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Sialylglycoconjugates and sialyltransferase activity in the fungus *Cryptococcus neoformans*

Marcio L. Rodrigues[1], Andrey S. S. Dobroff[2], José Nelson dos S. S. Couceiro[1], Celuta S. Alviano[1]*, Roland Schauer[3] and Luiz R. Travassos[2]

[1] *Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil,* [2] *Disciplina de Biologia Celular, Universidade Federal de São Paulo, São Paulo, Brazil,* [3] *Biochemisches Institut, Christian-Albrechts-Universität, Kiel, Germany*

***Cryptococcus neoformans* is a fungal pathogen associated with systemic mycoses in up to 10% of AIDS patients. *C. neoformans* yeasts express sialic acids on the cell wall, where they play an anti-phagocytic role, and may represent a virulence factor at the initial phase of infection. Since the nature of the sialic acid-carrying components is undefined in *C. neoformans*, our aim in the present work was to identify sialylated molecules in this fungus and study the sialylation process. *C. neoformans* yeast forms were cultivated in a chemically defined medium free of sialic acids, to search for autologous sialylglycoconjugates. Sialylated glycolipids were not detected. Two glycoproteins with molecular masses of 38 and 67 kDa were recognized by *Sambucus nigra* agglutinin, an α2,6-sialic acid-specific lectin. The 67 kDa glycoprotein also interacted with Influenza C virus, but not with *Limax flavus* agglutinin, suggesting the presence of the 9-*O*-acetylated sialic acid derivative as a constituent of the oligosaccharide chains. A partially purified protein fraction from cryptococcal yeast forms was able to transfer sialic acid from CMP-Neu5Ac to both *N*-(acetyl-1-^{14}C)-lactosamine and asialofetuin. Additional evidence for a sialyltransferase in *C. neoformans* was obtained through the reactivity of fungal proteins with rabbit anti-rat α2,6 sialyltransferase polyclonal antibody. Our results indicate that sialic acids in *C. neoformans* are linked to glycoproteins, which are sialylated by the action of a fungal sialyltransferase. This is the first demonstration of this biosynthetic step in pathogenic fungi.**
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***Keywords: Cryptococcus neoformans*, sialylglycoconjugates, *O*-acetylated sialic acid, sialyltransferase, lectins**

Introduction

Cryptococcus neoformans is a yeast-like fungus commonly infecting immunosuppressed hosts causing cryptococcal meningitis, a serious and often fatal clinical condition [1]. Infection by *C. neoformans* begins by inhalation of fungal basidiospores or poorly encapsulated yeasts [2]. The capsular polysaccharide of *C. neoformans*, its major virulence factor, protects yeasts against phagocytosis by alveolar macrophages [3]. The cryptococcal capsule, however, reaches its complete expression at least 5 hours after the initial contact of the fungal propagules with the host [4]. The mechanisms by which *C. neoformans* resists phagocytosis and destruction by host cells during this period are not defined.

Sialic acids are members of a family of around 50 derivatives of neuraminic acid that have many functions in a wide range of biological systems [5,6]. During the last two decades, sialic acids have been characterized in several fungal species [7–17], but little progress has been made in the identification of sialic acid-containing molecules and their biosynthesis. Their role in fungal pathogenicity is still unclear.

In a previous work, we reported on the occurrence of sialic acids at the cell surface of *C. neoformans* [8]. Both *N*-acetylneuraminic acid (Neu5Ac) and its 9-*O*-acetylated derivative (Neu5,9Ac$_2$) were detected in *C. neoformans* yeast cells. Neu5Ac is the most common sialic acid derivative found in nature [5], and its presence in other fungal species has been recently reviewed [7]. The occurrence, however, of Neu5,9Ac$_2$ in *C. neoformans* represents the only report on the expression of this sialic acid derivative in pathogenic fungi. Evidence for

*To whom correspondence should be addressed: Dr Celuta S. Alviano, Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro. Cidade Universitária, CCS, Bloco I, sala 050. 21941 590, Rio de Janeiro—RJ, Brazil. Tel.: 55 21 2562 6711; Fax: 55 21 2560 8344; E-mail: alviano@micro.ufrj.br

the presence of Neu5,9Ac_2 on the surface of *C. neoformans* was obtained by agglutination of yeast forms with influenza C virus, which specifically recognizes this sialic acid derivative at 4°C [18].

Sialic acids were identified in *C. neoformans* as antiphagocytic cell wall structures [8], which raises the possibility that they could play a relevant role on the initial defense against host effector cells, before the full expression of the capsule. A possible role of Neu5,9Ac_2 in the pathogenicity of *C. neoformans* is still unknown, but natural substitutions of Neu5Ac, as by 9-*O*-acetylation, can abrogate the cell reactivity with certain ligands such as factor H [19], which negatively regulates the alternative pathway of complement.

Neu5Ac residues α2,6-linked to galactose and 9-*O*-acetylneuraminic acid units were detected after cultivation of cryptococcal cells in a chemically defined medium free of sialic acids [8], indicating that sialic acids in *C. neoformans* are synthesized and transferred to glycoconjugates independently of their presence in culture media. This also suggests that biosynthesis of sialoglycoconjugates involves the action of glycosyltransferases, well described in mammalian systems [20]. In this work, we describe the occurrence of sialylglycoconjugates in aqueous, but not lipidic, extracts of *C. neoformans* yeasts. Sialylated glycoproteins containing 9-*O*-acetylated sialic acids and sialyl-α2,6-galactosyl sequences were detected, in agreement with our previous results using whole cryptococcal cells. We also suggest that sialylation of these molecules is catalyzed by the action of a CMP-sialic acid-dependent sialyltransferase, since protein preparations from *C. neoformans* reacted with anti-sialyltransferase antibodies and showed specific enzymatic activity.

Materials and methods

Chemicals

Culture media components, sialidases from *Vibrio cholerae* and *Clostridium perfringens*, nitrocellulose membranes, salts, protease inhibitors, standards of sialic acids and gangliosides, *N*-(acetyl-1-^{14}C)-lactosamine, detergents and secondary antibodies were obtained from Sigma Chem. Co. (St Louis, MO). Organic solvents and the chromatographic apparatus were purchased from Merck (Rio de Janeiro, Brazil). Centricon-3 was from Amicon and lectins were obtained from EY Laboratories (San Mateo, CA). Human fetuin and standard rat sialyltransferase were purchased from Calbiochem (San Diego, CA) and the rabbit anti-α2,6 sialyltransferase antibody was a kind gift from Dr Karen J. Colley (University of Illinois, College of Medicine, Chicago, Illinois). Chemiluminescence reagents were obtained from Amersham Pharmacia Biotech.

C. neoformans

The encapsulated *C. neoformans* strain HEC3393 (serotype A) was obtained from the Laboratorio de Micologia Medica, Hospital Evandro Chagas, FIOCRUZ, Rio de Janeiro. This strain was isolated from a human case of meningoencephalitis. Stock cultures were maintained in Sabouraud dextrose agar under mineral oil and kept at 4°C. *C. neoformans* cells were grown in a chemically defined medium, as previously described [8], containing (g/liter): 6.25 glycerol, 0.001 of a purine-pyrimidine mixture (adenine, thymine, cytosine, guanine and hypoxantine, 1:1:1:1:1. w/w/w/w/w), 0.002 calcium pantothenate, 0.5 cystein, 1.5 $(NH_4)_2SO_4$, 1.8 K_2HPO_4, 0.1 $MgSO_4 \cdot 7H_2O$; 0.05 mg/liter biotin and 0.05 mg/liter thiamin·HCl. Cells were cultivated at room temperature for 5 days, collected by centrifugation and washed twice in 0.01 M PBS, pH 7.2.

Sialic acid analysis in *C. neoformans* glycolipids

To evaluate the possible association of sialic acids with lipid molecules, *Cryptococcus* yeast cells were first extracted at room temperature successively with chloroform/methanol 2:1 and 1:2 (v/v). The crude lipid extract was partitioned according to Folch et al. [21]. Lipids in the Folch's upper phase were first examined for the presence of sialic acids by high performance thin layer chromatography (HPTLC) on silica gel plates developed with chloroform/methanol/0.02% calcium chloride (60:40:9, v/v/v), and revealed with the resorcinol-HCl reagent. Alternatively, the Folch's upper phase was lyophilized, resuspended in water, and acidified to pH 2.0 with concentrated formic acid. A control system consisted of the same preparation supplemented with 100 μg of a standard ganglioside (monosialoganglioside—GM3). These preparations were heated for 1 h (80°C) and hydrolyzed units of sialic acids purified by ultrafiltration in Centricon 3 (Amicon), followed by passage through 1 ml anionic column of Dowex 1 × 8 (100 to 200 mesh, formate form). Eluted samples were then derivatized with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and finally analyzed by gas chromatography (GC) as previously described [12].

Sialic acids were also released by acid hydrolysis from control or lipid-free *C. neoformans* cells, which had been treated with chloroform/methanol 2:1 and 1:2 (v/v). *C. neoformans* cells (10^{10} cells) were suspended in 2 mL of water, and the pH of this suspension was adjusted to 2 with 50% (v/v) formic acid, followed by incubation for 1 h at 70°C. The mixture was cooled in ice and centrifuged for 10 min at 900 g. The supernatant containing free sialic acids was removed and the cell pellet re-extracted for 1 h at 80°C (pH 1.0). The combined supernatants were then ultrafiltered in a Centricon 3 micropartition system from Amicon and the lower layer passed through 2 mL cation column of Dowex 50WX8 (100 to 200 mesh, hydrogen form). The eluate was finally purified by gel filtration in BioGel P-2 column (100 × 0.5 cm, minus 400 mesh), eluted with 0.01 M acetic acid, keeping the flow rate at 1 mL/h. The presence of sialic acid in the fractions was monitored by HPTLC on silica plates with the solvent mixture *n*-propanol-1 M ammonia-water (6:2:1 v/v). Spots were visualized by reaction with resorcinol-HCl. Samples containing sialic acids were finally quantified colorimetrically by Warren's thiobarbituric acid method [22].

Protein extracts

Extracts from *C. neoformans* yeast cells (HEC3393) were prepared as described by Peñalver et al. [23], with minor modifications. Yeast cells (10^{10}) were suspended in a lysis buffer, consisting of 0.01 M PBS (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA, inhibitor of metalloproteinases), 1 mM trans-epoxysucciny-L-leucylamido-(4-guanidino) butane (E-64, inhibitor of cysteine proteinases), 1 μg/ml aprotinin (serine proteinase inhibitor), 1 μg/ml pepstatin (aspartyl proteinase inhibitor), and 5 mM dithiothreitol (DTT). Glass beads (0.3 mm in diameter) were then added to the suspension, and yeasts were broken in a cell homogeneizer (type 853023/8, B. Braun Biotech International, Germany) by alternating 1 min shaking periods and 2 min cooling intervals. Cell disruption was assessed in a light microscope. After removal of the glass beads, the suspension was centrifuged (1,200 g, 10 min, 4°C) and the supernatant concentrated in Centricon 10. Proteins in this extract were then quantified by the method of Lowry et al. [24].

Influenza C virus and rabbit immune sera

A standard sample of influenza C (influenza C/Taylor/1233/47) virus with known affinity for 9-*O*-acetyl-Neu5Ac (Neu5,9Ac_2) was used as a probe to detect this sugar on fungal glycoproteins [25]. Virus preparations were adjusted to 512 haemagglutination units (HAU) for use. Rabbit immune serum was obtained through subcutaneous inoculation of virus-adsorbed rabbit erythrocytes in a white male rabbit, at weekly intervals, for 3 weeks. The serum was tested by the haemagglutination inhibition test and adjusted to 1:500 dilution for Western blotting analysis.

Detection of sialylated glycoproteins

Protein extracts from *C. neoformans* were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 12% gels) and silver-stained or transferred to nitrocellulose membranes, following previously described protocols [26]. Blotted proteins were blocked in PBS containing 0.1% Tween 20 (PBS-T) and 5% bovine serum albumin (BSA). After blocking, membranes were exposed to peroxidase-labeled *Limax flavus* agglutinin (LFA), which binds sialic acid-containing glycoconjugates in all linkages, or *Sambucus nigra* agglutinin (SNA), which recognizes sialic acids α2,6 linked to galactose or *N*-acetylgalactosamine. The lectins (1 μg/mL) were incubated with membranes for 1 h at room temperature, followed by washing (three times) in PBS-T and detection by chemiluminescence. In these analyses, control of specificity was achieved by pre-treatment of protein extracts with sialidase from *V. cholerae*, as described elsewhere [15]. For detection of 9-*O*-acetylated sialoglycoproteins, proteins were separated in SDS-PAGE gels (12%) and transferred to nitrocellulose membranes as described above. Membranes were blocked in PBS-T containing 5% low fat dry milk and incubated for 1 h on ice in the presence of a suspension of influenza C virus, which specifically recognizes 9-*O*-acetylated sialic acids at 4°C [25]. After washing in cold PBS, the membranes were incubated with rabbit anti-influenza C serum (at 1:500 dilution) at 4°C, followed by washing in cold PBS and incubation with peroxidase-labeled anti-rabbit antibody. To control specificity of virus binding, extracts were pre-treated with sialidase as described before [15] or, alternatively, blots were treated with 0.1 N NH_4OH for 30 min at 4°C for de-*O*-acetylation. Reactive molecules were detected by chemiluminescence.

Sialyltransferase activity

Cryptococcal protein extracts were obtained as described above, except for the addition of Triton CF-54 at 0.5% to the lysis buffer. These extracts (50 mg of protein diluted in 5 mL of PBS) were fractionated by incubation with galactose-Sepharose beads (1 mL of the swollen resin) for 1 h at 4°C. Unbound proteins were removed by centrifugation followed by extensive washing of beads with 10 mM sodium cacodylate, pH 6.5, 0.1% Triton CF-54, and 0.25 M NaCl. The beads were then incubated with 0.5 M galactose in 25 mM sodium cacodylate, pH 5.3, 0.1% Triton CF-54, and 0.15 M NaCl for 1 h at 4°C to elute bound proteins. Enzymatic assays were performed using CMP-Neu5Ac as the donor substrate, whereas asialofetuin or *N*-(acetyl-1-^{14}C)-lactosamine (25 mCi/mmol) were the acceptor substrates. In radioactive detections of enzyme activity, a reaction mixture (pH 6.0) containing the galactose-binding molecules obtained as described above (100 μg/mL), 0.1 M sodium cacodylate, 10 mM $MgCl_2$, 0.5% Triton CF-54, 1 mM CMP-Neu5Ac, and 60,000–80,000 cpm of the radioactive substrate in a final volume of 100 μL were incubated for 1 h at 37°. Reactions were stopped by lowering the temperature of incubation to 4°C, followed by purification of the sialylated radioactive products by passage through a 1 ml anionic column of Dowex 1 $\times$ 8 (100 to 200 mesh, formate form). After the sample was applied, the resin was washed with 16 mL of 0.01 M formic acid, followed by elution of bound fractions with 1 M formic acid. The eluate and washings were collected in fractions of 1 mL, which had their ^{14}C radiation measured in a liquid scintillator. As a control, *N*-(acetyl-1-^{14}C)-lactosamine was passed through the anionic column under the same conditions described above. Fractions with the highest counts of ^{14}C were combined, lyophilized and analyzed by HPTLC on silica gel plates with the solvent mixture *n*-propanol–1 M ammonia–water (6:2:1, vol/vol/vol). For R_f determination, each lane of the HPTLC plate was divided into 12 strips of 5 mm, which were separately scraped off the plate, suspended in scintillation liquid, and counted for radioactivity. Lyophilized fractions from the Dowex resin were re-suspended in 100 μL of PBS, pH 6.0, and incubated overnight at 37°C in the presence of *C. perfringens* or *V. cholerae* sialidases (50 mU). These samples were passed through the anionic column as described above, followed by HPTLC analysis. Alternatively, dried samples were suspended in 100 μL of water and the pH adjusted to 2 with 50% (vol/vol) formic acid. This solution was heated for 1 h at

80°C, followed by purification on the anionic resin and analysis by HPTLC.

Sialyltransferase activity was also assayed by Western blotting with peroxidase-labeled lectins, using asialofetuin as the acceptor substrate. Asialofetuin was prepared by treatment of human fetuin (1 mg) with sialidase from *V. cholerae* (0.02 units) for 12 h, at 37°C (pH 6.0). The protein profile generated after treatment was composed of three bands with molecular masses of 60, 58, and 45 kDa (not shown), and their degrees of sialylation were monitored by Western blot with peroxidase-labeled LFA (not shown). For examination of sialyltransferase activity, a reaction mixture (pH 6.0) containing the cryptococcal galactose-binding molecules (100 μg/mL), 0.1 M sodium cacodylate, 10 mM $MgCl_2$, 0.5% Triton CF-54, 1 mM CMP-Neu5Ac, and 1 mg/mL asialofetuin in a final volume of 100 μL was incubated for 1 h at 37°C. Control systems contained all the reagents, except CMP-Neu5Ac or asialofetuin. Samples were then dried by centrifugation *in vacuo*, resuspended in 15 μL and subjected to Western blotting and detection of sialylated products by sequential incubation with biotinylated-LFA and peroxidase-labeled streptavidin. Reactive molecules were visualized by chemiluminescence detection.

Immunodetection of sialyltransferase

Crude extracts or partially purified molecules, obtained as described above, were separated by SDS-PAGE (12% gels) and transferred to nitrocellulose membranes, followed by blocking with PBS-T containing 5% low-fat dry milk. Blocked membranes were then incubated for 1 h in the presence of rabbit polyclonal antibodies (at 1:100 dilution) raised against rat liver α2,6 sialyltransferase, provided by Karen J. Colley (University of Illinois). Antibody production and purification were described before [27,28]. Membranes were washed in PBS and incubated with a peroxidase-labeled anti-rabbit antibody, followed by washing in PBS and immunodetection of reactive molecules by chemiluminescence assay. As a positive control, purified α2,6 sialyltransferase from rat liver was submitted to the same analysis. Antibody specificity in all systems was evaluated by incubating secondary antibodies directly with fungal or animal proteins. Proteins that were not blotted were visualized by silver staining.

Results

Sialylglycoplipids are not detectable in *C. neoformans*

A crude lipid extract from *C. neoformans* was obtained and partitioned according to the method of Folch and co-workers [21]. The upper phase, containing essentially polar lipids, was concentrated and separated by HPTLC, followed by reaction with resorcinol-HCl. In this analysis, sialic acid-containing molecules would typically be visualized as blue-purple bands. Analysis of the polar lipid profile, however, revealed the absence of sialic acid-containing spots (data not shown). In addition, the Folch's upper phase was submitted to acid hydrolysis, followed by purification of acidic components and analysis by GC. Reference peaks of sialic acids were obtained by analysis of control preparations, which consisted of hydrolyzed units obtained from a similar Folch's polar phase supplemented with GM3 or, alternatively, with a standard of Neu5Ac. In our experimental conditions, no peaks with retention times similar to those observed in the control systems were observed (data not shown). To confirm that sialic acid units are not present in cryptococcal lipids, sialic acids were extracted by acid hydrolysis and purified from delipidated cells, and the results compared with those obtained after liberation from control cells. Purified bands with Rfs similar to that of a standard of Neu5Ac were detected in both preparations (not shown). Quantitative analysis using the method of Warren revealed that the sialic acid content in both control and organic solvent-treated cells were very similar, consisting of 3.0 and 3.1×10^6 residues per cell, respectively.

Sialylglycoproteins in *C. neoformans*

Protein extracts were separated by SDS-PAGE (12% gels), blotted onto nitrocellulose membranes and incubated at 4°C with influenza C, a condition in which 9-*O*-acetylated sialic acids are specifically recognized by this virus. After incubation with a rabbit anti-influenza C serum, a fungal component with molecular mass of 67 kDa was detected (Figure 1). This reaction was abolished by previous treatment of blotted glycoproteins with alkali, which promotes de-*O*-acetylation [29], and by incubation at 37°C, a temperature in which the influenza C virus shows acetylesterase cleaving activity [18] (data not shown). Treatment of extracts with sialidase was poorly effective, which may reflect the greater resistance of 9-*O*-acetylated sialic acids to enzymatic hydrolysis [29]. When the fungal proteins were incubated in the presence of peroxidase-labeled SNA, bands with molecular masses corresponding to 67 and 38 kDa were detected, demonstrating the presence of sialyl-α2,6-bonds. The former molecule was less sensitive to sialidase treatment than the 38 kDa component, which could also be explained by the presence of *O*-acetyl groups [19,29]. When peroxidase-labeled LFA was used as the probe to detect sialoglycoconjugates, the 38 kDa glycoprotein was again detected, which is in agreement with the occurrence of 9-*O*-acetyl groups, which mask recognition by LFA but not SNA, in sialic acid units of the 67 kDa glycoprotein [19]. Proteins from *C. neoformans* were not reactive with *Maackia amurensis* agglutinin (data not shown), which confirmed the previous study showing that α2,3-sialylgalactosyl sequences are not detectable in cryptococcal yeasts [8].

Sialyltransferase in *C. neoformans*

Previous reports have demonstrated that several fungal species synthesize the sialic acids Neu5Ac, Neu5Gc or Neu5,9Ac_2 after growth in a chemically defined medium, in the absence of sialic acid-containing substrates [8–14]. This suggested that the acidic

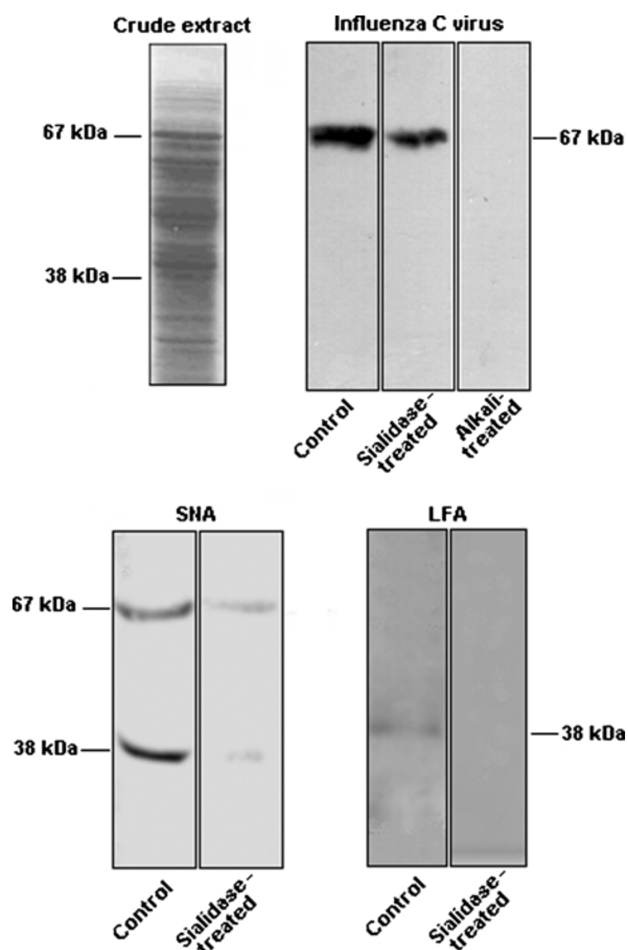

Figure 1. Detection of sialylglycoproteins in *C. neoformans* extracts. Proteins in the crude extract were silver-stained or blotted onto nitrocellulose membranes. Extracts were then incubated with influenza C virus and a rabbit anti-influenza C serum or with peroxidase-labeled lectins (LFA and SNA). A 67 kDa 9-*O*-acetylated sialylglycoprotein and a 38 kDa sialylated component were detected, the former being poorly sensitive to sialidase treatment. De-O-acetylation of blotted glycoproteins by alkali treatment abolished interactions with influenza C virus.

carbohydrate units were transferred to terminal ß-galactosyl residues by CMP-sialic acid-dependent sialyltransferases. To evaluate this possibility, *C. neoformans* yeasts were suspended in a lysis buffer containing Triton CF-54 and disrupted in a cell homogenizer. Based on the galactose-binding properties of sialyltransferases [27], a partially purified preparation from *C. neoformans* was obtained by affinity chromatography on a galactose column. The cryptococcal galactose-binding fraction was then incubated in a mixture containing CMP-Neu5Ac and *N*-(acetyl-1-^{14}C)-lactosamine, rendering a strongly negatively charged radioactive compound, which eluted from the anionic column Dowex 1 × 8 with 1 M formic acid (Figure 2A). *N*-(acetyl-1-^{14}C)-lactosamine did not bind to the resin, with all its radioactivity being eluted by washing the column with 0.01 M formic acid. Column fractions 1–4 (corresponding to unmodified *N*-[acetyl-1-^{14}C]-lactosamine) and 18–26 (to the product formed after incubation in the presence of CMP-Neu5Ac) were pooled, lyophilized, and analyzed by HPTLC. This assay revealed that a highly polar product was in fact generated in the presence of CMP-Neu5Ac, as shown by R_f determination of radioactive fractions and comparison with the *N*-(acetyl-1-^{14}C)-lactosamine standard (Figure 2B). The negatively charged compound was treated with sialidase, followed by purification of the products by Dowex chromatography. A major peak corresponding to a negative molecule was still detected, although a minor one obtained in early fractions was generated (Figure 2C). To confirm that de-sialylation induced the regeneration of *N*-(acetyl-1-^{14}C)-lactosamine, the products of the enzymatic hydrolysis were analyzed by HPTLC. As shown in Figure 2D, this analysis resulted in the detection of a less polar fraction, actually corresponding to the migration of *N*-(acetyl-1-^{14}C)-lactosamine, and a highly polar peak, probably resulting from non-hydrolyzed molecules. Similar profiles of binding to Dowex 1X8 and migration rates in HPTLC were observed when sialidase treatment was replaced by hydrolysis with formic acid (data not shown). These results strongly indicate that a sialyltransferase activity is expressed in *C. neoformans* cells.

The cryptococcal galactose-binding proteins were also incubated in the presence of CMP-Neu5Ac and asialofetuin. To detect possibly sialylated glycoproteins, products were electrophoresed, blotted and incubated with biotinylated-LFA. Results (Figure 3) demonstrated that molecules in this fraction were in fact able to transfer sialic acid units from CMP-Neu5Ac to asialofetuin, since an LFA-reactive band was detected after incubation. In the absence of donor or acceptor substrates, the reaction did not occur, which indicates the specificity of sialyl transfer and lectin binding.

Immunodetection of sialyltransferase

Extracts or partially purified galactose-binding proteins from *C. neoformans* were separated by SDS-PAGE and silver-stained or, alternatively, blotted onto nitrocellulose membranes and incubated with antibodies raised against rat α2,6-sialyltransferase [27,28]. Figure 4 shows that, in crude extracts, a single band with apparent molecular mass of 60 kDa is recognized by anti-sialyltransferase antibodies, suggesting that a fungal protein with epitopes similar to those observed in the catalytic sites of animal sialyltransferases occur in *C. neoformans*. Silver staining of the galactose-binding fraction revealed the presence of three bands (Figure 4). Two of these proteins were recognized by the anti-α2,6-sialyltransferase antibody, indicating the exposition of a previously non-reactive molecule to the antibodies in the purified fraction. As expected, the standard animal sialyltransferase strongly reacted with anti-sialyltransferase antibodies. Anti-IgG (secondary) antibodies were not reactive against fungal or animal proteins.

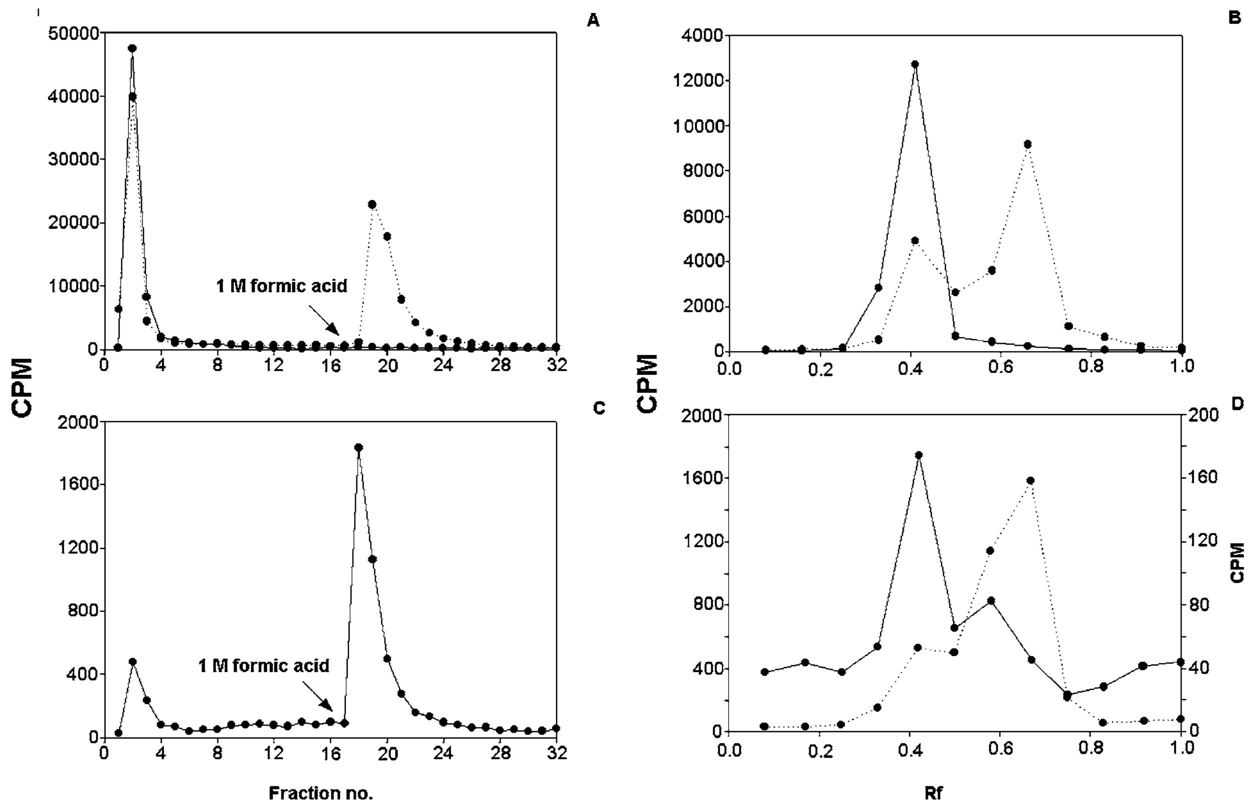

Figure 2. Transfer of sialic acid from CMP-Neu5Ac to *N*-(acetyl-1-^{14}C)-lactosamine is catalyzed by *C. neoformans* proteins. A. Chromatographic profile of *N*-(acetyl-1-^{14}C)-lactosamine (solid line) and the product generated after incubation in the presence of CMP-Neu5Ac (dotted line). The latter compound is a negatively charged molecule, since it was eluted with 1 M formic acid. Fractions 1–4 (*N*-[acetyl-1-^{14}C]-lactosamine separation, solid line) and 18–26 (separation of the negatively charged product, dotted line) were combined and analyzed by HPTLC (B). The acidic product (dotted line) had a higher migration rate, as expected for sialylated *N*-acetyl-lactosamine. The solid line is representative of the chromatographic profile of *N*-(acetyl-1-^{14}C)-lactosamine. The product obtained by pooling fractions 18–26 was treated with sialidase and again purified on a Dowex 1 × 8 column, which resulted in the generation of a less polar compound corresponding to *N*-(acetyl-1-^{14}C)-lactosamine (fractions 1–4, C). This result was confirmed by the HPTLC (D) profile of fractions 1–6 and 18–25 (from Figure 2C), which were pooled and analyzed in silica plates after this purification.

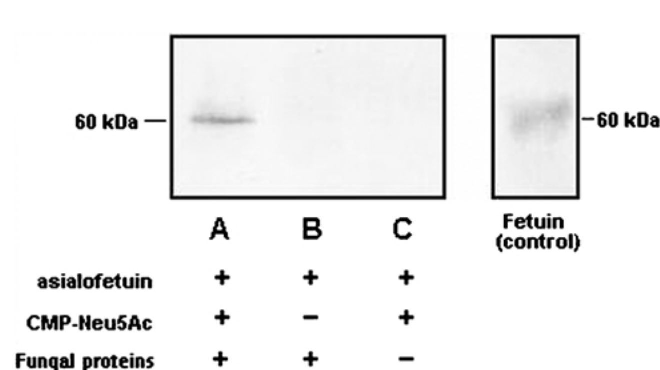

Figure 3. Sialyltransferase activity in *C. neoformans*. The Galactose-Sepharose fraction from protein extracts was incubated with asialofetuin and CMP-Neu5Ac (A) and analyzed by Western blotting with biotinylated-LFA. Removal of CMP-Neu5Ac (B) or fungal molecules (C) resulted in negative reactions. Fetuin was used as a positive control of lectin binding.

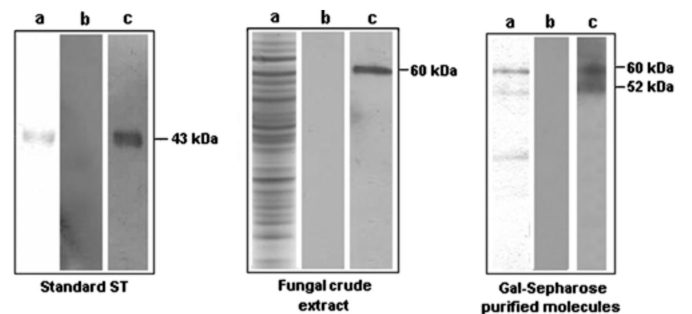

Figure 4. Fungal molecules are recognized by anti-sialyltransferase antibody. Standard α2,6-sialyltransferase (ST), crude *C. neoformans* extract and fungal galactose-Sepharose purified molecules were separated by SDS-PAGE, followed by silver staining (a) or blotting onto nitrocellulose membranes. Blotted proteins were incubated with secondary antibodies (b) or with the rabbit polyclonal anti-rat α2,6-sialyltransferase antibody (c).

Discussion

In the present work, we demonstrate that *C. neoformans* expresses sialoglycoproteins, but not sialoglycolipids, after growth in a sialic acid-free culture medium, which suggests that sialic acids are synthesized *de novo* and are transferred to terminal ß-galactosyl residues of glycoproteins probably by regular CMP-sialic acid-dependent sialyltransferases. These results confirmed our previous data showing that *C. neoformans* expresses sialic acids on the fungal cell wall, as inferred from yeast cell reactivity with sialic acid-specific lectins and the enhancement of lectin binding by inhibition of capsule production [8].

A polar lipid fraction from *C. neoformans* cells was analyzed by HPTLC and GC, revealing that sialic acids are not detectable in cryptococcal glycolipids. In fact, a comparison between the sialic content of *C. neoformans* cells and solvent-treated yeasts revealed that lipid removal does not affect the number of sialic acid residues, suggesting that sialylated glycolipids are not present in these cells. In contrast, two sialylglycoproteins were detected in crude extracts of *C. neoformans*, as inferred from their reactivity with peroxidase-labeled lectins and influenza C virus used as probes in Western blotting analyses.

Hamilton and co-workers [15] have shown that sialic acid is a component of a *C. neoformans* glycoprotein with a molecular mass corresponding to 38 kDa, produced as an exoantigen. Our analyses using cellular extracts demonstrate that probably the same glycoprotein, and an additional sialylated molecule of 67 kDa, are produced by *C. neoformans*. SNA recognized both molecules, while LFA only reacted with the 38 kDa component. This is in accordance with preceding reports showing that the presence of 9-*O*-acetyl groups in Neu5Ac can block the interaction between Neu5Ac and LFA, but not with SNA [19]. In fact, the 67 kDa glycoprotein reacted with influenza C virus at 4°C, was poorly sensitive to sialidase treatment, and its previous treatment with alkali prevented virus binding, all of these being characteristics of Neu5,9Ac_2-containing molecules [29]. In contrast, the 38 kDa component, which was recognized by both LFA and SNA, was sensitive to sialidase treatment and did not react with influenza virus, therefore containing unsubstituted α2,6-linked Neu5Ac.

Sialic acids are cell surface components of a wide variety of organisms. In several biological models, their distribution and functions are well established [5,6]. Sialic acids are also synthesized by fungi but, although they have been described as surface components of *C. neoformans* [8,15], *Sporothrix schenckii* [9], *Paracoccidioides brasiliensis* [10,11], *Candida albicans* [12], *Fonsecaea pedrosoi* [13], *Aspergillus fumigatus* [14], and *Pneumocystis carinii* [16,17], their biological significance in these cells has not been unequivocally determined. In the specific case of *Aspergillus*, *Sporothrix*, and *Cryptococcus* species, sialic acids seem to contribute to microbial pathogenesis; for instance, Neu5Ac expression on the conidial cell wall of the pathogen *A. fumigatus* was 3–20 times greater than that on the cell wall of the non-pathogenic species *A. auricomus*, *A. wentii* and *A. ornatus* [14], and this was correlated with the increased ability of *A. fumigatus* to bind to proteins of the basal lamina. In *S. schenckii* [30] and *C. neoformans* [3,8], sialic acid units protect yeast cells against phagocytosis, which may represent an early mechanism of fungal defense against host destruction. Besides influencing the interaction with the host, sialic acids may have additional functions in fungal cells: in *F. pedrosoi* [13], they seem to play a structural role in the preservation of cell morphology.

The apparent roles of sialic acids in pathogenic fungi stimulate studies on their biosynthesis, which is a completely unexplored field in fungal biology. As reviewed by Angata and Varki [6], fungal enzymes comparable to those known to be involved in the biosynthesis, activation, or transfer of sialic acids in bacteria and mammals have not been described. Fungal cells apparently synthesize sialoglycoconjugates in a process catalyzed by the action of CMP-Neu5Ac-dependent sialyltransferases, since they express sialic acids after growing in culture media depleted of these sugars [8–14]. Moreover, sialic acid expression can be modulated by environmental conditions, as in the case of *A. fumigatus* [14]. In this pathogen, expression of Neu5Ac in conidia grown on a sialic acid-free minimal medium was significantly less than in conidia cultivated in a complex medium, which suggests a regulation of synthesis by components of the medium [14]. Presumably, a putative sialyltransferase activity could be negatively modulated by limiting concentration of CTP in the minimal medium, which would therefore control the formation of CMP-Neu5Ac, the donor substrate for sialyltransferase activity. Nevertheless, the lack of reports on the transfer of sialic acids in fungal cells does not discard the possibility that they have alternative pathways to synthesize and express sialic acids or acquire them from external sources [6].

Using different approaches, we currently demonstrate that a preparation of partially purified galactose-binding proteins from *C. neoformans* is able to use CMP-Neu5Ac as the donor substrate for the transfer of sialic acid to different acceptors. After incubation in the presence of the cryptococcal proteins, asialofetuin became reactive with LFA. When *N*-[acetyl-1-^{14}C]-lactosamine was used as the acceptor substrate, a radioactive acidic compound binding with high affinity to the anionic resin Dowex 1 $\times$ 8 was formed. This strongly negative molecule had higher migration rates on HPTLC plates when compared to the original substrate *N*-[acetyl-1-^{14}C]-lactosamine, and was partially sensitive to sialidase treatment. The generation of a negative radioactive compound after incubation in the presence of CMP-Neu5Ac strongly indicates that the galactose-binding preparation from *C. neoformans* presents sialyltransferase activity. Interestingly, the sialylated compound was poorly sensitive to sialidase treatment and hydrolysis with formic acid. This could be explained by the formation of an alternative linkage between sialic acid and the galactose residue in *N*-acetyl-lactosamine, which may have a parallel with previous results using the model pathogen *A. fumigatus*, whose

surface is strongly recognized by LFA but not SNA or MAA lectins [14].

Additional evidence corroborating the occurrence of a sialyltransferase in *C. neoformans* came from the fact that rabbit polyclonal antibody raised against mammalian sialyltransferase recognized cryptococcal proteins. These antibodies, that recognize the catalytic site of the enzyme, reacted with a single band with an apparent molecular mass of 60 kDa in crude extracts. When the galactose-binding molecules were exposed to the anti-α2,6-sialyltransferase antibody, an additional band was detected, suggesting that the latter could be somewhat inaccessible to antibodies in the crude extracts because of co-migration and superposition. These results are suggestive that a fungal protein with domains (epitopes) similar to those observed in the catalytic sites of animal sialyltransferases can occur in *C. neoformans*.

In the specific case of the encapsulated pathogen *C. neoformans*, the antiphagocytic properties of sialic acids could be related with fungal protection against host cells at the initial phase of infection. Infection by *C. neoformans* starts by inhalation of desiccated, poorly encapsulated haploid yeast cells or basidiospores [3]. In the alveolar space, inhaled fungal cells are initially confronted by resident alveolar macrophages and, in this context, synthesis of the capsule is generally regarded as a major protection mechanism against phagocytosis [1]. Fully encapsulated yeast cells, however, can be found in the lung tissue only 5 to 10 h after the introduction of poorly encapsulated organisms [4]. The presence of antiphagocytic residues of sialic acids on the fungal cell wall could therefore protect yeasts before the full expression of the capsular polysaccharide takes place. Sialic acid residues in *C. neoformans* are mainly linked to galactose [8], therefore they may protect yeast cells against endocytosis by blocking their interaction with galactose-recognizing receptors of host macrophages [5]. They can also promote cell-cell repulsion by contributing to the cryptococcal negative charge before the capsule is formed.

The results described in this study provide novel information about sialyltransferases in pathogenic fungi and stimulate studies aiming at the characterization, at the molecular and structural levels, of sialylated molecules and the enzymes responsible for their biosynthesis in fungal cells. Specific inhibitors of this glycosylation step would allow evaluating the role of sialic acids in pathogenic fungi.

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