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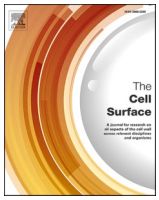

Understanding *Aspergillus fumigatus* galactosaminogalactan biosynthesis: A few questions remain

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ABSTRACT

Half a century after their discovery, polymers of *N*-acetylgalactosamine produced by the Aspergilli have garnered new interest as mediators of fungal virulence. Recent work has focused on the *Aspergillus fumigatus* secreted and cell wall-associated heteropolymer, galactosaminogalactan (GAG). This polymer, composed of galactose (Gal) and partially deacetylated *N*-acetylgalactosamine (GalNAc), plays a role in a variety of pathogenic processes including biofilm formation, immune modulation and evasion, and resistance to antifungals. Given its many potential contributions to fungal pathogenesis, GAG is a promising therapeutic target for novel antifungal strategies. As such, several studies have sought to elucidate the biosynthetic pathways required for GAG production and secretion. Herein we review the progress made in the understanding of the molecular mechanisms underlying GAG synthesis and identify several gaps in our understanding of this process.

Introduction

The *A. fumigatus* exopolysaccharide galactosaminogalactan has been reported to mediate a number of functions in host-pathogen interactions. As a cationic exopolysaccharide located within the extracellular matrix, GAG serves as an adhesin that mediates adhesion to anionic surfaces such as human cells, macromolecules and plastic, and supports biofilm formation (Gravelat et al., 2013). GAG is adherent to the outer cell wall of hyphae, where it can serve to cloak cell wall polysaccharides like β-glucans from innate immune detection, and repel cationic molecules such as antimicrobial peptides associated with neutrophil extracellular traps (Gravelat et al., 2013; Lee et al., 2015). Secreted GAG can also directly modulate host immune responses through platelet activation, mediating neutrophil apoptosis, inducting of IL-1 receptor antagonist secretion, and activation of the inflammasome (Speth et al., 2019; Briard et al., 2020). Given the multiple roles that GAG plays in virulence, there has been great interest in elucidating the mechanisms by which GAG is synthesized and exported to the extracellular space. The current model of GAG biosynthesis has been largely based on the study of a cluster of five genes identified through a transcriptomic study of biofilm-deficient *A. fumigatus* regulatory mutants (Gravelat et al., 2013). Investigation of the five putative carbohydrate active enzymes encoded by this cluster has led to a synthase-dependent model of polymer synthesis (Fig. 1). In this model, GAG synthesis is initiated intracellularly with the interconversion of UDP-*N*-acetylglucosamine to UDP-*N*-acetylgalactosamine, and UDP-glucose to UDP-galactose by the bifunctional UDP-glucose-4-epimerase, Uge3 (Lee et al., 2014). Polymerization and extracellular export of the nascent macromolecule is thought to be mediated by a 300 kDa putative transmembrane glycosyltransferase, Gtb3 (Le Mauff, 2020). Release of fully acetylated GAG from the transferase is hypothesized to be mediated by Sph3, a membrane-anchored glycoside hydrolase (GH) with retaining *endo*-α-1,4-*N*-acetylgalactosaminidase activity (Le Mauff et al., 2019). Upon traversing the cell wall, fully acetylated GAG is processed by Agd3, a secreted carbohydrate esterase, which de-*N*-acetylates long stretches of GalNAc to galactosamine (GalN), rendering the polymer cationic and biologically active (Bamford et al., 2020). Ega3, a second membrane-anchored GH, has been found to exhibit α-1,4-galactosaminidase activity *in vitro* and is therefore able to cleave deacetylated GAG (Bamford et al., 2019).

Although this model is largely consistent with the published studies of GAG biosynthetic enzymes, several questions remain. Is Gtb3 a

Abbreviations: GAG, galactosaminogalactan; GalNAc, *N*-acetylgalactosamine; Gal, galactose; GH, glycoside hydrolase.

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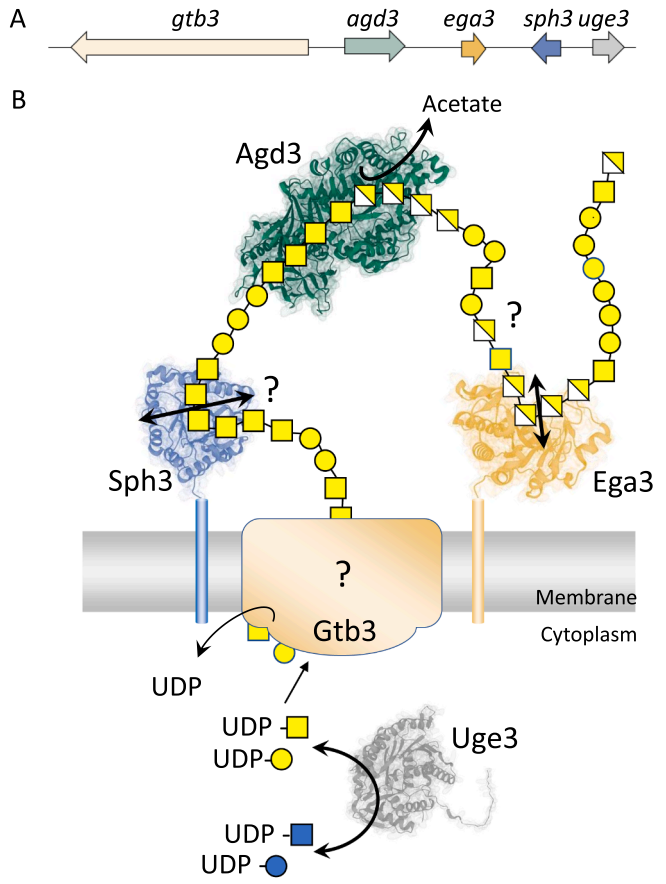

Fig. 1. GAG biosynthesis current model A. Graphical representation of GAG biosynthetic gene cluster located on the chromosome 3. B. Graphical representation of the current model of GAG biosynthesis. Enzyme domains are presented using the Alphafold2.0 prediction for Uge3 (Uniprot Q4WX18), the solved crystal structures of Sph3 (PDB 5C5G), Agd3 (PDB 6NWZ) and Ega3 (PDB 6OJ1). Due to its size, the structure of Gtb3 has yet to be modelled or confirmed. Unresolved questions in GAG biosynthesis are indicated with "?" marks. Monosaccharides were represented according to the international nomenclature: glucose: blue circle, galactose: yellow circle, *N*-acetylglucosamine: blue square, *N*-acetylgalactosamine: yellow square, galactosamine: half yellow square. UDP indicates Uridyldiphosphate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

bifunctional transferase that can mediate the polymerization of two different monosaccharides to form a heteropolysaccharide of variable composition? Why does deletion of a GH, Sph3, result in a loss of GAG production? Finally, why is Ega3, a GH specific for deacetylated GAG, located at the cell membrane if GAG is only deacetylated after secretion by the cell-wall localized Agd3? These questions are discussed below in further detail.

Could GAG synthesis be mediated by only one glycosyltransferase?

In the current model of GAG biosynthesis, the polymerization of Gal and GalNAc to form GAG heteropolymer is mediated by a single glycosyl transferase, Gtb3 (Fig. 1). By implication, this enzyme would be required to accept both UDP-Gal and UDP-GalNAc as donor sugars and also be able to use Gal and GalNAc as acceptors. This is somewhat at odds with the fact that the vast majority of glycosyltransferases that have been reported to adhere to the "one enzyme – one linkage" hypothesis developed in the early 2000's (Roseman, 2001). This hypothesis suggests that glycosyltransferases exhibit specificity for a single nucleotide sugar donor and a unique acceptor. Such a hypothesis argues against the promiscuous ability of Gtb3 to mediate the multiple reactions required to synthesis GAG. However, exceptions to the "one enzyme – one linkage" hypothesis have been documented. Of note, in one well characterized example, the bovine β1-4Gal-T1 galactosyltransferase was reported to be unable to discriminate between UDP-GalNAc and UDP-galactose as donor sugars following a single mutation of an amino acid in the donor binding site (Qasba et al., 2008). Biochemical and structure–function studies will be therefore invaluable in defining the donor and acceptor specificity of Gtb3, studies that have been hampered by the large size (>300 kDa), and membrane localization of this protein.

An alternate hypothesis to the single glycosyl transferase model is one in which other, yet uncharacterized, transferases or other enzymes are required for GAG biosynthesis. Given the presence of regions of GalNAc homopolymer, Gal homopolymer and Gal-GalNAc heteropolymer within the mature GAG polymer, GAG synthesis could require as many as four separate transferases, one specific for each combination of Gal and GalNAc as donor and acceptor sugars. In this case, Gtb3 would likely mediate the polymerization of UDP-GalNAc, given that four of the enzymes encoded within the 5-gene GAG biosynthetic cluster all exhibit GalNAc/GalN specificity. In this second model, the incorporation of Gal into GAG would then be mediated by one or more independent galactosyltransferases. It is also possible that GAG could be assembled from two independently synthesized sets of oligosaccharides: oligo-GalNAc and oligo-Gal. In this case only two transferases would be required for GAG synthesis, however this model requires other enzymes to assemble these components into mature heteropolymer GAG.

The reported phenotype of the Δ*gtb3* strain provides only limited insights into these hypotheses (Briard et al., 2020). Although this mutant failed to produce detectable GAG and lacked detectable secreted and cell wall-associated GalNAc, no changes were reported in the hexose content of the cell wall and secreted polysaccharides. It is therefore possible that deletion of Gtb3 resulted in the loss of poly-GalNAc, but that production of the galactan component of GAG was unaffected. Unfortunately, as the hexose composition of the cell wall in this strain was not characterized further, these findings could also simply indicate compensatory cell wall changes in response to the loss of GAG. Characterizing the cell wall and secreted hexose composition in greater detail might help distinguish between these two possibilities. Importantly, the absence of galactan production in the Gtb3-deficient mutant would not rule out the multiple transferase model. It is possible that GalNAc polymerised by Gtb3 may be required as a substrate for any Gal-specific transferase(s), or that Gtb3 itself is required for the formation of protein heterocomplex that mediates GAG synthesis. Ultimately, biochemical and structure function studies will likely be required to resolve these questions.

What is the role of Sph3 in GAG biosynthesis?

Structure-function studies of Sph3 have demonstrated that this enzyme exhibits retaining *endo*-α-1,4-*N*-acetylgalactosaminidase activity, and that a loss of Sph3 in *A. fumigatus* was associated with a complete loss of GAG production (Le Mauff et al., 2019; Bamford et al., 2015). These observations raise the question: how does loss of a GAG-degrading enzyme lead to a loss of GAG production? It has been hypothesized that as a membrane anchored glycoside hydrolase, Sph3 may serve to cleave the newly synthesized GAG polymer to allow for secretion into the cell matrix (Le Mauff, 2020). If true, it would be predicted that some GAG could still be produced, and detectable at the level of the plasma membrane. However, biochemical and microscopy studies examining the extracellular glycan composition of the Sph3-deficient mutant strain failed to detect any GalNAc-containing polymers (Bamford et al., 2015). It is of course possible that the techniques used in these studies were insufficiently sensitive to detect production of small amounts of cell-membrane associated GAG. Immune electron microscopy could prove a useful tool in determining if short-chain GAG is present at the plasma membrane in this strain.

Alternately, it is also possible that Sph3 does not function as a glycoside hydrolase during GAG synthesis. Several examples have been reported in which glycoside hydrolases can also function as transglycosylases. Under appropriate conditions, these enzymes can accommodate an oligosaccharide into their binding site instead of the water molecule which they normally use for the hydrolysis (Bissaro et al., 2015). These enzymes can then mediate polymer elongation or branching. Such enzymes have been reported to play important roles in *A. fumigatus* cell wall synthesis such as the cross linking of chitin and β-glucans by the Chr chitinases (Fang et al., 2019), and the elongation and branching of β-glucans by the Gel glucanases (Gastebois et al., 2010; Mouyna et al., 2000). It is therefore possible that Sph3 could function as a transglycosylase to link emergent GAG oligosaccharides together to form a longer polymer. Although such a function would be consistent with the loss of GAG production in the Sph3 mutant, it raises further questions. Given that Sph3 was found to have no activity against galactose-containing regions of GAG (Le Mauff et al., 2019), how would these regions be incorporated into the final polymer? Do other enzymes supporting galactose transglycosylation exist? Testing the ability of Sph3 to mediate transglycosylation of short GalNAc and Gal oligosaccharides *in vitro* may help shed light on these questions.

Why is Ega3 predicted to be localized at the plasma membrane while its substrate is found within the cell wall?

As with Sph3, the role of Ega3, the second glycoside hydrolase within the GAG cluster in GAG biosynthesis remains unknown. Although the phenotype of an Ega3-deficient *A. fumigatus* mutant has not yet been reported, biochemical studies have demonstrated that Ega3 exhibits α-1,4-galactosaminidase activity (Bamford et al., 2019). Ega3 is therefore dependent on the deacetylase activity of Agd3 for the generation of its substrate. However, Agd3 was found to be localized within the fungal cell wall, while Ega3 contains a transmembrane domain and is predicted to be found at the plasma membrane (Fig. 1). What could then be the function of a glycoside hydrolase specific for deacetylated GAG at the plasma membrane where GAG has yet to be deacetylated?

One possibility is that the predicted plasma membrane localization of Ega3 may be incorrect. Ega3 localization to the plasma membrane is predicted by the presence of a *N*-terminal transmembrane domain but has not been experimentally validated (Bamford et al., 2019). Of note, a highly disordered sequence of 20 amino acids links the transmembrane domain and the GH114 $(\beta/\alpha)_8$ enzymatic barrel of Ega3. It is possible that proteolytic cleavage of this region could allow release of Ega3 where it could transit to the cell wall and interact with its substrate (Bamford et al., 2019). Alternately, Ega3 could remain associated with membrane lipids but be transported to the cell wall via extracellular vesicles. The production of extracellular vesicles has been reported in *A. fumigatus*, and these structures were found to contain a wide array of enzymes and glycans involved in cell wall polymer biosynthesis (Rizzo et al., 2020). Indeed, proteomic analyses of extracellular vesicles of *A. fumigatus* formed during protoplast regeneration reported the presence of Ega3, albeit with low protein coverage identity (Rizzo et al., 2020).

Although no Ega3-deficient *A. fumigatus* mutant has been described, a mutant deficient in Ega3 was reported in another ascomycete, *Metarhizium robertsii* (Mei et al., 2021). In this insect pathogen, GAG synthesis is restricted to the appressorium, where it mediates adhesion to insect cuticles (Mei et al., 2021). In contrast to the loss of GAG that was observed with Sph3 deletion, disruption of Ega3 in *M. robertsii* did not abolish GAG formation nor result in reduced fungal adhesion (Mei et al., 2021). Although in a different species, these observations suggest that Ega3 is likely not required for GAG synthesis. The composition of GAG in this *M. robertsii* mutant strain was not analyzed in detail, and it is therefore possible that Ega3 could function to modify the composition or size of GAG after synthesis. The construction of an *A. fumigatus* Ega3-deficient mutant and the characterization of its GAG, as well as the confirmation of Ega3 localization, will be instrumental in improving our understanding of the function of this protein in GAG biosynthesis.

Conclusion

Although the current model of GAG biosynthesis has been helpful in understanding the role of this polymer in virulence, a number of questions remain unanswered. Completing the characterization of each of the proposed GAG biosynthetic enzymes through subcellular localization studies, biochemical analyses and construction of enzyme-deficient mutants will likely be required to resolve some of these questions and generate a complete and robust model of GAG biosynthesis. Given the emerging role of GAG in fungal virulence, and the prevalence of the GAG biosynthetic cluster across a wide range of fungal pathogens, a better understanding of the synthesis of this glycan may help guide future antifungal strategies targeting the role of this polymer in human, plant and animal fungal diseases.

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François Le Mauff: Conceptualization, Writing – review & editing.
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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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