Involvement of human CD44 during Cryptococcus neoformans infection of brain microvascular endothelial cells

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Summary
Pathogenic yeast Cryptococcus neoformans causes devastating cryptococcal meningoencephalitis. Our previous studies demonstrated that C. neoformans hyaluronic acid was required for invasion into human brain microvascular endothelial cells (HBMEC), which constitute the blood–brain barrier. In this report, we demonstrate that C. neoformans hyaluronic acid interacts with CD44 on HBMEC. Our results suggest that HBMEC CD44 is a primary receptor during C. neoformans infection, based on the following observations. First, anti-CD44 neutralizing antibody treatment was able to significantly reduce C. neoformans association with HBMEC. Second, C. neoformans association was considerably impaired using either CD44-knock-down HBMEC or C. neoformans hyaluronic acid-deficient strains. Third, overexpression of CD44 in HBMEC increased their association activity towards C. neoformans. Fourth, confocal microscopic images showed that CD44 was enriched at and around the C. neoformans association sites. Fifth, upon C. neoformans and HBMEC engagement, a subpopulation of CD44 and actin translocated to the host membrane rafts. Our results highlight the interactions between C. neoformans hyaluronic acid and host CD44 and the dynamic results of these interactions, which may represent events during the adhesion and entry of C. neoformans at HBMEC membrane rafts.

Introduction
Opportunistic fungal pathogens have emerged during the past decade as important causes of morbidity and mortality in immunocompromised patients (Chayakulkeeree and Perfect, 2006; Perfect, 2007). Among them, cryptococcal meningoencephalitis is the most common opportunistic infection and is one of the major causes of death in AIDS patients (Mitchell and Perfect, 1995; Bicanic and Harrison, 2005). In order to cause life-threatening meningitis, Cryptococcus neoformans must cross the blood–brain barrier (BBB), formed mainly by brain microvascular endothelial cells (BMEC). We have used human BMEC (HBMEC) as an in vitro BBB model for C. neoformans infection studies (Chen et al., 2003; Chang et al., 2006; Jong et al., 2007). We found that C. neoformans was able to induce significant membrane ruffling of HBMEC during its invasion. The yeast also causes actin re-organization, probably via the activation of ROCK, LIMK and phosphocofilin pathway(s) in HBMEC (Chen et al., 2003). The underlying mechanism is currently unclear.

Cryptococcus neoformans is encompassed by its capsule polysaccharide, which is a well-known major virulent factor in this yeast (Chang and Kwon-Chung, 1994; McFadden et al., 2006; Yauch et al., 2006). The capsule polysaccharide is composed of the primary component glucuronoxylomannan, together with minor components (galactoxylomannan, mannoproteins) plus other polysaccharide derivatives. Acapsular mutants are typically avirulent, whereas encapsulated isolates have varying degrees of virulence (Chang and Kwon-Chung, 1994; 1998; 1999; Chang et al., 1996; Buchanan and Murphy, 1998; Charlier et al., 2005). However, little is known regarding the interactions of any capsule component (or any surface ligand) with a host receptor to precipitate its invasion process. We have recently identified and characterized a C. neoformans gene, CPS1 (Chang et al., 2006). Deletion of CPS1 from C. neoformans cells causes...
alterations in the ultrastructures between cell wall and capsule in Δcps1 cells and results in reduction of their ability to associate with HBMEC. We have also demonstrated that hyaluronic acid (HA; also known as hyaluronan) can be detected in C. neoformans CPS1 wild-type strain, but not in the Δcps1 cells. We have further determined that the CPS1 gene encodes a HA synthase (Jong et al., 2007). These results suggest that HA plays a role as an adhesion molecule during infection by the yeast.

Hyaluronic acid is present in all vertebrates. It is also present in the capsule of some pathogenic bacteria such as Streptococcus sp. and Pasteurella (Pruimboom et al., 1999). Several cell membrane-localized HA binding proteins (or HA receptors) have been identified, including CD44, RHAMM, Ivd4, LEC receptor and others (Bontese et al., 2002). The most common HA receptor is CD44, but it plays different roles in different cell types. For example, tumor invasion can be enhanced by HA through CD44 association (Sneath and Maningham, 1998; Toole et al., 2002). Group A Streptococcus uses the capsular HA to promote its invasion through CD44-mediated cell signalling (Cybes and Wessels, 2001). It has been shown that the integrity of membrane rafts is required for efficient binding of the IpaB protein of pathogenic Shigella to the host via CD44 interaction and that the CD44 becomes membrane raft-associated (Lafont et al., 2002). We have previously observed the involvement of C. neoformans HA during pathogen–host interaction, raising the possibility that there is a host surface HA receptor at the sites on HBMEC membranes during infection (Chang et al., 2006; Jong et al., 2007). As CD44 is a major membrane HA receptor in many cell types, it is tempting to speculate that C. neoformans HA directly engages the HBMEC CD44 as a part of its invasion mechanism.

CD44 partitions into membrane rafts in cholesterol-rich Triton X-100-insoluble plasma membrane domains in human peripheral blood lymphocytes and other cells (Ilango et al., 1999). An important feature of rafts is their dynamic nature, which allows the transient formation of membrane platforms that build up molecular machineries implicated in cell signalling or targeting mechanisms. The extent of the lateral mobility of CD44 is dependent on the state of actin cytoskeleton and lipid composition of the plasma membrane. The lateral mobility of CD44 is significantly affected when either the actin cytoskeleton is disrupted or plasma membrane cholesterol is depleted. Involvement of membrane rafts in bacterial entry mechanisms has also been described for some Escherichia coli strains (Baorto et al., 1997; Guignot et al., 2000) that enter via caveolae, as well as for Mycobacterium spp. (Gatfield and Pieters, 2000), Chlamydia trachomatis (Norkin et al., 2001), Salmonella (Garner et al., 2002) and Shigella (Sansonetti, 2001).

In this report, we investigated the interaction between C. neoformans HA and host CD44, the potential receptor for C. neoformans in HBMEC. In agreement with this notion, blockage of CD44 function by antibodies, inhibitors or small hairpin RNA (shRNA) effectively abrogated C. neoformans association with HBMEC, a loss-of-function phenotype. Conversely, overexpression of CD44 in HBMEC increased C. neoformans association activity towards HBMEC, a gain-of-function phenotype. CD44 itself accumulated at the yeast–host interaction sites, in response to C. neoformans adhesion. Redistribution of CD44 and some actin molecules towards the membrane lipid rafts was evident. Our results suggest that the interaction between C. neoformans HA and HBMEC CD44 initiates the events of the entry process at the HBMEC membrane rafts. Subsequently, membrane signalling may be relayed through CD44 to the actin cytoskeleton inside the HBMEC cells.

Results

The presence of CD44 on the surface of HBMEC

In many cell types, CD44 is the major cellular surface receptor for HA. However, the CD44 molecules of HBMEC have never been explored. We first determined whether CD44 protein was expressed in HBMEC. Our protein blot showed that a single form of CD44 was observed in HBMEC with the apparent MW around 90 kDa, which was in agreement with the expected size for the standard (invariant) form of CD44 (Fig. 1A). CD44 protein level maintained about the same after 3 h incubation with C. neoformans cells. Actin was used as the loading control. The results suggested that total CD44 protein level in HBMEC did not change in the presence of yeast cells, at least for 3 h. We then performed immunofluorescence microscopy to detect the subcellular localization of CD44 on HBMEC. Our results showed that the CD44 was localized on the plasma membrane, shown as green fluorescence concentrated on the contour of the HBMEC cells. Weak green signals could be detected inside the cells; presumably, these were intracellular CD44 molecules (Fig. 1B, a). As a control, the secondary FITC-conjugated antibody alone did not show any non-specific staining on HBMEC (not shown). The cells were also stained with propidium iodide (PI) to locate their nuclei (Fig. 1B, b), and the overlaid image was shown in Fig. 1B, c. We detected no co-localized signals between the anti-CD44 and PI stains.

Effect of anti-CD44 antibodies on the association of C. neoformans with HBMEC

CD44 is the primary receptor of HA in many cell types. To investigate the potential role of CD44 in C. neoformans
We tested whether treatment with anti-CD44 antibodies prevented *C. neoformans* association with HBMEC. There are two types of anti-CD44 antibodies that are commercially available. The epitope of the first type is the HA binding domain, resulting in neutralizing HA–CD44 interaction (Santa Cruz Biotech, cat # sc-18882L, azide-free). The second type binds to CD44 molecules, but does not affect its HA binding activity (Santa Cruz Biotech, clone DF1485). To evaluate the involvement of CD44 in *C. neoformans* and HBMEC interaction, a concentration range of the neutralizing antibody was incubated with HBMEC prior to the *C. neoformans* in vitro association assay. The HA contents and their association ability with HBMEC of these tested strains have previously been examined (Jong *et al.*, 2007). They are all CPS1 wild-type strains that produce detectable amount of HA. As shown in Fig. 2, the neutralizing anti-CD44 antibody effectively blocked *C. neoformans* association with HBMEC in a dose-dependent manner, using both capsulated and acapsulated strains. No effect on the association activity was found, using the non-neutralizing anti-CD44 antibody (DF1485), purified mouse IgG (mock) or unrelated antibodies to proteins such as CD4 and CD47 as the controls (data not shown). Thus, the results support the involvement of CD44 in the process of *C. neoformans* adhesion to HBMEC.

**Effect of anti-CD44 shRNA on the *C. neoformans* association activity towards HBMEC**

Anti-CD44 shRNA is an effective way to silence endogenous mRNA and thus CD44 proteins in HBMEC. The
knock-down of CD44 allows us to evaluate its impact on
C. neoformans association with HBMEC. Five shRNA
sequences were used and their corresponding locations
were shown in Fig. 3A. Four were located inside the open
reading frame of CD44, and one was located at the 3′
end, near the poly(A) site of the CD44 message (Fig. 3A).
These shRNAs were carried on a lentivirus expression
vector to facilitate their transfection and expression.
Protein blots were used to detect CD44 levels in trans-
fected cells. As shown in Fig. 3B, those shRNA located
inside the open reading frame of CD44 were able to
reduce CD44 protein level significantly; only the one
(sample #67) near the poly(A) tail failed to knock down
CD44 proteins in HBMEC. Some residual signals were
detected in samples #63 to #66, probably because of
untransfected HBMEC in the pool. As a control, vector
bearing green fluorescent protein (GFP) or untreated
HBMEC revealed a normal level of CD44. Interestingly,
the CD44 level was proportional to the C. neoformans
association ability with HBMEC (Fig. 3C). Among them,
the anti-CD44 shRNA probe #64 resulted in the lowest cell
association activity (N = 4). Regardless of the transfection
efficiency, our results strongly suggested that CD44
in HBMEC played a major role as a receptor during
C. neoformans adhesion.

Relationship between C. neoformans CPS1 gene
functions and HBMEC CD44 during C. neoformans
invasion
We have previously demonstrated that the C. neofo-
rmans CPS1 wild-type strain contains HA. In the
CPS1 knockout mutant (Δcps1), the surface HA was dimin-
ished and its association with HBMEC was also signifi-
cantly reduced in vitro (Chang et al., 2006; Jong et al.,
2007). Here, we test the association efficiency of the
wild type versus the mutant Δcps1 yeast cells on normal
and CD44-knock-down HBMEC. As expected, strains
B-4500FO2 (CPS1 wild type) and C588 (a CPS1-
complemented strain) had a higher association activity
than C559 (Δcps1 strain) on normal HBMEC or the cells
transfected with lentivirus carrying GFP gene (Fig. 4,
group I: normal HBMEC, and group II: GFP/HBMEC). In
the case of CD44-knock-down HBMEC, the association
ability of B-4500FO2 and C588 yeast cells was reduced
to the same level as C559 on normal HBMEC (Fig. 4,
group III). Thus, yeast association activity was signifi-
cantly impaired by loss of either yeast adhesion mol-
ecule (presumably HA) or the putative host receptor

![Fig. 3. Knock-down of CD44 by shRNA results in a reduction of
C. neoformans association with HBMEC.](https://example.com/f3)

![Fig. 4. Differential association of CPS1 versus Δcps1 strains on
CD44/HBMEC or CD44/HBMEC cells. In vitro association assay
was performed and analysed as described in Experimental
procedures. C. neoformans isogenic strains, B-4500FO2 (CPS1
wild type), C588 (CPS1 complemented) and C559 (Δcps1),
were used in parallel. Untreated HBMEC or cells transfected with GFP
were used for comparison (groups I and II respectively). Clones of
#64 and #65 anti-CD44 shRNAs were pooled and used to eliminate
endogenous CD44 on HBMEC (group III). Data are expressed as
percentage of yeast cells associating with HBMEC and are the
mean of triplicates ± SD.](https://example.com/f4)
triplicates untreated HBMEC for 3 h to detect the yeast association activities. Data expressed as percentages of associated yeast cells are the means of one set of samples was used for protein blots to detect the CD44 expression (A), and another set of samples was used for C. neoformans association assay (B), in which C. neoformans strains B-4500F02 (cps1+) and C559 (Δcps1) (10^6 ml^-1) were incubated with transfected or untreated HBMEC for 3 h to detect the yeast association activities. Data expressed as percentages of associated yeast cells are the means of triplicates ± SD.

CD44. The results suggested that the association between C. neoformans cells and HBMEC involved the HA–CD44 interaction.

Overexpression of CD44 in HBMEC and its effects on C. neoformans association activity

To further examine the roles of CD44 during C. neoformans infection, we overexpressed CD44 in HBMEC. The CD44 expression vector contained the CMV promoter and a full-length CD44 with a C-terminal HA tag. The untreated HBMEC and the cells transfected with vector alone (without insert) were performed in parallel as the controls. As shown in the protein blot (Fig. 5A, row 1), a full-length CD44 (~92 KDa) can be detected by the anti-HA-tagged antibody in CD44-transfected cells but not in the empty vector control or untreated HBMEC. Similarly, immunoblotting using the anti-CD44 antibody also showed a greater quantity of CD44 in transfected cells when compared with controls. Densitometric analysis of the scanned protein bands revealed that the expression levels of CD44 increased about five- to sixfold in our expression system (Fig. 5A, row 2). CD44 overexpression in HBMEC resulted in four- to fivefold increase in the association activity of C. neoformans strain B-4500F02 (cps1+), while little or no increase was observed using strain C559 (Δcps1). Thus, the increase of the association activity in the overexpressed cells is a CPS1-dependent process.

CD44 is enriched at the C. neoformans association site on HBMEC

To investigate whether CD44 was present at the yeast entry sites, double-immunofluorescence staining of CD44 (green) and yeast cells (red) was carried out. A section of the top view of infected HBMEC was shown by the confocal microscopy, in which clusters of CD44 signals (green) on the surface of HBMEC were observed (Fig. 6A, a). Clusters of CD44 signals were quite different from the untreated HBMEC (Fig. 1B, a), but were consistent with our previous transmission electron microscope (TEM) studies showing that C. neoformans induces significant membrane ruffling of HBMEC (Chen et al., 2003). One potential entry site with dense CD44 staining (box) was enlarged (Fig. 6A, b). In parallel, C. neoformans cell surface polysaccharides were stained by the fluorescent dye calcofluor white and displayed as a pseudo-red colour, showing a budding yeast cell with a dumbbell shape (Fig. 6A, c). In a combined image, a region with high density of CD44 signal and a budding cell signal overlapped (Fig. 6A, d). The image was rotated approximately 45 degrees clockwise and analysed by the ScanLine software (Fig. 6A, e). CD44 showed a significant recruitment at the yeast entry foci, in cellular extensions that were raised in the vicinity of the site of yeast interaction with the cell membrane (Fig. 6A, e, right chart). These data showed that C. neoformans cells adhered to HBMEC where CD44 was enriched. Consistently, no such recruitment of CD44 was observed when the Δcps1 strain was used (data not shown).

Another confocal image showing a side view of the CD44/yeast interaction site was displayed in Fig. 6B. In this case, a single C. neoformans cell was observed (Fig. 6B, a). In an enlarged image, protrusion of CD44 signals were observed, encompassing the yeast cell (Fig. 6B, b, d). The densest region of CD44 overlapped with the yeast signal (Fig. 6B, d). Scanning intensities of CD44 (green curve) and C. neoformans (red curve) in the optical section corresponded to the CD44 and yeast cell

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respectively. Notice that a higher density of CD44 signal on the membrane was observed surrounding the putative yeast entry site (Fig. 6B, d, arrow). Together, the results showed that *C. neoformans* cells could adhere to HBMEC where CD44 was recruited, presumably at the entry site of invasion.

**Association of HA-coated beads with CD44 signals on the surface of HBMEC**

To further confirm whether HA was mediating the association with CD44, we coated HA on fluorescent beads and examined whether these beads could interact with...
CD44. HBMEC was probed with anti-CD44-rhodamine (red) and DAPI (blue), whereas the fluorescence beads were displayed in green in these images. After incubation and washing, some HA-coated beads could still be observed in association with HBMEC. Interestingly, the association sites of HA-coated beads were predominantly co-localized with the CD44 signals, as yellow signals were observed at their interfaces (Fig. 7A). A few unattached green beads were observed; presumably these beads were non-specifically trapped on HBMEC after washing steps. When the untreated fluorescent beads were used as the control, very few green signals could be observed. We noticed that the uncoated beads were not co-localized with any CD44 signal (Fig. 7B). Similar negative results were observed when beads coated with dextran were used as the control (data not shown). Thus, specific interactions could be mediated between HA-coated beads and CD44 on HBMEC surface in these studies.

C. neoformans adhesion to HBMEC is impaired after cholesterol depletion by filipin

Filipin can effectively extract cholesterol from membrane rafts, resulting in disorganized membrane raft microdomains and redistributed raft-associated proteins to detergent-soluble fractions (Shen et al., 2000). We addressed the question whether the integrity of membrane rafts of HBMEC was required for efficient association of C. neoformans cells. A control experiment was first performed to ensure that there was no detectable effect on the C. neoformans growth rate by adding filipin up to 1 mM (data not shown). We then examined the effect of filipin on C. neoformans adhesion using the in vitro association assay. Our results showed that the association activity between C. neoformans and HBMEC decreased as the concentration of filipin increases (from 0 to 1 μM) (Fig. 8A).

To complement the above cell association assay, we performed immunofluorescence microscopy to examine the morphology of HBMEC after filipin treatment. Untreated HBMEC showed CD44 to be primarily distributed on the membrane (Fig. 8B, a), as had been observed in Fig. 1B. However, after filipin treatment (0.5 μM), the membrane CD44 signals in many areas of cultured HBMEC cells became disappeared and cytosolic CD44 signals were also dimming (Fig. 8B, b). After increasing the filipin concentration to ~1 μM, the CD44 signals of most HBMEC were barely detected (Fig. 8B, c). Thus, filipin could alter membrane structure and appeared to strip CD44 signals from HBMEC.

Similar CD44 images could be observed with filipin treatment (0.5 μM) in the presence of C. neoformans B-4500FO2 cells (Fig. 8C). DAPI (blue) staining was used to locate nuclei of HBMEC cells (Fig. 8C, a), and FITC (green) was used to probe the CD44 signals (Fig. 8C, b). The image showed a mixed population of HBMEC, i.e. some still exhibited membrane CD44 localization but most showed weak signals throughout the cells. In general, the number of associated yeast cells was proportional to the intact membrane sites generating CD44 signals. Thus, the number of C. neoformans cells associated with HBMEC decreased as the filipin concentration increased. When associated C. neoformans cells were observed (Fig. 8C, c), the yeast cells were found to co-localize with the membrane CD44 signal (Fig. 8C, d). Together, both the cell association assay (Fig. 8A) and the immunofluorescent images (Fig. 8B and C) were consistent with each other in that filipin treatment interfered the association of C. neoformans cells with HBMEC, presumably through the removal of CD44 from HBMEC membrane. We speculate that CD44 may locate on membrane rafts, which are enriched in cholesterol, and the ability of CD44 to cluster in the membranes of HBMEC in response to exposure to cryptococcal cells may be reduced after treatment with filipin, resulting in the reduction of associated cryptococcal cells. We tested the validity of our speculation below.

Redistribution of CD44 to membrane rafts during C. neoformans invasion

Membrane lipid rafts can be biochemically distinguished from detergent-insoluble cytoskeleton-associated material by density gradient centrifugation. As a result of their high lipid content, they float at a low density, while detergent-insoluble cytoskeleton assemblies remain at higher densities (Ilangumaran et al., 1999). As CD44 could be a membrane receptor on HBMEC, we used density gradient centrifugation to fractionate membrane rafts. We addressed two questions: (i) whether CD44 of HBMEC was membrane raft-associated and (ii) whether there was a redistribution of CD44 during C. neoformans adhesion.

Fig. 7. Association of HA-coated beads with CD44 signals on HBMEC. Green fluorescent beads, coated with HA (A) or blank (B), were incubated with HBMEC for 2 h. After washing with PBS three times, the HBMEC cultures on the slides were further probed with anti-CD44-rhodamine (red) and DAPI (blue), and then examined using a fluorescence microscope. The size of the fluorescent beads is ~1.0 μm in diameter.
HBMEC was lysed in the buffer containing 1% Triton X-100 and floatation was performed in Optiprep gradients. After centrifugation in this gradient, detergent-insoluble, glycosphinogolipid-rich membrane fractions representing the lipid rafts as expected floated to the interphase between 0% and 20% OptiPrep layers, peaking at fraction 2 in our studies. The loading buffer floated to the top (fraction 1), but soluble proteins or cytoskeleton-associated, detergent-insoluble proteins remained in the bottom fractions of the gradient (fractions 3–9).

Initially, we tested our HBMEC samples in the Optiprep floatation gradients by the dot blots. In a normal HBMEC, Filipin (μM) 

![ Filippin effects on C. neoformans association with HBMEC.](image)

A. Association assays were performed using C. neoformans B-4500FO2 strain, except that HBMEC were pre-incubated with 0, 0.25, 0.5 and 1 μM Filipin (shaded boxes). Filipin was dissolved in methanol to make a 10 mg ml⁻¹ (~64 mM) of a stock solution and diluted with sterile water or medium to final concentrations as shown. Equal concentrations of methanol in the medium were added in parallel as the controls (open bars). Data are expressed as percentage of inoculum using mean of triplicates ± SD. Data analysis revealed a significant dose-dependent effect in the Filipin group (P = 0.003) and with no difference in the control group respectively (P > 0.05). For immunofluorescence microscopic studies, HBMEC were pre-incubated with 0, 0.5 and 1 μM Filipin and probed with CD44-FITC (green) and DAPI (blue) in the absence (B) or presence (C) of C. neoformans B-4500FO2.

**Fig. 8.** Effect of Filipin on the C. neoformans association with HBMEC.

**A.** Association assays were performed using C. neoformans B-4500FO2 strain, except that HBMEC were pre-incubated with 0, 0.25, 0.5 and 1 μM Filipin (shaded boxes). Filipin was dissolved in methanol to make a 10 mg ml⁻¹ (~64 mM) of a stock solution and diluted with sterile water or medium to final concentrations as shown. Equal concentrations of methanol in the medium were added in parallel as the controls (open bars). Data are expressed as percentage of inoculum using mean of triplicates ± SD. Data analysis revealed a significant dose-dependent effect in the Filipin group (P = 0.003) and with no difference in the control group respectively (P > 0.05). For immunofluorescence microscopic studies, HBMEC were pre-incubated with 0, 0.5 and 1 μM Filipin and probed with CD44-FITC (green) and DAPI (blue) in the absence (B) or presence (C) of C. neoformans B-4500FO2.
about 50% of cellular caveolin, a lipid raft marker (Krajewska and Maslowska, 2004), was found in the low-density raft-associated fractions (Fig. 9A, lane a). This distribution of caveolin was similar to the one observed in HeLa cells (Lafont et al., 2002). In the presence of C. neoformans strain B-4500FO2, a significant amount of caveolin upshifted to the low-density fractions (Fig. 9A, lane b). In untreated HBMEC, CD44 was primarily associated with soluble fractions (fractions 6–8) (Fig. 9A, lane c). For the cells treated with C. neoformans, a significant portion of CD44 had apparently relocated to the membrane rafts as observed in the fraction 2 (Fig. 9A, lane d). The result suggested that there was a reorganization of membrane rafts taking place during C. neoformans infection, and CD44, as the yeast putative receptor, became enriched in these membrane rafts on the surface of HBMEC.

We further tested whether C. neoformans HA played any role in the redistribution of CD44 to membrane rafts during the pathogen-host engagement. To do this, C. neoformans strain B-4500FO2 (CPS1 wild type)–treated HBMEC was incubated with Triton X-100 to isolate detergent-resistant membranes from the floatation gradient centrifugation. In parallel, strain C559 (Δcps1) was used for comparison. We observed that incubation with strain B-4500FO2 led to an increased association of CD44 with membrane rafts (Fig. 9B, row 1; asterisk on top indicates the membrane raft fraction). In contrast, incubation with isogenic strain C559 (Δcps1) resulted in much less CD44 detected in the low-density fraction detergent-resistant membranes (Fig. 9B, row 2). This result was similar to the untreated HBMEC samples (data not shown). In the filipin-treated HBMEC, CD44 was hardly recovered from the Triton-resistant fractions and soluble fractions (Fig. 9B, row 3). As filipin extracts membrane cholesterol, the treatment may lead to the loss of CD44 during the washing process. This possibility could also explain why filipin-treated HBMEC lost their ability to associate with C. neoformans cells (Fig. 8).

When the anti-actin antibody was used, an increased subpopulation of actin was detected in the membrane raft fraction of cells treated with CPS1 wild-type yeast (Fig. 9B, row 4). Actin was barely observed in the membrane raft fraction when strain C559 (Δcps1) (Fig. 9B, row 5), filipin (row 6) or buffer (data not shown) was used to treat HBMEC. These results suggested that a direct linkage might extend the action of CPS1 (presumably through HA) to HBMEC CD44 molecules and subsequently to actin molecules.

One of the major functions of profilin is to regulate actin polymerization at the barbed end and thereby promote actin filament growth. We therefore used an increased level of profilin as an indication of actin filament activity. The same blots from above were re-probed with anti-profilin antibodies in this set of experiments. Interestingly, the results show that much more profilin could be detected in the B-4500FO2-treated HBMEC than the C559-treated one (Fig. 9B, rows 7 and 8 respectively). In the filipin-treated HBMEC, only a slower migrating band of
proliferation of cells; presumably, this was a phosphorylated (inactivated) form of profilin (Fig. 9B, row 9). The results suggest that HA-host CD44 engagement might stimulate actin polymerization.

Cryptococcus neoformans B-4500FO2 increased CD44 redistribution to the membrane rafts, whereas filipin removed CD44 signal from HBMEC (Fig. 9B, CD44 panel). We performed additional experiment to further examine the effect of filipin on CD44 distribution in the presence of C. neoformans. Control experiments performed without filipin in the presence and absence of yeast cells reproduced the results shown in Fig. 9B (CD44 panel). When HBMEC was treated with filipin, the CD44 signals were significantly reduced and were displayed in a very similar pattern, whether in the absence (Fig. 9C, panel 3) or presence (Fig. 9C, panel 4) of C. neoformans B-4500FO2. These results suggested that C. neoformans could not revert nor attenuate the filipin effect on HBMEC. Furthermore, these results were consistent with our immunofluorescence microscopic images (Fig. 8C), which suggested that filipin perturbed membrane structure, extracted CD44 from HBMEC and prevented C. neoformans cells from association with HBMEC.

Discussion

Little is known how C. neoformans invades into the brain and causes devastating central nervous system infection. We have previously demonstrated that C. neoformans surface HA may play a role as a ligand to interact with HBMEC (Chang et al., 2006; Jong et al., 2007). One of the key questions here is whether there is a host receptor for C. neoformans HA binding. There are several HA binding proteins (or receptors) that have been identified, such as CD44, RHAMM, ivd4, LEC and others from different cell types (Bono et al., 2001; Turley et al., 2002). We could easily detect CD44 (Fig. 1), but barely detect RHAMM on HBMEC (data not shown). Our results showed that blockage of CD44 either by antibody or by shRNA substantially reduced C. neoformans association activity towards HBMEC. Some residual association could still be observed in our in vitro assay after anti-CD44 antibody and anti-CD44 shRNA treatments (Figs 2–4). It is not clear whether other HA binding proteins (or co-receptors) are present in HBMEC, nor whether they play any role during yeast HA engagement. As the role of CD44 is predominant (Figs 2–7), our results suggest that CD44 is the major HA receptor on HBMEC during C. neoformans infection.

One characteristic of CD44, as the major cell surface receptor for HA, is that the protein localizes to detergent-resistant cholesterol-rich microdomains, or membrane rafts, in fibroblasts, blood cells, mammary epithelial cells and other cells (Oliferenko et al., 1999). However, in Madin–Darby canine and bovine kidney epithelial cells, CD44 was completely soluble in Triton X-100 and no association of CD44 with the cytoskeleton could be demonstrated (Neame et al., 1995). In specific cell types, CD44 is able to translocate to the membrane rafts to associate with HA (Tammi et al., 1998; Oliferenko et al., 1999; Simons and Toomre, 2000). It has been shown that the integrity of membrane rafts is required for efficient association of Shigella to HeLa and CHO cells via CD44 interaction (Lafont et al., 2002). This association process is involved in the redistribution of CD44 to membrane rafts in mammary epithelial cells (Oliferenko et al., 1999; Skoudy et al., 2000). Our study revealed a similar observation. In the absence of C. neoformans, the majority of CD44 was in a detergent-soluble form, and in the presence of C. neoformans with the wild-type CPS1, we observed the induction of CD44 redistribution to the membrane rafts in HBMEC (Fig. 9). Confocal microscopic images support this notion (Fig. 6). The involvement of membrane rafts is also supported by the results of the filipin treatment experiments (Figs. 8, 9B and C). It is known that HBMEC has an extremely low rate of induced phagocytosis. The structure and functions of HBMEC membrane rafts may be different from other cell types. The behaviour of CD44 in HBMEC in response to C. neoformans invasion might differ in time, duration and extent. An in-depth investigation of the CD44 of HBMEC would reveal more information about whether and how C. neoformans uses this mode of entry during infection.

There are several precedents regarding the involvement of CD44 in infections caused by other microorganisms. For example, the hepatitis B virus X protein-induced association of CD44 with moesin causes the pseudopodial tips of F-actin-binding proteins in a Rho and Rac-dependent manner (Legg et al., 2002). Invasion of Neisseria meningitides into vascular endothelial cells follows type IV pilus-mediated adhesion, in which CD44 and cortical actin polymerization co-localizes within these membrane protrusions (Eugene et al., 2002). CD44 is also involved in the entry of Shigella into epithelial cells by the binding of a secreted bacterial protein, IpaB (Skoudy et al., 2000). The entry of Listeria monocytogenes into cells seems to take advantage of a co-receptor function of CD44 (Chang et al., 1997). CD44 has been implicated in mediating the activation of Met by InIB. Also, CD44 is involved in the cell-to-cell transfer of bacteria after the replication phase. Once Listeria reaches the inner surface of the plasma membrane, CD44 concentrates at that region of the membrane before protrusion formation (Sechi et al., 1997). It is apparent that none of the above pathogens uses HA for CD44 interaction. On the other hand, Streptococcus pyogenes attaches to cells through its HA association with the host CD44 (Cywes and Wessels, 2001). This interaction triggers tyrosine
phosphorylation of several host-cell proteins as well as cytoskeletal rearrangements that cause ruffles and the extension of lamellipodia (Cywes and Wessels, 2001). Consistent with these data, CD44-deficient mice are resistant to oropharynx colonization by *S. pyogenes* (Cywes and Wessels, 2001). Another case is *Pasteurella* which also contains HA molecules for host CD44 interaction during its invasion (Pruimboom et al., 1999). Based on our results, invasion of *C. neoformans* seems follow a mechanism similar to that of *S. pyogenes*.

The interaction between *C. neoformans* HA and HBMEC CD44 may play crucial roles in the virulence of *C. neoformans*. First, *C. neoformans* cells disseminate through the bloodstream, and traversal of *C. neoformans* across the BBB is the key step for the pathogen neurotropism. HBMEC CD44 is a surface protein that can interact with its principal ligand HA from *C. neoformans* at the N-terminal extracellular domain. This interaction is likely to provide a force in withstanding blood flow in vivo and retain the yeast cell on the surface of HBMEC. Presumably, this step may be the reason, or one of reasons, that *C. neoformans* has predilection for the brain. Second, CD44 protein contains a transmembrane domain that interacts with membrane rafts. The dynamic nature of the membrane rafts may allow recruitment of additional CD44 molecules, actin and other components to secure yeast adherence to the host cells. In this way, *C. neoformans* may establish its docking sites to facilitate its entry. Third, it is known that the cytoplasmic tail of CD44 interacts with cytoskeletal linker proteins to promote cytoskeletal rearrangement. Our previous TEM studies found that *C. neoformans* induced significant morphological changes in HBMEC, suggesting that cytoskeletal reorganization occurred during the invasion process. Scanned electron microscopic studies from another group also showed microvillus-like projections embracing the yeast cell at the docking site of HBMEC, and subsequently, lamellipodia-like engulfment of the yeast cell took place (Chang et al., 2004). How *C. neoformans* triggers cytoskeletal reorganization in HBMEC is unclear. As CD44 is known to participate in signalling responses regulating cytoskeleton organization (Hall and Nobes, 2000), it is tempting to speculate that the cytoplasmic tails of the CD44 molecules are involved in the *C. neoformans* cell-induced cytoskeletal rearrangements in HBMEC with the accumulation of actin at the site of yeast entry. Some types of cytoskeletal activities in infected HBMEC may be induced through CD44 signalling, resulting in utilization of the actin network to pull the yeast into the cytoplasm. However, the molecular mechanism(s) of this process remains to be demonstrated. It has been documented that the intracellular domain of CD44 may bind to several cytoskeletal linker proteins such as ERM and N-WASP (Bourguignon et al., 2007). CD44 also interacts with other proteins, such as ankyrin, in transformed prostate tumors (Zhu and Bourguignon, 1998) and Her2 in breast and ovarian cancer cells (Bourguignon et al., 1997). The interactions eliciting intracellular signalling may be varied in different cell type restricted. In any case, CD44, as a potential host receptor for *C. neoformans*, provides an avenue for further studies of the mechanisms involved in the infection of HBMEC. We are currently attempting to determine the relaying signals that induce reorganization of membrane rafts and actin bundle formation at the yeast entry site.

**Experimental procedures**

**Strains, media and cultures**

*Cryptococcus neoformans* strains B-4500FO2 and C559 are isogenic strains with wild type and CPS1-deleted gene respectively, and strain C588 is the CPS1-complemented strain (Chang et al., 2006; Jong et al., 2007). Strain B-3501 is a highly encapsulated strain (Chen et al., 2003). Strain 602 is a cap64 mutant strain, and strain TYCC38-602 is an encapsulated transformant of 602 with wild-type CAP64 gene (Chang et al., 1997). Strain TYCC33 is generated by gene deletion of CAP59 and is an isogenic strain of B-4476FO5 (CAP59). Yeast cells were grown aerobically at 30°C in 1% yeast extract, 2% peptone and 2% dextrose (YPD broth) or Sabouraud medium (Difco Laboratories, Detroit, MI). Cells were harvested at early log phase, washed with phosphate-buffered saline (PBS) and resuspended in Hams-F12/M199 (1:1, v : v), 5% heat-inactivated fetal bovine serum (experimental medium) and 1% human serum. The *Cryptococcus* cell number was determined by direct counting from a hemocytometer (Wang et al., 1999).

**In vitro association assay with HBMEC**

The in vitro association assay of *C. neoformans* to HBMEC was previously described (Chen et al., 2003; Chang et al., 2006). Briefly, HBMEC were grown until confluence in collagen-coated 24-well tissue culture plates (Costar, Corning, New York). An inoculum of 10⁶ yeast cells suspended in 0.5 ml of medium was added to the HBMEC monolayer (multiplicity of infection of 10) for 2 h at 37°C. Unattached yeast cells were then removed from the HBMEC monolayer by washing with medium four times. HBMEC were lysed with 0.5% Triton, diluted and plated onto blood or YEPD agar plates to determine the colony forming units that had been associated with HBMEC. Results are presented as the per cent adhesion of inoculum: [(number of *Cryptococcus* recovered)/(number of *Cryptococcus* inoculated)] × 100%. The association assay for each experiment was usually reproduced three to five times. A representative result was shown in the figures. For HA-coated bead study, the microsphere beads (F8823) were physically associated with HA molecules according to the manufacturer’s instruction (Molecular Probes, Eugene, Oregon). Briefly, an equal volume of bead solution (2% solid solution) and HA (0.01 mg ml⁻¹) was mixed overnight with rocking. The resulting mixture was washed with PBS three times and then resuspended into the original volume of PBS. For the association assay, five microlitres of coated (or uncoated) microsphere beads was usually used for each well of the chamber slides.
CD44 knock-down experiments

Human CD44 cDNA sequence (GenBank NM_000610) shows a ~4 kb mRNA (Fig. 3A). The CD44 shRNA kit of MISSION TRC-Hs 1.0 (Human) was purchased from Sigma Chem. It includes five CD44 small hairpin fragments (TRCN0000057563 to TRCN0000057567) cloned into the lentiviruses expression vector pKLO.1-puro. We assigned the clone number from #63 to #67 in our studies. The hairpin sequences were comprised of a 21-base pKLO.1-puro. We assigned the clone number from #63 to #67 in our studies. The hairpin sequences were comprised of a 21-base and 6-base loop, and the sequences are listed as below. They are marked in Fig. 3A.

#63: CCGGGGCTATTAGTTCTCAACTGAGTTTGGAATACTAATAGGGCTTTTTG
#64: CCGGGCTCCAGATGACATATCTCTGAGAATATGTGTCATACGAGGAGTTTTTG
#65: CCGGCGGAAGTGCTACTTCAAGACAACTCGAGTTGTCTGAAAGTAGCACTTCCGTTTTTG
#66: CCGGCGAACTCTAATGTCAATCGTTCTCGAGAACGATTGAAGTAGCACTTCCGTTTTTG
#67: CCGGCCAATCTAATGTTCTCTGAGAAGACGATTGACCATTAGGTTGGTTTTTG

The vector alone and the one with an insert containing GFP were used as the controls. Experiments were performed according to the manufacturer's instructions. Briefly, 10 μg of purified plasmid was transfected into HBMEC in a T75 flask at ~80% confluence. After 2 days, transfected HBMEC were cultured in the presence of puromycin (1 μg/ml). After two more days, the cells were harvested, ready for protein blots to examine the CD44 protein level (Fig. 3B). In parallel, another set of transfected HBMEC was subjected to the C. neoformans in vitro association assay (Fig. 3C).

Immunofluorescence microscopy

Samples for immunofluorescence microscopy were prepared as follows. HBMEC were plated onto glass coverslips (22 mm, square), which had been previously coated with type I collagen from rat tail (Upstate, 5–10 μg/cm²) in an 8-well square culture system (Nalgen Nunc). HBMEC (~5 x 10⁴ cells) were seeded onto one coverslip 24 h prior to the experiment. HBMEC were pre-washed four times with PBS, then fixed with 4% paraformaldehyde/PBS (v:v) for 30 min at room temperature. After an additional three washes with PBS, the HBMEC were blocked with 5% milk/PBS for 30 min and then incubated with anti-CD44 monoclonal antibody (Santa Cruz, 1:500 dilution) at 4°C overnight. The coverslips were then washed four times with PBS, and PI (1 μg/ml) (Sigma Chemical, St. Louis, MO) in 1% BSA/PBS and/or anti-mouse IgG FITC conjugate (1:100 dilution) was added into each well for 1 h at 4°C. Another three washes were applied before sealing the coverslips onto slides. C. neoformans cells were stained with the fluorescent dye calcoflour white and displayed as red pseudo-colour in this study (Figs 6 and 8C). Samples were examined under a fluorescence microscope at the Congressman Dixon Cellular Imaging Core Facility, Children’s Hospital Los Angeles.

Preparation of membrane lipid rafts or microdomains from HBMEC

The Caveolae/Rafts Isolation kit was purchased from Sigma Chemical (cat # CS0750). For each sample, HBMEC was seeded and grown in 60 mm Petri dish for 2 days. On the day of experiment, the cells were individually incubated with either medium (control), 3 x 10⁻⁶C. neoformans B-4500FO2 or C559, or filipin (0.5 μM) individually for 3 h in the experimental medium with 1% human serum. After incubation, the cells were washed with PBS three times, scraped in PBS and spun down at 2000 r.p.m. at 4°C. C. neoformans cells were lysed in 200 μl of TN solution (25 mM Tris-HCl, 7.5, 1 mM DTT, cocktail of protease inhibitors, 10% sucrose and 1% Triton-100) on ice and incubated for 30 min on ice. Samples were mixed with 1.16 ml of cold Optiprep, transferred into SW40 centrifuge tubes and overlaid with 2 ml each of 40%, 30%, 25%, 20% and 0% Optiprep in TN. The gradients were spun at 35 000 r.p.m. in an SW40 rotor for 5 h at 4°C. Nine fractions (0.5 ml) were collected from the top to the bottom of the centrifuge tubes. Forty microlitres of each fraction was used for dot blots, or 30 μl was used for protein blots. The antibody dilution was chosen as follows: caveolin (BD610059, 1:1000 dilution), CD44 (Santa Cruz, sc-7964, 1:300 dilution), actin (Santa Cruz, sc-8432, 1:400 dilution), profilin-1 (Cell Signaling, #3237, 1:500 dilution) and anti-rabbit-HRP conjugate (1:2000 dilution). For subsequent probings, the membrane was stripped with 62.5 mM Tris, pH 6.8, 20% SDS, 100 mM β-mercaptoethanol at 70°C for 30 min, then washed with PBS/0.05% Triton three times before probing with another antibody.

Statistical analysis

The statistical analysis of the data from our in vitro studies involved analysis of variance (ANOVA). The dependent variable was the associated per cent of cells or colony-forming unit while the independent fixed factors were the treatments (CAP versus cap mutant, or CPS1 isogenic strains, etc.). Raw data were entered into EXCEL files and automatically converted to statistical packages. ANOVA and covariates were followed by a multiple comparison test, such as the Newmann–Keuls test, to determine the statistical significance between the control and treatment groups. P < 0.05 was considered to be significant.

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