Biosynthesis of the Fungal Cell Wall Polysaccharide Galactomannan Requires Intraluminal GDP-mannose

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Background: Understanding the mechanisms of cell wall biogenesis is important for development of antifungal strategies.

Results: Biosynthesis of the fungal polysaccharide galactomannan requires import of GDP-mannose into the Golgi apparatus.

Conclusion: It differs from the biosynthesis of other fungal cell wall polysaccharides.

Significance: This provides a new paradigm for cell wall polysaccharide biosynthesis.

Fungal cell walls frequently contain a polymer of mannose and galactose called galactomannan. In the pathogenic filamentous fungus Aspergillus fumigatus, this polysaccharide is made of a linear mannan backbone with side chains of galactofuran and is anchored to the plasma membrane via a glycosylphosphatidylinositol or is covalently linked to the cell wall. To date, the biosynthesis and significance of this polysaccharide are unknown. The present data demonstrate that deletion of the Golgi UDP-galactofuranose transporter GlfB or the GDP-mannose transporter GmtA leads to the absence of galactomannan, respectively. This indicates that the biosynthesis of galactomannan probably occurs in the lumen of the Golgi apparatus and thus contrasts with the biosynthesis of other fungal cell wall polysaccharides studied to date that takes place at the plasma membrane. Transglycosylation of galactomannan from the membrane to the cell wall is hypothesized because both the cell wall-bound and membrane-bound polysaccharide forms are affected in the generated mutants. Considering the severe growth defect of the A. fumigatus GmtA-deficient mutant, proving this paradigm might provide new targets for antifungal therapy.

The fungal cell wall is a complex and dynamic entity essential for growth and development of fungi. It consists of an alkali-insoluble polysaccharide network, with which glycoproteins are covalently or non-covalently associated (1–3). A common trait is the presence of a β-1,6-branched β-1,3-glucan that forms a continuous, three-dimensional scaffold to which other components of the alkali-insoluble fraction are covalently linked. Most commonly, these are chitin, a β-1,4-N-acetylglucosamine polymer occurring in all fungi with the sole exception of the fission yeast Schizosaccharomyces pombe (4), and other glucans, such as the β-1,6-glucans in Saccharomyces cerevisiae (5) or the mixed β-1,3/β-1,4-glucans in Aspergillus fumigatus (6). In the latter fungus, galactomannan, a linear α-1,2/α-1,6-mannan with short side chains of β-1,5-galactofuran, also substitutes the cell wall β1,3/1,6-glucan (6, 7). This polysaccharide can additionally be anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI)4 (8) or be part of the alkali-soluble fraction of the cell wall, containing the non-covalently associated polysaccharides α-1,3-glucan and galactosaminogalactan as well as secreted glycoproteins (5). Finally, circulating galactomannan is also present in patient blood, where it can be detected by the monoclonal antibody EB-A2 for clinical diagnostic of invasive aspergillosis (9, 10). Galactofuranose (Galf) is widely distributed among fungi (11), and galactomannan itself has been reported in many fungi, although structural variations exist.

The polysaccharides β-glucan and chitin are both synthesized at the plasma membrane by integral membrane complexes that use cytosolic nucleotide sugars and extrude the growing polysaccharide chains into the extracellular space (12–14). Biosynthesis of β-glucan is essential for fungal viability, and the likely catalytic subunit of the glucan synthase complex, FKS1, is the target of a recent class of antifungals, the echinocandins (15). In contrast, galactomannan biosynthesis as well as its biological role and importance for fungal viability are currently undefined. However, the recent characterization of a Golgi-localized UDP-Galf transporter and its subsequent deletion in A. fumigatus (16) and Aspergillus nidulans (17) suggested its implication in galactomannan galactofuranosylation. Indeed, a soluble cell extract of the transporter-deficient mutant was unreactive to the anti-galactofuran antibody EB-A2 (16).

Nucleotide sugar transporters form a family of structurally conserved integral membrane proteins that supply the endoplasmic reticulum or Golgi apparatus of eukaryotic cells with donor substrates of glycosyltransferases (18, 19). GDP-mannose (GDP-Man) transporters have been studied in various organisms, such as the protozoan parasite Leishmania (20), S. cerevisiae (21), plants (22), and various fungi (23–25), including two Aspergilli (26, 27). Mammalian cells do not possess any

4The abbreviations used are: GPI, glycosylphosphatidylinositol; Galf, galactofuranose; Man, mannose; IPC, inositolphosphorylceramide; GIPC, glycosylinositolphosphorylceramide.
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GDP-Man transporter becausemannosylation is restricted to the conserved core glycosylation events in the endoplasmic reticulum that use lipid-activated mannose or cytosolic GDP-Man. In fungi, GDP-Man transport is indispensable for modification of protein N- and O-glycans (28–30), glycolipid biosynthesis (31), and GPI anchor substitution (32, 33). It has been reported to be essential in Saccharomyces (21), Candida (23), and Aspergillus niger (26).

Here, we provide genetic and biochemical evidence for the implication of nucleotide sugar transporters in cell wall-bound galactomannan biosynthesis and in fungal growth through characterization of A. fumigatus mutants deficient in UDP-Gal or GDP-Man transporters.

EXPERIMENTAL PROCEDURES

A. fumigatus Strains—A derivate of the A. fumigatus clinical isolate D141 deficient in non-homologous end-joining (AF3S5) was used (34). A. fumigatus gmtA was replaced by a pGpdA::ble/tk::trpCt cassette (35), using electroporation of conidia (36). Generation of the replacement cassette is described in the supplemental material and primers used are listed in Table S1. The resulting A. fumigatus ΔgmtA was grown in minimal medium containing 30 mg/liter phleomycin. For carbohydrate analysis, strains were grown in Sabouraud medium supplemented with 1.2 M sorbitol as osmotic stabilizer.

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In order to investigate the importance of GDP-Man in A. fumigatus, we constructed a ΔgmtA knockout mutant by CRISPR/Cas9 gene truncation (37). A. fumigatus ΔgmtA was grown in minimal medium containing 30 mg/liter phleomycin. For carbohydrate analysis, strains were grown in Sabouraud medium supplemented with 1.2 M sorbitol as osmotic stabilizer.

Digestion of Alkali-insoluble Fraction by β-Glucanase—For digestion with β-glucanase (Quantzyme ylg™, Qbiogene), 1 ml of cell wall suspensions was incubated with 200 units/ml β-glucanase in 50 mM triethylamine/acetate buffer, pH 7.4, 5 mM sodium azide at 37 °C for 5 days. Samples were centrifuged for 30 min at 13,000 × g to remove residual cell wall material. Sugar quantification was performed as described previously (40).

Galactomannan Quantification—Ten μg of the β-glucanase-released material were dissolved in 100 μl of TBS, and 10× dilution series of this stock were applied to a Platelia Aspergillus enzyme immunoassay plate (Bio-Rad). Galactomannan was quantified from reactivity to EB-A2 antibody according to the kit manual.

Determination of the Monosaccharide Composition—Defined amounts of freeze-dried alkali-insoluble cell walls (from 500 μl of suspension, yielding 2–4 mg of dry mass) were hydrolyzed with sulfuric acid as described previously (39). Alternatively, for hydrolysis of the β-glucanase-released material, 10-μg aliquots were dissolved in 130 μl of 4 M TFA and incubated at 100 °C for 4 h. Internal standard (25 μl of a 1 mM L-fucose solution) was added, and the hydrolysate was dried in a centrifugal vacuum concentrator. Labeling with anthranilic acid and separation by RP-HPLC (Prominence UFLC XR, Shimadzu with a LiChroCART® 250-4 Superspher® 100 RP-18 end-capped cartridge; Merck) was as described (41).

Purification and Analysis of Lipagalactomannan—Purification of lipagalactomannan and fractionation on octyl-Sepharose was essentially as described (8). Details are provided in the supplemental material. Reactivity to EB-A2 was tested using 1:500 dilutions of the fractions with the Platelia Aspergillus enzyme immunoassay kit (Bio-Rad) according to the kit manual.

RESULTS

A. fumigatus GmtA Is an Active GDP-Man Transporter—The A. fumigatus genome contains a single ortholog of the S. cerevisiae GDP-Man transporter Vrg4p. The gene AFUA_5G05740, named here gmtA, encodes a 382-amino acid protein displaying 50% identity with S. cerevisiae Vrg4p on the amino acid sequence level and clustering with other characterized GDP-Man transporters in phylogenetic analyses (26). The ability of GmtA to complement the lethal phenotype of a conditional S. cerevisiae VRG4 knock-out strain, generated by replacing the endogenous VRG4 promoter region with a galactose-inducible promoter, demonstrates that it is an active GDP-Man transporter (supplemental Fig. S1).

Deletion of A. fumigatus GmtA Results in a Severe Growth Defect—In order to investigate the importance of GDP-Man transport for cell wall biogenesis, we generated an A. fumigatus null mutant called ΔgmtA by replacement of the gmtA ORF with the phleomycin resistance gene ble (35), using swollen conidia electroporation. Correct replacement of the ΔgmtA genomic locus was confirmed by Southern blot analysis (supplemental Fig. S2).
At the macroscopic level, the ΔgmtA mutant is severely impaired in growth and sporulation, both on minimal medium and Sabouraud medium. It forms tiny, compact colonies fundamentally different from the aerial appearance of the wild type and is unable to sporulate or spread (Fig. 1, A and B), even after several weeks of incubation at 37 °C. Sporulation and growth can be slightly improved by the addition of 1.2 M sorbitol to the medium (Fig. 1, C and D). This requirement for osmotic stabilization suggests a weakened cell wall in the ΔgmtA mutant.

Cell wall weakening of ΔgmtA was further indicated by time lapse recordings of swelling and germinating spores. Resting conidia were transferred to Sabouraud medium at 37 °C, and bright field microscopy pictures were captured every 15 min. Fig. 2 shows a representative germination of a ΔgmtA spore. The conidia swelled up to 8-fold the volume of swollen wild type conidia and frequently burst before or shortly after the emergence of the germ tube.

Deletion of the A. fumigatus GDP-Man Transporter Arrests Glycosylinositolphosphorylceramide (GIPC) Biosynthesis—GDP-Man transporters have been localized to the Golgi apparatus in several organisms, including A. niger (26). Deletion of gmtA is thus expected to abrogate transport of GDP-Man in the Golgi apparatus and interfere with biosynthesis of glycoconjugates. GIPCs, whose structures have been comprehensively described (42, 43), have thus been analyzed by MALDI-TOF mass spectrometry. The core structure Man₁–3Man₁–2IPC (Hex,–IPC) can be modified by mannose and/or galactofuranose,

![Fig. 1. Colony morphology of A. fumigatus ΔgmtA. Conidia of the A. fumigatus ΔgmtA mutant and the mother strain were plated on minimal medium (A and C) or Sabouraud medium (B and D), supplemented or not with 1.2 M sorbitol as indicated. Plates were incubated at 37 °C for 56 h.](image1)

![Fig. 2. Swelling and burst of ΔgmtA conidia. Swelling and germination of conidia were observed in bright field time lapse recordings at 37 °C in Sabouraud medium. Four representative snapshots with time indicated are depicted (bar, 10 μm).](image2)

![Fig. 3. Negative ion matrix-assisted laser desorption ionization spectra of GIPCs isolated from wild type and ΔgmtA mutant. GIPCs were extracted by solvent partitioning and recovered in the aqueous phase (A and C) or organic phase (B and D) from the wild type strain (A and B) or ΔgmtA mutant (C and D). Each mono- or oligosaccharide is linked to IPC containing C18:0 or C20:0 phytosphingosine conjugated to a fatty acid varying in chain length and hydroxylation state, giving rise to peak series. The masses of the main species containing monohydroxylated lignoceric acid are indicated. Hex, hexose; HexN, hexosamine; HexNAc, N-acetylhexosamine.](image3)
yielding GIPCs with three or four hexoses (Hex$_3$- and Hex$_4$-IPCs). These acidic GIPCs, contained in the aqueous phase after solvent partitioning, were observed in the mother strain (Fig. 3A) but were absent from the $\Delta$gmtA mass spectra (Fig. 3C). Similarly, the zwiterionic glycolipid Man$_1$–3Man$_1$–6GlcN$_\alpha$1–2IPC (Hex$_3$–HexN-IPC) that associates with the organic phase during partitioning is only visible in the wild type strain (Fig. 3, B and D). Spectra obtained from the $\Delta$gmtA mutant display intense peaks corresponding to the biosynthetic precursors of GIPCs: IPC, hexosamine-IPC, and N-acetyhexosamine-IPC (Fig. 3, C and D). It should be noted that in the conditions used, the desorption/ionization efficiency is much higher for acidic glycolipids than zwiterionic glycolipids. The relative intensity of HexNAC-IPC molecular ions observed in Fig. 3C reflects the properties of this glycolipid rather than its accumulation. In conclusion, deletion of the gmtA gene results in an arrest of glycolipid biosynthesis consistent with the absence of GDP-mannose in the Golgi lumen.

**Biosynthesis of Galactomannan Is Blocked in A. fumigatus $\Delta$gmtA and $\Delta$glfB Mutants**—The generation and partial characterization of an $A. fumigatus$ mutant deficient in the UDP-Galf transporter GlfB, named $\Delta$glfB, has been reported previously (16). To determine if galactomannan biosynthesis requires GlfB or the GDP-Man transporter GmtA, we first investigated the monosaccharide composition of alkali-insoluble cell wall fractions from the $\Delta$gmtA, $\Delta$glfB, and wild type strains of $A. fumigatus$. The complex polysaccharide network of the cell wall alkali-insoluble fraction is essentially made of four monosaccharides: glucose and N-acetylglucosamine, the monomers of $\beta$-glucan and chitin, respectively, as well as mannosamine and galactose, the monosaccharide units of galactomannan. Hydrolysis of the cell wall fractions was achieved using sulfuric acid (39), whereby the chitin monomer N-acetyl glucosamine is deacetylated to glucosamine. The analyses revealed that the cell wall of the $\Delta$glfB mutant lacked galactose, whereas mannosamine was still present (Fig. 4A). This result is consistent with the expected absence of galactofuranosyl side chains and the presence of the underlying mannan. Other monosaccharides were essentially unchanged when compared with the parental strain, reflecting unaltered $\beta$-glucan and chitin levels in this mutant. As anticipated, the alkali-insoluble fraction of $\Delta$gmtA was virtually devoid of mannose (Fig. 4A), indicating galactomannan deficiency in the cell wall of this mutant. Interestingly, $\Delta$gmtA cell wall fractions contained high levels of galactose and galactosamine, although amounts were unsteady and therefore not statistically significant as compared with wild type. This might be due to increased levels of precipitated galactosaminoglactan, which is a known contaminant of the alkali-insoluble fraction (6). In addition, glucosamine was approximately twice as abundant as compared with wild type, whereas the glucose content was reduced by $\sim$65%. This strongly suggests alterations in $\beta$-glucan and chitin content.

To confirm absence of galactomannan in the $\Delta$gmtA cell wall, we also analyzed the monosaccharide composition of the fractions after $\beta$-glucanase digestion, a treatment that releases galactomannan covalently linked to $\beta$-glucan and enables a more precise quantification of its components. Mannosamine, but no galactose, was present in solubilized oligosaccharides from $\Delta$glfB, whereas $\Delta$gmtA contained neither mannosamine nor galactose (Fig. 4B). Finally, the presence of galactomannan was also assessed by a highly sensitive ELISA using the anti-galactofuranosyl antibody EB-A2 (9) (Fig. 5). When using solubilized oligosaccharides from $\Delta$gmtA, only the highest concentration of 100 $\mu$g/ml triggered a moderate signal, whereas wild type material reached saturation already at concentrations of 0.1 $\mu$g/ml. Concentrations yielding the half-maximal signal ($V_{50}$) were calculated based on non-linear curve fitting ($R^2 > 0.98$) and were $\sim$3000 times higher for $\Delta$gmtA (111.7 $\mu$g/ml) than for wild type (0.039 $\mu$g/ml). Samples from $\Delta$glfB were negative at all concentrations. The very low signal observed with fractions from the $\Delta$gmtA mutant may reflect an extremely limited synthesis of galactomannan (less than 0.1%) due to the “leakiness” of the Golgi toward GDP-Man or be due to contamination by galactofuranosylated glycoproteins that are also recognized by the EB-A2 antibody (44). Together, these data clearly establish a
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![Graph showing OD460 vs sugar concentration for wild type, ΔgltB, and ΔgmtA mutants](image)

FIGURE 5. Detection of cell wall galactomannan from A. fumigatus wild type and ΔgltB and ΔgmtA mutants by quantitative ELISA. The alkaline-insoluble cell wall fractions from A. fumigatus wild type and ΔgltB and ΔgmtA mutants were solubilized by β-glucanase digestion, and the sugar concentration was adjusted to 100 μg/ml 10-fold dilutions were applied to ELISA and detected with the anti-galactofuran antibody EB-A2. Data points represent mean values of two biological replicates.

![Graph showing OD460 vs Fraction for wild type and ΔgmtA](image)

FIGURE 6. Purification of GPI-anchored galactomannan from A. fumigatus wild type and ΔgmtA mutant. GPI-anchored galactomannan was prepared from membranes of A. fumigatus wild type (diamonds) and ΔgmtA mutant (inverted triangles) and purified by hydrophobic interaction chromatography on octyl-Sepharose. The presence of galactomannan in the 2-ml fractions was investigated by ELISA using the anti-galactofuran antibody EB-A2.

galactofuran deficiency or galactomannan deficiency in the cell walls of ΔgltB or ΔgmtA, respectively.

At last, we investigated the presence of membrane-bound galactomannan in the ΔgmtA mutant. Using established conditions that allowed purification of lipogalactomannan from the wild type strain, we were not able to isolate any galactomannan from the ΔgmtA mutant (Fig. 6). Thus, both membrane-bound and cell wall-bound galactomannan are essentially absent from the GDP-Man transporter-deficient strain of A. fumigatus.

DISCUSSION

The GDP-Man transporter of the protozoan parasite Leishmania donovani was one of the first nucleotide sugar transporters to be characterized (20). The authors demonstrated that the gene LPG2 is required for uptake of GDP-Man into the Golgi lumen and for synthesis of the GPI-anchored polysaccharide lipophosphoglycan. The biosynthesis of this unique polysaccharide has been partially elucidated. The GPI anchor biosynthesis is initiated in the endoplasmic reticulum and completed in the Golgi apparatus, where the polysaccharide repeating units are then assembled (45). Together with Leishmania lipophosphoglycan and Crithidia fasciculata lipoarabinogalactan (46), A. fumigatus lipogalactomannan constitutes one of the rare examples of GPI-anchored polysaccharides (8) and may also be synthesized along the secretory pathway. An absence of reactivity of the anti-galactofuran antibody EB-A2 toward cell extract of the Golgi UDP-Galf transporter-deficient mutant ΔgltB, which suggested an impaired biosynthesis of this polysaccharide, supported this assumption (16). The present data clearly establishes that the Galβ side chains of galactomannan are also absent from the cell wall of this mutant. Similarly, both the GPI-linked galactomannan and cell wall-bound galactomannan are absent from an A. fumigatus GDP-Man transporter-deficient mutant named ΔgmtA. A compensatory chitin up-regulation and decreased glucan content seem to be associated with the lack of galactomannan. These cell wall alterations generated by unavailability of GDP-Man in intraluminal vesicles contrast with the limited changes in the ΔgltB cell wall composition. The latter is deficient in galactose but retains wild type levels of other monosaccharides and thus probably only lacks galactofuran. Modification of the cell wall composition of the ΔgmtA mutant might indicate that the mannan backbone of galactomannan, in contrast to the galactofuran side chains, plays an essential role in cell wall architecture. Moreover, the presence of GDP-Man in the Golgi apparatus is required for elongation of N- and O-glycans, biosynthesis of GIPCs, and “decoration” of protein GPI anchors. Therefore, the possibility that defective mannosylation in the Golgi leads to mislocalization or impaired trafficking of cell wall-remodeling enzymes cannot be excluded. Remodeling of β-glucan, for example, is mediated by extracellular GPI-anchored glucansulfotransferases of the GAS/GEL family containing several potential glycosylation sites (47, 48).

In contrast to filamentous fungi, ascomycetous yeasts express abundant cell wall mannoproteins carrying N-glycans with up to 200 mannose residues. A pool of intraluminal GDP-Man is required for their biosynthesis and may be responsible for the essentiality of the GDP-Man transporter in S. cerevisiae (21, 28) and the pathogenic yeast Candida albicans (23). Despite its very severe growth and sporulation defect, an A. fumigatus ΔgmtA mutant was obtained by spore electroporation. It represents the first A. fumigatus strain deficient in galactomannan. It is, however, difficult to ascribe the dramatic growth phenotype of the ΔgmtA mutant to a specific carbohydrate, because the absence of the transporter has pleiotropic effects on glycosylation. The addition of mannose to N-glycans may occur in the Golgi apparatus of filamentous fungi, but this maturation step seems limited and dispensable for growth (49, 50). Likewise, mannosylation of IPC is not required for growth (51). In contrast, the initiation of O-glycans biosynthesis by dolichol-phosphomannose-dependent mannosyltransferases is essential for yeast and fungal growth, but, at least in yeast, their elongation in the Golgi apparatus is dispensable (52, 53). It remains thus possible that the manifest cell wall weakening observed in the ΔgmtA mutant results from the sole absence of
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galactomannan and is responsible for the severe growth phenotype.
A critical question raised by the present work is the site of assembly of galactomannan. So far, fungal cell wall polysaccharides (such as glucan and chitin) have been shown to be synthesized at the cell surface by type III integral membrane synthases (54). This study demonstrates, however, that both the backbone and side chain assembly of galactomannan require nucleotide sugar transporters and thus provide indirect evidence of the involvement of the Golgi apparatus in the biosynthesis of a fungal cell wall polysaccharide. In plants, the contribution of the Golgi apparatus to the elaboration of hemicellulosic and pectic polysaccharides has long been recognized, and it has recently been demonstrated that type II glycosyltransferases can elaborate the polysaccharide backbone (55, 56). Similarly, biosynthesis of the unique polysaccharide capsule of the basidiomycete Cryptococcus neoformans requires intraluminal GDP-mannose, and capsule material has even been observed within intracellular vesicles (25, 57, 58). The particularity of A. fumigatus galactomannan is its occurrence as membrane GPI-anchored polysaccharide, in addition to the cell wall-bound polysaccharide (8). Both forms require the GDP-Man and UDP-Galf transporters for their synthesis. These observations support a biosynthetic model according to which galactomannan is assembled on a GPI anchor in the Golgi apparatus and secreted to the plasma membrane before being cross-linked to cell wall β-glucan by extracellular transglycosidases. Transglycosidases mediating the remodeling of the cell wall β-glucan scaffold have already been characterized in A. fumigatus and S. cerevisiae (47, 48, 59, 60). Others are thought to be involved in the transfer of GPI-anchored mannoproteins to the cell wall of ascomycetous yeasts but remain elusive (1). Hitherto, very little attention has been given to GPI-anchored polysaccharides. This paradigm, however, may not be limited to Aspergillus galactomannan, and future studies will determine if it may also apply to other extracellular polysaccharides and organisms.

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