Interaction of surface molecules on *Cryptococcus neoformans* with plasminogen

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Abstract

Microbial pathogens are known to express molecules that interact with host proteins, leading to invasion and colonization. For example, some pathogenic microorganisms express proteins that bind to and enhance the activity of plasminogen. In this way, pathogens utilize the host fibrinolytic system to promote invasion. We found that triosephosphate isomerase (TPI), a glycolytic enzyme produced by *Staphylococcus aureus*, bound to mannooligosaccharides from the pathogenic capsulated fungus *Cryptococcus neoformans* and human plasminogen, suggesting that TPI is a moonlighting protein. Several *C. neoformans* surface proteins are thought to be plasminogen-binding proteins. Here, we examined the ability of surface polymers (including polysaccharides) to bind plasminogen. Heat-killed *C. neoformans* cells transformed plasminogen into plasmin in a dose-dependent manner in the presence of tissue plasminogen activator. Soluble polysaccharides were found to bind plasminogen based on surface plasmon resonance (SPR) analysis. Neutral polysaccharides fractionated using DEAE column chromatography bound and activated plasminogen. However, the fraction containing glucuronoxylomannan (the primary component of the capsule) did not activate plasminogen. In addition, binding between glucuronoxylomannan and plasminogen was weak. Components of the neutral polysaccharides were identified as mannose, galactose, glucose and xylose. In conclusion, neutral polysaccharides that may affect fibrinolysis were detected on the surface of *C. neoformans*.

Introduction

Microbial pathogens are known to express molecules that interact with host proteins, leading to invasion and colonization. For example, some pathogenic microorganisms express proteins that bind to and enhance the activity of plasminogen (Bhattacharya *et al.*, 2012; Sanderson-Smith *et al.*, 2012; Fulde *et al.*, 2013; Godier & Hunt, 2013; Magalhaes *et al.*, 2013). Thus, pathogens utilize the host fibrinolytic system to promote invasion. We found that triosephosphate isomerase (TPI), a glycolytic enzyme produced by *Staphylococcus aureus*, interacted with mannooligosaccharides from the pathogenic capsulated fungus *Cryptococcus neoformans* (Ikeda *et al.*, 2007; Furuya & Ikeda, 2009) and human plasminogen (Furuya & Ikeda, 2011), suggesting that TPI is a moonlighting protein (Henderson & Martin, 2011). Several *C. neoformans* surface proteins are thought to be plasminogen-binding proteins (Stie *et al.*, 2009). Furthermore, plasmin may play a role during blood–brain barrier (BBB) invasion (Stie & Fox, 2012). Here, we examined the ability of surface polymers (including polysaccharides) to bind plasminogen.

Materials and methods

Preparation of the polysaccharide fraction

Cryptococcus neoformans B-3501 (serotype D) was cultured at 37 °C for 5 days in yeast nitrogen base broth with 2% glucose, 1% casamino acids, and 0.01% streptomycin. Soluble polysaccharides were obtained from the

supernatant by ethanol precipitation followed by protease treatment, as described previously (Ikeda *et al.*, 1991; Ikeda & Maeda, 2004). Polysaccharides were dissolved in water and fractionated using anion-exchange column chromatography (IEC DEAE-2025, 20ϕ × 150 mm). Bound polysaccharides were washed with 0.01 M sodium phosphate buffer (PB), pH 7.4, and eluted with a linear gradient of 0–0.4 M NaCl in PB. Total carbohydrate contents of the effluent were measured using the phenol-H_2SO_4 method.

Plasminogen activation by tissue plasminogen activator (t-PA)

To examine the effects of polysaccharides on plasminogen activation, plasminogen was activated to plasmin using t-PA. Reagents were diluted in 50 mM Tris-HCl buffer, pH 7.4. First, 40 μL of 500 nM plasminogen and 10 μL of polysaccharide at various concentrations were incubated for 30 min at 37 °C on a microplate. Next, 10 μL of t-PA (recombinant human; Technoclone GmbH; 10 μg mL^{-1}) was added and incubated for 10 min at 37 °C, followed by addition of the chromogenic substrate S-2251 (40 μL of 0.5 mM solution; Chromogenix, Chapel Hill, NC). The absorbance at 405 nm (which depended on the release of *p*-nitroaniline from the substrate by plasmin activity) was monitored for 3 h at 10-min intervals.

Effect of intact *C. neoformans* cells on plasminogen activation

To examine the effects of intact *C. neoformans* on plasminogen activation, cells were added to the above plasminogen activation assay systems. Cell suspensions (10 μL) at various concentrations (10^8, 10^7, 10^6, and 10^5 cells mL^{-1}) were incubated with 40 μL of 500 nM plasminogen for 30 min at 37 °C. After incubation with t-PA for 10 min at 37 °C, substrate S-2251 was added and the absorbance at 405 nm was recorded.

Interaction between polysaccharide fractions and plasminogen

To examine the interaction between polysaccharide fractions and plasminogen, SPR analysis was performed using a Biacore 3000 (GE Healthcare, Milwaukee, WI).

Plasminogen (human; Enzyme Research Laboratories, South Bend, IN) was diluted with 10 mM sodium acetate buffer (pH 5.0) to a concentration of *c.* 20 μg mL^{-1} and immobilized on a standard sensor chip (CM 5) using an amine-coupling kit according to the manufacturer's instructions. Running buffer containing 10 mM HEPES

(pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20 was used. The flow rate was maintained at 10 μL min^{-1} for immobilization and 20 μL min^{-1} for analysis.

Results

Plasminogen activation by *C. neoformans* cells

To determine whether molecules on the surface of *C. neoformans* interacted with plasminogen, *C. neoformans* cells were added to the plasminogen activation system. Heat-killed cells were used to examine carbohydrate heat stability. As shown in Fig. 1, the cells facilitated plasminogen activation to plasmin in the presence of t-PA in a dose-dependent manner. When cells were used at a final concentration of 10^7 mL^{-1}, the reactions were significantly accelerated. At cell concentrations of 10^4 mL^{-1}, reactions proceeded similarly to the control without *C. neoformans*. These results suggested that heat-resistant molecules on *C. neoformans* play a role in plasminogen activation.

Interaction between cryptococcal soluble polysaccharides and plasminogen

To explore the role of polysaccharides located on the cell surface in plasminogen activation, soluble polysaccharides were examined using SPR. For these experiments, plasminogen was immobilized on the sensor tip as the ligand.
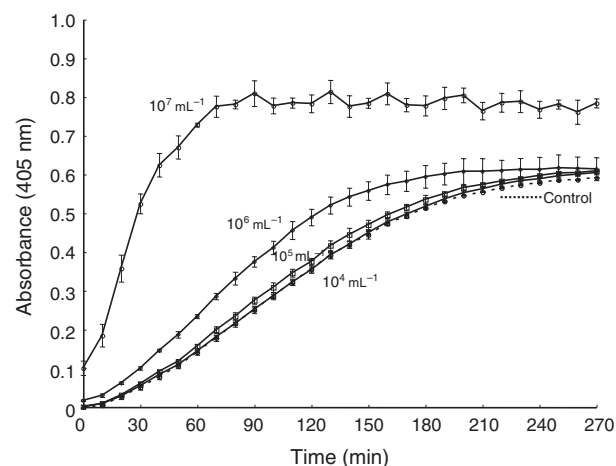

Fig. 1. Effect of heat-killed *Cryptococcus neoformans* cells on plasminogen activation by t-PA. Plasminogen was pre-incubated with *C. neoformans* cells for 30 min at 37 °C, after which t-PA was added. After incubation for 10 min, substrate S-2251 was added. The absorbance at 405 nm was monitored for 4.5 h at 10-min intervals.

Soluble polysaccharides at concentrations of 125, 250, 500, 1000, and 2000 µg mL^{-1} responded to plasminogen in a dose-dependent manner (Fig. 2). SPR sensorgrams showed rapid binding and dissociation between cryptococcal molecules and plasminogen. Based on these results, the molecules that activate plasminogen are likely carbohydrates, and not proteins.

Interaction between glucuronoxylomannan and plasminogen

Capsule is a representative surface structure of *C. neoformans*, and the primary component of the capsule is glucuronoxylomannan. Therefore, the glucuronoxylomannan fraction isolated from soluble molecules was applied to SPR. However, the binding of glucuronoxylomannan to plasminogen decreased compared with crude polysaccharides (Fig. 3). To assess whether glucuronoxylomannan affected plasmin activation, glucuronoxylomannan was added to the plasminogen activation system. As shown in Fig. 4, no significant enhancement of plasminogen activation by glucuronoxylomannan (250, 500 and 1000 µg mL^{-1}) was observed ($P > 0.05$).

Effects of the neutral fraction of polysaccharides on the interaction and activation of plasminogen

We next examined neutral polysaccharides fractionated using DEAE column chromatography. SPR showed binding between the neutral fraction and plasminogen in a dose-dependent manner (Fig. 5a). Furthermore, the neutral components activated plasminogen to plasmin in the presence of t-PA (Fig. 6).
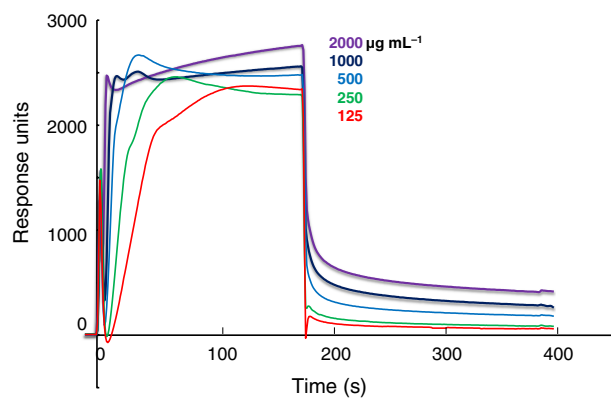

Fig. 2. Interaction between soluble polysaccharides from *Cryptococcus neoformans* and plasminogen based on SPR. Human plasminogen was used as the ligand, and analytes were cryptococcal polysaccharides (125–2000 µg mL^{-1}).
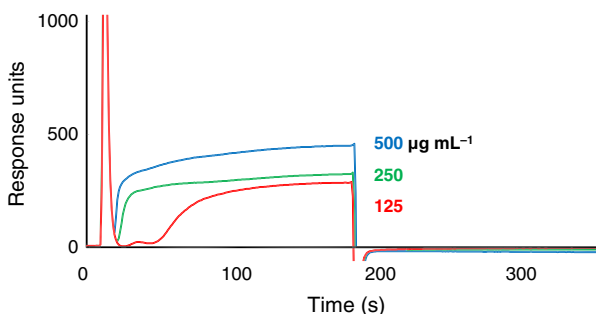

Fig. 3. Binding between glucuronoxylomannan and plasminogen based on SPR. Human plasminogen was used as the ligand, and the analyte was the glucuronoxylomannan fraction (125–500 µg mL^{-1}).
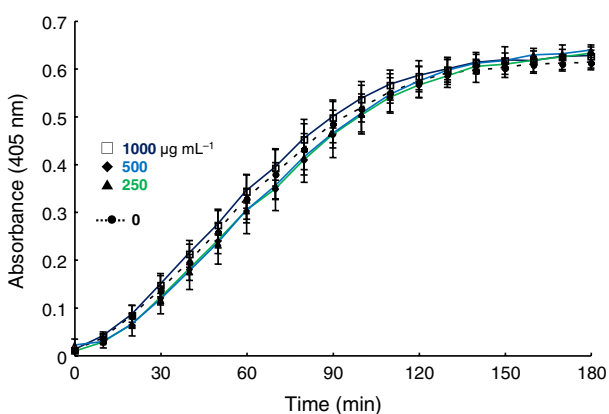

Fig. 4. Effect of glucuronoxylomannan (250–1000 µg mL^{-1}) on plasminogen activation in the presence of t-PA. Plasminogen was pre-incubated with glucuronoxylomannan. Closed circle; 0, closed triangle; 250, closed diamond; 500, open square; 1000 µg mL^{-1} for 30 min at 37 °C, after which t-PA was added. After incubation for 10 min, substrate S-2251 was added. The absorbance at 405 nm was monitored for 3 h at 10-min intervals.

Contribution of carbohydrates to plasminogen activation

The neutral polysaccharide fraction eluted with water using DEAE column chromatography was acid hydrolyzed (0.5 M sulfuric acid, 100 °C, 18 h) and neutralized with barium carbonate. The sugar components were identified by HPLC after labeling with 4-aminobenzoic acid ethyl ester as mannose, galactose, glucose, and xylose at ratios of 3.42 : 1.09 : 1.00 : 0.26, respectively. To explore the role of carbohydrates in the interaction with plasminogen, polysaccharides were oxidized by sodium metaperiodate (0.02 M, 4 °C, 120 h) and the interaction with plasminogen was compared with native polysaccharides using SPR. As shown in Fig. 5b, the interaction was sensitive to periodate oxidation, suggesting that carbohydrates play a role in the interaction with plasminogen. To determine
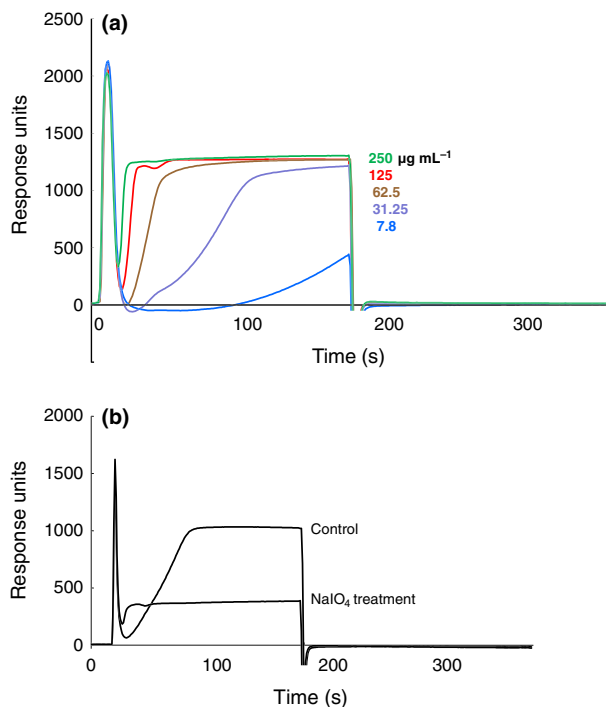
Fig. 5. Sensorgrams from SPR analysis of polysaccharides. Human plasminogen was used as the ligand. (a) Binding between the polysaccharide fraction eluted from the DEAE column and plasminogen. The analytes were the unbound fraction (7.8–250 µg mL^{-1}). (b) Comparison of sensorgrams between plasminogen and the neutral fraction untreated and treated with NaIO$_4$. Polysaccharide (10 µg mL^{-1}) before and after treatment was used as the analyte.
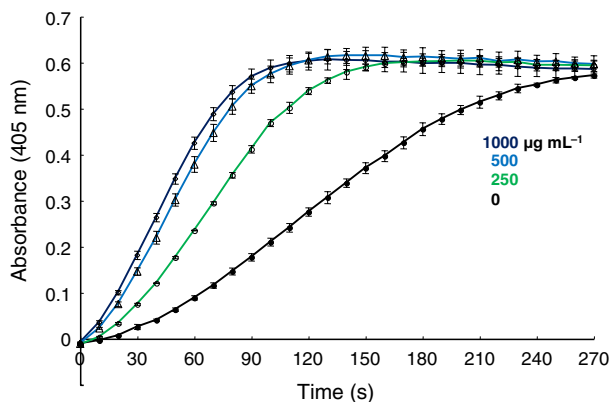

Fig. 6. Activation of plasminogen by the unbound fraction (250–1000 µg mL^{-1}) in the presence of t-PA. Plasminogen was pre-incubated with polysaccharide for 30 min at 37 °C, after which t-PA was added. After incubation for 10 min, substrate S-2251 was added. The absorbance at 405 nm was monitored for 4.5 h at 10-min intervals.

whether the fraction contained proteins, SDS-PAGE analysis of the polysaccharide fraction followed by silver staining was also performed. However, no protein band

was detected (data not shown). Furthermore, proteins extracted from the cell surface using 3 M LiCl hardly reacted with plasminogen according to dot immunobinding assays and SPR (data not shown).

Discussion

Microbial pathogens utilize host-derived molecules to invade target organs. Several bacteria bind extracellular matrix proteins including collagen, fibronectin, fibrin, and laminin (Singh *et al.*, 2012). Coagulation and fibrinolytic systems are recognized by microbial proteins, such as enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, which are glycolytic enzymes) in *Staphylococcus* and *Streptococcus*, M protein of *Streptococcus*, plasminogen-binding proteins (PgbA/PgbB) in *Helicobacter pylori*, and flagella in *Escherichia coli* (Bhattacharya *et al.*, 2012). In our previous study, TPI, a glycolytic enzyme, was shown to interact plasminogen (Furuya & Ikeda, 2011). TPI also interacted with α-1, 3-linked mannooligosaccharide from *C. neoformans* and contributed to the adherence of *C. neoformans* and *S. aureus* cells (Ikeda *et al.*, 2007; Furuya & Ikeda, 2009). Based on these results, TPI was suggested to be a moonlighting protein.

Cryptococcus neoformans is an encapsulated yeast that causes life-threatening meningitis. The polysaccharide capsule is mainly composed of glucuronoxylomannan (Bhattacharjee *et al.*, 1992) and is considered a pathogenic factor. However, specific virulence factors (such as exotoxins produced by several bacteria) have not been identified. During *C. neoformans* infection, molecules derived from *C. neoformans* can 'hijack' host factors during dissemination and interact with plasminogen on the cell surface to accelerate invasion. Several proteins from *C. neoformans* have been identified as candidate plasminogen-binding proteins (Stie *et al.*, 2009) and could play a role in crossing the BBB (Stie & Fox, 2012). Proteomic research showed molecular and cellular changes induced by *C. neoformans* in brain endothelial cells (Vu *et al.*, 2013). The role of the carbohydrate moiety in recognition and interaction between molecules has been reported previously (Liu & Pedersen, 2007; Springer & Gagneux, 2013). However, the association between carbohydrate and plasminogen remains unknown. Here, we examined the role of carbohydrates in the interaction with plasminogen and suggested that polysaccharide (rather than glucuronoxylomannan) interacts with and enhances plasminogen activation in the presence of t-PA.

Heat-killed *C. neoformans* accelerated plasminogen activation in the presence of t-PA, suggesting that molecules (rather than heat-labile proteins) play an important role in this process. Plasminogen activation activity was observed in the fraction containing polysaccharides

composed of neutral sugars, including mannose, galactose, glucose, and xylose. Mannose accounted for the majority of the fraction (59.3%). Glucuronoxylomannan, the primary component of the cryptococcal capsule, did not interact with or activate plasminogen. The plasminogen-activating molecules were sensitive to periodate oxidation, which suggested that carbohydrates were involved in this process. However, after treatment with sodium metaperiodate, one-third of the plasminogen-activating activity was maintained. The neutral polysaccharide fraction contained some periodate-resistant moieties that played a role in plasminogen binding. Glucuronoxylomannan contained peroxidation resistant α-1, 3-linked mannan in the backbone. Based on this information, mannose residues may play a role in plasminogen activation. Although the definitive chemical structures of the active substances have not been obtained, molecules that may function as plasminogen receptors on microorganisms could be predicted.

To determine whether our findings were limited to the strain used in the current study, we tested the interactions between plasminogen and fractions from *C. neoformans* CDC 551 (serotype A) and NIH 52 (serotype D) (Ikeda *et al.*, 1982) obtained by DEAE column chromatography. We confirmed that the unbound fraction responded clearly; however, fractions containing glucuronoxylomannan hardly reacted (data not shown). To confirm that the plasminogen receptor(s) is the virulence factor, further studies will be required using more virulent strains (e.g., H99) and/or clinical isolates.

In conclusion, on the surface of *C. neoformans*, neutral polysaccharides (excluding glucuronoxylomannan) were detected that affect fibrinolysis. Many molecules on the surfaces of microorganisms, as well as tumor cells (Andreasen *et al.*, 2000; Plow *et al.*, 2012; Godier & Hunt, 2013), have been identified as plasminogen receptors. Thus, microbial pathogens may utilize host plasminogen for colonization and dissemination using protein and carbohydrate components on the cell surface.

Acknowledgement

References

Andreasen PA, Egelund R & Petersen HH (2000) The plasminogen activation system in tumor growth, invasion, and metastasis. *Cell Mol Life Sci* **57**: 25–40.

Bhattacharjee AK, Kwon-Chung KJ & Glaudemans CP (1992) The major capsular polysaccharide of *Cryptococcus neoformans* serotype B. *Carbohydr Res* **233**: 271–272.

Bhattacharya S, Ploplis VA & Castellino FJ (2012) Bacterial plasminogen receptors utilize host plasminogen system for effective invasion and dissemination. *J Biomed Biotechnol* **2012**: 482096.

Fulde M, Rohde M, Polok A, Preissner KT, Chhatwal GS & Bergmann S (2013) Cooperative plasminogen recruitment to the surface of *Streptococcus canis* via M protein and enolase enhances bacterial survival. *MBio* **4**: 00629.

Furuya H & Ikeda R (2009) Interaction of triosephosphate isomerase from the cell surface of *Staphylococcus aureus* and alpha-(1→3)-mannooligosaccharides derived from glucuronoxylomannan of *Cryptococcus neoformans*. *Microbiology* **155**: 2707–2713.

Furuya H & Ikeda R (2011) Interaction of triosephosphate isomerase from *Staphylococcus aureus* with plasminogen. *Microbiol Immunol* **55**: 855–862.

Godier A & Hunt BJ (2013) Plasminogen receptors and their role in the pathogenesis of inflammatory, autoimmune and malignant disease. *J Thromb Haemost* **11**: 26–34.

Henderson B & Martin A (2011) Bacterial virulence in the moonlight: multitasking bacterial moonlighting proteins are virulence determinants in infectious disease. *Infect Immun* **79**: 3476–3491.

Ikeda R & Maeda T (2004) Structural studies of the capsular polysaccharide of a non-*neoformans Cryptococcus* species identified as *C. laurentii,* which was reclassified as *Cryptococcus flavescens*, from a patient with AIDS. *Carbohydr Res* **339**: 503–509.

Ikeda R, Shinoda T, Fukazawa Y & Kaufman L (1982) Antigenic characterization of *Cryptococcus neoformans* serotypes and its application to serotyping of clinical isolates. *J Clin Microbiol* **16**: 22–29.

Ikeda R, Matsuyama H, Nishikawa A, Shinoda T & Fukazawa Y (1991) Comparison of serological and chemical characteristics of capsular polysaccharides of *Cryptococcus neoformans* var. *neoformans* serotype A and *Cryptococcus albidus* var. *albidus*. *Microbiol Immunol* **35**: 125–138.

Ikeda R, Saito F, Matsuo M, Kurokawa K, Sekimizu K, Yamaguchi M & Kawamoto S (2007) Contribution of the mannan backbone of cryptococcal glucuronoxylomannan and a glycolytic enzyme of *Staphylococcus aureus* to contact-mediated killing of *Cryptococcus neoformans*. *J Bacteriol* **189**: 4815–4826.

Liu J & Pedersen LC (2007) Anticoagulant heparan sulfate: structural specificity and biosynthesis. *Appl Microbiol Biotechnol* **74**: 263–272.

Magalhaes V, Andrade EB, Alves J, Ribeiro A, Kim KS, Lima M, Trieu-Cuot P & Ferreira P (2013) Group B streptococcus hijacks the host plasminogen system to promote brain endothelial cell invasion. *PLoS One* **8**: e63244.

Plow EF, Doeuvre L & Das R (2012) So many plasminogen receptors: why? *J Biomed Biotechnol* **2012**: 141806.

Sanderson-Smith ML, De Oliveira DM, Ranson M & McArthur JD (2012) Bacterial plasminogen receptors:

mediators of a multifaceted relationship. *J Biomed Biotechnol* **2012**: 272148.

Singh B, Fleury C, Jalalvand F & Riesbeck K (2012) Human pathogens utilize host extracellular matrix proteins laminin and collagen for adhesion and invasion of the host. *FEMS Microbiol Rev* **36**: 1122–1180.

Springer SA & Gagneux P (2013) Glycan evolution in response to collaboration, conflict, and constraint. *J Biol Chem* **288**: 6904–6911.

Stie J & Fox D (2012) Blood-brain barrier invasion by *Cryptococcus neoformans* is enhanced by

functional interactions with plasmin. *Microbiology* **158**: 240–258.

Stie J, Bruni G & Fox D (2009) Surface-associated plasminogen binding of *Cryptococcus neoformans* promotes extracellular matrix invasion. *PLoS One* **4**: e5780.

Vu K, Eigenheer RA, Phinney BS & Gelli A (2013) *Cryptococcus neoformans* promotes its transmigration into the central nervous system by inducing molecular and cellular changes in brain endothelial cells. *Infect Immun* **81**: 3139–3147.