Sphingolipids of the mycopathogen *Sporothrix schenckii*: identification of a glycosylinositol phosphorylceramide with novel core GlcNH$_2$α1→2Ins motif

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Abstract  Acidic glycosphingolipid components were extracted from the yeast form of the dimorphic mycopathogen *Sporothrix schenckii*. Two minor and the major fraction from the yeast form (Ss-Y1, -Y2, and -Y6, respectively) have been isolated. By a combination of 1- and 2-D 1H-nuclear magnetic resonance (NMR) spectroscopy, electrospray ionization mass spectrometry (ESI-MS), and gas chromatography/mass spectrometry (GC/MS), Ss-Y6 was determined to be tritylcosylinositol phosphorylceramide with a novel glycan structure, Man$_6$GlcnH$_2$α1→2Ins1-P-1Cer (where Ins = myo-inositol, P = phosphodiester). While the GlcNH$_2$α1→6Ins1-P-motif is found widely distributed in eukaryotic GPI anchors, the linkage GlcNH$_2$α1→2Ins1-P has not been previously observed in any glycolipid. Ss-Y1 and Ss-Y2 were both found to have the known glycan structure Man$_3$α1→3Man$_1$α1→2Ins1-P-1Cer. Together with the results of a prior study [Toledo et al. (2001) Biochem. Biophys. Res. Commun. 280, 19-24] which showed that the mycelium form expresses GIPCs with the structures Man$_3$α1→6Ins1-P-1Cer and Man$_3$α1→3Man$_1$α1→6Ins1-P-1Cer, these results demonstrate that *S. schenckii* can synthesize glycosylinositol phosphorylceramides with at least three different core linkages. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Sphingolipid; Glycolipid; Dimorphism; Mycopathogen; Nuclear magnetic resonance; Mass spectrometry

1. Introduction

The risk of systemic fungal disease among growing populations of immunosuppressed or -compromised patients [5], along with recent work showing that fungi are vulnerable to inhibitors of sphingolipid biosynthesis (reviewed in [6]), has led to an increased interest in the structure, biosynthesis, and functional roles of fungal sphingolipids as potential targets for antifungal agents. A particularly interesting target is the fungal inositol phosphorylceramide (IPC) synthase [6], inhibitors of which are highly toxic to many mycopathogens, but exhibit low toxicity in mammals [7–10]. IPC synthase catalyzes the transfer of myo-inositol-1-phosphate from phosphatidylinositol to ceramide; further processing of IPC by glycosyltransferases yields glycosylinositol phosphorylceramides (GIPCs). A number of studies have pointed to potential interactions of GIPCs of fungi with the mammalian immune system [11–13]. Elucidation of such interactions calls for detailed knowledge of the structures of these compounds, which is still limited in comparison to what is known about glycosphingolipids (GSLs) of animal species. Also, as occurred earlier with mammalian studies, expanding knowledge of fungal GIPC structures can be expected, by extension, to reveal a great deal about the activities and expression patterns of the enzymes responsible for their synthesis. The detection of novel glycosyl linkages, and therefore of novel glycosyltransferases, is an important complement to molecular and cell biological approaches to glycoconjugate function; in addition, specific GIPC processing enzymes could offer further potential targets for development of inhibitors as antifungal agents.

In this paper, we report on the structure elucidation of GIPCs from the dimorphic mycopathogen *Sporothrix schenckii*. Among other observations, these studies revealed that a major component of the yeast form has the structure Man$_3$α1→3Man$_1$α1→6GlcnH$_2$α1→2Ins1-P-1Cer$^2$. To our knowledge, this is the first report of any glycolipid with the GlcnH$_2$α1→2Ins1-P- structural motif, although the isomeric GlcnH$_2$α1→6Ins1-P- linkage is commonly encountered in eukaryotic GPI anchored proteins and phosphoglycans [14,15].

2. Materials and methods

2.1. Fungal isolate and growth conditions

The culture of *S. schenckii*, strain 65 (originally obtained from cutaneous footpad lesion of an otherwise healthy individual), was

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2 A preliminary description of these studies has been presented in poster form [1].
provided by Dr. Olga Gompertz, Department of Cellular Biology, Universidade Federal de São Paulo/Escola Paulista de Medicina, São Paulo, SP, Brazil. Mycelium and yeast forms of *S. schenckii* strain 65 were grown in brain-heart infusion (BHI; 37 g/l water), incubated at 25 and 37°C, respectively, and harvested as described [16].

2.2. Extraction, purification, and thin layer chromatographic analysis of GSLs

Extraction and purification of GSLs were carried out as described previously [11,17,18]. Briefly, GIPs were extracted by homogenizing yeast or mycelium forms (25–35 g wet weight) in an Omni-mixer (Sorvall Inc. Wilmington, DE, USA), three times with 200 ml of isopropanol/hexane/water (55:20:25, v/v/v, upper phase discarded), and twice with 200 ml of chloroform/methanol (2:1, v/v). The five extracts were pooled, dried on a rotary evaporator, dialyzed against water, lyophilized, re suspended in chloroform/methanol/water (30:60:8, v/v/v; solvent A), and applied to a column of DEAE-Sephadex A-25 (Ac⁺ form). Neutral GSLs were eluted with five volumes of solvent A. Acidic GSLs were eluted with five volumes of 0.5 M sodium acetate in MeOH. The acidic fraction was dried, dialyzed exhaustively against deionized water, redried and further purified by repetitive preparative-scale high performance liquid chromatography (HPLC) (50 cm × 4.6 mm Spherelone (Phenomenex, Torrance, CA, USA) 10 μm porous spherical silica; elution Z-propanol-hexane-water gradient from 55:40:5 to 55:25:20 over 120 min, then isocratic for 45 min; flow rate 0.5 ml/min; 80–1 ml fractions collected). The identity and purity of each peak was assessed by analytical high performance thin layer chromatography (HPTLC), performed on silica gel 60 plates (E. Merck, Darmstadt, Germany) using chloroform/methanol/water (60:35:10, v/v/v) as mobile phase, with detection by Bial’s orcinol reagent.

2.2.1. HPTLC profile of *S. schenckii* GIPCs

HPTLC profiles of GIPCs from mycelial and yeast forms of *S. schenckii* differed considerably, as shown in Fig. 1. In the mycelial form, five bands were visible with orcinol staining, designated Ss-M1 through Ss-M5 (lane M). Of these, the first three appeared to be major components, with decreasing amounts of Ss-M4 and Ss-M5. Characterization of Ss-M1, -M2, and -M3 has been reported [4]. The appearance of GIPCs from the yeast form was more complex, with five very minor bands, designated Ss-Y1 through Ss-Y5, migrating above a single major band, Ss-Y6, having a very low Rf (lane Y). Several more closely spaced minor band were visible below this one. Of these yeast form fractions, only Ss-Y1, -Y2, and -Y6 were purified in sufficient quantity and were homogeneous enough for detailed characterization.

2.2.2. Characterization of *S. schenckii* yeast form GIPC profiles

1-D 1H-NMR spectra of *S. schenckii* yeast form fractions Ss-Y1 and Ss-Y2 (not shown) were both essentially identical to that of Man3Man3Man2IPCer previously isolated and characterized from *Paracoccidioides brasiliensis* [3]. At least with respect to glycan structure, the identities of Ss-Y1 and -Y2 were therefore considered to be known.

3. Results

3.1. HPTLC profile of *S. schenckii* GIPCs

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Component analysis by GC/MS indicated that fraction Ss-Y6 contained mannose, glucosamine (detected as GlcNAc), and myo-inositol in ratio 2:1:1; other components detected were t18:0 and t20:0 4-hydroxysphinganines (phytosphingosines) and h24:0 fatty acid (major). The relevant downfield section of a 1-D 1H-NMR spectrum of fraction Ss-Y6 is reproduced in Fig. 2; a section of the TOCSY spectrum corresponding to this region is reproduced in Fig. 3A. Chemical shift/connectivity assignments of all 1H signals, as well as approximate measurements of $J_{i,j}$ coupling constants, in the monosaccharide, inositol, and proximal part of the ceramide were obtained from sequential application of 2-D $^1$H-$^1$H gradient-COSY and TOCSY experiments. Complete assignments of $^1$H resonances derived from this analysis are listed in Table 1. The myo-inositol residue was recognized as a cyclic spin system in which all $J_{i,j}$ are large except for $J_{1,2}$ and $J_{2,3}$, as H-2 is the only equatorial proton in the 1,2,3,4,5,6-hexahydroxy-cyclohexane ring. The three monosaccharide residues were recognized by their connectivity/coupling patterns [28] starting from the most downfield signal (H-1) of each spin system. Aside from the presence of two Man residues recognizable by their signature small values for $J_{1,2}$ and $J_{2,3}$, the NMR spectrum of Ss-Y6 is characterized in particular as exhibiting a sugar H-2 signal shifted far upfield at 2.380 ppm, part of a spin system, originating from H-1 at 5.011 ppm, having $J_{i,j}$ coupling patterns consistent with an α-glucopyranosyl configuration. The upfield shifted signal is consistent with H-2 attached to a carbon atom bearing a free amino group. The linkage assignments were established by observation in the NOESY spectrum (Fig. 3B) of strong interresidue correlations between H-1 of the GlcNH$_2$ residue and Ins H-2 (3.982 ppm), between Man H-1 at 4.623 ppm and GlcNH$_2$ H-6 (3.568 ppm), and between Man H-1 at 4.885 ppm and H-3 of the first Man residue (3.614 ppm). Weak interresidue cor-

![Fig. 2. Downfield sections of 1-D 1H-NMR spectra of GIPC fractions Ss-Y6 (A) and Ss-M3 (B). Key reporter signals are labeled.](image)

![Fig. 3. Sections of 2-D 1H-NMR spectra of GIPC fraction Ss-Y6; 1H-1H-TOCSY (A) and 1H-1H-NOESY (B). Key through-bond chemical shift correlations for the myo-inositol and three monosaccharide residues are labeled in A, key interresidue NOE correlations are boxed and labeled in B.](image)
relations were also observed from Man H-1 to GlcNH2 H-5 (4.046 ppm) and other H-6 (3.645 ppm). Based on NMR spectroscopic analysis, the structure of Ss-Y6 could be proposed as Manα3Manα6GlcNH2α2IPC.

In "ESI-MS analysis two major M-Li⁺ for Ss-Y6 were observed at m/z 1423 and 1451, in ~ equal abundance (Fig. 4A), consistent with a composition of HexαHexNH2Ins-P linked to Cer composed of t=18:0 and t=20:0 phytosphingosines with h=24:0 fatty acid. Some minor ceramide variants differing by 14 Th (CH2) increments from the major species were also detectable. Product ion mode "ESI-MS/CID-MS of the molecular species at both m/z 1423 and 1451 (data not shown); designations of fragments are illustrated in Scheme 1) yielded significant fragments at m/z 255 [Ins-P-Li⁺]⁺ ([Y₁/B₄PO₃-Li⁺]), 331 [B₅-Li⁺], 416 [HexNH₂-Ins-P-Li⁺]⁺ ([Y₂/C₄PO₃-Li⁺]), 434 [HexNH₂-Ins-P-Li⁺]⁺ ([Y₂/C₄PO₃-Li⁺]), 492 [B₅-Li⁺] or [Y₃/B₅Li⁺], 510 [C₆-Li⁺] or [Y₃/C₆Li⁺], 578 [HexNH₂-Ins-P-Li⁺]⁺ ([Y₃/B₅PO₃-Li⁺]), 596 [HexNH₂-Ins-P-Li⁺]⁺ ([Y₃/C₄PO₃-Li⁺]), 654 [B₆-Li⁺], 672 [C₇-Li⁺], 740 [HexαHexNH2-Ins-P-Li⁺]⁺ ([B₆PO₃-Li⁺]), and 758 [HexαHexNH2-Ins-P-Li⁺]⁺ ([C₇PO₃-Li⁺]). Ceramide-related ions were observed at m/z 690 [Cer-Li⁺]⁺ ([Y₄/Li⁺]+ and 776 [CerPO₂-Li⁺]⁺ ([Y₅PO₂-Li⁺]) in the product spectrum from m/z 1423. In the product spectrum from m/z 1451, these were shifted to m/z 718 and 802, respectively.

The identity and placement of the HexNH2 residue in the sequence was confirmed by treating an aliquot of Ss-Y6 with acetic anhydride under conditions known to yield selective N-acetylation. Following this treatment, the two major molecular ion species were observed in "ESI-MS profile at m/z 1465 and 1493, respectively (Fig. 4B), consistent with addition of one acetyl group, i.e. conversion of the HexNH2 residue to HexNAc. Although the high OR "ESI-MS spectrum was too noisy to interpret fully, fragments were clearly observable at m/z 255 [Ins-P-Li⁺]⁺ ([Y₃/B₅PO₃-Li⁺]), 690 and 718 [Cer-Li⁺]⁺ ([Y₃-Li⁺], and 800 [HexαHexNH2-Ins-P-Li⁺]⁺ ([C₇PO₃-Li⁺]) (data not shown). "ESI-MS/CID-MS precursor ion scanning from the m/z 800 fragment (Fig. 5A) clearly showed its origins from the major molecular ions at m/z 1465 and 1493; minor molecular species at m/z 1479, 1507, and 1521 were also detectable (~5-10% the abundance of m/z 1493). "ESI-MS/CID-MS product ion scanning from the m/z 800 fragment (Fig. 5B) yielded abundant fragment characteristic for the glycan sequence, i.e. at m/z 111 [H₂PO₂-Li⁺], 169 [B₅-Li⁺] or [Y₁/B₅-Li⁺], 187 [C₆-Li⁺] or [Y₁/C₆Li⁺], 255 [Ins-P-Li⁺]⁺ ([Y₁/B₅PO₃-Li⁺]), 273 [Ins-P-Li⁺]⁺ ([Y₁/C₄PO₃-Li⁺], 331 [B₆-Li⁺], 349 [C₇-Li⁺], 372 [Y₃/B₅-Li⁺], 390 [Y₃/C₆Li⁺], 398, 458 [HexαIns-P-Li⁺]⁺ ([Y₂/B₅PO₃-Li⁺]), 476

Table 1

<table>
<thead>
<tr>
<th>Hex linkages ppm</th>
<th>monosaccharide, inositol, ceramide sphingoid and fatty-acyl (in parentheses) residues of Manα3Manα6GlcNH2α21PC (Ss-Y6) from S. schenckii in DMSO-d₆</th>
<th>2% D₂O at 35°C</th>
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<tbody>
<tr>
<td>H-1</td>
<td>8.885</td>
<td>6.423</td>
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<tr>
<td>(J₂, J₃)</td>
<td>(~ 2.0)</td>
<td>(1.8)</td>
</tr>
<tr>
<td>H-4</td>
<td>3.422</td>
<td>3.508</td>
</tr>
<tr>
<td>H-5</td>
<td>3.602</td>
<td>3.432</td>
</tr>
<tr>
<td>H-6</td>
<td>3.47</td>
<td>3.47</td>
</tr>
</tbody>
</table>

"J₂ (Hz) for monosaccharide residues are given in parentheses.

Scheme 1. Molecular ions and fragmentation of Ss-Y6 and N-acetylated Ss-Y6 in positive ion mode ESI-MS and -MS/CID-MS.

Discussion

Analysis of GIPCs in the dimorphic mycopathogen S. schenckii (in [4] and the present study) has revealed (i) the expression of a variety of glycan structures, some of which have not been previously reported in fungi; (ii) different patterns of both glycan and ceramide expression in the yeast and mycelial forms of this fungus; and (iii) that, between the two forms, S. schenckii is capable of creating at least three different glycosyl linkages with the myo-inositol ring of Inpcer. These are Manα1→2Ins, Manα1→6Ins, and GlcNH2α1→2Ins; of these, the first has been confirmed to be widely distributed in GIPCs of fungi [2,3,12,29] and the second has been detected only recently in S. schenckii mycelium form GIPCs [4]; the third linkage has not, to our knowledge, been reported in any eukaryotic glycoconjugate or carbohydrate. The isomeric structure, GlcNH2α1→6Ins, is of course well known as the common core linkage found in glycosylinositol phospholipids (GIPs) of parasites and in the GPI protein membrane anchors widely distributed among eukaryotes [14,30]. The occurrence of non-N-acetylated glucosamine in another linkage to myo-inositol is particularly interesting, since it is known from studies with mammalian and trypanosomatid cells that two steps are required for formation of GlcNH2α1→6Ins found in GPI anchors, transfer of GlcNAc from UDP-GlcNAc to Ins of phosphatidylinositol, and subsequent de-N-acetylation [30]. That both reactions could occur together in forming an alternate glycosyl linkage is an intriguing possibility.

From the results of this and other studies [2-4,12,29,31], a picture is emerging of GIPC structural diversity which may result from the diversity of C-1,2-linked Man-, GlcNAc-, and GlcNH2-containing "Manα3Manα6GlcNH2α21PC (Ss-Y6), which has been shown to have significant structural diversity [4]. This diversity may contribute to the broad range of biological activities of GIPCs, which include cell signaling, cell adhesion, and immune modulation [4,12,29,31]. The ability of GIPCs to adopt different structures may allow them to interact with a variety of cellular receptors and contribute to their biological activities.

Although Barr et al. [2] did not specify precisely the linkage between Man and Ins in their compounds, but characterized it as Manα1→2/6Ins, NMR spectroscopy of H. capsulatum GIPCs shows that the linkage is Manα1→2Ins-P-1Cer as reported previously for GIPCs of P. brasiliensis [3] and for Ss-Y1 and -Y2 as reported herein.
ultimately rival that found in animal GSLs. As in the animal kingdom, some structures appear to be shared in common by two or more fungal species, while the overall expression repertoire may be unique for each species. A GIPC having the structure Manα3(Galβ4)Manα2IPC has been found in both yeast and mycelium forms of *Histoplasma capsulatum* [2,12] and in the mycelium form of *S. schenckii* [4]; and the common intermediate structures Manα2IPC and Manα3Manα2IPC have been isolated from several species [2,3,12,29]. As with their mammalian counterparts, patterns of GIPC expression within a particular fungal species can also be expected to depend in a complex manner on a variety of factors, such as strain, culture conditions, and stage of development.

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Fig. 4. Singly charged Li⁺ adduct molecular ion profiles of Ss-Y6 (A) and N-acetylated Ss-Y6 (B) in positive ion mode ESI-MS.
Fig. 5. Positive ion mode ESI-MS/CID-MS of m/z 800 ion in spectrum of N-acetylated Ss-Y6. A: Precursor ion scan (molecular ion region). B: Product ion scan.

References


