac1 in *C. neoformans* penetration into the brain, mice that received NSC23766 or vehicle control were injected with B-3501A (1×10^5 cells in 100μl PBS) via the tail vein. 24 hours later, the brains of the mice were removed, homogenized and examined for CFUs by culturing on YPD agar plates. The yeast counts were expressed as CFUs/gm. Kidneys, lungs and spleens were also determined for yeast counts (expressed as CFUs/gm). *$p<0.05$ compared to PBS-treated animals.

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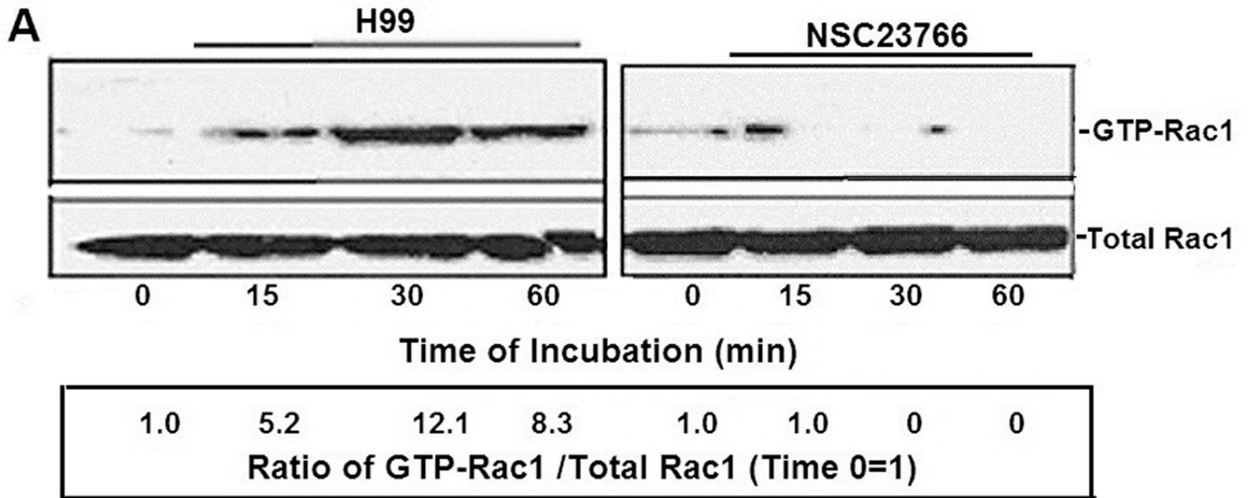

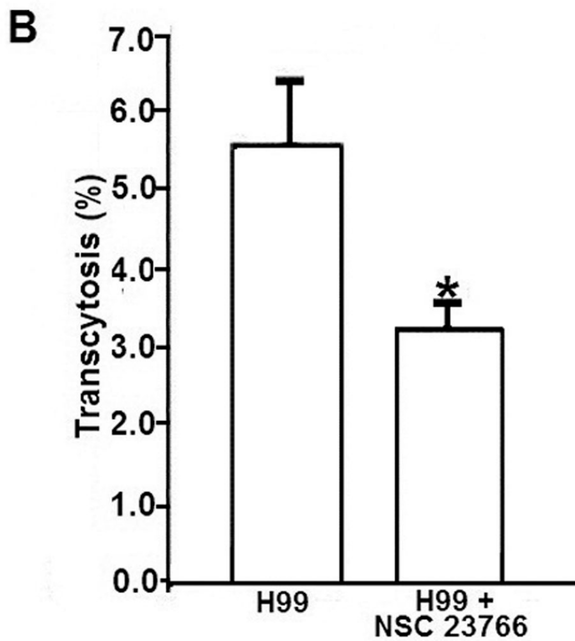

Figure 3. Role of Rac1 in *C. neoformans* serotype A strain H99 transcytosis of HBMEC monolayer

A. To examine Rac1 activation of host cells by strain H99, HBMEC treated with Rac1 inhibitor or vehicle control were incubated with strain H99 for various time periods, after which lysates were analyzed for pull-down assays for GTP-Rac1. Numbers represent the ratios of GTP-Rac1 to total Rac1 with control (Time point 0 assumed as 1).

B. The role of Rac1 in strain H99 transcytosis, HBMEC monolayers grown on transwells were treated with NSC23766 or vehicle and incubated with strain H99. After 9 hour incubation, transcytosed CFUs were determined as described in *Materials and Methods* *$p<0.05$ compared to vehicle treated HBMEC.

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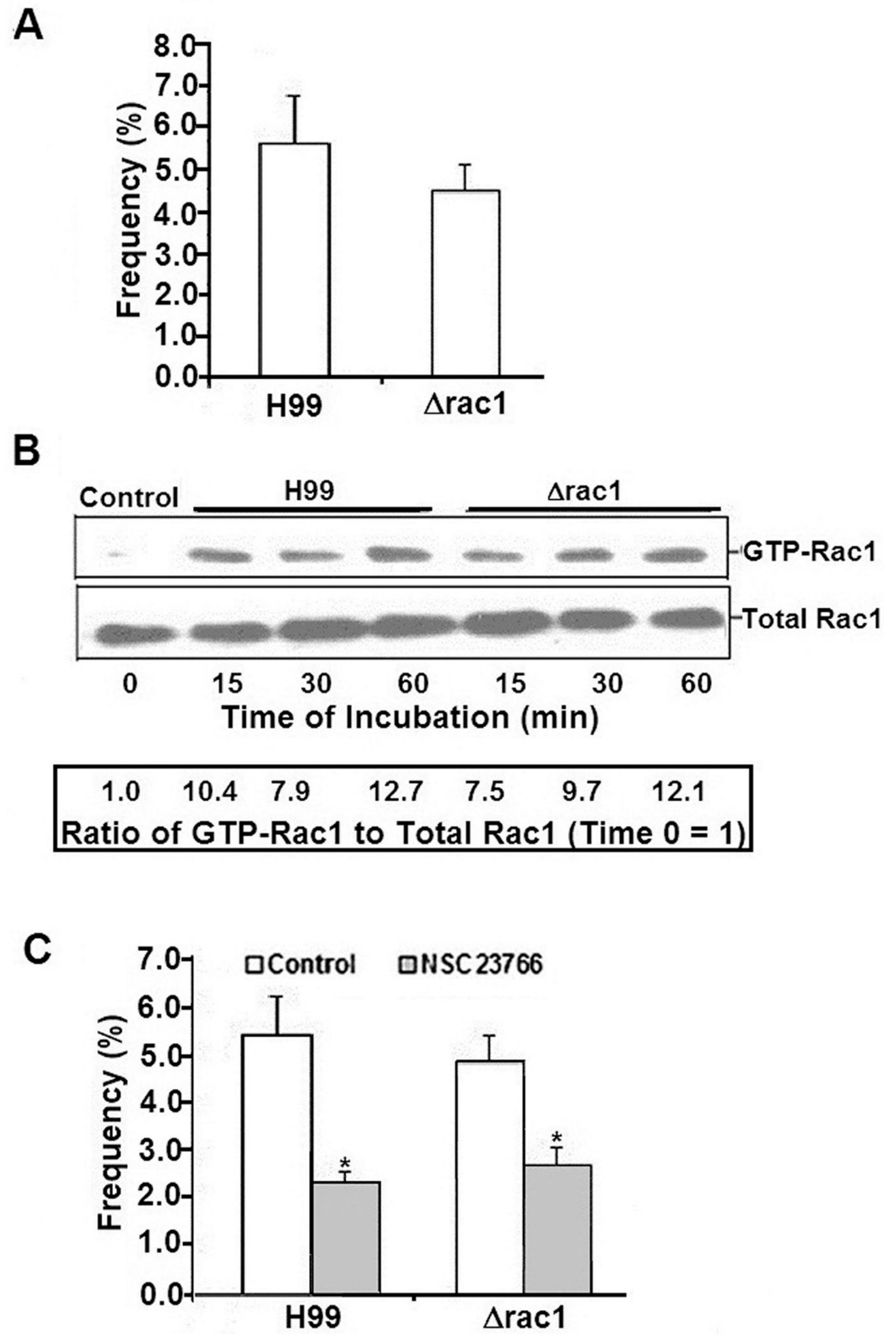

Figure 4. Role of *C. neoformans RAC1* in activation of host Rac1 in HBMEC

A. To determine whether *C. neoformans* Rac1 played a role in trancytosis, the *Δrac1* mutant along with the parent strain H99 were examined for transcytosis across HBMEC monolayers after 9 hour incubation.

B. To analyze the role of *C. neoformans* Rac1 in activating host Rac1, HBMEC were incubated with strain H99 or *Δrac1* mutant for various time periods, after which lysates were analyzed for GTP-Rac1 or total Rac1 in Western Blot assays.

C. To examine differences in transcytosis between strain H99 and the *Δrac1* mutant in the presence of Rac1 inhibitor, HBMEC were treated with NSC23766 or vehicle, and then

examined for transcytosis after 9 hour incubation. *p<0.05 compared to vehicle-treated HBMEC

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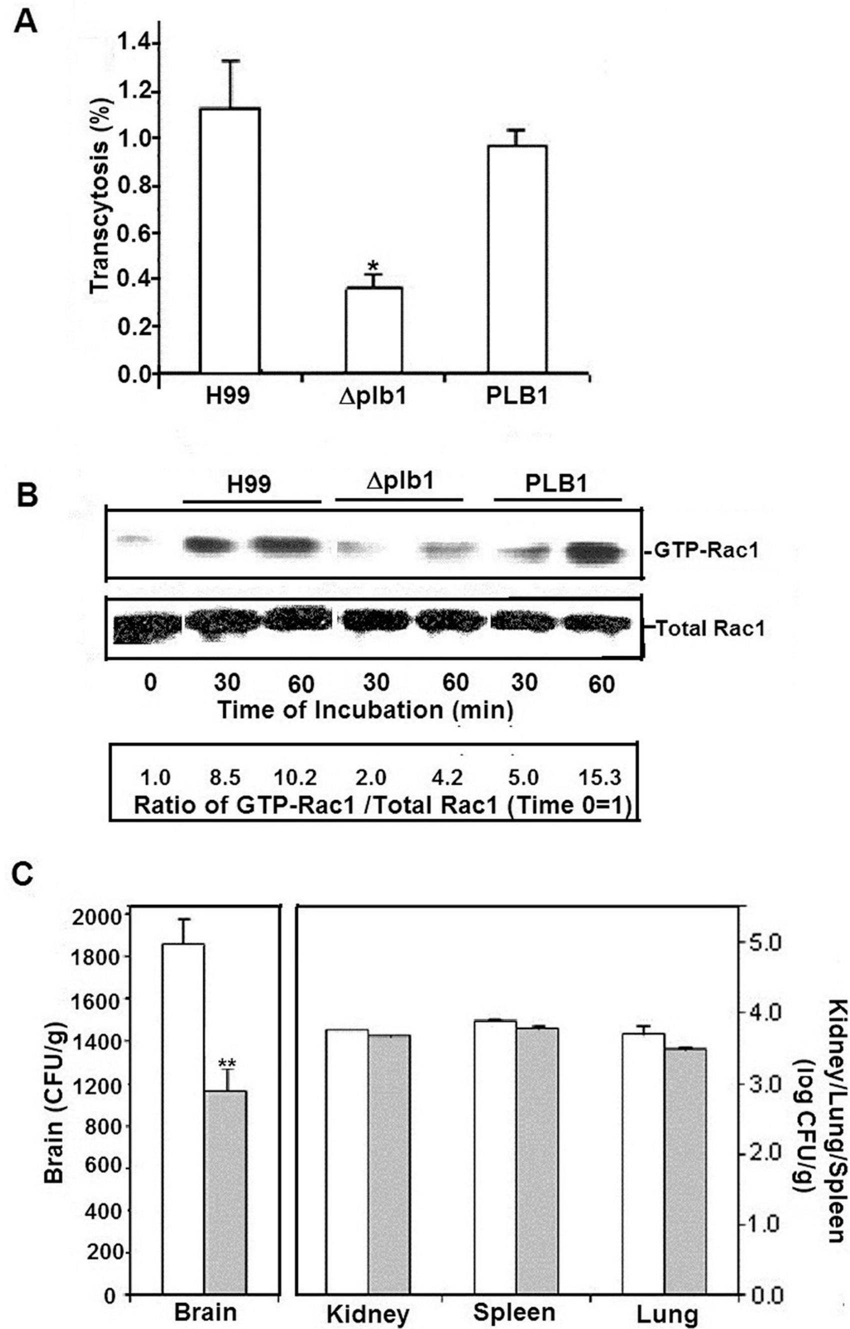

Figure 5. Role of *C. neoformans PLB1* in activation of host Rac1

A. To analyze the role of *C. neoformans PLB1* in transcytosis, HBMEC were incubated with strain H99, the *Δ plb1* mutant or the mutant reconstituted with *PLB1*. Transcytosed *C. neoformans* was determind after 9 hour incubation as described in *Materials and Methods*. *p<0.05 compared to H99 or the reconstituted strain.

B. To analyze the role of *PLB1* in activation of host Rac1, HBMEC were incubated with either strain H99, *Δ plb1* or the mutant reconstructed with *PLB1* for various time points, after which lysates were analyzed for GTP-Rac1 or total Rac1 in Western Blot assays. Numbers represent ratios of GTP-Rac1/ total Rac1 assuming 0 time point as 1.

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C. Mice were injected with either strain H99 (n=6) or the *Δ plb1* mutant (n=6) via the tail vein. After 24 hours, the brains, spleens, kidneys and lungs were removed, homogenized and cultured for count of CFUs. Yeast numbers were expressed as CFUs per gram of tissue. *$p<0.05$ compared to H99.

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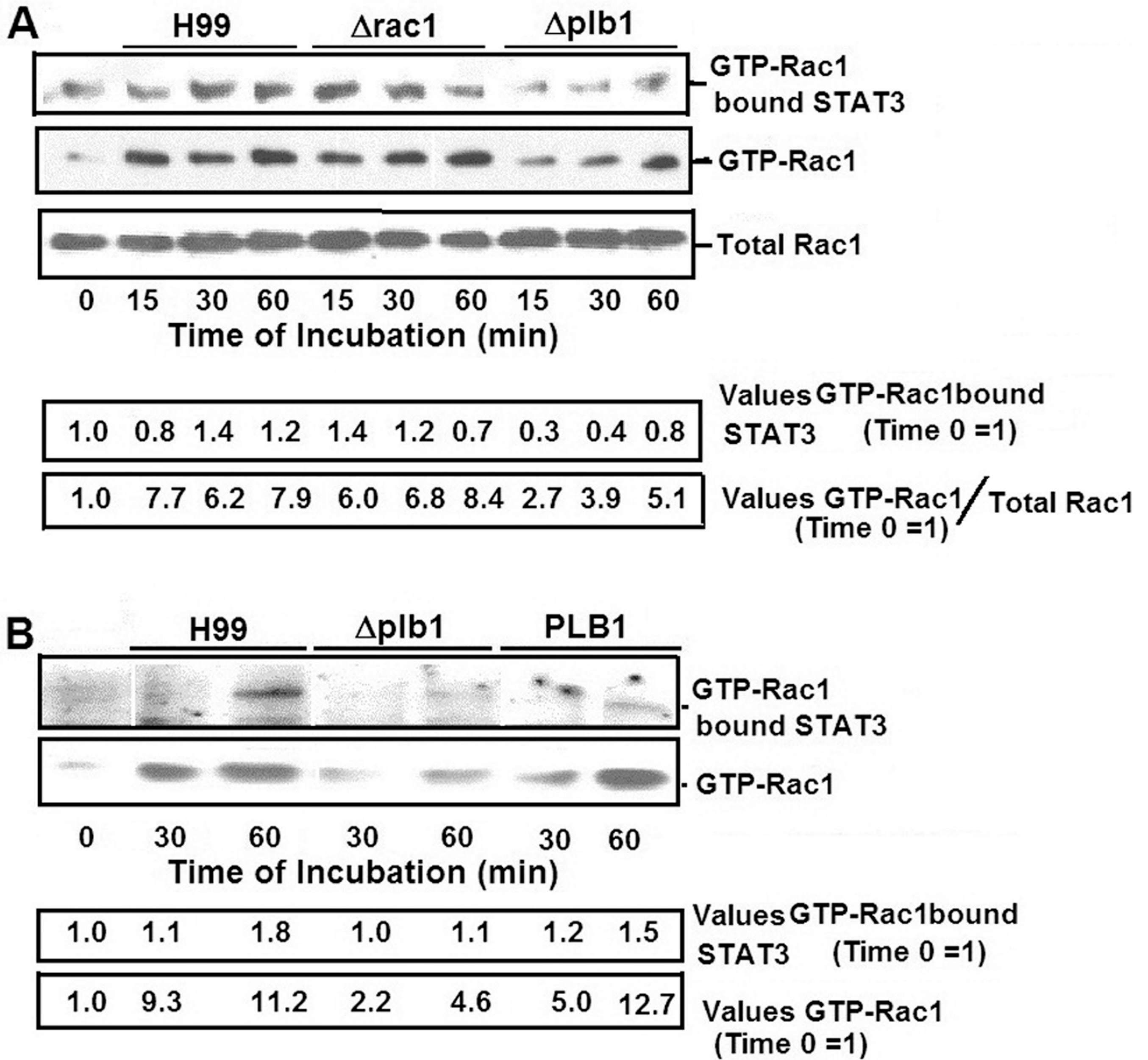

Figure 6. Analysis of STAT3 association with GTP-Rac1 in lysates of HBMEC incubated with *C. neoformans*

A. The lysates of HBMEC incubated with either strain H99, *Δrac1* or *Δ plb1* for various time periods were examined for the levels of GTP-Rac1 by pull-down assays. Nitrocellulose membranes that were used to resolve GTP-Rac1 in the lysates were examined for STAT3 using a specific monoclonal antibody.

B. In other experiments the nitrocellulose membranes on which GTP-Rac1 was resolved were assessed for levels of GTP-Rac1 bound STAT3. Bands were estimated densitometrically and represented as a ratio of values (STAT3/GTP-Rac1).

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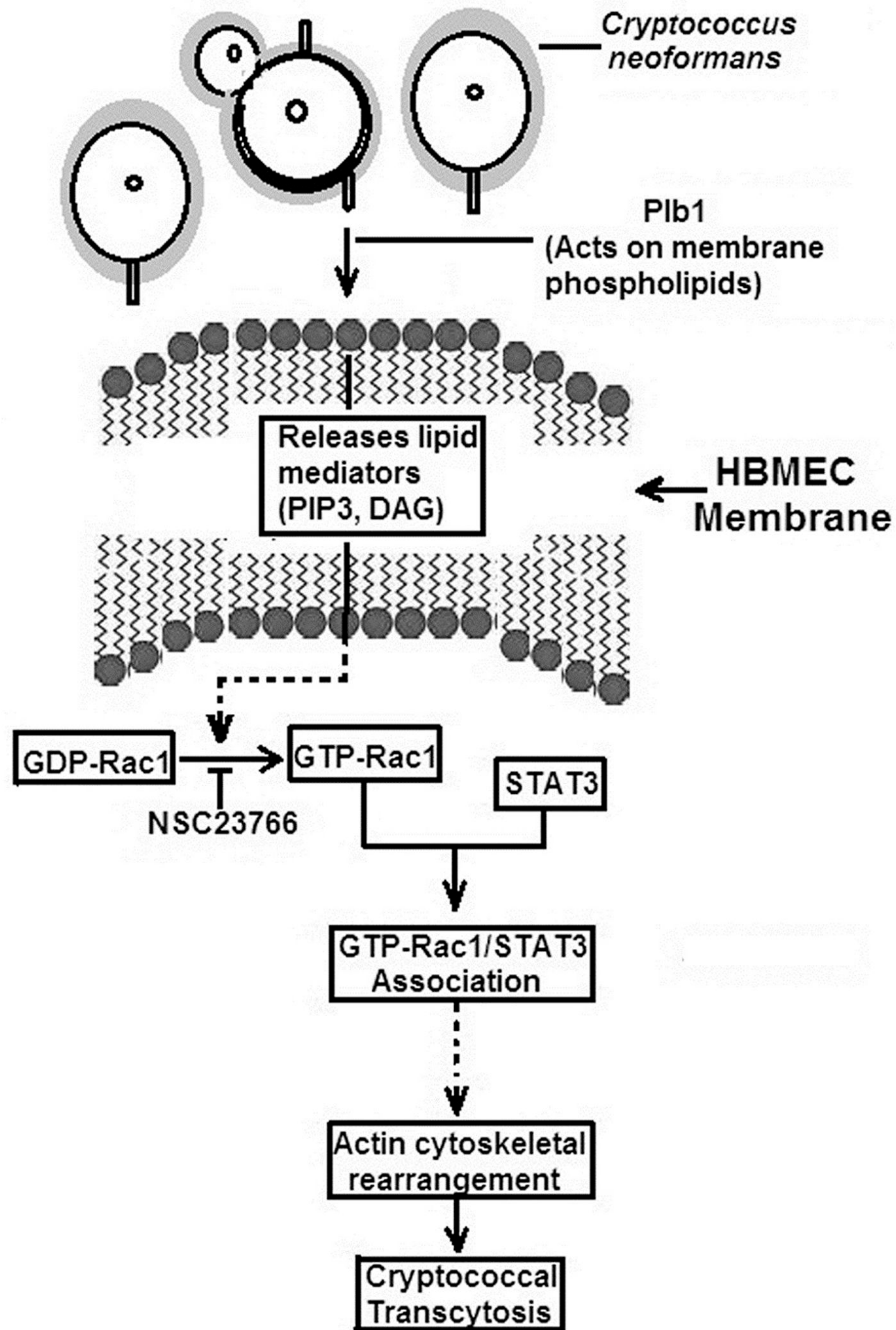

Fig 7. Diagrammatic representation of the host cell signaling pathways activated by *C. neoformans* Plb1 for transcytosis of HBMEC monolayer

For transcytosis of HBMEC monolayer, *C. neoformans* Plb1 is likely to interact with the blood-brain barrier and activate host cell Rac1. Activated Rac1 (GTP-Rac1) in turn interacts with STAT3, which we hypothesize aids in actin cytoskeleton rearrangements involved in *C. neoformans* transcytosis across HBMEC monolayer. We also hypothesize that the cytosolic phospholipase-like domain present in the C-terminal of Plb1 is likely to act on host cell phospholipids to release lipid mediators, which are involved in activating Rac1.