Fundamental research on the biosynthesis of fungal-type galactomannan (FTGM) as a new drug target

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Research aims

Aspergillus fumigatus is the major causative fungus of invasive pulmonary aspergillosis. The structure of galactomannan (GM) produced by *A. fumigatus* was described in the 1930s and GM presence has been used clinically as an index for the early diagnosis of invasive aspergillosis. GM consists of α-(1→2)-/α-(1→6)-mannosyl and β-(1→5)-/β-(1→6)-galactofuranosyl residues, and it is located in the outermost layer of the *A. fumigatus* cell wall[1)]. There are two types of GM in *A. fumigatus*, namely fungal-type (FTGM) and *O*-mannose-type (OMGM) [2)]. FTGM contains a linear α-mannan structure called core mannan in which 9 or 10 α-(1→2)-mannotetraose units are concatenated by α-(1→6) linkages. Moreover, FTGM contains galactofuran side chains consisting of β-(1→5)-galactofuranotetraose units concatenated by β-(1→6) linkages that are bound to core mannan by β-(1→2), β-(1→3), and β-(1→6) linkages. OMGM consists of β-(1→5)-galactofuranosyl chains bound to the nonreducing terminal side of an *O*-mannose-type glycan, in which mannosyl chains are attached to a serine or threonine hydroxyl group in the proteins. Similar to FTGM, the galactofuranosyl residues of OMGM are elongated by β-(1→6)-galactofuranose[2)]. Because plants and higher animals, including humans, do not produce GM, inhibition of the GM biosynthetic pathway represents a potential mode of action for medicines and agricultural chemicals without the risk of side effects. However, information on the glycosyltransferase involved in GM biosynthesis is limited. We identified and characterized the galactofuranosyltransferases (GfsAs) responsible for OMGM and FTGM biosynthesis in the model filamentous fungi *A. nidulans* and *A. fumigatus* in 2013[3)]. Therefore, in this research, we will proceed with the detailed enzymatic functional analysis of *A. fumigatus* GfsA (AfGfsA), AfGfsC, a paralogue of AfGfsA, and AfCmsA, a putative mannosyltransferase. We hope these findings will facilitate the

development of novel antifungal agents by elucidating the functions of glycosyltransferases responsible for FTGM synthesis in filamentous fungi.

Methods

We obtained recombinant AfGfsA, AfGfsC, and AfCmsA proteins using an *Escherichia coli* expression system. The genes encoding the soluble forms of AfGfsA, AfGfsC, and AfCmsA, lacking the N-terminal region, which harbors the putative signal sequence and a transmembrane domain, were introduced into an *E. coli* expression vector fused in frame with a 6× histidine (6×His) tag at the N-terminus. Recombinant 6×His-tagged proteins were purified via Ni^{+} affinity chromatography.

GM was extracted from *A. fumigatus* mycelia via autoclaving at 121°C for 120 min. The β-elimination reaction was performed by exposing the fractionated GM to generate FTGM. Glycosidic linkage investigation of the polysaccharides and oligosaccharides was performed using methylation analysis. The Nuclear Magnetic Resonance (NMR) spectra were recorded using a JNM-LA600 spectrometer (JEOL).

Results

The galactofuranosyltransferase activity of the purified AfGfsA and AfGfsC proteins was assayed using uridine-5'-diphosphate (UDP)-galactose, UDP-galactose mutase, 10 mM sodium dithionite, 1 mM Mn^{2+}, and 1 mM *para*-nitrophenyl (pNP)-β-D-galactofuranose. The reacted mixture was analyzed using HPLC with an amino column. As a result, AfGfsA and AfGfsC could synthesize pNP-β-(1→5)-galactofuranotetraose (unpublished data). GMs were prepared using WT and Δ*gfsAC* strains and characterized via ^{13}C-NMR spectroscopy and methylation analysis. Interestingly, the signal for β-(1→5)-galactofuranose disappeared in the ^{13}C-NMR spectrum for ΔAfgfsAC-GM (unpublished data). In addition, no 5-galactofuranose produced by ΔAfgfsAC-GM was detectable via GC-MS of the *O*-methyl alditol acetate derivatives from the methylation analysis (unpublished data). These observations indicate that all β-(1→5)-galactofuranosyl residues of FTGM and OMGM in *A. fumigatus* are synthesized by AfGfsA and AfGfsC[1)].

The mannosyltransferase activity of the purified AfCmsA protein was assayed using guanosine 5'-diphosphate-D-mannose, 1.5 mM Mn^{2+}, and 1.5 mM pNP-α-D-Man-. The reacted mixture was analyzed using HPLC with an amino column. As a result, AfCmsA

could synthesize pNP-α-(1→2)-mannobiose, indicating that AfCmsA is an α-1,2-mannosyltransferase (Fig. 2)[4]. Next, FTGMs were extracted from WT, Δ*cmsA*, and Δ*cmsA*::*cmsA* strains and subjected to acid hydrolysis to remove galactofuranosyl residues from FTGM, and the products (designated as FTGM-HCls) were analyzed via ^{1}H-NMR spectroscopy. The H-1 signals for the chemical shifts of the α-(1→2)-mannan backbone appeared from 5.0 to 5.2 ppm in the ^{1}H-NMR spectra of WT-FTGM-HCl. Conversely, these core mannan signals were absent or substantially suppressed in the ^{1}H-NMR spectra for ΔcmsA-FTGM-HCl, indicating that core mannan structures were altered and/or lost in the absence of *cmsA* (Fig. 3). Next, these FTGM-HCls were analyzed via gel filtration chromatography and detected using the phenol-sulfuric acid method. The peak FTGM-HCl content in the WT strain was detected in fractions 35 to 50. In the Δ*cmsA* strain, the molecular weights of FTGM-HCls were shifted downward, whereas the gene-complemented Δ*cmsA::cmsA* strain displayed restoration of the molecular masses of FTGM-HCls similar to those of the WT strain (Fig. 4). These results indicate that the average molecular weights of FTGM-HCl are reduced in the absence of *cmsA*. Taken together, these findings identify CmsA as the α-1,2-mannosyltransferase responsible for FTGM core mannan biosynthesis.

Conclusion

All β-(1→5)-galactofuranosyl residues of FTGM and OMGM in *A. fumigatus* are biosynthesized by AfGfsA and AfGfsC. CmsA is an α-1,2-mannosyltransferase that is responsible for FTGM core mannan biosynthesis in *A. fumigatus*. These enzymes represent promising targets for novel antifungal therapies.

References

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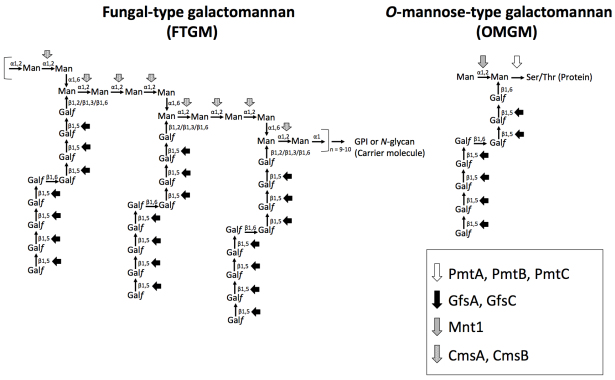

Figure 1. Structures and biosynthesis of fungal-type (FTGM) and *O*-mannose–type galactomannan (OMGM) in *Aspergillus fumigatus*.

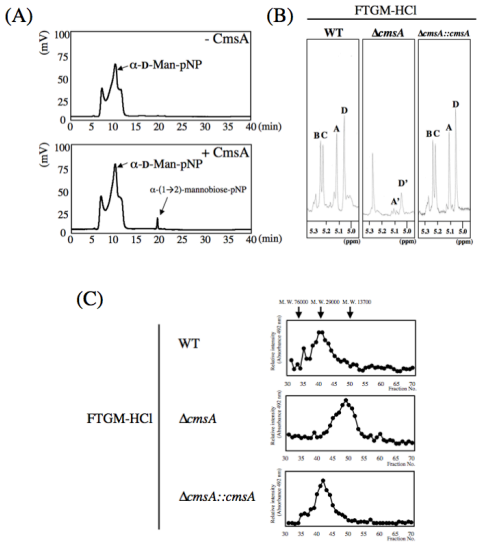

Figure 2. **(A)** *In vitro* assay of CmsA mannosyltransferase activity. Chromatograms show typical results of the assay without (-CmsA, upper panel) and with CmsA (+CmsA, lower panel). **(B)** ^{1}H-NMR analyses of FTGMs without galactofuranose sugar chains (FTGM-HCl) from the WT, Δ*cmsA*, and Δ*cmsA::cmsA* strains. Total GM (FTGM+OMGM) was extracted, purified FTGMs were prepared via β-elimination, and FTGM-HCl was prepared via acid hydrolysis of the purified FTGMs (0.1 M HCl for 60 min). Signals A (5.104 ppm), B (5.233 ppm), C (5.216 ppm), and D (5.054 ppm) of the ^{1}H-NMR spectra are derived from H-1 at the C-1 position of each mannose residue. The signals A′ and D′ at 5.1 and 5.05 ppm of the ^{1}H-NMR spectra are from H-1 at the C-1 position of the underlined mannose residue in t-Man-(1→6)-α-Man- and t-Man-(1→2)-α-Man-. **(C)** Gel filtration analyses of FTGM-HCl from the WT, Δ*cmsA*, and Δ*cmsA::cmsA* strains. FTGM-HCls were prepared and analyzed via gel filtration chromatography using a Sephacryl S-200 HR (1 × 75 cm) column and 0.8 M NaCl for elution.