Short Report

Identification of Heparin-binding Proteins on the Cell Surface of *Cryptococcus neoformans*

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ABSTRACT

Interactions between virulence factors of pathogens and host responses play an important role in the establishment of infection by microbes. We focused on interactions between *Cryptococcus neoformans* proteins and heparin, which is abundant on host epithelial cells. Surface proteins were extracted and analyzed. Fractions from anion-exchange column chromatography interacted with heparin in surface plasmon resonance analyses. Heparin-binding proteins were purified and then separated by gel electrophoresis; and were identified as transaldolase, glutathione-disulfide reductase, and glyoxal oxidase. These results imply that multifunctional molecules on *C. neoformans* cells, such as those involved in heparin binding, may play roles in adhesion that trigger responses in the host.

Key words : *Cryptococcus neoformans*, heparin, transaldolase

Introduction

Host-microbe interactions at the molecular level play a key role in pathogenicity, particularly in opportunistic infections. In the case of *Cryptococcus neoformans*, inhalation of fungal cells leads to an initial pulmonary infection. This species exhibits several virulence factors, including a capsule primarily composed of acidic heteropolysaccharides, melanin synthesized by the enzyme laccase, growth capacity at 37°C, the calcineurin pathway, and suppression of host immunity. Although the mechanism by which *C. neoformans* invades the central nervous system is not fully understood, adherence to epithelial cells, including respiratory and alveolar cells, might be an important initiating event^{1,2}. Together with use of biomolecules in their host, these events might result in pathogenesis in this fungus. Cell surface glycosaminoglycans, such as heparan sulfate, play roles in the adhesion of several viruses, bacteria, and fungi. For example, the adsorption process of herpes simplex virus (HSV) requires binding of heparan sulfate to target cells. A mutation of glycosaminoglycan synthesis in Chinese hamster ovary cells increases host resistance to the virus^{3,4}. The trans-activator of transcription

(Tat) associated with human immunodeficiency virus (HIV) is a heparin receptor⁵. During poxvirus infection, heparan sulfate acts as a receptor, which implies a potent target for antiviral agents⁶. In bacteria, heparin-binding hemagglutinin (HBHA) in *Mycobacterium*⁷⁻⁹, the Fim2 subunit of fimbriae in *Bordetella pertussis*¹⁰, and many proteins including metabolic enzymes from *Mycoplasma*¹¹ and *Staphylococcus aureus*¹² have been identified. Among eukaryotic pathogens, heparin-binding motifs in proteins from *Candida* have been described¹³, as have a *Blastomyces* adhesin-1 called BAD-1¹⁴ and other heparin-interacting proteins, including an elongation factor of *Cryptosporidium*¹⁵. Many of these proteins may have roles in adherence, biofilm formation, invasion, or stress responses. Previously, we identified heparinase as a plasminogen-binding protein on the cell surface of *Trichosporon asahii*, a yeast pathogen¹⁶. In this study, molecules on the surface of *C. neoformans* that could bind heparin were analyzed to elucidate how this pathogen utilizes biochemical pathways in its infection process.

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Materials and methods

Preparation of extracts from the cell surface of *C. neoformans*

C. neoformans B-3501 was cultured at 37°C for 2 days in yeast nitrogen base broth supplemented with 2% glucose and 1% casamino acids and subjected to shaking (total volume, 5.4 l). After centrifugation (9,000 rpm, 20 min), the cells were washed once with phosphate-buffered saline and suspended in 3 M LiCl to obtain cell surface proteins. After mixing gently on ice for 15 min, the cell-free extract was dialyzed against water and the surface proteins were collected¹⁷. Crude extracts were fractionated using anion-exchange column chromatography with a diethylaminoethanol (DEAE) column (Toyoparl DEAE 650; Tosoh Bioscience, Tokyo, Japan). Unbound molecules were washed with water, and bound molecules were eluted with 0.01 M sodium phosphate buffer, pH 7.4 (PB), then eluted stepwise with 0 – 1.0 M NaCl in PB. The eluted fractions were subjected to further analyses.

Interactions between DEAE fractions and heparin

To examine the interactions between fractions obtained from DEAE anion-exchange column chromatography and heparin, surface plasmon resonance (SPR) analyses were performed using a Biacore 3000 biosensor (GE Healthcare, Milwaukee, WI, USA). Heparin lithium (Wako, Osaka, Japan) was dissolved in 0.1 M NaHCO₃ (50 mg/2.5 ml), 15 mg NHS-LC-biotin (ProteoChem, Hurricane, UT, USA) was added, and the mixture was kept overnight at 4°C. The solution was dialyzed against water to remove any NHS-LC-biotin reagent that was not bound to heparin. Biotinylated heparin was diluted in buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P20 (HBS-EP) to a concentration of 20 µg/ml and immobilized on a streptavidin-coated sensor chip (Sensor Chip SA; GE Healthcare) according to the manufacturer's instructions. The flow rate was maintained at 5 µl/min for immobilization and 20 µl/min for analysis.

Heparin affinity chromatography

Affinity chromatography was performed using a heparin-agarose column (Sigma-Aldrich, St. Louis, MO, USA). Briefly, the gel (0.5 ml) was equilibrated with Tris-HCl buffer (10 mM, pH 7.8). DEAE fractions that reacted with heparin based on SPR analysis were applied to the column, then eluted with 0 M, 0.1 M, 0.2 M, 0.4 M, 1.0 M, 1.5 M, and 2.0 M NaCl in Tris-HCl buffer.

Identification of heparin-binding proteins

The fractions found to bind with heparin were identified via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry. Proteins were separated using SDS-PAGE (10%, Multigel Mini10; Cosmo Bio, Tokyo, Japan) and visualized with silver staining (Silver Stain MS Kit; Wako). The molecular mass of each protein was

determined based on a set of protein markers (ColourPlus Prestained Protein Marker, Broad-range; New England BioLabs, Ipswich, MA, USA).

The protein bands of interest were excised and subjected to in-gel digestion for mass spectrometric analysis as described previously^{18,19}. The gel pieces were then destained, reduced with dithiothreitol, alkylated with iodoacetamide, and incubated in digestion buffer [50 mM NH₄HCO₃, 2 µM trypsin (Trypsin Gold; Promega, Madison, WI, USA) and 0.01% ProteaseMax (Promega)] at 42°C for 3 h. The resulting extracted peptides were desalted and concentrated using StageTips that were made in-house using the solid phase extraction disk, EmporeTM 2215 (FF)-C-10 Fast Flow Disk, 47 mm (3M Company, Maplewood, MN, USA)²⁰.

The peptides were analyzed using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system (Orbitrap Q-Exactive plus MS connected with Easy nLC 1000; Thermo Fisher Scientific, Rockford, IL, USA), as described previously²¹. After the samples were loaded onto the pre-column, Acclaim PepMap 100 (75 µm × 2 cm, C18, 3 µm, 100 Å; Thermo Fisher Scientific), at a flow rate of 300 nl/min, the peptides were isolated by the gradient program using mobile phases A (water containing 0.1% formic acid) and B (acetonitrile containing 0.1% formic acid), in the following sequence: 0% B (0 min) – 30% B (30 min) – 100% B (31 min) – 100% B (40 min). LC-MS/MS data were processed with Proteome Discoverer (ver. 1.4.1.14; Thermo Fisher Scientific). Database searching was done using the embedded Sequest HT server and was performed against *C. neoformans* sequences in the NCBI database (version 04/2017 with 58,568 sequences).

Results

Fractionation of heparin-binding proteins

To search for heparin-binding proteins on the surface of *C. neoformans*, DEAE anion-exchange column chromatography was applied to the extract in 3 M LiCl. Binding assays based on SPR demonstrated that the fractions eluted with 0.1 M and 0.2 M NaCl interacted with heparin, as shown in Fig. 1. Of these two fractions, 0.1 M NaCl appeared to have higher binding affinity.

Furthermore, the 0.1 M and 0.2 M NaCl fractions were applied to a heparin agarose column. The eluents from 0.1 M NaCl in Tris-HCl buffer were analyzed using SDS-PAGE followed by silver staining. The fractions were observed to contain several proteins, as shown in Fig. 2. The broad band labeled C was present in both fractions, but B was hardly detected in lane 2 (0.2 M, DEAE) and A was undetectable in lane 2. The protein bands A, B, and C from lane 1 (0.1 M, DEAE) were applied to LC-MS/MS analysis to identify the proteins.

Fig. 1. Sensorgrams showing the interactions between immobilized heparin and protein fractions obtained through anion-exchange (Toyopearl DEAE 650) column chromatography with surface plasmon resonance (SPR) from *C. neoformans*. Biotinylated heparin was immobilized on a streptavidin-coated sensor chip. The elution rate was 20 μ l/min.

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of heparin agarose fractions eluted with 0.1 M NaCl. Lane 1, diethylaminoethanol (DEAE) fraction eluted in 0.1 M NaCl; Lane 2, DEAE fraction eluted in 0.2 M NaCl.

Table 1. Identification of heparin-binding proteins

SDS-PAGE Band	Protein	Accession #	Score	% Coverage	PSMs	Amino acids	MW (kDa)	Identity (%)
A	Hypothetical protein CNBK0310	XP 772657.1	115.73	74.30	73	323	35.3	96
	Transaldolase	XP 012052888.1	72.00	41.49	46	323	35.3	
B	Glutathione-disulfide reductase, putative	AAW43464.1	185.12	59.92	102	479	52.1	97
	Glutathione-disulfide reductase	XP 012050124.1	135.29	44.05	83	479	52.3	
C	Glyoxal oxidase, putative	AAW43726.1	130.88	27.07	66	676	72.0	52.3
	Hypothetical protein CNBA3760	XP 777907.1	110.25	25.00	65	664	70.8	

Identification of heparin-binding proteins

To identify the proteins derived from bands A, B, and C, peptides obtained by in-gel digestion were subjected to LC-MS/MS analysis followed by database searching against *C. neoformans* sequences (58,568 sequences). Table 1 shows the identified proteins. Band A was identified as hypothetical protein CNBK0310 (Accession; XP 772657.1), with sequence coverage of 74.30%, a score of 115.73, and 73 peptide spectrum matches (PSMs); and transaldolase (Accession; XP 012052888.1), with sequence coverage of 41.49%, a score of 72.00, and 46 PSMs. The identity between CNBK0310 and transaldolase was 96%. This implies the possibility that there are transaldolase paralogues with very high identity. However, it is also possible that the uniformity of the microbial population might have been lost after repeated passages. Transaldolase is an enzyme involved in the pentose phosphate pathway. Band B was identified as two glutathione-disulfide reductases with very high identity (97%) (Accession; AAW43464.1 and XP 012050124.1). Glutathione-disulfide reductase catalyzes the reduction of glutathione disulfide to

glutathione (GSH). Band C was identified as glyoxal oxidase, putative (AAW43726.1) and hypothetical protein CNBA3760 (XP 777907.1). The identity was 52.3%; thus, band C may contain several proteins.

Discussion

Adherence of microbes to the epithelial cells of their host using biomolecules may be an important process in the initial phase of infection. We analyzed the role of heparin in the interaction of *C. neoformans* with respiratory organs, as it is an abundant glycosaminoglycan on the surface of human epithelial cells. Transaldolase and glutathione reductase were identified as heparin-binding proteins. These are known proteins; the former is an enzyme in the pentose phosphate pathway, and the latter catalyzes the reduction of glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH). They may play alternative roles²²⁾ as heparin receptors on the surface of cells. Previously, Missall et al. detected transaldolase and glutathione reductase proteins that were regulated by

nitric oxide stress in *C. neoformans*²³⁾, as well as nitrosative-stress-activated transcription of transaldolase. It is unclear whether the environmental conditions we used for cultivation led to oxidative stress. In addition, Missall et al. prepared a *glr1* Δ mutant of *C. neoformans* deficient in glutathione reductase, which was less virulent than the wild type in a mouse model. In the pentose phosphate pathway, where transaldolase plays a role, nicotinamide adenine dinucleotide phosphate (NADPH) is generated and then used in the reduction of GSSG to GSH by glutathione reductase. Notably, both transaldolase and glutathione reductase were detected as heparin-binding proteins on the cells of *C. neoformans* in our experiments. To investigate the localization of glutathione reductase on the cell surface, we tested its enzymatic activity in whole cells of *C. neoformans* using a glutathione reductase assay kit (Japan Institute for the Control of Aging, Nikken Sell, Co. Ltd., Kyoto, Japan). Glutathione reductase activity was detected, although the results are not direct evidence of protein localization. In another important fungal pathogen, *Candida albicans*, glutathione reductase activity was inhibited by sulfated polysaccharides including heparin²⁴⁾. In the medically important *Candida* species *C. parapsilosis* and *C. tropicalis*, transaldolase was identified as a fibronectin- and laminin-binding protein²⁵⁾. In bacteria, a role of extracellular transaldolase in adhesion to mucin and aggregation of cells has been demonstrated in *Bifidobacterium bifidum*, which implies that transaldolase is an important colonization factor in the gut²⁶⁾. These results imply that multifunctional molecules, the so-called moonlighting proteins²²⁾, of *C. neoformans* could exert an adhesive effect that triggers interactions with the host.

Glyoxal oxidase has been described as a glycosylphosphatidylinositol (GPI)-anchored protein in *C. neoformans*²⁷⁾. This enzyme is produced by the fungus *Phanerochaete chrysosporium*, which uses it in lignin degradation pathways²⁸⁾. The degradation products of this process contain polyphenols, which are the substrate for cryptococcal laccase and are related to the virulence factor of melanin formation.

Glycosaminoglycans play a role in infection as adhesion molecules for a number of pathogens, including viruses, bacteria, eukaryotic parasites, and fungi. Heparin binding and infectivity have been investigated in viruses, including HSV^{3, 4)}, HIV⁵⁾, and mouse adenovirus type 1 (MAV-1)²⁹⁾, and these studies showed that the viruses utilized cell-surface glycosaminoglycans such as heparan sulfate, which implies that cell-surface heparan sulfate glycosaminoglycans are important in the process of infection. In the case of HSV, binding depends on the sulfate content and requires at least 10 oligosaccharides³⁾.

In mycobacterial culture supernatant and cell extracts of *Mycobacterium bovis* and *M. tuberculosis*, a 28-kDa HBHA was found to contribute to adherence on epithelial cells; HBHA differed from fibronectin-binding proteins⁷⁾. Furth-

ermore, HBHA is required for dissemination of *M. tuberculosis*⁸⁾. In *Staphylococcus aureus*, heparin enhances biofilm formation in conjunction with secreted and cell-wall proteins¹²⁾. *Mycoplasma pneumoniae* possesses heparin-binding proteins¹¹⁾, and fimbriae of *Bordetella pertussis* bind heparin¹⁰⁾.

Among fungi, 34 proteins from *Candida albicans* are heparin-binding proteins¹³⁾. A surface protein of *Blastomyces dermatitidis* named BAD-1 binds heparin and is related to pathogenicity in a mouse model¹⁴⁾. Here, we identified three cryptococcal proteins that possibly bind heparin; other proteins remain to be identified. Going forward, we will conduct further studies using different culture conditions and preparation methods, and analyze other related species.

Plasmodium falciparum, the protozoon that causes malaria, binds heparin only at the apical tip of the merozoite surface and blocks interactions with the erythrocyte membrane, implying the potential of heparin as a new therapy against malaria^{30, 31)}. Heparin also interacts with elongation factor 1 α of *Cryptosporidium*¹⁵⁾.

Heparin binds to many microorganisms with a large variety of proteins as receptors. This binding could be instrumental to their colonization and dissemination processes. There are two possible binding mechanisms for enabling heparin to bind with so many proteins. Heparin and heparin sulfate are negatively charged polymers, and heparin columns are available for cation exchange chromatography. On the other hand, several reports have described heparin-binding motifs on receptor proteins. In HBHA (198 amino acid residues), a heparin-binding protein of *M. tuberculosis*, a cationic lysine-rich region (160 to 198) that comprises one of its four domains is essential for heparin binding. This domain contains two types of lysine-rich repeats, R1 and R2. R1 contains a sequence repeated (KKAAPA) three times, whereas R2 contains a sequence (KKAAAKK) repeated twice⁹⁾. The Fim2 subunit of fimbriae expressed by *B. pertussis* Lys-186 and Lys-187 is important in binding¹⁰⁾. Lysine residues have also been suggested to be essential for *C. albicans* heparin-binding protein (Int1p)¹³⁾, such as the K805/K806 of motif 1 in Int1p. BAD-1 of *Blastomyces* has a tandem repeat loop that may represent a putative heparin-binding motif¹⁴⁾. The mechanisms of heparin/protein binding remain unknown.

Interactions between microbes and airway epithelial cells involving heparin may induce host responses. HBHA of *Mycobacterium* increases broad cytokine and chemokine responses, including those of IFN- γ , TNF- α , IL-5, IL-10, IL-13, IL-17, MIP-1 α , and MIP-1 β following BCG vaccination^{23, 32)}. Our findings were obtained using a strain of *C. neoformans*. In addition to *C. neoformans*, *C. gattii* is an emerging pathogen responsible for cryptococcosis^{1, 33)}. The identification of molecules involved in adherence could provide insights into the mechanisms that these pathogenic

yeasts use to establish infection and colonization.

Conflict of Interest

None declared.

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