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Aspergillus GDP-Man transporter STD NMR

Direct investigation of the Aspergillus GDP-mannose transporter by STD NMR spectroscopy

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Aspergillus species, predominantly *A. fumigatus* and *A. flavus*, represent some of the most important air-borne fungal pathogens world-wide. Found ubiquitously in nature, *Aspergillus* spp. cause a variety of diseases depending on the immune status of the host.^[1] Most cases of invasive aspergillosis (IA) are associated with HIV infections, haematological malignancies, leukaemia or lymphoma.^[2] The harmless status of *Aspergillus* has changed over the past two decades, with the frequency of IA increasing 14-fold in the past 10 years.^[3] IA has a mortality of over 95%, and the fungus is able to cause several forms of disease in humans of which IA is the most severe. The high mortality rate of this disease warrants increased efforts towards understanding the basic principles of *Aspergillus* pathogenicity.^[1, 4] New antifungal agents have aided in both the treatment of fungal infections and in the prevention of disease in susceptible individuals, but resistance is an increasing concern.^[5] Only a few potential antifungal targets have been exploited to date and there is a critical need for the discovery and development of novel antifungal agents that will result in improved therapy in this ever-expanding patient population.

A novel class of antifungal agents called the echinocandins target the synthesis of β -(1,3)-glucan, the a major cell wall component (galactomannan, Figure 1a).^[6] Galactomannan is composed of a linear mannan core branched with short α 1,5-linked galactofuranose chains, bound covalently to the cell wall β -(1,3)-glucan, anchored to the lipid membrane by a glycosylphosphatidylinositol (GPI), or released in the environment during tissue invasion or growth in culture.^[7, 8] Besides being an abundant component of the extracellular matrix, secreted galactomannans are a serological diagnostic of IA.^[9, 10] The efficient biosynthesis of the fungus galactomannan cell wall requires the transport of nucleotide sugars, the donor substrates for glycosyltransferases, from the cytosol into the Golgi lumen.

This critical task is carried out by a group of proteins termed nucleotide-sugar transporters (NSTs).^[11] Some NST activities are found in all organisms, however some are restricted to certain lineages.^[12, 13] For example, a Golgi resident GDP-mannose (GDP-Man) transporter (Figure 1b) is not found in mammalian cells but in yeast, *Leishmania*, and *Aspergillus*.^[12, 13] Since NST activity is required prior to glycosylation, there has been great interest in better understanding NST specificity, regulation, and cellular targeting. At present, there is relatively little information on the structure of the NST protein complex, the basis for NST specificity, and the targeting and organization of NSTs within the cell. Loss of expression of all Man-containing molecules in *A. fumigatus* by a targeted deletion of the genes involved in GDP-Man biosynthesis resulted in complete loss of virulence in macrophages and susceptible mice.^[14, 15] In addition a more recent study has shown that the GDP-Man transporter (encoded by the *gmtA* gene) is essential in *Aspergillus niger*.^[16] Therefore, the *Aspergillus* GDP-Man transporter (*AspGMT*), which is not found in mammalian cells,^[12, 13] represents a promising drug target for the development of novel anti-fungal drugs that inhibit GDP-Man translocation into the Golgi lumen required for the biosynthesis of the fungal virulence factor galactomannan.

NST's are Golgi- or Endoplasmic Reticulum (ER)-resident type III transmembrane proteins with 8–10 predicted membrane-spanning domains. There is currently no 3D- or 2D-crystal structure available for any known NST. Therefore, a conventional structure-based drug design approach is currently not feasible. Work previously performed in our laboratory showed that it is possible to detect the binding of nucleotide sugars to endogenous NSTs present in isolated Golgi-enriched fractions (GeFs) isolated by *P. pastoris*.^[17] Here we report the first direct investigation of *AspGMT* substrate binding by STD NMR spectroscopy and the identification of important moieties critical for *AspGMT* interaction with GDP-Man. This information will significantly assist the design of novel inhibitors of the *AspGMT*, and potentially galactomannan synthesis.

Figure 1. (a) The cell wall of *Aspergillus* sp. is composed of β -(1,3)-glucan, chitin and galactomannan. Galactomannan is covalently attached to β -(1,3)-glucan or anchored to the lipid membrane through a GPI anchor. (b) The biosynthesis of the fungus galactomannan cell wall requires the transport of GDP-Man, the donor substrates for mannosyltransferases, from the cytosol into the Golgi lumen. GDP-Man transported into the Golgi is coupled with the export of GMP in an antiporter mechanism.

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GDP-Mannose interacts with the *Aspergillus* GDP-Mannose Transporter

Based on our initial observation that binding of nucleotide sugars to endogenous NSTs present in isolated GeFs can be monitored by STD NMR spectroscopy,^[17] we focused on investigating the binding of GDP-Man to GeFs isolated from *Aspergillus* (*Asp*-GeF). Our aim was firstly to determine if it is possible to detect ligand binding to NSTs that are present in *Asp*-GeFs at endogenous levels, and secondly to establish a preliminary binding/affinity model for GDP-Man interaction with the *Asp*GMT.

Figure 2. ¹H (a) and STD NMR spectra (b-e) of GDP-Man in complex with *Asp*-GeF (b), *Asp*-GeF-DN (c), human-GeF (d) and no protein (e). The on-resonance frequency was set to -1.00 ppm and the off-resonance to 300 ppm. The total saturation was 3 sec. The residual water signal was suppressed using a gradient selective WATERGATE sequence. All NMR experiments were performed at 600 MHz and 283 K in 10 mM deuterated TRIS containing 2 mM MgCl₂, pH 7.5. The uncorrected STD spectrum (b) was used to determine the epitope map and relative STD NMR effects were calculated according to the equation $A_{STD} = (I_0 \times I_{sat})/I_0 = I_{STD}/I_0$.

Figure 2 shows the ¹H (Figure 2a) and STD NMR spectra of GDP-Man in the presence of Golgi preparation from *Aspergillus* (*Asp*-GeF) (Figure 2b). It is immediately apparent, due to strong STD NMR signals that GDP-Man binds to proteins present in the Golgi preparation. Most striking is the observation that the H1 proton of the ribose moiety received the most saturation and hence was set at 100% to calculate relative STD NMR intensities. The importance of the H1 proton of the ribose moiety for the interaction of nucleotide sugars with their corresponding binding protein has

been previously reported and our finding is in good agreement with the literature.^[17-20]

To address the question of whether the observed interaction of GDP-Man with *Asp*-GeF is due to binding of the endogenous GMT, a number of control NMR experiments were performed. Figure 2c shows a clear decrease in STD signals upon denaturing of protein associated with *Asp*-GeF (*Asp*-GeF-DN) by heating the Golgi preparation to 70 °C for 15 min prior to the addition of GDP-Man. However, residual STD signals remained suggesting either incomplete denaturation or non-specific binding to the Golgi membrane. To investigate this further, the binding of GDP-Man to GeFs prepared from the human cell line HT-29 (human-GeF; mammalian cells are known not to express a GMT^[12]) was studied. Figure 2d shows that GDP-Man complexed with human-GeF gave similar STD signal intensities to that observed for *Asp*-GeF-DN. This suggests that the STD signals observed using *Asp*-GeF represented the interaction of GDP-Man to the endogenous *Aspergillus* GMT (*Asp*GMT). However, the procedure for preparation GeFs can occasionally lead to the production of inside-out vesicles where proteins that normally face the Golgi lumen (inside) now face the cytosol (outside). Therefore, mannosyltransferases that catalyses the transfer of Man from GDP-Man to glycoconjugates (Figure 1b), which would normally face the lumen, could become exposed in inside-out GeFs. To rule out the possibility that the observed STD effect was due to the interaction of GDP-Man with mannosyltransferases, a GDP-Man consumption assay was performed. In this experiment if GDP-Man was able to interact with an exposed mannosyltransferase, GDP-Man consumption over time should be observable. *Asp*-GeFs were incubated with GDP-Man for 2 h at 37 °C, and the consumption of GDP-Man monitored in real time by ¹H NMR spectroscopy. The ¹H NMR spectrum of GDP-Man in the presence and absence of *Asp*-GeF at 37 °C clearly shows that GDP-Man was not consumed, and therefore it is unlikely that GDP-Man was interacting with exposed mannosyltransferases (see supporting information). Our data strongly suggest that GDP-Man binds specifically to the endogenous *Asp*GMT. The low STD NMR signal intensities that were observed for GDP-Man in complex with denatured Golgi fractions (*Asp*-GeF-DN) are likely a direct result of an incomplete denaturation of Golgi proteins. On the contrary, the residual STD NMR signals of the GDP-Man:human-GeF complex (Figure 2d) are probably the result of GDP-Man binding to other non-GMT proteins. Therefore, both STD NMR control experiments cannot be used to generate double difference STD NMR spectra (STDD) to eliminate background binding. A more suitable control experiment would be the complexing of GDP-Man with GeF's derived from an *Aspergillus* GMT deficient mutant. However, Carvalho and co-workers have shown recently^[16] that a knock-out deletion of the GMT gene in *Aspergillus niger* is lethal. Therefore we determined the binding epitope for the interaction of GDP-Man with the *Asp*GMT (Figure 2 upper panel) using the uncorrected STD NMR spectrum as shown in Figure 2b. In addition we also generated two STDD NMR spectra. The first was obtained by subtracting the GDP-Man:*Asp*-GeF-DN complex (Figure 2c) from the spectrum obtained for the GDP-Man:*Asp*-GeF complex (Figure 2b), and the second by subtracting the GDP-Man:*Asp*-GeF-DN complex (Figure 2c) from the spectrum obtained for the GDP-Man:human-GeF complex (Figure 2d) (see supporting information). A comparison of relative STD NMR effects reveals that the epitope map obtained from the uncorrected STD NMR spectrum differs only marginally from an epitope map based on both STDD NMR spectra (see Table S2, supporting information).

The nucleotide sugar interactome reveals that the nucleotide portion of GDP-Man receives the greatest amount of saturation

relative to the sugar moiety, suggesting that the GDP moiety is strongly interacting with *Asp*GMT. However, some saturation is also being received by the mannose (Man) component, indicating that the sugar moiety is likewise important for binding. A similar observation has also been reported for the interaction of CMP-sialic acid with CMP-sialic acid transporter,^[17] and points to a key role for the sugar moiety in mediating NST specificity.

GDP, GMP and Mannose compete with GDP-Mannose for the *Aspergillus* GDP-Mannose Transporter binding site.

To further probe the interaction of nucleotide sugars and nucleotides with the *Asp*GMT competition STD NMR experiments using GMP-Man, GMP, GDP and Man were acquired. First, a STD NMR spectrum of GDP-Man in the presence of *Asp*-GeF was acquired followed by the consecutive addition of GMP and GDP. The extent of competition was determined by measuring the reduction in signal intensity of the H8 proton of the guanidine residue of GDP-Man. For example, this specific STD NMR signal (H8 Gua, GDP-Man) is reduced by 46% following the addition of an equimolar concentration of GMP (Figure 3a). A further addition of GDP to the *Asp*-GeF:GDP-Man:GMP mixture revealed that the STD NMR signal intensity of the H8 proton of the guanidine residue of GDP-Man was reduced by an additional 29%, leading to a total decrease of 75% in comparison to the STD spectrum acquired with only GDP-Man. Interestingly, an identical decrease of all GDP-Man STD NMR specific signals was also detected (Gua H8, 7.85 ppm; Man H1, 5.25 ppm; Man H2 3.75 ppm; and Man H3-H6, 3.7-3.4 ppm, see supporting information). This observation clearly suggests that GMP and GDP are able to displace GDP-Man, and thus GDP-Man, GMP and GDP are binding to the same site of the *Asp*GMT in a competitive manner. This is supported by the fact that the first addition of an equimolar amount of GMP lead to a mean reduction in GDP-Man specific signals by 47±1.4% (see supporting information). This compares favourably with the expected 50% reduction if GDP-Man and GMP competed for the same binding site with equal affinity. The subsequent addition of GDP to GDP-Man and GMP lead to a total reduction in GDP-Man specific signals by 77±2.1% (see supporting information). However, in this instance the mean reduction in GDP-Man specific signals is greater than the 66% expected if all three ligands interacted with equal affinity. Our data suggests that GDP may bind to the *Asp*GMT with higher affinity because its addition resulted in a greater displacement of GDP-Man than would be expected.

The effect of GMP and GDP on GDP-Man interaction with *Asp*-GeF, lead us to explore the effect of D-mannose (D-Man) on GDP-Man binding in a subsequent STD NMR competition experiment. Figure 3b shows the competitive effects of D-Man on the binding of GDP-Man. A small reduction in signal intensity of the H8 proton of the guanidine residue of GDP-Man (about ~15%) indicates that D-Man does not readily displace the tightly bound GDP-Man sugar nucleotide, suggesting only a weak interaction of Man with the *Asp*GMT. Interestingly, STD signals of D-Man (H1 Man) are detectable, although with very low intensity (see supporting information). It is important to note that D-Man in solution exists as a mixture of 67% α -D-Man and 33% β -D-Man resulting in a non-equimolar mix. Therefore, it is likely that the actual displacement potency of D-Man is higher than the measured 15%.

Figure 3. Absolute STD NMR signal intensities of the H8 proton of the guanidine residue of GDP-Man in complex with *Asp*-GeF (a and b, blue spectrum), after addition of an equimolar concentration of GMP (a, red spectrum), GDP (a, green spectrum) and mannose (b, red spectrum)

The data present herein shows for the first time the direct analysis of nucleotide sugar, nucleotide and free sugar interactions with the *Aspergillus* GDP-Man transporter (*Asp*GMT), a protein that represents a potential novel target for drug discovery. We revealed through competition STD NMR experiments that GDP appears to bind tighter to the *Asp*GMT than GMP and GDP-Man, with Man found to be the weakest binding ligand. Taken together our data permits the relative importance/affinity of individual ligand moieties that bind *Asp*GMT to be summarized as follows:

$$\text{GDP} \geq \text{GMP} \approx \text{GDP-Man} \gg \text{Man}$$

As shown in Figure 1b the natural antiporter substrates for the GMT are GDP-Man and GMP, and our data showed that both make intimate contacts with the *Asp*GMT binding site. However, the observation that in fact GDP may bind the GMT with higher affinity than the natural substrates can now be exploited in the design of novel GMT inhibitors.

Experimental Section

General – GDP-Man, GMP, GDP and D₂O (99.9%) were purchased from Sigma Aldrich (Australia).

***Aspergillus* culture and subcellular fractionation** – *Aspergillus niger* ATCC 1004 were cultured on complete-agar YMPG media (0.3% w/v yeast extract, 0.3% w/v malt extract, 0.5% w/v peptone, 1% w/v glucose, and 1.5% w/v agar) for 10 days at 25 °C in 75 cm² tissue culture flasks fitted with filtered lids. Spores were harvested as described by Tiralongo *et al.* 2009^[21] with glass beads in the presence of 20 mM PBS, pH 7.4 containing 0.05% Tween-20 (PBST) by vigorous shaking. Subsequently, the dislodged spores were filtered through sterile glass wool, collected by centrifugation (1000 g, 5 min), resuspended in 20 mM PBS and counted. Liquid YMPG cultures (1 L) were inoculated with 0.5-2x10⁶ spores/mL and incubated for 19 h at 37 °C with shaking at 150 rpm. The mycellium was harvested and Golgi-enriched fractions isolated according to Record *et al.*^[22] and Engel *et al.*^[23] Briefly, the mycellium was collected on Whatman no. 4 filter paper washed with milliQ water, removed from the filter paper and weighed. The mycellium was ground in a mortar with 1 - 1.5 volumes of sterile sand and 30 mL of

lysis buffer (10 mM HEPES/Tris, pH 7.4, 0.8 M sorbitol, 1 mM EDTA, supplemented with EDTA-free Protease Inhibitor (Roche). Large particles were removed by filtration through plain gauze that was rinsed with 20 -50 mL lysis buffer and subsequently squeezed to recover all lysed material. The filtrate was centrifuged at 10,000 g for 30min, with the resulting supernatant further centrifuged at 27,000 g for 20min. A Golgi-enriched fraction (GeF) was isolated from supernatant by ultracentrifugation at 100,000 g for 45min. The resulting pellet was resuspended in 800 µL of lysis buffer, frozen using liquid nitrogen in 100 µL aliquots and stored at -80 °C until required.

NMR experiments – All NMR experiments were performed on a Bruker Avance 600 MHz NMR spectrometer, equipped with a 5 mm cryoprobe using Shigemi tubes. Chemical shifts of GDP-Man, GDP and GMP were assigned using COSY, TOCSY and HSQC experiments.

STD NMR experiments – STD NMR experiments were performed essential as described by Maggioni *et al.* 2008.^[17] The frozen GeFs were thawed slowly on ice and buffer exchanged. Briefly, 100 – 200 µL of NMR buffer (10 mM deuterated Tris/DCI pH 7.5, supplemented with 2 mM MgCl₂ in D₂O) was used to suspend the GeFs. Following centrifugation at 18,000 x g for 10 min the supernatant was removed, the pellet resuspended in NMR buffer, and centrifugation repeated. This procedure was performed twice more to assure that the exchange from lysis-buffer to NMR-buffer was complete. All NMR spectra were acquired using a Bruker 600 MHz ultrashield NMR apparatus equipped with a ¹H/¹⁵N/¹³C cryoprobe. NMR signal assignment of GDP-Man, GDP, GMP was achieved by

Each STD NMR experiment was performed in Shigemi NMR tubes containing 200 µg equivalent of protein as GeF's in NMR buffer and 100-fold molar excess of ligand (200 µL final volume). To monitor any changes in the ligand during an STD NMR acquisition, proton spectra before and after the STD NMR acquisition was made using 8 scans each. Protein saturation was achieved using a Gaussian pulse train cascade for 3 sec at the on-resonance frequency of -1 ppm and at the off -resonance frequency of 300 ppm. To suppress residual HDO signals water suppression by gradient tailored excitation (WATERGATE) sequence was used. A total of 1024 scans (1K) were acquired. The on- and off- resonance spectra FIDs were stored and processed separately and the subtraction of the on-resonance and the off-resonance spectrum resulted in the STD NMR spectrum. To determine specific binding of ligands to protein saturation transfer double difference (STDD) spectra were generated by superimposing two individual STD NMR spectra followed by a subtraction. STDD NMR spectra were used to calculate the relative STD NMR effects according to the formula

$A_{STD} = (I_0 \times I_{sat})/I_0 = I_{STD}/I_0$. using the H1 proton of the Ribose (H1 Rib) as 100 %.

Monitoring GDP-Man consumption by NMR spectroscopy – The consumption of GDP-Man was determined using a ¹H-acquisition with 16 scans following incubation for 2 h at 37 °C in the presence and absence of Asp-GeF.

Keywords: GDP-Mannose transporter, Aspergillus, Fungi, Saturation Transfer Difference (STD) NMR, Nucleotide sugar transporters

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Entry for the Table of Contents

Layout 1:

COMMUNICATIONS

Here we report on the direct analysis of the GDP-Man transporter in isolated *Aspergillus* Golgi-enriched fractions by STD NMR spectroscopy. STD NMR spectra reveal that the guanidine and ribose moiety of GDP-Man receives more saturation than the mannose sugar residue. Competition STD NMR experiments revealed that GDP appears to bind tighter to the GDP-Man transporter than GMP and GDP-Man, with Man found to be the weakest binding ligand. Taken together our data permits the relative importance/affinity of individual ligand moieties that bind the *Aspergillus* GDP-Man transporter to be summarized as follows:

$\text{GDP} \geq \text{GMP} \simeq \text{GDP-Man} \gg \text{Man}$.

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Supporting Information

Figure S1. ^1H NMR spectra of GDP-Man (a) and GDP-Man incubated for 2h at 37°C in the presence of Asp-GeF (b). The spectra clearly indicate that GDP-Man was not being consumed.

Table S1. Apart from H8 Gua signal of GDP-Man, the extent of competition was also determined by measuring the reduction of STD NMR signal intensity of the H1, H2 and H3-H6 protons of the mannose moiety. The mean reduction and standard deviation (SD) across all these signals are shown. The addition of equimolar GMP led to a mean reduction in GDP-Man signals by $47 \pm 1.4\%$. The subsequent addition of GDP to GDP-Man and GMP led to a total reduction in GDP-Man specific signals by $77 \pm 2.1\%$

Competing nucleotide	% Reduction in GDP-Man specific STD signals					SD
	H8 Gua (7.85 ppm)	H1 Man (5.25 ppm)	H2 Man (3.75 ppm)	H3-H6 Man (3.7-3.4 ppm)	Mean	
GMP	46	49	46	46	47	1.4
GMP + GDP	75	79	75	78	77	2.1

Figure S2. a) STD NMR spectra acquired of GDP-Man complexed with Asp-GeF. b) STD NMR spectra acquired of GDP-Man complexed with Asp-GeF following the addition of equimolar Man. For the H8 Gua signals and for the H3, H4, and H5 Rib signals a reduction in STD signal intensity is visible. The only STD NMR signal of the free mannose that could be detected is the H1 (α -anomer) signal (4.89 ppm), although with very low intensity.

Table S2. Relative STD NMR values of GDP-Man. The STD NMR spectrum of the Asp-GEF:GDP-Man complex was used to calculate the relative STD NMR effects for GDP-Man. STDD spectra (*Asp-GeF*:GDP-Man – *Asp-GeF*-DN:GDP-Man) and (*Asp-GeF*:GDP-Man – *human-GeF*:GDP-Man) were also used to calculate relative STD NMR effects for GDP-Man. All STD NMR effects were calculated according to the formula $A_{STD} = (I_o \times I_{sat})/I_o = I_{STD}/I_o$ using the H1' (Rib) proton to 100%.

	H8 (Gua)	H1' (Rib)	H1 (Man)	H2' (Rib)	H3' (Rib)	H4' (Rib)	H5/5' (Rib)	H2 (Man)	H3, H5, H6'/H6'' (Man)	H4 (Man)
<i>Asp</i> -GEF (STD)	92	100	23	n.d.	31	39	39	18	15	24
(<i>Asp</i> -GeF)-(<i>Asp</i> - GeF-DN) (STDD)	61	100	37	n.d.	40	40	22	22	15	21
(<i>Asp</i> -GeF)- (<i>human</i> -GeF) (STDD)	95	100	37	n.d.	31	39	24	18	19	n.d.