Isolation of Sporothrix schenckii MNT1 and the biochemical and functional characterization of the encoded α1,2-mannosyltransferase activity

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Sporothrix (Sp.) schenckii is a pathogenic fungus that infects humans and animals, and is responsible for the disease named sporotrichosis. The cell wall of this fungus has glycoproteins with a high content of mannose and rhamnose units, which are synthesized by endoplasmic reticulum- and Golgi-localized glycosyltransferases. Little is known about the enzymatic machinery involved in the synthesis of these oligosaccharides in Sp. schenckii, or the genes encoding these activities. This is in part because of the lack of an available genome sequence for this organism. Using a partial genomic DNA library we identified SsMNT1, whose predicted product has significant similarity to proteins encoded by members of the Saccharomyces (Sa.) cerevisiae KRE2/MNT1 gene family. In order to biochemically characterize the putative enzyme, SsMNT1 was heterologously expressed in the methylotrophic yeast Pichia pastoris. Recombinant SsMnt1 showed Mn²⁺-dependent mannosyltransferase activity and the ability to recognize as acceptors α-methyl mannoside, mannoside, Man₉GlcNAc₂ oligosaccharide and a variety of mannobiosides. The characterization of the enzymic products generated by SsMnt1 revealed that the enzyme is an α1,2-mannosyltransferase that adds up to two mannose residues to the acceptor molecule. Functional complementation studies were performed in Sa. cerevisiae and Candida albicans mutants lacking members of the KRE2/MNT1 gene family, demonstrating that SsMnt1 is involved in both the N- and O-linked glycosylation pathways, but not in phosphomannan elaboration.

INTRODUCTION

Sporothrix (Sp.) schenckii is the causative agent of sporotrichosis, a subacute or chronic subcutaneous mycosis. The disease has a worldwide distribution, is generally acquired by traumatic inoculation into subcutaneous tissues, and in immunocompromised individuals can cause death (López-Romero et al., 2011).

The cell wall of this fungus is composed of alkali-soluble and -insoluble glucans linked by β1,3-, β1,4- and β1,6- bonds (Previto et al., 1979), and glycoproteins composed mainly of mannose, rhamnose and galactose named peptidorhamnomannan, galactomannan and peptidohamnoglactan (Lloyd & Bitoon, 1971; Mendonça et al., 1976; Mendonça-Previto et al., 1980; Nakamura, 1976).

At present, the structure of the oligosaccharides attached to cell wall glycoproteins is not known in detail, but there are reports indicating that some rhamnomannans have an α1,6-mannose backbone oligosaccharide, with side chains composed of one or two rhamnose units (Lloyd & Bitoon, 1971). Another rhamnomannan identified has an α1,4-linked mannose backbone oligosaccharide with lateral branches of a single α1,2-linked mannose residue, which can also be monorhamnosylated (Travassos et al., 1973). The major O-linked glycans present within the peptidohamnogomannan contains an α1,2-mannobiose core followed by one α-glucuronic acid residue, which can be mono- or di-rhamnosylated with a length of up to five monosaccharide units (Lopes-Alves et al., 1992), and has been demonstrated to be an important antigenic determinant (Lopes-Alves et al., 1994).

Although there have been significant advances in the identification and characterization of glycan structures from this fungus, the enzymes participating in the biosynthetic pathways have been poorly studied.

Glycosylation is a major post-translational modification of proteins. There are three main types of protein glycosylation in eukaryotes: glycosylphosphatidylinositol (GPI)
anchors, in which the non-reducing end of the glycan moiety of a GPI group is attached to the protein, and N- and O-linked glycosylation, in which an oligosaccharide is attached to the amide group of asparagine, and to the hydroxy group of serine and threonine residues, respectively. Studies in the human pathogenic fungus Candida albicans indicate that proper elaboration of glycoproteins is required for adhesion, virulence and host–fungus interaction (Bates et al., 2005, 2006; Munro et al., 2005; Netea et al., 2006; Cambi et al., 2008; Harris et al., 2009; Mora-Montes et al., 2007, 2009, 2010a; McKenzie et al., 2010; Sheth et al., 2011).

The structure of the O- and N-linked glycans is determined by glycosyl hydrolases and transferases localized in the endoplasmic reticulum and the Golgi complex. Most of the Golgi mannosyltransferases are encoded by the KRE2/MNT1 and MNN gene families (Lussier et al., 1999; Häusler & Robbins, 1992; Romero et al., 1999). These enzymes are able to transfer mannose from a GDP-mannose donor to the hydroxyl group of an oligosaccharide acceptor (Paulson et al., 1987).

In Saccharomyces (Sa.) cerevisiae, the KRE2/MNT1 gene family is composed of nine members. ScKre2, ScKtr1 and ScKtr3 are α1,2-mannosyltransferases involved in the addition of the second and third mannose to the O-linked glycans, and also participate in the proper elaboration of the N-linked glycan outer chain (Lussier et al., 1997), whereas ScYur1 and ScKtr2 encode mannosyltransferases only involved in N-linked mannosylation (Lussier et al., 1996). Furthermore, ScKtr6 is the main enzyme involved in phosphorylomannosylation of both N-linked and O-linked glycans (Wang et al., 1997; Nakayama et al., 1998). The role of ScKtr4, ScKtr5 and ScKtr7 is currently unknown.

In C. albicans, the MNT1 gene family has five members with more specialized functions: CaMnt1 and CaMnt2 have redundant activities in the addition of the second and third mannose residues to O-linked mannans (Munro et al., 2005; Buurman et al., 1998; Thomson et al., 2000), both CaMnt4 and CaMnt5 participate in the synthesis of N-linked mannan outer chains, and CaMnt3 and CaMnt5 have redundant participation in the synthesis of the phosphomannan moiety (Mora-Montes et al., 2010a). Furthermore, this gene family is important for proper cell wall composition, virulence and interaction with immune cells (Mora-Montes et al., 2010a).

In the opportunistic mould Aspergillus fumigatus, only three predicted MNT1 gene family ORFs have been identified, and the encoded proteins are putative α1,2-mannosyltransferases. The lack of AfMnt1 leads to a thinner hyphal cell wall, an increased sensitivity to cell wall stressors, temperature-restricted growth, and reduced sporulation and virulence (Wagener et al., 2008).

To date, the mannosyltransferases described in fungi do not have any human orthologues; therefore, the study of these enzymes could potentially unveil new antifungal targets. In this paper we report the isolation of Sp. schenckii MNT1 and the biochemical and functional characterization of the encoded α1,2-mannosyltransferase activity.

**METHODS**

**Strains, media and culture conditions.** The organisms used in this work are listed in Table 1 and were maintained at 28 °C in YPD medium [1 % (w/v) yeast extract, 2 % (w/v) mycological peptone, 2 % (w/v) glucose]. Solid plates were prepared with 2 % (w/v) bacteriological agar. The mycelial form of Sp. schenckii was propagated in YPD broth, pH 4.5, as described previously (Ruiz-Baca et al., 2005), while transition to yeast morphology was achieved in YPD medium, pH 7.4, as reported previously (Mora-Montes et al., 2010b). The C. albicans strains were grown at 28 °C in YPD medium or in SC medium [0.67 % (w/v) yeast nitrogen base without amino acids, 2 % (w/v) glucose, 0.077 % (w/v) complete supplement mixture minus uracil] supplemented with 50 μg uridine ml⁻¹ when necessary. For β-N-acetylhexosaminidase (Hex1) induction, the cells were grown at 28 °C in medium containing 25 mM glucosamine as described previously (Mora-Montes et al., 2010a). For expression of ScMNT1 in Sa. cerevisiae, strains were grown at 28 °C in SC + GAL medium [0.67 % (w/v) yeast nitrogen base without amino acids, 0.077 % (w/v) complete supplement mixture minus uracil, 2 % (w/v) galactose and 3 % (w/v) raffinose].

**ScMNT1 isolation.** A 767 bp fragment of ScMNT1 was firstly isolated by PCR using genomic DNA from Sp. schenckii and degenerate primers (5′-GCNACNTTGYTNACNYTG-CN-3′ and 5′-RTCNCCC-CANCKYCTCRTARAARAA-3′), whose design was based on conserved regions of putative amino acid sequences of proteins belonging to the KRE2/MNT1 family from Neurospora crassa (GenBank accession no. XP_962248.1), Magnaporthe oryzae (XP_363108.1), Podospora anserina (XP_003437245.1) and Chaetomium globosum (XP_001223342.1), organisms closely related to Sp. schenckii (Teher et al., 2003).

In order to obtain the whole ORF we generated a partial genomic DNA library as follows. Sp. schenckii genomic DNA was digested with HindIII, SalI, EcoRI, PstI or BamHI, the digest was used to perform a Southern blot analysis with the radiolabelled 767 bp fragment, and the DNA from the hybridization area was isolated and cloned into the pBluescript-KS II (+) vector (Stratagene). The library was screened by PCR using the primer pair 5′-AGGACAGGCGCAAAAAGG-3′ and 5′-GGTTGACGACTCAGAAGAA-3′. By following this strategy we isolated a HindIII-restricted −5 kbp DNA fragment that was sequenced and found to contain the entire ScMNT1 ORF.

**SpMNT1 expression in Pichia pastoris.** The DNA sequence encoding the putative SsMnt1 soluble domain (nucleotides 170–1515) was cloned into the expression kit (Invitrogen). Strains HMP1 and X-33_pPICZ A with the inserts at the XbaI sites of the pPICZ A expression vector (Invitrogen) were used to transform P. pastoris X-33 (Invitrogen) by electroporation, as described by the manufacturer. Transformants were selected on YPDS plates (1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) glucose, 1 M sorbitol, 2 % (w/v) agar) containing 100 μg Zeocin ml⁻¹ (Invitrogen). Strains HMP1 and X-33_pPICZ A are X-33 with SacI-digested pPMNT1 and SacI-digested pPICZ A inserted at the AOX1 locus, respectively. This recombination event was confirmed by PCR using the primer pair included in the P. pastoris expression kit (Invitrogen).

The P. pastoris strains were grown at 30 °C with shaking (200 r.p.m.) in MGY medium [1 % (w/v) yeast extract, 2 % (w/v) peptone, 1.34 % (w/v) yeast nitrogen base without amino acids, 4 × 10⁻³ % (w/v) uracil].
Table 1. Strains used in this study

<table>
<thead>
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<th>Strain</th>
<th>Organism</th>
<th>Genotype</th>
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<tr>
<td>EH-206</td>
<td><em>Sp. schenkii</em></td>
<td>Clinical isolate</td>
<td>WDCM*</td>
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<td><em>P. pastoris</em></td>
<td>Wild-type</td>
<td>Invitrogen</td>
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<td>X-33_pPICZaA</td>
<td><em>P. pastoris</em></td>
<td>As X-33 but pPICZaA inserted at AOX1</td>
<td>This study</td>
</tr>
<tr>
<td>HMP1</td>
<td><em>P. pastoris</em></td>
<td>As X-33 but pPICZaA-SsMNT1 inserted at AOX1</td>
<td>This study</td>
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<td>CAI4</td>
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<td><em>C. albicans</em></td>
<td>as RPS1/psiαΔ::CpI10</td>
<td>Brand et al. (2004)</td>
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<td>NGY337</td>
<td><em>C. albicans</em></td>
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<td>Munro et al. (2005)</td>
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<tr>
<td>NGY527</td>
<td><em>C. albicans</em></td>
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<td>Mora-Montes et al. (2010a)</td>
</tr>
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<td><em>S. cerevisiae</em></td>
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*World Data Center for Microorganisms; accession number BMFM-UNAM (834).  

biotin, 1% (w/v) glycerol] until they reached saturation (typically 18 h), harvested by centrifugation and resuspended in 100 ml MMY medium, which is similar to MGY, except that it contains 1% (v/v) methanol instead of glycerol, to induce expression of recombinant protein. Methanol was added to a final concentration of 1% (v/v) every 24 h to maintain the protein induction during a 4 day period.

**SsMNT1 expression in C. albicans**. The DNA sequence encoding the entire ORF of SsMNT1 was codon-optimized for expression in *C. albicans* and synthesized by DNA 2.0, adding the restriction sites HindIII and Xhol at the 5’ and 3’ ends, respectively. This ORF was cloned into the HindIII–Xhol sites of the pACT1-GFP vector (Barelle et al., 2004), replacing the GFP ORF and under the control of the ACT1 promoter, generating pACT-MNT1. The Stul-digested plasmid was used to transform *C. albicans* strains. The integration into the RPS1 locus was verified by PCR using the primer pair 5’-CACCAG-GATTTATTGCAAC-3’ and 5’-GAAGGAGTAGAATGTAC-3’.

**SsMNT1 expression in S. cerevisiae**. The optimized DNA sequence for expression in *C. albicans* was used for expression in *S. cerevisiae*, adding the restriction sites HindIII and Xhol at the 5’ and 3’ ends, respectively. The ORF was cloned into the HindIII–Xhol sites of the pYES2 vector (Invitrogen), under the control of the GAL1 promoter. The construction generated was used to transform the *S. cerevisiae* krrΔ null mutant (Wang et al., 1997). As a control, the cells were transformed with the empty vector.

**Mannosyltransferase assay**. Mannosyltransferase enzyme activity was assayed as described by Nakajima & Ballou (1975). Assay mixtures contained 50 mM PIPES-NaOH, pH 7.0, 10 mM MnCl2, 50 mM *z*-methyl mannoside, 0.38 μM GDP-[14C]mannose [5 μCi (185 kBq); specific activity 262 μCi mmol⁻¹ (96.9 GBq mmol⁻¹)] and 45–50 μg of soluble recombinant protein previously concentrated and desalted by an Amicon System (Millipore). Reactions were performed for 24 h at 30 °C in a final volume of 50 μl. The reaction mixtures were added to a 1.5 ml Eppendorf tube containing 0.4 ml Dowex 1-X2 (Bio-Rad) anion exchange resin to remove non- incorporated, labelled GDP-mannose. The neutral products were eluted with 1.5 ml deionized water and radioactivity was measured in a Beckman LS 6500 scintillation counter. Control assays were conducted without protein or with protein from strain X-33_pPICZaA. For the acceptor preference assays, reactions were carried out as described, but the acceptor was substituted by 4 mM of each of the following sugars: mannos, *z*-methyl mannoside, *z*1,2-mannobiose, *z*1,3-mannobiose, *z*1,6-mannobiose and Man,GlcNAc2 (all from Sigma), or 3 mg of cell walls from *C. albicans* strains: wild-type NGY152, Camnt1-Camnt2A and Camnt3-Camnt4-Camnt5Δ null mutants. These cell walls were obtained as previously described (Mora-Montes et al., 2007).

**Protein electrophoresis and quantification**. Protein was quantified using the Bradford assay (Bradford, 1976). Analytical (12%) SDS-PAGE gel electrophoresis and Coomassie blue staining were utilized to separate and visualize proteins, respectively (Laemmli, 1970; Merrill, 1990).

**Analysis of the products synthesized by SsMnt1**. Incubation mixtures contained mannos and *z*1,2-mannobiose as acceptors, and...
control reactions were conducted with protein from strain X-33_pPICZaA. TLC was performed on silica gel 60 F254 plates (Merck) in propanol:butanol/water 12:3:4 (v/v) as mobile phase. The ascending liquid face was left to reach the edge of the plates, which were dried and then the separation process was repeated three times. After running, the silica plate was cut into 1 cm fractions and the radioactivity was measured. The standard sugars used were mannose, α1,2-mannobiose, raffinose, maltotetraose and maltopentaose (all from Sigma). Sugar detection was performed with diphenylamine-aniline-phosphoric acid reagent (Anderson et al., 2000) with incubation at 100 °C until spots appeared.

Aspergillus saitoi α1,2-mannosidase treatment. The products of the enzymic reaction, using mannose as acceptor, were concentrated by evaporation and resuspended in 14 μl deionized water containing 15 units α1,2-mannosidase (Glyko) and the appropriate buffer to a final volume of 20 μl. Digestions were performed at 37 °C for 24 h and the products were applied to a silica gel 60 F254 plate. TLC was performed and radioactivity measured as described above. A reaction without α1,2-mannosidase was incubated simultaneously as a control.

Alcian blue binding assays. The phosphomannan content was determined as described elsewhere (Hobson et al., 2004).

Hex1 electrophoretic mobility shift assays. Cell-free homogenates were prepared using glass beads, as described previously (Mora-Montes et al., 2010a). The homogenate was centrifuged for 10 min at 13,206 g and 4 °C, and the supernatant was retained. The samples were loaded onto a 4% PAGE gel and run for 1.5 h at 20 V under native conditions. The N-acetylatedhexosaminidase activity was determined by incubation with 0.4 mM 4-methylumbelliferyl-β-N-acetylglucosaminide (Sigma) in 0.1 M citrate-KOH buffer (pH 4.5) for 30 min at 37 °C, and the results were observed by exposing the gel to UV light.

β-Elimination of C. albicans cell walls. The C. albicans cell walls tested as acceptors in standard mannosyltransferase reactions were dissolved in 3 ml 0.1 M NaOH and incubated for 18 h at room temperature with gentle shaking. The samples were centrifuged for 2 min at 13,206 g and the supernatant was neutralized with HCl, concentrated by lyophilization and counted for radioactivity.

Bioinformatics. The translation start site of SsMNT1 was predicted using the software WebGene (http://www.ith.cnrt.fr/webgene/), whereas the transmembrane region of SsMnt1 was determined with the program TMHMM v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

**RESULTS**

Isolation and bioinformatics analysis of the Sp. schenckii MNT1 gene

Since no genome sequence is available for this organism, we used classic strategies for gene isolation, involving the generation of a partial genomic DNA library. Degenerate primers were designed based on the conserved regions of mannosyltransferases belonging to the KRE2/MNT1 gene family from fungi closely related to Sp. schenckii (Teherl et al., 2003). These primers were used to amplify a 0.76 kbp fragment from Sp. schenckii genomic DNA by PCR, which was sequenced and identified as a portion of an ORF with similarity to members of the KRE2/MNT1 gene family present in other fungi. This DNA fragment was used as a radiolabelled probe in a Southern blot analysis, and a ~5 kbp HindIII fragment hybridized with the probe. This analysis also revealed that the genome of Sp. schenckii has only one copy of this gene (data not shown).

A partial genomic library of Sp. schenckii HindIII-digested genomic DNA was constructed and screened as described in Methods. Upon screening with the 0.76 kbp fragment described above, we identified a positive construct with a ~5.0 kbp insert, which was fully sequenced. The construct contained the entire SsMNT1 ORF spanning 1515 bp along with 394 bp of the upstream region. In addition, we found a small 660 bp ORF downstream of MNT1 which encodes a putative 37S ribosomal protein S12, followed by a truncated portion of a 612 bp ORF that encodes a putative acyl-CoA dehydrogenase. This gene organization is also present in fungi phylogenetically related to Sp. schenckii (see Fig. S1 available with the online version of this paper).

Bioinformatics analysis indicated the presence of the consensus sequence for a Kozak box at position −6 to +4 (Fig. S2) (Kozak, 1987). Analysis by RT-PCR using Sp. schenckii cDNA from either hyphae or yeast cells and specific primers for SsMNT1 confirmed gene expression in both morphologies (not shown). In addition, the presence of two introns was detected: one spanning 97 bp (position +49 to +145) and the second containing 68 bp and localized at position +1109 to +1177 (Fig. S2). Both introns are flanked by the consensus splicing sites 5′GT-3′AG (Reed & Maniatis, 1985).

The SsMNT1 ORF encodes a predicted protein of 449 amino acids with a predicted molecular mass of 51.6 kDa. Similar to other mannosyltransferases, SsMnt1 is a putative type II membrane protein with a short six amino acid cytosolic tail, a single transmembrane region of 18 residues followed by a stem portion, and a catalytic domain of 425 amino acids likely to reside within the Golgi lumen. Bioinformatics analysis showed that this protein belongs to the superfamily 15 of glycosyltransferases and has 59, 55 and 50 % identity to CaMnt2, ScKtr1 and CaMnt1, respectively, and also displays high identity with other members of the S. cerevisiae KRE2/MNT1 gene family (Fig. 1). A pIle-up analysis revealed a high degree of conservation in the mannosyltransferase domain, which contains the CHFWSNFEI motif (Fig. 1). Many glycosyltransferase families possess a DXD-like motif; this sequence binds the cofactors necessary for enzymic activity (Wiggins & Munro, 1998). In ScKre2, the only member of the KRE2/MNT1 gene family crystalized thus far (Lobsanov et al., 2004), the 247EPD249 sequence forms the predicted ‘DXD’ motif, and similarly, SsMnt1 has the same 258EPS262 cofactor-binding sequence (Fig. S2).

**Heterologous expression of SsMnt1 in P. pastoris**

In order to characterize the biochemical properties of the SsMnt1 product, the DNA sequence encoding the soluble domain of this protein (amino acids 1–24 were removed) was codon-optimized for expression in P. pastoris and
cloned into the pPICZaA expression vector, generating pPMNT1 (see Methods). In this construct the ORF was placed in-frame with the z-factor export sequence and under the control of the AOX1 promoter. P. pastoris X-33 was transformed with the SacI-linearized pPMNT1, and the vector was inserted within the AOX1 locus, generating strain HMP1 (see Methods). As control, strain X-33 was transformed with the empty SacI-linearized pPICZaA, generating strain X-33_pPICZaA.

Both HMP1 and X-33_pPICZaA strains were grown in MGY medium until they reached saturation, and were then transferred to MMY medium to induce gene expression. A ~43 kDa protein was expressed in strain HMP1, and its amount increased depending on the induction time with methanol (Fig. 2). The predicted molecular mass for this protein is 48 kDa; this difference may be explained by proteolytic processing by endogenous proteases released into the culture medium (Macauley-Patrick et al., 2005). We decided to characterize the protein secreted after 48 h of incubation, and found that the culture medium of HMP1 showed mannosyltransferase activity [3.3 ± 0.2 c.p.m. min⁻¹ (mg protein⁻¹)] while in the control strain X-33_pPICZaA, activity was barely detectable [5.8 ± 0.1 c.p.m. min⁻¹ (mg protein⁻¹)].

**Biochemical properties of recombinant SsMnt1**

The enzyme activity showed an optimal pH between 6.8 and 7.6 in 50 mM Pipes-NaOH (data not shown), and a dependence on Mn²⁺, with an optimum concentration of 15 mM, while other cations such as Ca²⁺, Co²⁺, Zn²⁺ and Mg²⁺ failed to stimulate mannosyltransferase activity, indicating that Mn²⁺ is an essential cofactor for enzyme activity (Fig. 3a). The enzyme showed a $K_m$ and $V_{max}$ of 30 μM GDP-Man and 3.3 μmol α-methyl mannose-mannose h⁻¹ (mg protein)⁻¹, respectively. The ability of the enzyme to use acceptors other than α-methylmannoside was also assessed and the results showed that the highest enzyme activity was obtained when α-methylmannoside was used as acceptor, but the enzyme could use α1,2-, α1,3- or α1,6-mannobiose, mannose and Man$_3$GlcNAc$_2$ to a lesser extent (40, 30, 19 and 17 % of activity with respect to α-methylmannoside, respectively; Fig. 3b).

**Characterization of products generated by recombinant SsMnt1**

In order to determine the number of mannose residues added by recombinant SsMnt1, the products obtained after enzymic reactions were applied onto silica gel plates and then subjected to TLC as described in Methods. The results indicated that SsMnt1 was able to form a disaccharide and a trisaccharide when mannose was used as acceptor, and a trisaccharide when α1,2-mannobiose was used as acceptor (Fig. 4). To determine the linkage of the mannose transferred to the acceptor by SsMnt1, the reaction products were digested with α1,2-mannosidase from A. saitoi and then analysed by TLC as described in Methods. After incubation the enzymic products were separated as free mannose (data not shown), therefore indicating that SsMnt1 is an α1,2-mannosyltransferase.

**Complementation studies in Sa. cerevisiae and C. albicans mnt null mutants**

In order to determine the biosynthetic pathway in which SsMnt1 plays a role, we decided to express SsMNT1 in C. albicans strains harbouring disruptions of members of the MNT1 gene family. We analysed the level of cell wall

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**Fig. 1.** Pile-up analysis of the deduced amino acid sequence of SsMnt1 and those of ScKre2, ScKtr1, ScKtr3, ScYur1, CaMnt1 and GaMnt2. Amino acids with complete identity are indicated with asterisks, semicolons indicate conserved substitutions, full stops indicate semiconservative substitutions. The black line denotes the mannosyltransferase domain.

**Fig. 2.** Analytical SDS-PAGE showing production of SsMnt1 in P. pastoris. P. pastoris transformed with pPMNT1 was grown in the presence of methanol as described in Methods. Upon incubation for 0, 24, 48, 72 and 96 h, the medium was collected and concentrated, and aliquots containing 50 μg protein were taken and analysed by SDS-PAGE in a 12 % gel. The control lane (C) contains a protein sample collected from cells transformed with the empty vector.
phosphomannan as an indirect parameter of the status of glycosylation pathways (Bates et al., 2005, 2006; Mora-Montes et al., 2007, 2010a). For this purpose we tested the ability of cells to bind the cationic dye Alcian blue (AB), which binds to the phosphate group of phosphomannans (Hobson et al., 2004). The triple null mutant Cammt4-Cammt5Δ showed an AB binding of 60.6 ± 0.4 % of the wild-type level (106.6 ± 8.0 µg AB bound per cells at OD600 1.0), as reported previously by Mora-Montes et al. (2010a), whereas the same null mutant expressing the SsMNT1 gene was fully restored in ability to bind the dye (93.0 ± 9.2 %). The triple null mutant reintegrated with any of the native genes showed lower AB binding than that observed with SsMNT1 (68.6 ± 0.4 %, 69.1 ± 3.1 % and 76.02 ± 4.0 %, with CaMNT3, CaMNT4 and CaMNT5, respectively). These results demonstrate that SsMnt1 restores the phenotype of the triple null mutant to wild-type levels, although this mutant has defects in both N-linked glycosylation and phosphomannosylation pathways (Mora-Montes et al., 2010a); therefore, we could not properly establish the role of SsMnt1 in any of these pathways. Accordingly, to determine whether SsMnt1 is involved in N-linked glycosylation, we expressed SsMNT1 in the double Cammt4-Cammt5Δ null mutant, which has defects only in this biosynthetic pathway (Mora-Montes et al., 2010a), and assessed the N-linked glycosylation status by electrophoretic mobility shifts of Hex1, as a molecular marker for this pathway (Bates et al., 2006; Mora-Montes et al., 2007, 2010a). Hex1 from the double Cammt4-Cammt5Δ null mutant migrated faster than the protein from wild-type control cells, but this was restored in the double mutant strain complemented with SsMNT1 (Fig. 5), strongly suggesting that SsMnt1 is involved in N-linked mannosylation. In addition, cell walls from C. albicans wild-type cells were tested as acceptors, and we found that SsMnt1 was able to transfer mannose residues, incorporating 13.3 ± 1.14 c.p.m. min⁻¹ (mg protein)⁻¹. When cell walls were prepared from either Cammt1-Cammt2Δ, which generates truncated O-linked mannan (Munro et al., 2005), or Cammt3-Cammt4-Cammt5Δ null mutants and used in similar experiments, a significant increase in the amount of mannose residues was recorded, with 51.5 ± 2.13 and 165.4 ± 3.24 c.p.m. min⁻¹ (mg protein)⁻¹, respectively. Subsequently, these radiolabeled cell walls were subjected to β-elimination, and we observed that 80, 70 and 95 % of transferred sugars were β-eliminated from walls of the Cammt1-Cammt2A and Cammt3-Cammt4-Cammt5Δ null mutants, and wild-type control cells, respectively (data not shown), confirming that SsMnt1 transferred mannose units to N-linked glycans, and also revealing that it has the ability to add sugar units to O-linked glycans.

Next, to determine the participation of SsMnt1 in the elaboration of cell wall phosphomannan, we expressed SsMNT1 in the Sa. cerevisiae ktr6Δ null mutant, which lacks the main phosphomannosyltransferase (Wang et al., 1997). The data indicated that the Sp. schenckii gene could not restore the cell wall phosphomannan content to a level similar to that of wild-type control cells, although we demonstrated SsMNT1 expression by RT-PCR (not shown). Therefore it is unlikely that the enzyme participates in the phosphomannosylation pathway.

**DISCUSSION**

Here, using a reverse genetic approach and screening of an Sp. schenckii genomic DNA library we isolated SsMNT1, which has all the known sequence signatures to be a new member of the superfamily 15 of glycosyltransferases. Interestingly, the gene organization at this locus, including intron positions, is highly conserved among phylogenetically related organisms, suggesting a lower degree of recombination at this site of the genome. In other fungi,
the KRE2/MNTI gene family varies in size, with a range from three to nine members, but here our Southern blot results showed one hybridization band. Although the family members are very similar at the protein sequence level, they do not have a high similarity at the nucleotide level, which is likely to explain our results. Less stringent conditions for hybridization and probing other ORFs would be required to detect other family members in this organism.

We expressed the soluble domain of SsMnt1 as a secreted protein in the methylotrophic yeast P. pastoris. The recombinant enzyme showed an absolute requirement of Mn$^{2+}$ for activity, at an optimal concentration of 15 mM, similar to ScKre2 and ScKtr1, which utilize only this metal ion as cofactor (Romero et al., 1997), whereas CaMnt1 can also utilize to a lesser extent Zn$^{2+}$ and Co$^{2+}$ (Thomson et al., 2000).

SsMnt1 has preference for $\alpha$-methyl mannoside over $\alpha$1,2-, $\alpha$1,3- or $\alpha$1,6-mannobiose when used as acceptors. Similar results have been obtained for the recombinant forms of CaMnt1, ScKre2 and ScKtr1 (Thomson et al., 2000; Romero et al., 1997). The similarity in both protein sequence and acceptor preference could not help us to establish the role of this enzyme in the protein glycosylation pathways, as the C. albicans enzyme is exclusively involved in O-linked mannosylation (Munro et al., 2005), while the Sa. cerevisiae enzymes participate in both N- and O-linked mannosylation (Romero et al., 1997). The recognition of Man$_5$GlcNAc$_2$ as acceptor suggests that SsMnt1 might have a role in N-linked glycan outer chain elongation; however, recombinant CaMnt1 shows the same ability but has no role in this pathway in vivo (Thomson et al., 2000; Munro et al., 2005).

The preference for $\alpha$-methyl mannoside as acceptor might indicate that the enzyme prefers mannose units with non-available anomeric carbon like those that are part of oligosaccharides.

Functional complementation studies were performed in Sa. cerevisiae and C. albicans null mutants to elucidate the glycosylation pathways in which SsMnt1 participates. Our results strongly indicate that this enzyme participates in the N-linked glycosylation pathway, as it restored the phenotype of the Camnt4-Camnt5Δ null mutant, a strain with defects only in the N-linked mannosylation pathway (Mora-Montes et al., 2010a). Results using whole cell walls from a Camnt3-Camnt4-Camnt5Δ null mutant as acceptors confirmed this observation, as a significant amount of radiolabelled material remained attached at the cell walls upon $\beta$-elimination. Furthermore, SsMnt1 has the ability to add mannose units to O-linked glycans, as a significant

Fig. 4. TLC of the enzymic products synthesized by SsMnt1. The reaction products of SsMnt1 using either mannose (●) or $\alpha$1,2-mannobiose (○) as acceptor were applied onto silica gel 60 plates and separated using propanol/butanol/water (12:3:4) as mobile phase. Portions of 1 cm were cut off and radioactivity was measured. A mixture of standards was run simultaneously. The standard sugars used were: (1) maltopentaose, (2) maltotetraose, (3) raffinose, (4) $\alpha$1,2-mannobiose and (5) mannose; the positions at which they run are indicated by arrows. The graph is representative of at least three independent experiments.

Fig. 5. Electrophoretic mobility shift assays of Hex1. Hex1 activity was induced as described in Methods. Samples of 100 µg protein were separated in native PAGE gels. The C. albicans strains used were: wild-type (NGY152, lane 1), Camnt4-Camnt5Δ (NGY516, lane 2), Camnt4-Camnt5Δ+CaMNT4 (NGY517, lane 3), Camnt4-Camnt5Δ+CaMNT5 (NGY518, lane 4) and Camnt4-Camnt5Δ+SsMNT1 (HMY50, lane 5). The continuous and dashed lines represent the electrophoretic mobility of normal and underglycosylated Hex1, respectively.
amount of the radiolabelled material was released by β-elimination from wild-type or CaMnt1-ΔCaMnt2Δ null cell walls. In summary, we report here the biochemical and functional characterization of what is believed to be the first member of the MNT1 gene family from Sp. schenckii.

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REFERENCES


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