Functional characterization of the *Candida albicans* MNT1 Mannosyltransferase Expressed Heterologously in *Pichia pastoris*

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Short title: Characterization of *Candida* Mnt1p

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SUMMARY

The $\alpha$1,2-mannosyltransferase gene $MNT1$ of the human fungal pathogen *Candida albicans* has been shown to be important for its adherence to various human surfaces and for virulence (Buurman, E.T., Westwater, C., Hube, B., Brown, A.P.J. and Gow, N.A.R. (1998). *Proc. Natl. Acad. Sci. USA* 95, 7670-7675). The CaMnt1p is a type II membrane protein which is part of a family of proteins which are important for both $O$ and $N$-linked mannosylation in fungi and which represent a distinct sub-class of glycosyltransferase enzymes. Here we use heterologous expression of Ca$MNT1$ in the methylotrophic yeast *Pichia pastoris* to characterize the properties of the CaMnt1p enzyme as an example of this family of enzymes and to identify key amino acid residues required for coordination of the metal co-factor and for the retaining nucleophilic mechanism of the transferase reaction. We show that the enzyme can use both Mn$^{2+}$ and Zn$^{2+}$ as metal ion co-factors and that the reaction catalyzed is specific for $\alpha$-methylmannoside and $\alpha$1,2 mannobiose acceptors. The N-terminal cytoplasmic tail, transmembrane domains and stem regions were shown to be dispensable for activity while truncations to the C-terminal catalytic domain destroyed activity without markedly affecting transcription of the truncated gene.
INTRODUCTION

In *Saccharomyces cerevisiae*, the process of glycosylation is essential for growth (1-3) affecting protein folding and stability, protection against proteolysis, intracellular trafficking and the biophysical properties of the cell wall (4-7). In the pathogenic fungus, *Candida albicans*, glycosylated outer cell wall mannoproteins form direct interactions with the host and are therefore critical for immunological reactivity, colonization and adhesion of host tissues (8-11). Both the protein and carbohydrate components of *Candida* mannoproteins have been implicated in mediating adhesion to host cells (12-17). Glycosylation is therefore important for pathogenicity and for host-fungal interactions.

The structure of the *O* and *N*-linked mannan-oligosaccharides to serine/threonine and asparagine residues respectively is determined by glycosyltransferases which in fungi include enzymes encoded by the *MNT* and *MNN* gene families (18). These enzymes transfer a mannose from a GDP-mannose donor to the hydroxyl group of an oligosaccharide acceptor (19). Oligosaccharides are assembled by the sequential and concerted action of an array of glycosyltransferases as proteins pass through the secretory system. In the yeast-like fungi including *S. cerevisiae* and *C. albicans*, the outer mannose chains of *N*-linked glycans form extensive branched structures consisting of an *α*1,6- backbone on to which, *α*1,3-, *α*1,2- and *β*1,2-mannan side chains are attached (20, 21). In contrast *O*-glycosylation of *C. albicans* involves addition of short, linear chains formed by 3 or more mannose sugars (22-25). The mannan structures in *C. albicans* may vary in different strains, and serotypes (11).

Mutants with disrupted genes that function in the synthesis of mannan provide a route to assess how glycosylation contributes to the pathogenicity of *C. albicans*. Strains deleted in the protein mannosyl transferase gene *CaPMT1*, which initiates *O*-linked glycosylation, were unable to form hyphae on solid Spider medium, were less adhesive to colon carcinoma cells,
hypersensitive to a range of antifungals and avirulent in a mouse systemic infection model (26). The CaMNT1 also participates in O-glycosylation by adding the second mannose sugar to the first (25). Null mutants in CaMNT1 had reduced adherence to human buccal cells and were attenuated virulence in systemic and vaginal rodent models of disease. Normal O-glycosylation therefore is essential for Candida pathogenesis.

CaMNT1 is one of five related genes identified in C. albicans to date (25, 27). It was cloned by virtue of its homology to ScMNT1/KRE2, an α1,2-mannosyltransferase involved in O-glycosylation of S. cerevisiae (28-30). However, the CaMNT1 is more closely homologous to ScKTR1 than ScMNT1 (27). The completed sequence of the yeast genome revealed ScMNT1/KRE2 belongs to a family of nine related genes, ScKTR1-7 and ScYUR1 (31-33) which play a role in both O- and N-linked mannosylation. These genes encode proteins which are all type II membrane proteins, sharing a common domain structure of glycosyltransferases (34-36). An MNT1-like protein has a short amino-terminal cytoplasmic tail of 3-27 amino-acids, a 14-21 amino-acid transmembrane domain, and an extended stem region followed by a large carboxy-terminal catalytic domain (25, 31, 33). Primary amino-acid sequence alignment of the MNT1 gene families in these two yeasts revealed two highly conserved regions in the catalytic domain (27, 37). However, the protein sequence of the MNT1 gene families does not contain the DXD motif recently found to be essential for catalysis in the Mnn1p and Och1p families of yeast glycosyltransferases (38). Since this key motif is not present in the MNT1 gene family it was important to determine the mechanism of catalysis of this family of enzymes that play critical roles in pathogenesis and may serve as targets for rational drug design (39). The proposed mechanism of action of non-processing, retaining glycosyltransferases such as mannosyltransferase enzymes involves two nucleophilic substitutions mediated by acidic amino acid residues (36, 40-42). Golgi mannosyltransferases also require an essential Mn²⁺ cofactor (43, 44).
Here we examine the catalysis of CaMnt1p, expressed heterologously in *Pichia pastoris* as an example of the *MNT1/KRE2* gene family in fungi. We determine the key amino acid residues essential for CaMnt1p enzyme activity *in vitro* and examine the metal ion and *in vitro* acceptor specificity.
EXPERIMENTAL PROCEDURES

Construction of expression plasmids - The DNA encoding the soluble domain of MNT1 (amino acids 31-432) was amplified from Candida genomic DNA by polymerase chain reaction (PCR). The primers included restriction sites XhoI and BamHI at the 5’ends for cloning. Primer sequences were: 5’-TACACCTCGAGCTCTCGGTCATCATTCCA-3’ and 5’-TACACGG-ATCCTTAAGCAGTGTACTTTTCCC-3’. The digested PCR product was cloned into the BamHI-XhoI sites of the pHIL-S1 expression vector (Invitrogen, Groningen, The Netherlands), in frame with the PHO1 signal sequence generating pHMNT1. To generate the deleted MNT1 constructs, primers were designed from the point of truncation (Fig. 3A) and each truncated MNT1 was cloned into the pHIL-S1 expression plasmid as described above. Plasmid DNA was linearised with BglII before transformation into P. pastoris GS115. To construct the ScMNT1 expression plasmid, the soluble domain of KRE2/MNT1 was amplified by PCR and cloned into pHIL-S1 as described above using the primer sequences: 5’-TACCTCGAGCTCAGCAATATATTCCGAGT-3’ and 5’-TACGGATCCCTACTCACGGAATTTTTTCCC-3’.

Expression in Pichia pastoris - P. pastoris GS115 (his4) was transformed by electroporation with 10 µg of linear DNA as described in the Pichia expression kit manual vs 2.0 (Invitrogen). Transformants were selected on histidine-deficient medium and tested for His^+ Mut^a phenotype by plating on minimal medium containing methanol (1.34% (w/v) YNB without amino acids, 1.64 µM biotin, 0.5% (w/v) methanol). Positive transformants were grown to near saturation at 30°C (OD_{600nm} = 14) in 50 ml buffered glycerol medium (0.1 M potassium phosphate buffer pH 6.0 with 1% (w/v) yeast extract, 1.34% (w/v) YNB without amino acids, 2% (w/v) peptone, 1% (v/v) glycerol and 1.64 µM biotin). Cells were harvested
and resuspended in 1/5 original culture volume (10 ml) of buffered methanol medium with 0.5% methanol instead of glycerol. Methanol was added to a final concentration of 0.5% every 24 h to maintain protein induction.

A buffered synthetic complete medium was used for experiments in which site specific mutagenised proteins were expressed for analysis of protein structure by circular dichroism. This was necessary to replace peptone in buffered methanol medium which interfered with CD spectra. Positive *Pichia* transformants were grown to near saturation at 30°C in 300 ml buffered synthetic complete glycerol medium (0.1M potassium phosphate buffer pH 6.0 with 1.34% YNB without amino acids (BIO 101), 1.64µM biotin, 0.079% complete supplement mixture (BIO 101) and 1% glycerol). Cell were harvested and resuspended in 3 times 10 ml buffered synthetic complete methanol media with 0.5% methanol instead of glycerol. Methanol was added to 0.5% every 24 hours to maintain protein expression. Optimum expression was reached after 5 days expression, yields were 15-20µg/ml of culture supernatant.

**Assay of CaMnt1p Enzyme Activity** - Mannosyltransferase activity was assayed as described previously (43). Assay mixtures contained 50 mM Tris-Cl (pH 7.2), 10 mM MnCl₂, 64 nM GDP-[³H]mannose (0.02 µCi; specific activity 6.4 Ci/mmol), 50 mM α-methylmannoside acceptor, or other mannan oligosaccharides, 5 mg/ml BSA and 1 µl culture supernatant as enzyme source. For experiments on the C and N terminal truncated proteins 0.7 µM GDP- [¹⁴C] mannose (0.01 µCi; specific activity 286 mCi/mmol) was used. Results were expressed as specific activities normalized per mg of protein in the culture supernatant. Standard reactions were performed for 30 min at 30°C in a volume of 50 µl. The reaction mixtures were passed through 0.6 ml QAE-Sephadex anion exchange resin to remove labeled GDP-mannose. The neutral products were eluted with 0.75 ml of water and radioactivity
counted in 3 ml scintillation fluid. Controls, using supernatants of *P. pastoris* transformed with the vector lacking *CaMNT1* inserts, were subtracted from all measured activities.

*Protein quantification and analysis* - Culture supernatants were analysed by SDS-PAGE (45) on a 12.5% separation gel and stained with Coomassie Brilliant Blue R-250. Expressed protein was quantified by the Bradford assay (46) using BSA in culture medium as a standard. The Bio-Rad GS-700 imaging densitometer software, ‘Molecular Analyst’ vs 1.5, was used to quantify protein from SDS-page gels. Known amounts of purified hen ovalbumin (48 kDa) were run as standards to calibrate the densities of bands.

*Circular dichroism* – Secreted proteins in buffered synthetic complete methanol medium were concentrated 30 fold to a final concentration of around 600 µg/ml using a Amicon ultrafiltration stirred cell with a 10 kDa cut-off PM10 membrane filter (Millipore, Watford, UK). The buffer was switched by washing the cell through three times with 30 ml of 10 mM sodium phosphate buffer pH 7.2. CD spectra were recorded using a JASCO J-600 spectropolarimeter at the Scottish Circular Dichroism Facility, University of Stirling. Near UV (260-320 nm) and far UV (190-260nm) spectra were measured in a cell of path length 0.5 ml and 0.02 ml respectively at 25ºC. Three spectra were averaged for each sample.

*Northern Analysis* - Total RNA was extracted from *P. pastoris* as described in Invitrogen manual (47). Northern blot analysis was performed as previously described (48).

*Site-Directed Mutagenesis* - Mutations were made by PCR using the Quick change™ site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands). Using the wild-type *MNT1* construct pHMNT1 as a template, the following mutations were made: D350A, E318A, H312A, H377A, S315A and D328A. All mutations were confirmed by dideoxy chain termination DNA sequencing. Each mutant plasmid was then transformed to *P. pastoris* as described above.

*Biochemical Analysis of CaMnt1p* - Metal ion dependency of CaMnt1p and ScMnt1p was
performed using heterologously expressed enzyme from *P. pastoris* culture supernatants. The amount of each cation was varied from 5 mM to 20 mM in the standard assay using 50 mM α-methyl mannoside as acceptor. To compare substrate specificity of ScKre2p and CaMnt1p, reactions were carried out with 25 mM of each of the following acceptors: methyl α-D-pyranoside (1) (Sigma), 1-α-3-D-mannobiose (2), α-1-4-mannobiose (3), 1-α-6-D-mannobiose (4), 1-α-3, 1-α-6-D mannotriose (5), (1-α-3,1-α-6-D)(1-α-3,1-α-6)-D mannopentose (6), 2-0-(α-D-mannopyranosyl)-D-mannopyranose (7), Man5GlcNAc-core hexasaccharide (8) (2-8, Funakoshi, Tokyo) in a 50 µl final reaction volume. Reactions were carried out for 5 h before measuring enzyme activity as described above. For determination of apparent K_m and V_max, incubation was for 30 min and the concentration of GDP-[3H]mannose was kept constant in the enzyme assay (64 nM, 0.02 µCi) while the concentration of cold GDP-mannose was varied from 5 - 200 µM.
RESULTS

_Heterologous Expression of Mnt1p in Pichia pastoris_ - CaMNT1 encodes a membrane bound Golgi mannosyltransferase with a short N-terminus, a single membrane spanning domain and a large lumenal C-terminal domain. In order to examine the biochemistry of the enzyme, the _P. pastoris_ expression system was used to produce soluble, secreted protein. The soluble domain of CaMNT1, minus the cytoplasmic tail and TMD, (amino acid residues 31-432) was cloned into the pHIL-S1 expression vector in frame with the PHO1 signal peptide sequence. _P. pastoris_ GS115 (his4) was used to transform construct pHMNT1 (Fig. 1) and a copy of MNT1 was inserted behind the AOX1 promoter in the _P. pastoris_ genome via homologous recombination.

Positive transformants were grown in a buffered medium to saturation and then transferred to methanol containing medium to induce protein expression. A single band of Mnt1p was detected on SDS-PAGE gels from 10-30 µl directly applied culture supernatants. No other proteins were detectable by Coomassie staining of gels. Expression was detected one day after methanol induction and increased over time (Fig. 2). Quantification of expressed protein indicated a yield of approximately 150 µg/ml after 3 days in methanol. The culture supernatant was assayed directly for CaMnt1p activity. The _P. pastoris_ expression system could therefore be used for further analysis of CaMnt1p. In contrast, when the _E. coli_ pET expression system was used to produce the same CaMnt1 construct, the expressed protein was again expressed at high levels but was inactive (results not shown).

_The Stem Region of Mnt1p is Not Required for Enzyme Activity_ - To define the catalytic domain of CaMnt1p, 5’ and 3’ deletions of CaMNT1 corresponding to non-conserved domains were constructed and expressed in _P. pastoris_ to generate a series of truncated proteins (Fig.
The different truncated genes were amplified by PCR and cloned into the pHIL-S1 expression vector. When *P. pastoris* was transformed with these constructs, protein could only be detected for the full-length CaMnt1p control and for two *N*-terminally truncated proteins (Fig. 3B) in which the first 106 amino acids were deleted. Northern analysis performed on *P. pastoris* confirmed all deleted constructs were still capable of Ca*MNT1* transcription, suggesting that deletions to the catalytic domain may reduce the stability of the expressed protein (Fig. 3C). A mannosyltransferase assay showed that the first 106 amino acids could be deleted with relatively little effect on enzymatic activity. This suggests that the *N*-terminal cytoplasmic domain, transmembrane domain and stem regions are dispensable for enzyme activity. (Fig. 3D).

*Properties of Recombinant CaMnt1p* - The pH dependency of enzyme activity was determined in Tris/Maleate buffer between pH 5.5 and 8.0. The enzyme showed maximum activity between pH 6.5 and 7.5 with a peak at pH 7.2 (data not shown). Sequence comparisons suggested that the CaMnt1p is not the closest homologue to ScMnt1p (27). Aspects of the properties of the two enzymes were therefore compared. To test the dependency of CaMnt1p and ScMnt1p on a cofactor, the concentration of divalent cations was varied in the enzyme assay. For ScMnt1p, the preferred cofactor was Mn$^{2+}$ and the optimum concentration was around 10 mM (Fig. 4). The CaMnt1p also showed highest activity at 10 mM Mn$^{2+}$ but could also use Zn$^{2+}$ and, to a lesser extent, Co$^{2+}$ (Fig 4). The K$_{m}$ and V$_{max}$ of CaMnt1p for GDP-mannose, were 55 µM and 86.2 pmol/min/mg respectively and the velocity of the reaction linear up to 30 min. The K$_{m}$ was similar to that observed for ScMnt1p (49).

*Mnt1p acceptor specificity* - Different disaccharide acceptors were tested using the heterologously expressed CaMnt1p (Fig. 5). In this case, reactions were incubated over 5 h to enable low efficiency reactions to be detected (49). These reactions were not linear over this
period. The enzyme could utilise α-methyl mannoside and α-1,2 mannobiose efficiently, confirming that CaMnt1p is specific for an α-mannose receptor with a preference for Man α-1,2 Man acceptor disaccharide. In contrast, α-1,3 mannobiose, α-1,6 mannobiose or α1,4 mannobiose gave lower activities. These results reinforce the view that CaMnt1p is involved in the addition of α1,2 linked mannose residues in O-glycosylation (25). However, transfer to N-linked core analogue GlcNAcMan₅ oligosaccharide showed CaMnt1p could employ this acceptor in an in vitro reaction.

Site-Directed Mutagenesis Identifies Key Residues For Catalysis - Bioinformatic analysis using the ClustralW program members identified twenty eight completely conserved amino-acids in two main clusters of the Candida and Saccharomyces MNT1 gene family (Fig. 6). Each domain was searched for strictly conserved amino acids with appropriate acid side chains and two residues were identified for potential nucleophilic reactions (Glu₃¹⁸, Asp₃⁵⁰) and two for metal ion binding capacity (His₃¹² and His₃⁷⁷). The histidines were conserved in all but one of these genes (ScKTR6/MNN6) which is an outlying, distantly related member of the MNT1 gene family that is likely to encode an enzyme that catalyses mannosylphosphate transfer (18). The selected amino acids, were replaced individually with alanine by site-directed mutagenesis (Fig. 7A) and each mutant mnt1 was transformed into the Pichia genome. Transformants were sequenced to confirm correct replacement of target amino acids and to ensure no other mutations were introduced. Enzymes containing the single mutated residues were then expressed and specific activities compared to wild-type protein (Fig. 7B & C). Activity was abolished for mutants: D350A, H312A and H377A and was close to zero for the E318A mutant. As controls, the strictly conserved, amino-acid Ser₃¹⁵ which is close to the proposed active center, and acidic non-conserved Asp²³⁸ were also mutated to alanine. Specific activity of these mutants was near wild-type Mnt1p level. These results indicate that
the acidic amino acids, Asp$^{350}$ and Glu$^{318}$ may act at the active site with one acidic residue acting to accept the proton from the hydroxyl of the GDP-mannose and the other as a nucleophilic center. The two essential histidines are likely to be involved in metal ion binding but this cannot be formally proved at this stage. It is unlikely that the mutations had a detrimental effect on folding of the enzyme since a non-denaturing protein gel showed migration of the mutant proteins was normal (data not shown). In addition, the near and far UV circular dichroism spectra of the wild type and D350A, H312A and H377A and E318A mutants were nearly identical indicating that no measurable changes in the tertiary and secondary structure of the proteins had resulted due to the point mutations (Fig.8).

DISCUSSION

We used the *Pichia pastoris* protein expression system to characterize CaMnt1p –a key α1,2 mannosyltransferase of *C. albicans* involved in glycosylation of cell wall proteins and in virulence of this fungal pathogen (25). We show that the cloned enzyme is an α1,2 mannosyl transferase which employs two conserved acid amino residues and is likely to use two conserved histidines to coordinate the metal ion cofactor and to create the reactive nucleophilic center required for a non-processing, GDP-mannose-dependent, retaining glycosyl transferase reaction.

Acceptor specificity studies showed highest activity for α-methyl-mannoside, α1,2 mannobiose and low activity for α1,3 and α1,6 mannobiose acceptors. High activity towards α methyl-mannoside was expected since Mnt1p has been shown to add the second mannose to the first in O-glycosylation (25). Mnt1p also showed high activity with α-1,2 mannobiose as acceptor suggesting it could also be involved in adding the third mannose to a lesser
extent. In general, glycosyltransferases exhibit flexibility in their recognition of acceptor substrates, but each enzyme has a high degree of specificity for the linkages they form. Addition of the second and third mannose residues by Mnt1p is possible because they are both α-1,2 linked. This type of functional redundancy exists among the enzymes involved in O-glycosylation of *S. cerevisiae*. ScMNT1, ScKTR1 and ScKTR3 are all capable of adding both the second and third mannose in O-glycosylation (32) Functional redundancy of ScMnt2p, ScMnt3p and ScMnn1p has also been shown for the addition of the two terminal α 1,3 mannose residues in *Saccharomyces* O-glycosylation (50).

The acceptor study also shows that CaMnt1p could accept an N-glycan core structure in an *in vitro* reaction. However, CaMnt1p is a type II Golgi-located enzyme which is unlikely to function in the assembly of the N-glycan core in the ER. It is also unlikely to function in the assembly of Man₅,₉ of N-glycan which uses dolichol-phosphate mannose as the substrate. However, CaMnt1p may play a role in N-glycosylation *in vivo*, perhaps in the extension of the α-1,2 containing outer chain branches. The CaMNT1 null mutant showed a dramatic reduction in adhesion to buccal epithelial cells and was dramatically attenuated in both vaginal and systemic models of infection (25). Participation of Mnt1p in both O- and N-linked outer branch mannosylation would be consistent with the marked nature of the mnt1 null mutant phenotype.

CaMnt1p required Mn²⁺ at an optimal concentration of 10 mM, but, unlike ScMnt1p/Kre2p it could also utilize Zn²⁺ to a lesser extent and Co²⁺. Concentrations of Zn²⁺ higher than 10 mM may have reduced activity by precipitating protein rather than affecting the activity of the protein. In contrast to CaMnt1p, the ScMnt1p/Kre2p could only utilise Mn²⁺ and had decreased specific activity in the presence of Co²⁺ and Zn²⁺. The *S. cerevisiae* mannosyltransferase Mnn1p has also been shown to use Zn²⁺, Co²⁺ and Fe²⁺ as cofactors (38), showing that for some mannosyltransferases, a variety of divalent cations are capable of
acting as cofactors in catalysis.

Comparison of amino-acid sequence encoding glycosyltransferases reveals little overall sequence homology, however they all have a similar domain structure consisting of a short cytoplasmic tail, a 16 - 20 amino-acid transmembrane domain, an extended stem region and a large luminal catalytic domain. Deletion analysis of CaMnt1p has showed that the stem region did not contribute significantly to the specific activity of the enzyme. The stem may act as a flexible tether, allowing the catalytic domain to glycosylate carbohydrate groups of both membrane bound and soluble proteins of the secretory pathway (35). In contrast even small deletions at the C-terminus resulted in lack of expression and function. The C-terminally deleted proteins still transcribed truncated mRNAs at near normal levels suggesting that the deletions did not markedly affect the efficiency of transcription and that the deleted proteins may be unstable.

Glycosyltransfer from nucleotide diphospho sugars can proceed by either an inverting or retaining mechanism. In an inverting reaction, a single nucleophilic substitution leads to formation of a $\beta$-linkage from an $\alpha$-linked donor, while in a retaining reaction, two nucleophilic substitutions result in an $\alpha$-linkage from an $\alpha$-linked donor (36, 40-42, 51, 52). Since Mnt1p forms an $\alpha$-linkage, two amino-acids with charged side-chains are likely to act in catalysis. A pile-up of the Candida and Saccharomyces MNT genes showed two strictly conserved histidines, one conserved aspartate and one conserved glutamate. When these amino acids were replaced individually by alanine, enzyme activity was abolished for His$^{312}$, His$^{377}$, Asp$^{350}$ and was close to zero for Glu$^{318}$. It is likely that the two acidic amino acid residues are involved as nucleophiles since evidence suggests conserved amino acids with carboxyl side-chains are important in catalysis of glucosyltransferases. The proposed mechanism of retaining glycosyltransferases such as CaMnt1p involves a two step displacement. The first step involves attack on the sugar anomeric centre by one of the
carboxylates, then a second carboxylate acts as the active site nucleophile to displace the GDP from the sugar nucleotide leading to formation of an glycosyl-enzyme intermediate. Transfer of the mannose to the growing oligosaccharide is completed by displacement of the enzyme from the intermediate by the hydroxyl group of the acceptor (35, 42, 51-53). In enzymes where manganese is required as a co-factor, the metal ion can be co-ordinated by aspartate, glutamate or histidine residues (54, 55). The two aspartate residues in the essential DXD motif of Mnn1p and Och1p have been proposed to act in co-ordinating the Mn$^{2+}$ cofactor (38). However, CaMnt1p has no DXD motif and it is possible that His$^{312}$ and His$^{377}$ coordinate Mn$^{2+}$ in CaMnt1p since conserved histidines are also often involved in coordinating metal ion cofactors (54, 55). The recent report of the crystal structure of bovine β4galactosyltransferase was the first crystal structure of a eukaryotic glycosyltransferase to be resolved (56). The structure revealed the DXD motif to be involved in binding the β−phosphate group of the UDP portion of the substrate and did not detect involvement of the motif in binding the Mn$^{2+}$ cofactor. Crystallographic analysis of the 3D structure of CaMnt1p would help resolve the precise function of the four essential amino acids determined by site-directed mutagenesis.

The $MNT1/KRE2$ gene family are not found in the human genome and represent a unique class of isoenzymes classified as family 15 (40). Although no single $MNT$ gene has been found to be essential it is possible that strains harbouring combinations of mutations may not be viable and that drugs that cross-react with multiple enzymes may be fungistatic or fungicidal. This provides further impetus for the detailed characterisation of the properties of these glycosyltransferases.

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REFERENCES


Figure Legends

**Fig. 1.** Map of expression plasmid pHMNT1. 5’ AOX, *P. pastoris* alcohol oxidase (AOX) gene promoter; SS, secretion signal of the *P. pastoris* acid phosphatase gene (PHO1); MNT1, soluble domain of CaMNT1 (amino-acids 31-432) minus transmembrane domain and N-terminal tail; 3’ AOX(TT) AOX transcription terminator; HIS4, *P. pastoris* histidinol dehydrogenase gene; 3’ AOX, 3’ AOX downstream sequence; fl, origin of replication; Ampicillin, ampicillin resistance gene.

**Fig. 2.** SDS-polyacrylamide gel electrophoresis of expressed CaMnt1p from *P. pastoris* culture medium. Samples of 20 µl of culture supernatant were loaded directly from *P. pastoris* strains transformed with pHIl-S1 vector only control (lane 1) or pHMNT1 (lanes 2-5) after 1, 2, 3 and 5 days of methanol induction respectively.

**Fig. 3.** Deletion Analysis of Mnt1p. A, Schematic representation of truncated MNT1 constructs amplified by PCR. Control protein was full length Mnt1p minus the N-terminal tail and transmembrane domain only. B, Coomassie stained SDS-PAGE gel showing expression of protein deleted MNT1 constructs 1-6 (lanes 1-6). Culture supernatants (25 µl) from *P. pastoris* strains transformed with deletion constructs were loaded without further treatment. C, Northern analysis of total RNA obtained from *P. pastoris* strains transformed with deletion constructs. Radiolabeled CaMNT1 was used as a probe. D, Mannosyltransferase activity of full length MNT1 where only the TMD and N-terminal tail were removed (C), compared to truncated proteins 1-6. The results are means of duplicates, normalized for the amount of protein with control supernatant subtracted as background. For constructs 3-6 residual activity minus control background is given since insufficient protein was present for normalisation. Assays for Mnt1p activity used the standard 30 min. reaction described in the Methods.

**Fig. 4.** Comparison of metal ion dependency of CaMnt1p and ScMnt1p. The dependency of ScMnt1p (A) and CaMnt1p (B) on a metal cofactors was compared using recombinant protein in an α-1,2 mannosyltransferase assay using 50 mM α-methylmannoside as the acceptor. *P. pastoris* culture supernatant (1 µl) containing CaMnt1p or ScMnt1p was
incubated in the standard 30 min assay at 30°C, testing activity of a range of cations: Mn$^{2+}$ (○), Zn$^{2+}$ (□), Co$^{2+}$ (●), Mg$^{2+}$ (X), Ca$^{2+}$ (■).

**Fig. 5. Acceptor specificity of CaMnt1p.** Mannosyltransferase activity of CaMnt1p using a range of oligosaccharide acceptors: methyl α-D-pyranoside (1) 2-0-(α-D-mannopyranosyl)-D-mannopyranose (2) 1-α-3-D-mannobiose (3), α-1-4-mannobiose (4), 1-α-6-D-mannobiose (5), 1-α-3, 1-α-6-D mannotriose (6), (1-α-3,1-α-6-D)(1-α-3,1-α-6)-D mannopentose (7), Man$_5$GlcNAc-hexasaccharide (8). Results are means of triplicates, with control supernatant subtracted as background. Assays were run over 5 h at 30°C using standard assay concentrations of substrate and 25 mM of the various acceptors (see methods). Error bars represent the standard deviation of the mean.

**Fig. 6. Pile-up analysis of MNT1 gene family of C. albicans and S. cerevisiae.** Analysis of the deduced amino-acid sequence of CaMNT1 and ScMNT1 gene families using seqnet GCG program. Only the region of homology in the catalytic domain is shown. Positions of complete identity are indicated with asterisks, a semi-colon indicates conserved substitutions and a full stop shows a semi-conservative substitution.

**Fig. 7. Site Directed Mutagenesis of CaMnt1p.** A, Schematic representation of region in catalytic domain showing the amino acids that were mutated to alanine (mutated residues highlighted in bold). W/T Mnt1p (lane 1), D238A (lane 2), E318A (lane 3), H312A (lane 4), H377A (lane 5), S315A (lane 6), D350A (lane 7). B, Coomassie stained SDS-PAGE gel showing expression of mutated proteins. Expressed protein was quantified using a scanning densitometer to compare a serial dilution against a known protein standard. C, Specific activity of the mutated proteins measured using P. pastoris culture supernatant in the recombinant assay. Results are the mean of triplicates, with control supernatant subtracted as background. Standard 30 min assays were run (see Methods) at 30°C. Error bars represent the standard deviation of the mean.
Fig. 8. **Circular dichroism of Mnt1p and site directed mutants.** *A*, Near UV (260-320nm) and *B*, far UV (190-260nm) circular dichroism spectra of W/T CaMnt1p (1) and, D350A (2), E318A (3), H312A (4), H377A (5) site directed mutants expressed in *P. pastoris*. The molar ellipticity values (deg.cm$^2$.dmol$^{-1}$) were normalised for protein concentration.
FIG. 1 Thomson et al
FIG. 2 Thomson et al
FIG. 4 Thomson et al
FIG. 5 Thomson et al
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FIG. 7 Thomson et al
Fig. 8 Thomson et al
Functional characterization of the Candida albicans MNT1 Mannosyltransferase Expressed Heterologously in Pichia pastoris
Lynn M Thomson, Steven Bates, Soh Yamazaki, Mikio Arisawa, Yuki Aoki and Neil AR Gow

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