RESEARCH ARTICLE

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-H₂SO₄ 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 (10⁷, 10⁶, 10⁵, and 10⁴ 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 10⁷ mL⁻¹, the reactions were significantly accelerated. At cell concentrations of 10⁴ 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.
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).

**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...
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, mannan 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.

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