Identification and Characterization of CPS1 as a Hyaluronic Acid Synthase Contributing to the Pathogenesis of Cryptococcus neoformans Infection

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Received 11 April 2007/Accepted 21 May 2007

Cryptococcus neoformans is a pathogenic yeast that often causes devastating meningoencephalitis in immunocompromised individuals. We have previously identified the C. neoformans CPS1 gene, which is required for a capsular layer on the outer cell wall. In this report, we investigate the function of the CPS1 gene and its pathogenesis. We demonstrated that treatment of yeast with either 4-methylumbelliferone or hyaluronidase resulted in a reduction of the level of C. neoformans binding to human brain microvascular endothelial cells (HBMEC). Yeast extracellular structures were also altered accordingly in hyaluronidase-treated cells. Furthermore, observation of yeast strains with different hyaluronic acid contents showed that the ability to bind to HBMEC is proportional to the hyaluronic acid content. A killing assay with Caenorhabditis elegans demonstrated that the CPS1 wild-type strain is more virulent than the cps1Δ strain. When CPS1 is expressed in Saccharomyces cerevisiae and Escherichia coli, hyaluronic acid can be detected in the cells. Additionally, we determined by fluorophore-assisted carbohydrate electrophoretic analysis that hyaluronic acid is a component of the C. neoformans capsule. The size of hyaluronic acid molecules is evaluated by gel filtration and transmission electron microscopy studies. Together, our results support that C. neoformans CPS1 encodes hyaluronic acid synthase and that its product, hyaluronic acid, plays a role as an adhesion molecule during the association of endothelial cells with yeast.

Cryptococcus neoformans is an encapsulated pathogenic fungus that primarily afflicts immunocompromised individuals. Patients with T-cell deficiencies, including AIDS, lymphoma, corticosteroid therapy, and idiopathic CD4 lymphocytopenia, are the most susceptible to C. neoformans infection. Rates of infection by C. neoformans have increased considerably over the past few years (31, 32). The increasingly expanded use of immunosuppressive drugs and the onset of AIDS have contributed to this epidemic. The organisms show a remarkable propensity to spread to the brain and meninges, where meningocellitis develops (19, 22). C. neoformans meningocellitis causes extremely high mortality and morbidity in patients without adequate T-cell-dependent immune function. The existing arsenal of antifungal agents is limited. Toxic side effects and the emergence of drug resistance are significant impediments to effective therapy. In order to cause meningitis, C. neoformans must penetrate the blood-brain barrier (BBB) (13, 24). The BBB is a barrier between blood circulation and the brain parenchyma and consists mainly of specialized capillary endothelium, comprising brain microvascular endothelial cells (BMEC) (22, 35). It is characterized by the presence of tight junctions between cells and extremely low phagocytosis. It is responsible for maintaining the biochemical homeostasis within the central nervous system. The underlying mechanism(s) whereby C. neoformans crosses the BBB is largely unknown. We have previously studied the invasion of C. neoformans with human BMEC (HBMEC) by using in vitro binding and transcytosis assays (13). C. neoformans induces considerable morphological changes and actin reorganization in HBMEC through the phosphorylation regulation of coflin, LIMK, and ROCK-linked pathways. The yeast cells were able to traverse across the BBB via a transcellular pathway through HBMEC (12).

The major virulence factors of C. neoformans are the polysaccharide capsular components (1, 3, 8, 21, 31). The main component of the C. neoformans polysaccharide capsule is glucuronoxylomanann; there are also minor components (galactoxylomannan, mannoproteins), and other polysaccharide derivatives (14). The capsule has the ability to exert a broad spectrum of influences on the immune response. Acapsular mutants are typically avirulent, whereas encapsulated isolates have differing degrees of virulence (21). Some capsule-associated genes, such as CAP10 (10), CAP59 (8), CAP60 (9), and CAP64 (11), have been identified. CAP64 was found to have six homologues in the C. neoformans genome that play an important role in determining the position and linkage of xylose and/or O-acetyl residues on the mannose backbone of the glucuronoxylomanann. CAP59 and CAP60 have a putative...
transmembrane domain, and CAP60 protein is localized primarily to the nuclear membrane. The functions of these genes are currently unknown. It seems that any genes directly or indirectly associated with capsule formation are crucial for *C. neoformans* virulence. It is also possible that an intact capsule structure may retain an essential virulence component(s) that is required for adhesion and/or invasion. In addition to these capsule genes, several genes involved in the biosynthesis of a polysaccharide capsule precursor and the process of polymerization have been characterized, such as UDP-glucuronic acid (GlcUA) decarboxylase (2, 27), α-1,3-mannosyltransferase (39), and phosphomannomerase isomerase (45).

A missing link here is whether there is any capsular component playing a role as a ligand to adhere or bind to the host cells to precipitate the invasion process.

We have previously identified and investigated a *C. neoformans* gene, CPS1 (7). Deletion of CPS1 from *C. neoformans* resulted in alterations of several phenotypes important for the virulence of *C. neoformans*, including modification of the ultrastructure between the cell wall and the capsule, temperature sensitivity, and a reduced ability to associate with HMBEC in vitro. These results suggest that CPS1 plays an important role in the pathobiology of *C. neoformans*. The CPS1 gene shares similarity with the type 3 polysaccharide synthase gene, CAP3B, of *Streptococcus pneumoniae* (7). CAP3B is essential for the capsular lipopolysaccharide synthesis as well as for the virulence of *S. pneumoniae*. The type 3 synthase from *S. pneumoniae* belongs to the β-glycosyltransferase superfamily, including the hyaluronic acid synthases from prokaryotes and eukaryotes, the cellulose synthases from plants and bacteria, the chitin synthases from yeast, and the Nod factor synthases from *Rhizobium* (18, 26).

We have demonstrated that the yeast wild-type CPS1 strain contains hyaluronic acid molecules, while the cpsiΔ strain does not (7). Hyaluronic acid is a linear unbranched molecule with repeating disaccharide units of GlcUA (β1→3) and GlcNAc (β1→4). The molecular size of hyaluronic acid is usually between 1 and 10 million Da. Whether the hyaluronic acid molecule plays any role during *C. neoformans* infection remains to be demonstrated. In this report, we provide several lines of evidence to demonstrate that CPS1 encodes a hyaluronic acid synthase. It synthesizes cell surface hyaluronic acid, which is important for yeast infection in vitro and in vivo. Its role in the pathogenesis of cryptococcal central nervous system infection is discussed.

**MATERIALS AND METHODS**

**Strains, media, and cultures.** *C. neoformans* strains have been described in detail previously (7, 13). Briefly, strains B-4500FO2 and C559 are isogenic strains with the wild-type CPS1 gene and a deleted cpsiΔ gene, respectively. Strain C588 is C559 transformed with pYCC662; the CPS1 gene was cloned into the pCPS vector. Strain 602 is a cap60 mutant strain, and strain TYCC68-602 is an encapsulated transformant of 602 with the wild-type CAP64 gene. Strain TYCC63 is generated by deletion of the CAP59 gene and is an isogenic strain of B4476FO5 (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 Ham's F-12 medium–M199 (1:1, vol/vol), 5% heat-inactivated fetal bovine serum (experimental medium), phosphate-buffered saline (PBS), and resuspended in Ham's F-12 medium–M199.

**CPS1** generated by deletion of the CAP59 gene and is an isogenic strain of B4476FO5 (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 Ham's F-12 medium–M199 (1:1, vol/vol), 5% heat inactivated fetal bovine serum (experimental medium), and 1% human serum. Cryptococcus yeast cell numbers were determined by direct counting using a hemocytometer. *Saccharomyces cerevisiae* strain BJ2168 (a prel-107 prhl-1122 pep7-3 las21 tor1a2-52) (42) and *Escherichia coli* strain BL21(DE3) were used for heterologous expression of the CPS1 gene. All chemicals were ordered from Sigma Chemical Co. (St. Louis, MO), and the restriction enzymes were obtained from New England Biolabs (Beverly, MA).

**Binding of yeast cells to HMBEC.** The in vitro assay of the binding of *C. neoformans* to HMBEC has been described previously (7, 13). Briefly, HMBEC were grown until confluence in collagen-coated 24-well tissue culture plates (Costar, Corning, NY). An inoculum of 10⁶ yeast cells suspended in 0.5 ml experimental medium was added to the HMBEC monolayer (multiplicity of infection, 10) for 2 h at 37°C. Yeast cells unattached to the HMBEC monolayer were removed by washing the monolayer four times with experimental medium. HMBEC were lysed with 0.5% Triton, diluted, and plated onto a blood or YPD agar plate to determine the number of CFU that were associated with HMBEC. Results are presented as percentages of the level of adhesion of the inoculum, calculated as [(number of Cryptococcus cells recovered)/(number of Cryptococcus cells inoculated)] × 100.

**Hyaluronidase treatment of Cryptococcus.** Yeast cells were grown in a rich medium (YPD) at 30°C. Fresh overnight cultures (1 ml) were washed with 30 mM potassium acetate (pH 5.5) once, then resuspended in 100 µl potassium acetate buffer with 10 µl hyaluronidase, and shaken at 30°C for 1 h. After enzymatic treatment, the cells were washed with PBS three times. These cells were used for our in vitro HMBEC binding assay or for transmission electron microscopy (TEM) study.

**TEM.** *C. neoformans* cells were fixed in buffer P (0.2 M sodium phosphate, pH 7.2) with 2% pararormaldehyde and 2% glutaraldehyde for 2 h at 37°C in a vacuum oven. Cells were washed three times with buffer P and then incubated with 8 ml of 50% normal human serum in GVB buffer (5 mM sodium Veronal-buffered 142 mM saline, pH 7.3, containing 0.1% gelatin, 1 mM MgCl₂, and 0.15 mM CaCl₂) for 30 min at 37°C. Cells were then washed with buffer P five times, and 2 ml of buffer P incubated with 1/20 dilution of biotinylated hyaluronide binding protein at 0°C for 30 min. After four washes with buffer P, cells were incubated with 1 ml of a 1/50 dilution of a streptavidin-horseradish peroxidase (HRP) conjugate at 0°C for 30 min. Cells were washed three times with buffer P and incubated in a substrate solution of dianinosbenzidine (1 mg/ml) and hydrogen peroxide (0.01%) in ice-cold 0.05 M Tris, pH 7.0, for 5 min at 0°C. The cell pellet was washed with 0.144 M cacodylate buffer, pH 7.4, and resuspended in 0.01% warm gelatin (in cacodylate buffer). Solidified gelatin was cut into 1- to 2-mm blocks and sectioned (thickness, ~30 nm), and the grid was stained with 5% aqueous uranyl acetate for 15 min, followed by Satos' lead for 1 min. The resulting sections were observed and photographed with an FEI Tecnai Twin TEM.

**ELISAs for hyaluronic acid.** The hyaluronic acid enzyme-linked immunosorbent assay (ELISA) kit obtained from Corgenix, Inc. (Denver, CO), was used to assay hyaluronic acid. Yeast cells (10⁶ cells) in the exponential-growth phase were incubated in individual wells at room temperature to trap the surface polysaccharide. After 30 min, the wells were washed with washing buffer according to the manufacturer's instructions. A second solution containing a hyaluronic acid-binding protein–HRP conjugate was added to the wells. After incubation, the wells were rinsed, and a chromatographic substrate (tetramethyl benzidine/ H₂O₂) was added to develop the color reaction. The intensity of the color was measured in optical density units with a spectrophotometer at 450 nm. The concentrations of hyaluronic acid were calculated by comparing the absorbance of the sample against a reference curve prepared from the reagent blank and hyaluronic acid reference solutions.

**Expression of CPS1 in Saccharomyces cerevisiae and E. coli.** Coexpression of genes in *Saccharomyces cerevisiae* has been described previously (42). In this study, *C. neoformans* CPS1 cDNA was subcloned into plasmid YGp181-HA (selection marker, Leu2), and *C. neoformans* uridine diphosphoglucose dehydrogenase (UDPGL DH) cDNA (GenBank accession no. AF400554) was subcloned into YGp123-HA (selection marker, Trp1). Both vectors are multicopy plasmids with a hemagglutinin (HA) tag under the control of the Gal11 promoter. The yeast media YPD and CSM medium were purchased from Bio 101, Inc. The constructed plasmids were transformed, individually or together, into *S. cerevi- siae* strain BJ2168. For the expression experiments, 500 ml of cultures was induced with 2% galactose for 4 h. The cell pellets were disrupted by a Bead-Beater in PBS supplemented with 20 mM benzamidine, 20 mM phenylmethylsulfonyl fluoride, and 1 mM DNsase. The expressed HA-tagged Cps1p and UDPGH DH proteins were probed with an anti-HA antibody on protein blots. A monoclonal antibody against the HA tag was purchased from Roche Life Sciences (Indianapolis, IN).

For the expression of CPS1 in *E. coli*, a 1.35-kb CPS1 cDNA fragment was amplified from a *Z. dag* *C. neoformans* cDNA library using primers EP5 (CAA GAGTCCATGATATCCTACACGACAAAC) and EP3 (CGCAGCCT TGGCTTCTGCGCGACCTG). The DNA fragment was subcloned into the pET22b expression vector (Novagen, Madison, WI) at the NcoI and HindIII.
sites. The Cps1 protein was expressed and purified from *E. coli* strain Rosetta-gami B(DE3) (Novagen, catalog no. 71136) according to the manufacturer’s instructions. Briefly, the induced culture (250 ml) was harvested, and the cell pellet was resuspended in 10 ml buffer LY (50 mM sodium phosphate buffer [pH 7.0], 5% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 1 mM benzamidine, and 0.5% n-dodecyl-β-D-maltoside [Sigma D-4641]) and homogenized with a membrane disruptor (model 60 Sonic Disembrator; Fisher Scientific) at 15 W for 1 min in ice. The resultant supernatant was mixed with nickel-nitrilotriacetic acid resin for the purification of Cps1p as described by the manufacturer. The bound proteins were eluted with 0.2 ml PBS–20% glycerol–0.25 M imidazole. For the hyaluronic acid synthase assay, 20 μl purified proteins was added to the 100-μl reaction mixture, containing 50 mM Tris (pH 7.6), 20 mM MgCl2, 1 mM β-mercaptoethanol, 100 μM UDP-GlcUA, and 100 μM UDP-GlcNAc. The reaction proceeded at 30°C for 2 h, and results were analyzed with the hyaluronic acid ELISA kit (Corgenix, Inc., Denver, CO).

**FACE and gel filtration.** *C. neoformans* capsule polysaccharides were fractionated prior to fluorophore-assisted carbohydrate electrophoresis (FACE) analysis. Briefly, the growth of a *C. neoformans* culture is terminated by adding 5% trichloroacetic acid (TCA). This step can effectively separate capsule polysaccharides from cell debris (based on reference 2a). The supernatant is filtered through a 0.2-μm-pore-size filter and then mixed with 3 volumes of ethanol to precipitate the polysaccharides. The resulting crude preparation is resuspended in PBS and subjected to Dowex 1 (2X) chromatography (37). After application of the sample, elution is carried out using a 0.1 to 2.0 M gradient of NaCl. Based on the uronic acid fractions, the elution eluting at 0.5 to 0.65 M NaCl are first collected (6) and then subjected to FACE analysis, as described previously (33). For size fractionation, 1 ml of each Dowex 1 (2X)-purified fraction was applied to a Bio-Rad Bio-gel SEC-1000 high-performance liquid chromatography (HPLC) column with a flow rate of 1 ml/s PBS. The elution was monitored with a model RI-403 differential refractometer, showing negative peaks, and 1 ml per fraction was collected. Some fractions were used for a hyaluronic acid ELISA (see Fig. 7B, upper panel).

**Caenorhabditis elegans** killing assays. Cryptococcal yeasts were grown as a lawn for *C. elegans* killing assays (28). In brief, *C. neoformans* strains (B-4500FO2 and C559) were inoculated into 2 ml of YPD medium and grown at 25°C for 48 h. Cells from each culture were counted and adjusted to 106 cells per ml. Thirty microliters of the culture was spread on NGM (nematode growth medium, containing NaCl, agar, peptone, cholesterol, CaCl2, MgSO4, potassium phosphate, and 75 μg/ml carbencillin to prevent bacterial growth) agar in 35-mm-diameter tissue culture plates. The plates were incubated at 25°C overnight. Worms from the wild-type *C. elegans* strain N2 were transferred to the yeast plates. These worms were reared on *E. coli* OP50 on NGM until they were at the L4 developmental stage, and then they were moved to the yeast plates at a density of 10 to 15 nematodes per plate. At transfer, worms were dripped in an ampicillin solution (100 μg/ml) to prevent bacterial growth carried over from the *E. coli* OP50 plates. The experimental plates and a set of control plates on which the worms ate *E. coli* OP50 were incubated at 20°C and examined for viability at 24-h intervals under a stereomicroscope. The experimental N2 worms were fertile, but their hatching progeny were unable to eat *C. neoformans* and thus did not grow. The control worms fed OP50 required frequent transfer to fresh plates during their fertile period to remove them from their growing progeny. The mean life span of *E. coli* OP50 was about 18 days under our experimental conditions. The nematodes were considered dead when they did not respond to touch with a platinum wire pick. The percentage of worms that were still alive was plotted against time by using SPSS (Chicago, IL) 11.0 statistical software. Statistical analysis. The basic method of statistical analysis of the data from our in vitro studies involved analysis of variance, as well as analysis of covariance when there was a continuous covariate such as time or titration. The dependent variable was the percentage of adherent cells or CFU, while the independent fixed factor was the treatment (CAP versus cap mutant, or CPS1 isogenic strains, etc.). In addition, the experiment number was used as a blocking factor. When the covariant variable had several levels, we made a multiple-comparison adjustment. Transformation of the dependent variable was considered, if necessary, to stabilize variance. Raw data were entered into Excel files and automatically converted to statistical packages. Analysis of variance and covariates, followed by a multiple-comparison test such as the Newman-Keuls test, was used to determine the statistical significance of differences between the control and treatment groups. A P value of <0.05 is considered to be significant.

**RESULTS**

**Effect of 4-MU on *C. neoformans* adhesion.** We previously developed an in vitro *C. neoformans* binding assay that detects both adherent and invading yeast cells in HBMEC (7, 13). In order to test whether any hyaluronic acid is synthesized in *C. neoformans* and whether there is a role for hyaluronic acid in *C. neoformans* binding, we examined the effect of 4-methylumbelliferone (4-MU) on the binding of *C. neoformans* to HBMEC. 4-MU is a reagent that can extract cardiolipin from hyaluronic acid synthase complex, leading to the loss of hyaluronic acid synthase activity (41). It has been demonstrated that hyaluronic acid production in *Streptococcus* can be blocked by 4-MU without affecting bacterial growth (up to 2 mM) (25). Initially, we did a control experiment to ensure that there was no detectable effect on *C. neoformans* growth (up to 6 mM 4-MU) (data not shown). We then examined the effect of 4-MU on *C. neoformans* association by using our in vitro binding assay. A range of concentrations of 4-MU was added to examine the consequences of the treatments. As shown in Fig. 1, the binding activity of strain B-4500FO2 (wild-type CPS1) decreased as the 4-MU concentration increased; presumably, blockage by 4-MU of the formation of hyaluronic acid resulted in a decrease in the ability to bind to HBMEC. In parallel, the binding ability of strain C559 (cps1Δ) was low, but did not differ, at all 4-MU concentrations. The results suggest that there is an intimate correlation between the hyaluronic acid content and *C. neoformans* binding to HBMEC in strain B-4500FO2 but no difference in strain C559.

**Effect of hyaluronidase treatment on the in vitro binding of *C. neoformans* to HBMEC.** To further test whether hyaluronic acid was involved in *C. neoformans* adhesion, we used a specific enzyme, hyaluronidase, to treat different *C. neoformans* strains. Treated yeast cells were washed extensively with PBS to remove the enzyme prior to the in vitro binding assay. The control, without hyaluronidase treatment, was assigned a value of 100% binding for each individual *C. neoformans* strain. The results showed that, regardless of the strain, the binding activity for HBMEC decreased in a dose-dependent manner (Fig. 2). No effect could be observed when chitinase or heparinase was used (data not shown). Thus, the results suggest that the

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**FIG. 1.** Effect of 4-MU on *C. neoformans* binding activity to HBMEC. An in vitro binding assay was performed and analyzed as described in Materials and Methods. A range of concentrations of 4-MU (0, 0.5, 1.0, and 2.0 mM) was used to treat *C. neoformans* B-4500FO2 (solid bars) or C559 (open bars) cells for 2 h; then cells were washed with PBS three times prior to the in vitro binding assay. Statistical analysis, using linear regression, shows P values of 0.01 and 0.004 in comparison to the 0 mM group for strains B-4500FO2 and C559, respectively.
binding of *C. neoformans* to HBMEC is a hyaluronic acid-dependent process.

**Detection of hyaluronic acid from various *C. neoformans* strains.** Based on the hyaluronic acid ELISAs (Corgenix, Inc.), we have previously demonstrated that the yeast cells of the wild-type strain contain hyaluronic acid molecules, while a *cps1Δ* strain does not (7). To further explore the role of hyaluronic acid in the binding of *C. neoformans* cells to HBMEC, we examined several isogenic *CAP* versus *capΔ* strains, in addition to *CPS1* wild-type and disrupted strains. *CPS1* wild-type strain B-4500FO2, used as a control, contains a much stronger hyaluronic acid signal than the deletion strain C559 (*cps1Δ*) (Fig. 3A, lane 1 versus lane 2). The abilities of these strains to bind to HBMEC are also proportional to their hyaluronic acid contents (Fig. 3B, lane 1 versus lane 2). Encapsulated strains (*CAP64* and *CAP59*) always have a higher binding activity (Fig. 3B, lanes 3 and 5) than their isogenic mutants (Fig. 3B, lane 4 and 6). Interestingly, they also have higher hyaluronic acid contents (Fig. 3A, lanes 3 and 5) than their isogenic *cap* mutants (Fig. 3A, lanes 4 and 6). The results indicate that the HBMEC binding activities are proportional to the hyaluronic acid contents of different strains. This raises the interesting point that acapsular mutant strains have an incomplete capsule structure, which may fail to retain hyaluronic acid molecules on their surfaces, resulting in a decreased ability to bind to HBMEC. The hyaluronic acid ELISA kit used here does not detect other glucosaminoglycans, such as chitin, heparin, and chondroitin sulfate (data not shown). It is clear that C559 (*cps1Δ*) has the weakest hyaluronic acid signal of all the strains used. In addition, the *CPS1* protein sequence shares the highest homology to known hyaluronic acid synthases (see Discussion). We concentrated on the *CPS1* gene in subsequent experiments.

**Pathogenesis of *CPS1* by a *C. elegans* longevity assay.** C559 (*cps1Δ*) is a temperature-sensitive mutant strain. It does not grow well at 37°C, preventing study of its pathogenesis in a mouse model. On the other hand, an in vivo pathogenesis assay for *C. neoformans* has been established, based on the yeast killing rate on *C. elegans* (28). This assay is particularly useful for the temperature-sensitive C559 (*cps1Δ*) study, because *C. elegans* grows and multiplies at room temperature. Here worms were exposed to the yeast strains after they had reached the L4 developmental stage, and their longevity reflects, in part, the virulence of the yeast strains. The survival curves plotted in Fig. 4 show that the nematodes had a longer life span when feeding on *C. neoformans* *cps1Δ* lawn cells. The 50% lethal time (time for half of the worms to die) was calculated with SPSS 11.0 statistical software. The average life span is greater when worms are exposed to C559 (*cps1Δ*) (~12.8 days) than when they are exposed to B-4500FO2 (wild-type *CPS1*) (~8.8 days) (*P* < 0.026). Our results demonstrate that *CPS1* deletion decreases the virulence of yeast in the *C. elegans* animal model (Fig. 4).

**Expression of *CPS1* in *Saccharomyces cerevisiae*.** In order to demonstrate the *CPS1* gene function, we subcloned and expressed *C. neoformans* *CPS1* cDNA in *S. cerevisiae*. Hyaluronic acid biosynthesis requires hyaluronic acid synthase (presumably Cps1p) and two precursor enzymes, UDPG DH and glucosamine-6-phosphate acetyltransferase, to provide the sub-

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FIG. 3. *C. neoformans* ability to bind to HBMEC and the relationship to their hyaluronic acid contents. (A) Hyaluronic acid ELISA. The hyaluronic acid molecules from the surfaces of *C. neoformans* cells were detected using a hyaluronic acid ELISA kit (Corgenix, Inc.). (B) An in vitro binding assay was performed to examine the ability of *C. neoformans* cells to associate with HBMEC. The CFU of *C. neoformans* cells binding to HBMEC in vitro was analyzed. Columns in both panels: 1, strain B-4500FO2 (wild-type *CPS1*); 2, strain C559 (*cps1Δ*); 3, strain TYCC38-602 (wild-type *CAP64*); 4, strain 602 (*cap64*); 5, strain B4476FO5 (wild-type *CAP59*); 6, strain TYCC33 (*cap59Δ*).
C. neoformans UDPG DH and CPS1 (lane b), and both (lane c) from respectively. The cloned cDNA was induced by galactose. (A) Protein blots show the expected HA-tagged proteins: expressed UDPG DH (lane a), Cps1p (lane b), and both (lane c) from S. cerevisiae strains. The baker’s yeast S. cerevisiae contains only the gene encoding glucosamine-6-phosphate acetyltransferase. We therefore coexpressed the genes encoding UDPG DH and Cps1p in baker’s yeast to build the C. neoformans hyaluronic acid biosynthesis pathway.

The cDNAs of CPS1 and UDPGDH were isolated and subcloned into the yeast expression vectors YGp181 (selection marker LEU2) and YGp123 (selection marker Try1) to obtain YGp181-CPS1 and YGp123-UDPG.DH, respectively. These vectors are all under the control of a strong Gal110 inducible promoter. The expressed proteins were detected by an anti-HA antibody on the protein blot (Fig. 5A). The result showed that C. neoformans UDPGDH, CPS1, and both genes together could be expressed in S. cerevisiae strain B2168, a protease-deficient strain (42). Hyaluronic acid synthase is a membrane protein, and its product, hyaluronic acid, extrudes outside the plasma membrane during hyaluronic acid biosynthesis. We detected the hyaluronic acid contents on the surfaces of S. cerevisiae cells expressing C. neoformans CPS1 by using the hyaluronic acid ELISA kit. Our results showed that only coexpression of CPS1 and UDPGDH in S. cerevisiae displayed a high level of hyaluronic acid (Fig. 5B). The result strongly supported the hypothesis that the CPS1 gene encodes hyaluronic acid synthase.

Characterization of hyaluronic acid synthase activities from E. coli-purified Cps1p. The Streptococcus HAS gene has been expressed in E. coli with hyaluronic acid synthase activity. The C-terminal His6 tag of the recombinant hyaluronic acid synthase protein does not affect its enzymatic activity (40). We used a similar expression system (the pET22b vector) to express C. neoformans CPS1 cDNA with a His6 tag to facilitate its purification. In parallel, E. coli cells with the pET22b vector only were used as a control. The expressed cells were lysed, extracted with n-dodecyl β-maltoside (0.5%), and subjected to nickel-nitrilotriacetic acid affinity purification. The purified fraction was detected with an anti-His6 monoclonal antibody. As shown in Fig. 6A, a single band of ~55 kDa was found in CPS1-expressing cells but was absent in the control. The affinity-purified Cps1p–His6 fraction was subjected to a hyaluronic acid synthase enzymatic assay using UDP-GlcUA and UDP-GlcNAc as the substrates. After a 2-h reaction, the synthesized molecule was measured with the hyaluronic acid ELISA kit (Corgenix, Inc.). The results in Fig. 6B show that E. coli-purified Cps1p was able to synthesize hyaluronic acid in the presence of substrates (lane 1) and that the reaction could be enhanced in the presence of cardiolipin, which is known as a cofactor of hyaluronic acid synthase (lane 5). Only background readings were observed under control conditions, such as the use of one substrate only (Fig. 6B, lanes 2 and 3), the use of EDTA to chelate magnesium ions (lane 4), hyaluronidase treatment (lane 6), and the control derived from extracts containing vector only without the CPS1 gene (lane 7). Thus, E. coli-expressed Cps1p possesses hyaluronic acid synthase activity.

Identification of C. neoformans hyaluronic acid by FACE analysis. One fundamental question here is whether hyaluronic acid is present in C. neoformans. In addition to the hyaluronic acid ELISA kit (Corgenix, Inc.), an alternative hyaluronic acid detection method was used to confirm our observation. To identify hyaluronic acid molecules among the bulky capsule polysaccharides is a challenging task. However, a recently developed advanced methodology, FACE (6, 33), allowed us to detect hyaluronic acid in a rather straightforward way.

FIG. 4. C. neoformans pathogenesis assay using a C. elegans in vivo animal model. Survivorship curves of nematodes in the presence of yeast strains B-4500FO2 (■) and C559 (▲).

FIG. 5. Expression of C. neoformans UDPG DH and CPS1 cDNA in S. cerevisiae and detection of their ability to synthesize hyaluronic acid. C. neoformans UDPG DH and CPS1 cDNA were subcloned into the S. cerevisiae expression vectors YGp181 and YGp123 with an HA tag, respectively. The cloned cDNA was induced by galactose. (A) Protein blots show the expected HA-tagged proteins: expressed UDPG DH (lane a), Cps1p (lane b), and both (lane c) from S. cerevisiae. The expressed proteins were detected by an antibody against the HA tag. (B) The hyaluronic acid contents from induced yeast cells were subjected to the hyaluronic acid assay (Corgenix hyaluronic acid kits). Lane 1, buffer only; lane 2, standard control with 50 ng hyaluronic acid (no yeast); lane 3, hyaluronic acid contents from coexpression of UDPG DH and CPS1 cDNA in S. cerevisiae; lane 4, hyaluronic acid contents from CPS1 expression alone in S. cerevisiae; lane 5, expression of UDPG DH cDNA alone in S. cerevisiae. Each value is the average of triplicate experiments.
The unique feature of FACE is the use of specific enzymes to cleave glycosaminoglycan into small products, usually disaccharide units. Each cleavage exposes a new reducing terminus that is fluoro-tagged by reductive amination with 2-aminooxacridone. The tagged products are then displayed by electrophoresis, identified by their characteristic migration, and quantitated by their molar fluorescence. This is a very sensitive method allowing detection in the nanogram range. More importantly, it can leave the bulk of C. neoformans capsule polysaccharides invisible (large molecules, not fluoro-tagged) and specifically detect hyaluronic acid disaccharide units (small molecules, fluoro-tagged) when present (6). To examine C. neoformans samples, a partial purification step is necessary to remove small sugar molecules derived from the culture medium (see Materials and Methods).

The partially fractionated C. neoformans capsule polysaccharides and the pooled fractions were subjected to FACE analysis (Fig. 7A). Approximately 10 μg (wet weight) per sample from various fractions was used for each experiment. As a positive control, 5 μg pure hyaluronic acid was treated in parallel to produce the hyaluronic acid disaccharide unit (δ-diHA) as a marker (Fig. 7A, lane 1). No corresponding band could be clearly observed in a crude capsule preparation, and some nonspecific bands may be derived from reducing sugars from the culture medium (lanes 2 and 5); however, after being enriched by the anion-exchange resin, the FACE gel showed a δ-diHA band in the Dowex 1 (2X)-eluted fraction of B-4500FO2 (wild-type CPS1 strain) (lane 3). The enzyme-buffer mixture was used as a negative control (lane 4). The result demonstrated that hyaluronic acid was present in the C. neoformans capsule polysaccharide fraction. In parallel, no signal could be detected in the corresponding fractions from cps1Δ capsule polysaccharide (lanes 5 and 6).

C. neoformans capsular components were extracted from B-4500FO2 (wild-type CPS1 strain) and fractionated by HPLC gel filtration. The hyaluronic acid contents from different fractions were detected with the hyaluronic acid ELISA kit (Fig. 7B, top panel). Hyaluronic acid molecules were detected from...
several fractions. The highest molecular size (void volume) is around $10^7$ Da, corresponding to $\sim 30,000$ disaccharide subunits of approximately 3 μm. However, this high-molecular-weight fraction is most likely derived from hyaluronic acid complex, in which hyaluronic acid molecules are associated with each other or with other components, instead of a single long molecule (see Fig. 8). The peak of the hyaluronic acid signal was in the range of $10^5$ Da, corresponding to $\sim 300$ disaccharide subunits (approximately 30 nm long). Little hyaluronic acid (or a background reading) was detected below the size of $10^5$ Da (Fig. 7B).

**Staining of hyaluronic acid fibrous structures outside the C. neoformans cell wall.** The CPS1 gene is predicted to encode a hyaluronic acid synthase in *C. neoformans*, as indicated in Fig. 5 and 6. Since hyaluronic acid from other species is accumulated extracellularly, it would be of interest to locate hyaluronic acid on *C. neoformans* cells. We therefore used biotinylated hyaluronic acid-binding protein to locate hyaluronic acid molecules and then probed with a streptavidin-HRP conjugate. The typical morphology of the wild-type CPS1 strain (B-4500FO2), with numerous fibrous structures radiating from the cell wall, is displayed in Fig. 8A. However, no such structure

FIG. 8. Staining of hyaluronic acid fibrous structures outside the *C. neoformans* cell wall. Yeast strain B-4500FO2 (wild-type CPS1) and its isogenic cps1Δ strain (C559) were probed with biotinylated hyaluronic acid-binding protein and a streptavidin-HRP conjugate. Treated samples were subjected to TEM studies to determine their morphological features. (A) Strain B-4500FO2 shows fibrous structures radiating from the cell wall. Bar, 100 nm. (B) Strain C559 shows only a light patch background near the cell wall (no fibrous structure). Bar, 100 nm. (C) Strain B-4500FO2 cells show a high-density fibrous structure between cells. Bar, 1 μm. (D) Higher magnification of boxed area in panel C. (E) In contrast, strain C559 cells do not show any fibrous structure. (F) Higher magnification of boxed area in panel E.
was observed adjacent to the walls of the cpsiΔ cells (Fig. 8B). Neither the HRP conjugate nor biotinylated marker proteins alone showed any nonspecific staining on HBMEC in our control experiments (data not shown). More TEM images were used to further illustrate the differences between the wild-type CPS1 and cpsiΔ cells. In Fig. 8C, one can observe fibrous structures, mostly 300 to 500 nm long, surrounding the wild-type CPS1 cells. Figure 8D, a higher magnification of part of Fig. 8C, shows the area between the capsules of two adjacent yeast cells, presented as interlocking fiber structures. In limited regions, the hyaluronic acid molecules can extend longer than 1 μm, corresponding to ~1,000 disaccharide units. In parallel, no obvious fibrous structure was observed in the cpsiΔ strain (Fig. 8E and F); only some lightly shadowed background (presumably other capsular components) appeared in the corresponding regions. These results (Fig. 8), together with the result presented in Fig. 3, allow us to conclude that C. neoformans hyaluronic acid, presumably the product synthesized by CPS1, extends from the cell wall to the external part of the yeast cells. The presence of hyaluronic acid, therefore, appears to play a major role in the binding of C. neoformans yeast cells to HBMEC in vitro (Fig. 1, 2, and 3) and is important for virulence in C. elegans (Fig. 4).

**DISCUSSION**

Capsular polysaccharides are a major virulence factor of *C. neoformans*. The genetic approach to the isolation and characterization of capsule-deficient strains revealed several important genes in capsule biosynthetic pathways, encoding either precursors or molecules associated with the transport of essential components. However, it is still not clear whether there is any capsule component on the yeast cell surface that plays a role as an adhesion molecule during invasion. One direct way to identify potential adhesion molecules would be to search for candidate genes based on their sequences from genomic databases. This can be verified by deleting the candidate genes and examining the phenotypic consequences of the deletions. *C. neoformans* CPS1 was the one gene identified through this reverse genetic approach (7).

The *C. neoformans* Cps1 protein was homologous to *Streptococcus pneumoniae* type 3 β-glycosyltransferase CAP3B (39.4% similarity), and the two proteins displayed similar patterns in the hydrophilicity plot. The type 3 synthase from *S. pneumoniae* is a β-glycosyltransferase that functions in the hydrophilicity plot. The type 3 synthase from *S. pneumoniae* is a β-glycosyltransferase that functions in the hydrophilicity plot of the type 3 polysaccharide [3]-β-D-glucuronic acid-(1→4)-β-D-glucose-(1→3) (7). A further database search using NCBI BLAST alignment showed that the Cps1 protein sequence aligned with COG1215, a group of β-glycosyltransferases. The first 50 highest homologies are summarized in Fig. 9A in accordance with their annotated functions (36 proteins). The hypothetical proteins, or proteins with unknown function, were not included (14 proteins). Of the proteins with annotated functions, 16 out of 36 (or 44%) are hyaluronic acid synthases (Fig. 9A). Note that both hyaluronic acid synthase and chitin synthase are N-acetylglucosamine transferases, because they use N-acetylglucosamine as the substrate, and that β-glicosyltransferase is a generic term that includes all of these enzymes. The sequence alignments have identified invariant residues within the conserved multiple transmembrane domains and the predicted intracellular loop, the putative binding site of hyaluronic acid synthase (Fig. 9B and C). These motifs are the structural features of the hyaluronic acid synthase proteins (23, 40, 43). In contrast, multiple transmembrane domains are usually not found in chitin synthase. In general, the hyaluronic acid synthase family contains six transmembrane domains, and the C-terminal transmembrane domain is less conserved. Indeed, *C. neoformans* Cps1 protein also contains five conserved transmembrane domains, and the sixth one, at the C-terminal end, is less conserved (Fig. 9C) (43). Finally, the amino acid sequence of the encoded Cps1

**FIG. 9.** Molecular features of the *Cryptococcus CPS1* gene, encoding a putative hyaluronic acid synthase. (A) The number of homologues to *CPS1* is listed. Based on their annotated functions, hyaluronic acid (HA) synthase has the highest count (16/36 [44%]), followed by other enzymes as listed. (B) Predicted protein sequence of *CPS1* deduced from the cDNA sequence. The putative transmembrane domains are boldfaced and underlined. The conservative GDD motif (substrate site), presumably involving the metal ion and UDP-sugar binding, is shaded. (C) Diagram of the predicted protein sequence shows the five conserved transmembrane domains (stippled boxes, 1 to 5) and a large intracellular loop containing the hyaluronic acid synthase substrate sites. The lightly stippled box 6 shows the less conserved transmembrane domain. Asterisks above the diagram indicate the conserved amino acid residues among hyaluronic acid synthases. (D) Sequence homology comparison by the ClusterLw program shows that *Cryptococcus CPS1* is evolutionarily closest to *Streptococcus* hyaluronic acid synthase (HAS). Mus, mouse; Equ, horse; Hum, human; Crypto, *Cryptococcus*; Strepto, *Streptococcus*.

A. Name | Counts (%)  
--- | ---  
HA synthase | 16 (44%)  
N-acetylglucosamine transferase | 7 (19%)  
Glycosyltransferase | 6 (7%)  
Chitin synthase | 4 (11%)  
Type 3 capsule polysaccharide synthase | 2 (5%)  
Cellubiose acid synthase | 1 (2%)  
Hypothetical or unannotated proteins | 14

B. MLYLTADKFPVFPFGVGIEYRYLYWIRLARATYRPILPPENNPTVAYHEDTVIIVTIDAGEEFAKSWLAGKNPKIIVTEAKMEGFLQDLQANVDPERRVLTPANKRLQMSHGRNTTIDIFVADBODAWPEITLPPYALCFEDQGVirGVTSGQRKVASRMTVWEVLAAFLGRTRNIEIASHSHTDSG3PICL3GR7TAAYRTLKPDFLHGFTNDFWLGYKHYLNGLSDDKFLTRWMSHGWNYTVQVCEAOELTSMKPNSWLFIQLVRTRWRSDFRSIFTERVFYVTKHPPFAMDKINPLTTVGPCLVYLVKSTKDVLDGGYHNAAWAVVGVYVLMLCTRLKLPHWLNPKHYYPAPIAFGYYFAIMKLYALFTLHEGTGWRAGIIGNITEATMAEEKNEASNFARQGQPDQYPPAARRK*

C. Intracellular loop (7)  
|  |  |  |  |  |  |  |  |
|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 |

D. HAS-Mus  
HAS-Rat  
HAS-Equ  
HAS-Mus3  
HAS-Hum  
CPS1-Crypto  
HAS-A-Strepto
protein shares the highest homology (28% identity and 42% similarity) with the Streptococcus hyaluronic acid synthase, HasA (Fig. 9D). Based on this sequence information, C. neoformans CPS1 most likely encodes a hyaluronic acid synthase. The questions remaining now are whether the sequence homology represents its functional conservation and whether CPS1 plays any role in the pathogenesis of C. neoformans.

In this report, we first demonstrated that treatment of yeast with 4-MU, a specific inhibitor of hyaluronic acid synthase, resulted in a reduction of the level of CPS1 (Fig. 1). The ability of the hyaluronidase-treated yeast cells to bind to HBMEC was also reduced (Fig. 2). Furthermore, the hyaluronic acid contents of different yeast strains are proportional to their abilities to bind to HBMEC (Fig. 3). These data suggested the existence of C. neoformans hyaluronic acid on the yeast cell surface.

These results support the hypothesis that the function of CPS1 is closely associated with the biosynthesis of hyaluronic acid in C. neoformans cells, i.e., either CPS1 encodes the hyaluronic acid synthase or it enhances hyaluronic acid biosynthesis and/or accumulation. In either case, the presence of hyaluronic acid molecules is closely related to the ability of C. neoformans to bind to HBMEC in vitro and its pathogenicity in C. elegans (Fig. 3 and 4). It is known that capsule genes are required for capsule formation and virulence. Mutation or deletion of any capsule gene significantly reduces the size of the C. neoformans capsule (9, 10, 11). In this scenario, an incomplete capsule structure may fail to retain hyaluronic acid molecules on its surface, resulting in decreased ability to bind to HBMEC, as shown in Fig. 3. Since C559 (cps1Δ) is a temperature-sensitive mutant strain, we used a C. elegans model of the in vivo pathogenesis assay for C. neoformans. Since C. elegans grows at room temperature, it is suitable for characterization of temperature-sensitive mutants such as the cps1Δ strain. The studies clearly demonstrated that the wild-type CPS1 strain is more virulent than the cps1Δ strain in this experimental animal model.

To further determine whether C. neoformans CPS1 encodes hyaluronic acid synthase, we expressed CPS1 in S. cerevisiae (Fig. 5) and in E. coli (Fig. 6). We were able to detect a hyaluronic acid signal on the baker’s yeast cell surface, indicating that the hyaluronic acid biosynthesis pathway can be constituted in baker’s yeast. This result is interesting in view of the fact that S. cerevisiae is a nonpathogenic yeast. It exhibits a very clean background in terms of virulence and hyaluronic acid synthesis. Another line of study is that recombinant Cps1p expressed in E. coli possesses the ability to incorporate UDP-GlcUA and UDP-GlcNac into a product, which can be detected by the hyaluronic acid ELISA kit (Fig. 6). Taking these results together, our findings strongly support the idea that CPS1 is the C. neoformans hyaluronic acid synthase gene, which plays a major role in the binding of C. neoformans to HBMEC. The importance of CPS1 for polysaccharide metabolism and potential virulence warrants further investigation. On the other hand, one should not rule out the possibility that the binding of C. neoformans to HBMEC is not directly mediated by hyaluronic acid. Instead, lack of hyaluronic acid may induce some changes in the fungal cell, resulting in altered ability to bind to HBMEC. Identification of a hyaluronic acid receptor on HBMEC would solve this issue.

We have also characterized the product encoded by CPS1, presumably hyaluronic acid, via a biochemical approach. We used FACE analysis to demonstrate the presence of hyaluronic acid isolated from C. neoformans capsule polysaccharides. Initially, we used trichloroacetic acid to precipitate capsule polysaccharides from C. neoformans in the presence of ethanol. The resultant materials were further purified through a Dowex 1 (2X) column and eluted at 0.5 to 0.65 M NaCl fractions. This column chromatographic pattern of the C. neoformans hyaluronic acid is similar to that of bovine vitreous-body hyaluronate (37). FACE has been used to detect hyaluronic acid from tissue samples, such as cartilage and heart valve (4, 5, 20), but it has never been applied to the C. neoformans capsule polysaccharides. Our studies reveal that FACE can be used to investigate another dimension of C. neoformans capsule structures, complemented by conventional methodologies. It is difficult to accurately measure the amount of hyaluronic acid relative to other capsule components. However, the recovery of purified hyaluronic acid from FACE analysis accounts for less than 1% of the total crude capsule preparation, suggesting that hyaluronic acid is a minor component in C. neoformans. An HPLC gel filtration study indicated that C. neoformans hyaluronic acid could consist of very high molecular weight molecules or complexes, up to 10^7 Da. The peak of hyaluronic acid content was in the range of ~10^5 Da, corresponding to ~300 disaccharide subunits, or a length of approximately 30 nm. Our TEM study (Fig. 8) revealed that yeast hyaluronic acid molecules extend from 0.3 to ~1 μm, corresponding to 300 to 1,000 disaccharide units. All these hyaluronic acid molecules could be detected in the wild type CPS1 strain but not in the cps1Δ strain. Taken together, C. neoformans CPS1 encodes a hyaluronic acid synthase, which generates a long hyaluronic acid molecule.

Hyaluronic acid not only is an important structural component in vertebrates but also plays an important role in many biological processes. Recent observations indicated that the association of hyaluronic acid with cell surface receptors such as CD44 and RHAMM (receptor for hyaluronic acid-mediated motility) influences cellular proliferation, differentiation, migration, and adhesion of eukaryotic cells (29, 30). In addition, some previous studies revealed that bacterial hyaluronic acid is important for pathogenesis, i.e., it protects the microbes from host defenses, including complement and phagocytosis. Using genetically defined acapsular mutants of Streptococcus pyogenes, an increase in phagocytosis of bacteria by granulocytes and a decrease in virulence in mice were reported (44). Loss of the hyaluronic acid capsule in bacteria typically reduces virulence >90% (36). Transforming Streptococcus has1 (the hyaluronic acid synthase gene) back into either acapsular Streptococcus strains or Enterococcus faecalis conferred the ability to synthesize hyaluronic acid so as to form a capsule and regain virulence (16, 17). Pasteurella multocida uses its surface hyaluronic acid as a ligand to bind to the target cells of the host (34). Other studies have shown that group A streptococci also use their hyaluronic acid as a ligand to CD44 on human keratinocytes, suggesting that the Streptococcus hyaluronic acid plays a direct role in microbial adhesion. This interaction between hyaluronic acid and host CD44 may lead to the transmission of signals that participate in cytoskeletal rearrangement during pathogen entry (15, 38). We have previously...
observed that C. neoformans was able to induce significant morphological alterations and cytoskeletal reorganization in HBMEC (13). Plausibly, this response might take place via interaction between hyaluronic acid and the host receptor on HBMEC.

In summary, our current data demonstrate that C. neoformans CPS1 encodes hyaluronic acid synthase. Hyaluronic acid on the yeast cell surface may act as a ligand that interacts with a putative receptor on HBMEC during the entry process. This is the first demonstration that hyaluronic acid in yeast might function as a ligand facilitating adhesion to host cells, as is the case for Streptococcus or Enterococcus. An understanding of the yeast-host interaction could serve as an important starting point in dissecting the complicated mechanism of endothelial cell invasion by C. neoformans that causes meningocaphealitis. We are currently investigating the potential receptor of C. neoformans hyaluronic acid, which would allow us to determine the yeast-host interaction triggering yeast entry into HBMEC.

ACKNOWLEDGMENTS

We thank A. Tang for critical reading.

This project is financially supported by Public Health Service grants R01-NS047599 (to A.J.) and R01-AH04635 (to S.-H.H.).

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