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Unravelling Secretion in *Cryptococcus neoformans*: More than One Way to Skin a Cat

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Abstract Secretion pathways in fungi are essential for the maintenance of cell wall architecture and for the export of a number of virulence factors. In the fungal pathogen, *Cryptococcus neoformans,* much evidence supports the existence of more than one route taken by secreted molecules to reach the cell periphery and extracellular space, and a significant degree of crosstalk between conventional and non-conventional secretion routes. The need for such complexity may be due to differences in the nature of the exported cargo, the spatial and temporal requirements for constitutive and non-constitutive protein secretion, and/or as a means of compensating for the extra burden on the secretion machinery imposed by the elaboration of the polysaccharide capsule. This review focuses on the role of specific components of the *C. neoformans* secretion machinery in protein and/or polysaccharide export, including Sec4, Sec6, Sec14, Golgi reassembly and stacking protein and extracellular exosome-like vesicles. We also address what is known about traffic of the lipid, glucosylceramide, a target of therapeutic antibodies and an important regulator of *C. neoformans* pathogenicity, and the role of signalling pathways in the regulation of secretion.

Keywords *Cryptococcus neoformans* · Protein secretion · Polysaccharide secretion

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Secretion and Virulence in *C. neoformans*

The fungal pathogen, *C. neoformans,* causes life-threatening pneumonia and meningoencephalitis, especially in the immunocompromised host. The pathogenesis of *C. neoformans* is largely dependent on secretion activity as many of the most well-characterized molecules required by *C. neoformans* to cause disease are extracellular, including the major polysaccharide building block of the capsule [glucuronoxylomannan-GXM] (reviewed in [1, 2]), urease [3] and phospholipase B [4–6]. The enzyme laccase, required to synthesize melanin pigment, is also secreted to the fungal cell periphery and is essential for cryptococcal virulence [7–10]. Furthermore, a diverse family of secreted glycosyl hydrolases, glycosyl synthases and proteases are involved in the assembly, maintenance and integrity of the cell wall, attachment of the capsule or virulence [11–14]. In *Candida albicans*, secretory pathways have also

been linked to virulence associated with candidiasis. The predicted secretome of *C. albicans* contains more than 200 proteins, many of which have been confirmed experimentally to be hydrolytic enzymes (proteinases, phospholipases and lipases) that facilitate host–pathogen interactions and invasion, and the evasion of host defence mechanisms [15].

Despite fungal pathogenesis being so heavily reliant on the secretion machinery, little investigation has been carried out into the precise mechanisms involved. However, for the last three decades, secretion has been actively studied in the model yeast *Saccharomyces cerevisiae* (reviewed in [16]) that has served as an invaluable tool for investigating the mechanisms that regulate the traffic of polysaccharides, laccase and phospholipase B in *C. neoformans* [4, 6–8, 17–19]. Independent studies demonstrated that both conventional and non-conventional secretory pathways are required to export these molecules [4, 17, 19].

Conventionally secreted proteins possess a secretion leader peptide that provides a signal for the protein to traverse the endoplasmic reticulum (ER) and Golgi apparatus. Following folding and acquisition of N- or O-linked sugars, the proteins are transported to the cell periphery in vesicular structures. Conventional secretion is inhibited by brefeldin A (BFA), which causes resorption of the Golgi apparatus into the ER [20]. Non-conventionally secreted proteins lack a secretion leader peptide and are therefore secreted independently of the ER–Golgi pathway. Some examples are basic fibroblast growth factor (FGF), interleukin (IL)-1b, HIV-tat, galectin-3 and thioredoxin [21–26]. Thus, their secretion is not inhibited by BFA. Non-conventional secretion pathways are poorly understood in mammalian cells and in yeast. In addition to proteins, polysaccharides involved in the elaboration of extracellular structures, such as the capsule, are cargo exported within secretory vesicles. What is now understood about secretion mechanisms in *C. neoformans* will be discussed in this review.

Protein Secretion in *C. neoformans*

Studies of secretion pathways in *S. cerevisiae* were pioneered in the late 1970 and 1980 by Randy Schekman and colleagues as well as Bankaitis et al. with the characterization of the *SEC* group of genes [27–31]. By characterizing the phenotypes of several temperature-sensitive *sec* mutants, Sec proteins were demonstrated to be involved in protein traffic from the ER to the Golgi complex, from the Golgi complex to the cell surface and from the cell surface or endosome back to the Golgi (reviewed in [16]). So far in *C. neoformans,* three Sec protein homologues, Sec4/Sav1, Sec6 and Sec14-1, have been implicated in secretion processes essential to virulence [4, 8, 19]. This section focuses on the role of Sec6 and Sec14 in the secretion of protein virulence determinants, phospholipase B1 (Plb1), laccase 1 (Lac1) and urease, while the following section discusses the role of Sec4/Sav1 and sec6 in the secretion of capsular polysaccharide building block, GXM.

Plb1 is targeted to the cell wall and extracellular space [32], and extracellular Plb1 is the sole contributor of phospholipase activity in *C. neoformans* [5, 33, 34]. It is essential for binding to lung epithelium [35], maintaining fungal load in the lung [4, 36], non-lytic escape from macrophages (vomocytosis) [4], dissemination of *C. neoformans* from the lung to the central nervous system [4, 36, 37] and immunomodulation [36]. Laccase is also targeted to the cell wall and the extracellular environment [38] and is involved in the synthesis of a stress-protecting pigment, melanin, which is deposited in the cell wall and discharged into the extracellular environment. Unlike Plb1, laccase expression is induced by glucose derepression [39]. Due to the presence of an N-terminal secretion leader peptide, both Plb1 and laccase are conventionally secreted proteins. Laccase also contains a hydrophilic cell wall-targeting signal at the C-terminus [40], while Plb1 acquires a glycosylphosphatidylinositol (GPI) anchor at its C-terminus [6].

Urease is involved in nitrogen assimilation. It is the last enzyme in the uric acid degradation pathway, liberating ammonia from urea [3]. As well as allowing *C. neoformans* to utilize uric acid within ecological niches [41, 42], it plays a role in the migration of *C. neoformans* across the blood–brain barrier and therefore in the development of meningoencephalitis [3, 43]. Despite the relatively reduced availability of nitrogen sources such as uric acid and urea in the host environment and regulation of urease by nitrogen catabolite repression [44], most clinical isolates of *C. neoformans* produce large quantities of urease such that detection of urease activity is commonly used as diagnostic marker [45, 46]. Under physiological

conditions, urea hydrolysis products potentially increase pH and ammonium hydroxide can damage tissue [3]. Although urease lacks a secretion leader peptide, its mode of secretion is not entirely non-conventional as discussed later.

Regulation of Laccase and Urease Secretion

Role of SEC6

In *S. cerevisiae, SEC6* is an essential gene and encodes a component of a protein complex (the "exocyst") which is involved in polarized fusion of exocytic vesicles with the plasma membrane [28]. Using a *C. neoformans* strain with reduced expression of *SEC6* via RNAi (i*SEC6*), Panepinto et al. [8] were the first to demonstrate the existence of a *SEC6*-dependent secretion pathway for the transport of laccase and urease and its segregation from a pathway involving Plb1. Furthermore, secretion of soluble (non-cell-associated) GXM polysaccharide was reduced in the *iSEC6* mutant. However, capsule size was not affected, consistent with the presence of more than one secretion pathway for GXM. Similar to the *SEC6* temperature-sensitive mutant in *S. cerevisiae*, *SEC6* mRNA suppression in *C. neoformans* coincided with the accumulation of intracellular vesicles (100–200 nm) that, in *C. neoformans,* were enriched in GFP-laccase. The accumulation of intracellular GFP-laccase in the i*SEC6* mutant coincided with the absence of GFP-laccase in the cell wall and was consistent with a secretion block. *SEC6* mRNA suppression also caused a mild cell wall defect upon exposure to the glucan-binding dye, Congo Red, but not to the chitin-binding dye, calcofluor white, suggesting that synthesis of cell wall glucan, but not chitin, is sensitive to *SEC6* suppression. *SEC6*-dependent secretion pathways were also shown to be essential for virulence in a mouse model of cryptococcosis [8].

SEC6-dependent cargo is structurally diverse and contains both conventionally and non-conventionally secreted cargo. Secreted GXM is a polysaccharide, and laccase is a classical secreted protein, while urease is not. Furthermore, the cell wall defect in the i*SEC* mutant, as evidenced by sensitivity to calcofluor white, is consistent with the transport of a subset of cell wall biosynthetic enzymes potentially involved in chitin assembly, being *SEC6*-dependent [8]. Some of these enzymes have been reported to possess a GPI anchor motif [12] and thus could potentially contribute further to the structural diversity of *SEC6*-dependent cargo.

Interestingly, the i*SEC6* mutant did not secrete extracellular, exosome-like vesicles which, incidentally, contain *SEC6*-dependent cargo (laccase, urease and GXM) and are of a similar size to *SEC6*-dependent intracellular vesicles [18, 47]. This is consistent with *SEC6*-dependent endosomes being precursors of exosome-like vesicles. In mammalian cells, exosomes are derived from multivesicular bodies (MVB), transport a number of proteins without a leader peptide, such as Hsp70 [48], and become released to the extracellular space following fusion of the MVB with the cell surface [49]. Exosome-like vesicles in fungi are unique in that they provide a potential mechanism to transport virulence factors across the fungal cell wall and protect their cargo from host-derived proteases. As *SEC6*-dependent endosomes and exosome-like vesicles carry similar cargo of both a conventional and non-conventional nature, they potentially represent points of convergence between the two secretory pathways.

Regulation of Plb1 Secretion

Several factors regulate the secretion of Plb1 to the fungal cell periphery and into the external milieu, including the GPI anchor [6, 50, 51], N-linked glycosylation [51] [33] and sequestration into lipid rafts [50] (for a review, see [52]. Phosphatidylinositol (PI)-specific phospholipase C (Plc1) is also involved, as Plb1 secretion is blocked in a *PLC1* deletion mutant, *Δplc1,* with Plb1 accumulating in a membrane fraction [53]. Plc1 might be involved in Plb1 secretion directly by hydrolysing the GPI anchor, or indirectly, through an alternative mechanism involving maintenance of a membrane environment conducive to the release of GPI-anchored proteins from membranes.

Role of SEC14 Homologues

Until recently, the essential cytosolic phosphatidylcholine/phosphatidylinositol transfer protein (PITP), Sec14, was thought to globally regulate protein export from the Golgi in *S. cerevisiae*. Compromised

Sec14 function leads to decreased secretion of invertase and acid phosphatase (via endosomes) and decreased export of carboxypeptidase Y to the vacuole, the lysosome equivalent of mammalian cells [54]. However, Curwin et al. [55] demonstrated that Sec14 regulates specific endosome/vacuole-dependent or endosome/vacuole-independent secretion pathways that originate within the Golgi. Specifically, they identified a role of Sec14 in a non-endosome-dependent secretion pathway involving the glycosyl hydrolase, Bgl2, but not other cell wall-associated proteins including Exg1, Scw4, Scw10, Cts1 and Hsp150. Furthermore, they demonstrated the involvement of Sec14 in retrograde protein transport from endosomes to the Golgi and from the plasma membrane to the vacuole. The fission and fusion of secretory vesicles is dependent on regulation of the composition of the lipid membrane environment of the donor and acceptor membranes. *SEC14* is one of the few proteins that integrate lipid metabolism with vesicular export by regulating PI 4-phosphate and PC levels in the Golgi [56]. Although this involves the PITP function of Sec14, the mechanism is unclear but may involve a role in lipid sensing [56].

A clue that a Sec14 homologue regulates Plb1 secretion in *C. neoformans* was the observation of differential abundance of such a homologue in a proteomics comparison of wild type and *Δplc1* (J. T. Djordjevic et al., unpublished observation). As Plb1 secretion is impeded in *Δplc1*, this finding was consistent with Sec14 (a PI donor) and Plc1 (a PI recipient) cooperating to regulate Plb1 secretion. The role of Sec14 homologues in Plb1 secretion in *C. neoformans* was therefore investigated using a series of *SEC14* deletion mutants, and a role of one of the homologues, CnSec14-1, was demonstrated to be essential for the secretion of Plb1 and virulence, but not for the secretion of laccase or soluble GXM as discussed below [4].

Similar to *SEC6, SEC14* is essential in *S. cerevisiae* [31] despite the presence of five Sec fourteen-like homologues (ScSfh1-5), and ScSec14p and ScSfh1p being 79% similar [56, 57]. The *SEC14* homologue in *C. albicans* also appears to be an essential gene [58]. A BLAST search against the cryptococcal database using the *ScSEC14/ScSFH* gene sequences identified three Cn*SEC14* homologues. Two of these homologues were more similar to each other (86% amino acid identity) than ScSec14 and Sfh1 (79% amino acid identity) and were thus named Cn*SEC14-1* and Cn*SEC14-2*. Both homologues could complement the temperature-sensitive phenotype of the Scsec14^{TS} mutant, confirming a shared functional role. The third homologue was most similar to ScSfh5. All contained the cellular retinaldehyde-binding protein and TRIO guanine exchange factor (CRAL/TRIO) structural domain characteristic of PITPs. To study the role of Cn*SEC14* homologues in Plb1 secretion, each was deleted individually and in paired combinations. All combinations except Cn*SEC14-1/CnSEC14-2* produced transformants. This apparent synthetic lethality is consistent once again with a shared functional role. Despite the higher similarity of CnSec14-2 to ScSec14 (71% compared to 59% for CnSec14-1/Scsec14), the *SEC14-2* deletion mutant (Cn*Δsec14-2*) had a similar virulence profile to the WT.

Deletion of Cn*SEC14-1* alone, or in combination with Cn*SFH5* (Cn*Δsec14-1*, Cn*Δsec14/Δsfh5),* leads to reduced secretion of Plb1 protein and activity but had no effect on either the association of laccase with the cell wall or melanization. This was consistent with unimpeded laccase transport to the cell wall in the absence of Cn*SEC14-1* and laccase export via a non-*SEC14-1*-dependent route. This is also consistent with the finding that laccase, but not Plb1 export, is *SEC6*-dependent [8]. In contrast to *iSEC6,* the two Plb1 secretion-defective mutants, Cn*Δsec14-1* and Cn*Δsec14/Δsfh5,* were mildly sensitive to calcofluor white (CFW) and resistant to Congo Red, consistent with cell wall glucan and chitin biosynthetic machinery being transported by *SEC6*- and *SEC14-1*-dependent pathways, respectively.

Cn*Δsec14-1* and Cn*Δsec14/Δsfh* were both attenuated for virulence in a mouse inhalation model, despite forming larger capsules. Interestingly, the production of enlarged capsules was also observed for the *PLB1* deletion mutant, Cn*Δplb1,* which was also attenuated for virulence, but to a greater extent than Cn*Δsec14-1* and *Cnsec14-1/Δsfh* [5]. Whether enlarged capsules in Plb1 secretion-defective mutants occur as a result of increased secretion of GXM remains to be investigated. A recent study by Chrisman et al. [59] demonstrated capsule enlargement in *C. neoformans* in response to phosphatidylcholine, the major substrate of Plb1 [33]. Interestingly, and in contrast to the finding above, they also demonstrated that capsule enlargement required the expression of *PLB1*, which is

consistent with the utilization of phosphatidylcholine by Plb1 functioning as a trigger for the export of capsular components.

All of the attenuated virulence phenotypes demonstrated for Cn*Δsec14-1* were at least partially restored to that of the WT, when intact Cn*SEC14-2* was ectopically expressed in Cn*Δsec14-1*. This is consistent, once again, with a shared functional role of Sec14-1 and Sec14-2. Phenotype restoration coincided with Cn*SEC14-2* mRNA levels increasing to a level that was almost nine times higher than in WT. The Cn*SEC14-2* mRNA levels obtained were presumably due to the contribution of expression from the ectopically expressed and native Cn*SEC14-2* gene. Cn*SEC14-2* mRNA levels were also elevated threefold when Cn*SEC14*-1 was deleted. However, this increase was not sufficient to retain a WT virulence phenotype in Cn*Δsec14-1*. Interestingly, expression of Cn*SEC14-2* was dependent on Cn*SFH5*. In the absence of Cn*SFH5,* Cn*SEC14-2* mRNA levels dropped to 35 and 24% in Cn*Δsfh5* and Cn*Δsec14-1/*Δsfh5, respectively, confirming that these low *SEC14-2* expression levels were sufficient for *C. neoformans* to be viable in the absence of both *SEC14-1* and *SFH5*. This finding raises the question as to why so much *Sec14-2* is produced in the WT, an observation that has also been made for *SEC14* in *S. cerevisiae*. The answer may be related to a putative role of Sec14 homologues in lipid sensing [56].

Polysaccharide Secretion

Capsule formation is a key pathogenic determinant in *C. neoformans* and requires export of the capsular polysaccharides GXM and galactoxylomannan (GalXM) [1, 2, 60]. *C. neoformans* is very efficient at secreting these polysaccharides to the extracellular space. Both in vitro and during infection, the concentration of extracellular GXM reaches the microgram per millilitre range [1, 61]. Secreted GXM can remain in its soluble form in the extracellular space or be used to enlarge the capsule. Soluble GXM is also liberated into the cerebrospinal fluid, where it contributes to increased intracranial pressure/hydrocephalus [1]. Furthermore, *C. neoformans* that have been phagocytosed by macrophages secrete GXM into the phagolysosome which interferes with macrophage immunological function [62]. Despite the impact of polysaccharide secretion on *C. neoformans* pathogenesis, knowledge of the mechanisms by which GXM reaches the extracellular space is still preliminary.

Conventional Mechanisms

Although polysaccharides could be considered as non-conventional secretion cargo, two pieces of evidence support the role of conventional post-Golgi secretion pathways in their externalization. One of these secretion pathways involves a homologue of Sec4p. Sec4p was initially identified in *S. cerevisiae* and characterized as a member of the Rab8 family of GTPases essential for vesicle-mediated exocytic secretion [63]. Electron microscopy revealed that, as observed for other *sec* mutants, yeast cells lacking Sec4p accumulated secretory vesicles in the cytoplasm when grown at the restrictive temperature of 37°C [63]. More than two decades later, Yoneda and Doering [19] identified a gene in *C. neoformans* which shared homology with *S. cerevisiae SEC4* and, in doing so, characterized the first cryptococcal *SEC*-related gene. They called the gene *SAV1* because it could not complement the Sc*sec4* phenotype. However, mutation of *SAV1* produced a temperature-sensitive phenotype that closely resembled that observed for the *S. cerevisiae sec4* temperature-sensitive mutant, including the accumulation of cytoplasmic vesicles [63]. These vesicles, which were not endocytic, were smaller than the Sec6p-dependent vesicles identified by Panepinto et al. [8] and contained GXM, consistent with their involvement in the traffic of capsular polysaccharide [19]. Sav1p was also found to be essential for the secretion of proteins including acid phosphatase [19]. Furthermore, *SAV1* mutation coincided with the appearance of an unusual organelle termed the sav1 body (SB) which was similar to structures observed in wild-type cells with raised luminal pH [64]. Similar to the *SAV1*-dependent vesicles, the SBs produced in the *SAV1* mutant at the restrictive temperature of 37°C colocalized with GXM, highlighting the potential role of this organelle in polysaccharide synthesis and a possible link between luminal pH and GXM biosynthesis [64]. Finally, the *SAV1* mutant exhibited a cell separation defect at restrictive temperatures and accumulated vesicles at bud necks, consistent with a role of *SAV-1*-dependent cargo in cell wall homeostasis during growth [19].

The second secretion pathway involved in the traffic of capsular polysaccharide is the *SEC6*-dependent pathway identified a few years later by Panepinto et al. [8]. As previously mentioned, this mutant also accumulated cytoplasmic vesicles and showed defective secretion of soluble GXM. However, capsule size was similar to wild type, consistent with unimpeded transport of GXM intended for capsule biosynthesis. Thus, the results described in these two independent studies, and the observation that GXM export and capsule formation were impaired in *C. neoformans* cells exposed to brefeldin A [65], are in agreement with the involvement of conventional, post-Golgi mechanisms of secretion in polysaccharide release.

Non-Conventional Mechanisms

Capsule formation was normal in yeast cells lacking either Sec4p or Sec6p [8, 19] suggesting that defects in the *SEC*-related pathway for polysaccharide release are compensated by other secretion mechanisms. Considering that elements of the post-Golgi secretory pathway altered GXM traffic, but not capsule assembly, the effect of interference of non-conventional mechanisms of secretion on polysaccharide export was investigated in *C. neoformans*.

Numerous secreted proteins lack a leader peptide and are secreted in the presence of brefeldin A [66] and, therefore, exit the cell by non-conventional secretion mechanisms [67]. Recently, Golgi reassembly and stacking proteins (GRASPs) were shown to regulate non-conventional protein secretion in a number of eukaryotes, including *S. cerevisiae* and *Pichia pastoris* [17, 68–73]. GRASPs are primarily thought to be required for Golgi stacking [73]. However, GRASP was found in Golgi membranes where stacks are not usually visible [72, 74]. In these cells, Golgi apparatus was functional for transport and glycosylation, suggesting alternative roles for this protein. In fact, GRASP-related non-conventional mechanisms of protein secretion have been documented in different organisms [68–72], but, until very recently, the role of these secretion pathways in polysaccharide traffic was unknown.

GRASP has recently been associated with polysaccharide secretion in *C. neoformans* [17]. Deletion of *GRASP* did not affect classic virulence factors such as growth at 37°C, extracellular urease activity and melanin formation. The *grasp* mutant, however, had an impaired ability to secrete GXM, produced smaller capsules and capsular polysaccharide molecules and displayed capsular structures with reduced dimensions, in comparison with wild-type and reconstituted cells [17]. These alterations coincided with an increased susceptibility of the mutant to the antimicrobial activity of macrophages and were consistent with other studies involving mutants that were defective in GXM secretion and capsule formation [1].

The *C. neoformans* mutant lacking GRASP showed attenuated virulence in an animal model of cryptococcosis [17], consistent with only partial inhibition of capsule assembly and GXM secretion. Furthermore, the results suggest that molecules in addition to GRASP play redundant roles in polysaccharide secretion and capsule assembly. This supposition is in agreement with the results obtained with *sec* mutants of *C. neoformans* [8, 19].

The mechanism(s) by which GRASP regulates polysaccharide secretion in *C. neoformans* are still unknown. Release of polysaccharide-containing exosome-like vesicles to the extracellular space is thought to be associated with GXM export and capsule growth as these vesicles contain GXM [18]. In this context, lack of GRASP could affect the export of GXM-containing vesicles to the extracellular space. However, in contrast to what has been demonstrated for a *S. cerevisiae grasp* mutant [75], *C. neoformans* cells lacking GRASP showed normal release of extracellular vesicles [76]. Thus, despite cryptococcal extracellular vesicles containing leaderless proteins and GXM, consistent with their role in a non-conventional secretion pathway, they may in fact represent a merging point for conventional and non-conventional secretion mechanisms.

Rather than playing a role in extracellular vesicle release, GRASP could be required for loading GXM into secretory vesicles. As a first step to investigating this, future experiments will determine whether GXM and GRASP associate or colocalize. Alternatively, the role played by GRASP in polysaccharide traffic could be related to its presence in Golgi cisternae, a hypothesis supported by the finding that GRASP is required for correct Golgi morphology in *C. neoformans* [17]. Although it is well accepted that GRASP is a Golgi-associated protein, it has been proposed that it could also mediate vesicle fusion at the plasma

membrane [73]. This proposal is in agreement with GRASP localization at the plasma membrane during epithelial cell remodelling in *D. melanogaster* [72]. Remarkably, GRASP-deficient *D. discoideum* cells show defects in the final stage of vesicle fusion with the plasma membrane during non-conventional secretion [77].

The demonstration that both conventional and non-conventional pathways of secretion are involved in polysaccharide traffic in *C. neoformans* suggests that both mechanisms are required for GXM export and capsule formation. As discussed above, all secretion mutants characterized in the *C. neoformans* model had altered GXM traffic but remarkably still produced a capsule [8, 17, 19]. These observations support the above-mentioned hypothesis that multiple mechanisms exist in *C. neoformans* to export GXM as well as protein. Possibly, some of these mechanisms would be required for coating the cell wall with polysaccharide; these mechanisms would differ from those required for capsule enlargement and export of extracellular GXM. The generation of *C. neoformans* mutants defective in other steps of the secretory pathway will be enormously helpful to draw more concrete conclusions in this field.

Lipid Traffic and Extracellular Vesicles

Eukaryotic lipids are classic components of the plasma membrane. In fungal cells, however, early reports suggested that lipids could associate with the cell wall [78, 79]. These initial studies were, in fact, confirmed by demonstrating the presence of glucosylceramide (GlcCer), a target of therapeutic antibodies and important regulator of *C. neoformans* pathogenicity [18, 80–84], within the different layers of the cryptococcal cell wall, in addition to the plasma membrane [84, 85]. Some of the cell wall compartments in which GlcCer was localized contained membranous structures resembling secretory vesicles [84, 86], suggesting that GlcCer participates in a vesicle-dependent trans-cell wall transport system [18] and that lipids are transient components of the fungal cell wall. In agreement with this, GlcCer is a membrane component of extracellular vesicles produced by *C. neoformans* [18]. These extracellular vesicles also contained GXM, and their production was associated with capsule enlargement [18, 86–89] demonstrating that GXM export in *C. neoformans* is closely linked to lipid traffic. Both cell wall- and capsule-associated vesicles have been documented by transmission electron microscopy [18, 86].

The nature of the organelles involved in polysaccharide synthesis and traffic is largely unknown. A number of reports suggest that GXM is, at least, partially synthesized in cytoplasmic, vesicle-like structures [19, 90–92]. Electron microscopy studies revealed that GXM is in close proximity to vesicular structures and reticular membranes [92]. Serological, chromatographic and spectroscopic analyses of extracellular vesicles revealed a similar association of GXM with membrane lipids [92]. These results are indicative of an intimate association between GXM and lipids in both intracellular and extracellular compartments and are consistent with polysaccharide synthesis and transport occurring within membrane-associated structures.

Many questions relating to the relevance of lipid traffic and extracellular vesicle release in the physiology of *C. neoformans* are yet to be answered. It has been clearly demonstrated that vesicle release to the extracellular space occurs only in viable cells [18, 93, 94]. However, it is still unknown how vesicles that contain pigment [7], protein [47, 94], polysaccharide [18, 60, 92] and lipid [18, 92, 93] traverse the cell wall and which mechanisms regulate this event. Nevertheless, the observations made so far support the hypothesis that lipid traffic across the cell wall is part of an active and efficient system of extracellular secretion.

A second and crucial question is how fungal cells regulate the release of molecules from within extracellular vesicles as part of their normal physiology and/or pathogenesis. It would be reasonable to suppose that mechanisms that promote the lysis of extracellular vesicles may exist. In this regard, many hypotheses have been raised; most of them focused on the activity of lytic extracellular enzymes [89]. These hypotheses, however, still require experimental confirmation. In this context, occurrence of non-enzymatic mechanisms of vesicle lysis cannot be ruled out. In fact, Wolf and Casadevall suggested that soluble proteins with no evident enzymatic activity may promote the destabilization of cryptococcal extracellular vesicles [95]. In such a case, this would lead to a simple and efficient physical mechanism promoting the release of intravesicular components to

the extracellular space. Such mechanisms would allow *C. neoformans* to use, for instance, vesicular GXM for capsule enlargement, in agreement with the previous evidence correlating capsule enlargement with extracellular vesicle production.

Cell Signalling and Secretion

Several stress-responsive signal transduction pathways including the cAMP/Protein kinase A (PKA) and the Ca^{2+}/calcineurin pathways have been identified in *C. neoformans* [96]. These pathways also regulate virulence phenotypes, and evidence is emerging that some have a regulatory role in secretion. Furthermore, PI3 kinase signalling, usually involved in autophagy, also appears to play a role in the transport of laccase [97].

The cAMP/PKA Pathway

The cAMP/PKA pathway, which is activated in response to nutrient stress, regulates capsule, melanin and mating [98, 99]. A *C. neoformans* mutant defective in PKA catalytic activity has a smaller capsule and is attenuated for virulence in a mouse model, while a mutant lacking PKA regulatory activity produces a larger capsule and is hypervirulent [100]. Although PKA activity influences the transcription of genes involved in the secretion and phospholipid biosynthesis [65], it does not appear to regulate the secretion of Plb1 (J. T. Djordjevic et al., unpublished observation). Thus, its role in secretion appears to be specific for capsular polysaccharide via a mechanism that remains to be elucidated.

Ca^{2+}/Calcineurin Pathway

In *C. neoformans*, a Ca^{2+}/calmodulin-dependent phosphatase, calcineurin, is essential for high temperature growth, cell wall integrity and virulence [101, 102]. Several recent observations support a role of this pathway in secretion and vesicular traffic. Firstly, the calcineurin catalytic subunit, Cna1, copurifies with Sec13 and Sec28 proteins involved in anterior and retrograde vesicular traffic, respectively, and high temperature stress causes Cna1 to colocalize with Sec13 and Sec28 in the ER and in bud necks following its relocation from the cytosol (L. Kozubowski and J. Heitman et al., unpublished observation). Calcineurin also plays a role in endocytic traffic during thermal stress via an association with one of its substrates, cts1 (L. Kozubowski and J. Heitman et al., unpublished observation). Furthermore, Plb1 secretion is blocked in calcineurin mutants (J. T. Djordjevic et al. unpublished observation).

PI3Kinase-Mediated Signalling and the Multivesicular Body

In mammalian cells, the MVB is a hub organelle essential for the initiation of multiple cellular functions. It is the site of exosome formation and the generation of autophagosomes. Furthermore, protein sorting to late endosomes/lysosomes and the recycling endosome and retrograde endosomal transport to the Golgi are all initiated within the MVB [103]. All of these processes are coordinated within the MVB by the signalling activity of a class III PI3 kinase (PI3K) which generates PI(3)P and which is encoded by the gene *VPS34*. The Williamson laboratory previously demonstrated that a cryptococcal *VPS34* deletion mutant was defective in the formation of autophagic bodies and that this coincided with severely attenuated virulence in a mouse model [97]. Furthermore, this mutant was defective in melanization, consistent with defective laccase export to the periphery mediated via the MVB. Using GFP-Lac1, studies are ongoing in this laboratory to investigate how laccase trafficking is regulated by MVBs and PI(3)K signalling.

Conclusions and Perspectives

The current literature clearly demonstrates that secretion pathways in *C. neoformans* involve multiple mechanisms. Some of these mechanisms are analogous to those described in other eukaryotic models, while others show important differences. This observation, coupled with the fact that secretion plays a crucial role in fungal virulence, highlights the possibility that secretory steps could become attractive targets for therapeutic intervention. Although significant progress has been made in this field in the last decade, much remains to be discovered. Understanding more about how *C. neoformans* exports virulence-related molecules will undoubtedly lead to the identification of the most crucial steps.

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