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The putative flippase Apt1 is required for intracellular membrane architecture and biosynthesis of polysaccharide and lipids in *Cryptococcus neoformans*

Juliana Rizzo^{a,b}, Ana C. Colombo^{c,d}, Daniel Zamith-Miranda^e, Vanessa K.A. Silva^{a,b,f}, Jeremy C. Allegood^g, Arturo Casadevall^h, Maurizio Del Poeta^{c,i,j}, Joshua D. Nosanchuk^e, James W. Kronstad^k, and Marcio L. Rodrigues^{a,b,*}

^aCentro de Desenvolvimento Tecnológico em Saúde (CDTS), Fundação Oswaldo Cruz, Rio de Janeiro, Brazil

^bInstituto de Microbiologia Paulo de Góes (IMPG), Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

^cDepartments of Biochemistry and Molecular Genetics and Microbiology, Stony Brook University, New York, USA

^dPrograma de Pós-Graduação em Química Biológica do Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

^eDepartments of Medicine and Microbiology and Immunology, Albert Einstein College of Medicine, New York, USA

^fPrograma de Pós-Graduação em Biologia Parasitária do Instituto Oswaldo Cruz (IOC), Fundação Oswaldo Cruz, Rio de Janeiro, Brazil

^gDepartment of Biochemistry and Molecular Biology, Virginia Commonwealth University School of Medicine, Richmond, USA

^hDepartment of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, USA

ⁱVeterans Administration Medical Center, Northport, USA

^jDivision of Infectious Diseases, Stony Brook University, New York, USA

^kMichael Smith Laboratories, Department of Microbiology and Immunology, Faculty of Land and Food Systems, The University of British Columbia, Vancouver, Canada.

Abstract

Flippases are responsible for the asymmetric distribution of phospholipids in biological membranes. In the encapsulated fungal pathogen *Cryptococcus neoformans*, the putative flippase Apt1 is an important regulator of polysaccharide secretion and pathogenesis in mice by unknown

*Corresponding author at: Instituto Carlos Chagas (ICC), Fundação Oswaldo Cruz (Fiocruz), Rua Professor Algacyr Munhoz Mader, 2135-2261-Cidade Industrial de Curitiba, Curitiba, PR 81310-020, Brazil. marcio.rodrigues@fiocruz.br (M.L. Rodrigues).

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mechanisms. In this study, we analyzed the role of *C. neoformans* Apt1 in intracellular membrane architecture and synthesis of polysaccharide and lipids. Analysis of wild type (WT), *apt1* (mutant) and *apt1* Δ *APT1* (complemented) strains by transmission electron microscopy revealed that deletion of *APT1* resulted in the formation of irregular vacuoles. Disorganization of vacuolar membranes in *apt1* cells was accompanied by a significant increase in the amounts of intra-vacuolar and pigment-containing vesicles. Quantitative immunogold labeling of *C. neoformans* cells with a monoclonal antibody raised to a major capsular component suggested impaired polysaccharide synthesis. *APT1* deletion also affected synthesis of phosphatidylserine, phosphatidylethanolamine, inositolphosphoryl ceramide, glucosylceramide and ergosterylglucoside. These results reveal novel functions of Apt1 and are in agreement with the notion that this putative flippase plays an important role in the physiology of *C. neoformans*.

Keywords

Cryptococcus neoformans; Flippases; Vacuole; Polysaccharide secretion; Lipid biosynthesis

Introduction

The number of human patients with disseminated fungal diseases has substantially increased over the past decades. Recent estimates indicate that there are > 1.5 million human deaths each year because of invasive mycoses [1]. Within this group of patients, *Cryptococcus neoformans* is responsible for > 180,000 deaths annually, 75% occurring in the sub-Saharan Africa [2,3]. *C. neoformans* and its sibling species *C. gatii* are encapsulated yeast cells responsible for cryptococcosis, which usually manifests as pulmonary and brain diseases in immunocompromised patients [4]. These pathogens produce a range of virulence factors that regulate survival in the host (reviewed in [5]). Cryptococcal virulence factors include extracellular enzymes (laccase, urease and phospholipase B1), lipids and the secreted polysaccharide glucuronoxylomannan (GXM), a major capsular component [6–15]. GXM is thought to be constitutively secreted into fungal cultures and host tissues [12,16].

The mechanisms controlling the export of fungal molecules to the extracellular environment have been actively studied during the last decade [17–23]. Most of the extracellular molecules in fungi are secreted through non-canonical mechanisms that are regulated at multiple levels. Regulators of non-canonical secretion in fungi include Golgi reassembly and stacking protein (Grasp) [24], sucrose non-fermenting protein 7 (Snf7) [25], and the putative aminophospholipid translocase (flippase) Apt1 [26].

Aminophospholipid translocases (APT), flippases or P4-ATPases, are integral membrane proteins associated with the translocation of aminophospholipids, such as phosphatidylserine (PS) and phosphatidylethanolamine (PE), from the outer to the inner leaflet of the lipid bilayer, promoting the asymmetric distribution of lipids in biological membranes [27]. Since this asymmetry is fundamental for both intracellular and plasma membrane architecture [28], flippases directly impact endocytic and secretory pathways [29–36]. In *C. neoformans*, deletion of *APT1*, the gene coding for a putative flippase, resulted in disturbed GXM secretion *in vitro* and *in vivo*, increased cryptococcal susceptibility to antifungal agents,

reduced fungal survival inside macrophages and attenuated fungal virulence in a murine model of infection [26,37]. Similarly, deletion of cryptococcal *CDC50* (the gene encoding a non-catalytic subunit of flippases) affected iron acquisition, GXM secretion, susceptibility to macrophages and virulence in a mice model of cryptococcosis [38,39].

In this study, we evaluated ultrastructural properties of *C. neoformans* cells lacking AptI expression and observed important changes in the intracellular membrane architecture of *aptI* mutant cells. These changes were linked to decreased polysaccharide synthesis and marked alterations in the biosynthesis of different lipid classes. These results support the notion that AptI is a key regulator of cellular physiology in *C. neoformans*.

Material and methods

Strains and cultivation

C. neoformans strains used in this work were the wild-type (WT) isolate H99, which was the background for *APT1* deletion [37], the mutant *aptI* Δ 40, and a complemented strain (*aptI* Δ 40::*APT1*–25) generated and characterized in a previous study [37]. Stock cultures of WT cells were maintained on 2% agar YPD medium (1% yeast extract, 2% peptone, 2% dextrose). The *aptI* mutant and the complemented strains were maintained in the same medium supplemented with geneticin, G418 (200 μ g/ml) and nourseothricin (100 μ g/ml), respectively.

For electron microscopy and lipid analysis, the three *C. neoformans* strains were grown in YPD liquid medium (1% yeast extract, 2% peptone, 2% dextrose) at 30 °C for 24 h under shaking, washed twice with a minimal medium (MM) composed of dextrose (15 mM), MgSC₂ (10 mM), KH₂PO₄ (29.4 mM), glycine (13 mM) and thiamine-HCl (3 μ M), and incubated for additional 48 or 72 h in MM at 30 °C, with shaking. The cells were collected by centrifugation, washed 3 times with PBS and then processed for electron microscopy and lipid extraction.

Electron microscopy

For transmission electron microscopy (TEM) cryptococcal cells were fixed in 0.1 M sodium cacodylate buffer containing 2.5% glutaraldehyde and 2% paraformaldehyde for 1 h at room temperature. The samples were post fixed with 1% osmium tetroxide followed by 2% uranyl acetate, serially dehydrated in a graded series of ethanol concentrations, and then embedded in LX112 resin (LADD Research Industries, Burlington, VT). Ultrathin sections (70–80 nm thick) were cut on a Reichert Ultracut UCT and sequentially stained with 4% uranyl acetate and 0.1% lead citrate. Samples were observed on a JEOL 1200EX transmission electron microscope at 80 kV. For quantitative analyses after 48 h growth, 24 vacuoles of 10 different cellular sections were studied in WT cells. In the *aptI* mutant, 20 vacuoles of 12 different cellular sections were studied and 11 vacuoles of 11 cellular sections in complemented cells were analyzed. Analyses performed after 72 h of growth included 28 vacuoles of 12 different cellular sections in WT cells, 15 vacuoles of 10 different sections in the *aptI* mutant, and 13 vacuoles of 11 sections in complemented cells.

For immunolabeling, fungal cells were mixed 1:1 with 0.2 M sodium cacodylate containing 4% paraformaldehyde and 1% glutaraldehyde for 30 min at room temperature. After fixation, the cells were washed three times in PBS, enrobed in gelatin, cut into cubes of 1 mm and post fixed in 0.1 M sodium cacodylate containing 2.0% paraformaldehyde overnight. Samples were infiltrated with 2.3 M sucrose followed by a PVP sucrose mixture, frozen and cryosectioned on a Leica Ultracut UCT. The cryosections were washed, blocked and then incubated overnight in the presence of the murine monoclonal antibody (mAb) 18B7 (10 µg/ ml). MAb 18B7 recognizes cryptococcal glucuronoxylomannan (GXM) of all serotypes [40]. The cells were washed with PBS and incubated for 2 h with gold-labeled (15 nm) anti-mouse IgG. Control systems included cryosections incubated with PBS instead of mAb 18B7, followed by the gold-labeled anti-mouse IgG. After the final washing series, the sections were left for 15 min on 2% uranyl acetate and transferred to 0.75% methylcellulose droplets. After 30 s, the grids were looped out, the excess viscous solution was drained away and the sections were allowed to dry. The samples were observed on a JEOL 1200EX transmission electron microscope at 80 kV. Gold particles in electron micrographs were quantified as described [17]. For quantitative analysis of GXM labeling after 48 h of cultivation, sampling consisted of 14 cellular sections of WT cells and 17 sections of the *aptI* mutant. In samples obtained after 72 h of growth, 6 cellular sections of either WT or *aptI* cells were analyzed. For normalization, the average number of gold particles counted in control systems (no mAb 18B7 labeling) was subtracted from the total number of gold particles of each mAb 18B7-labeled section.

Analysis of glycosphingolipids and sterylglucoside

Fungal cells were grown for 48 or 72 h and adjusted to 5×10^8 cells for lipid extraction according to the Mandala's method [41]. Briefly, pellets were initially suspended in 1.5 ml of ethanol: dH₂O: ether: pyridine: 14.2 M NH₄OH (15:15:5:1:0.018, v:v:v:v) and total cells were extracted by sonication for 20 s, followed by incubation at 60 °C for 15 min. This procedure was repeated once and the extracts were centrifuged and vacuum-dried. The sediments were then extracted according to the Bligh and Dyer methodology [42], through the addition of 2 ml of methanol to each tube followed by vortexing and sonication. Chloroform (1 ml) was added to each sample followed by additional vortexing. The tubes were incubated at 37 °C for 1 h and suspensions were centrifuged. Supernatants were collected and supplemented with 1 ml of chloroform and 1 ml of water. After vortexing, the samples were centrifuged again and the lower phases were collected and vacuum-dried. Samples were hydrolyzed through the addition of 0.5 ml of chloroform and 0.5 ml of 0.6 M KOH in methanol, followed by vortexing and incubation for 1 h at 25 °C. Reactions were stopped by the addition of 0.325 ml of 1 M HCl and 0.125 ml of water. After centrifugation, the lower, organic phase was collected and vacuum-dried for structural analysis by mass spectrometry. For sphingo-lipid, sterylglucoside and IPC analyses, 150 µl of 1 mM ammonium formate supplemented with 0.2% formic acid in methanol was added to each sample. Samples were separated in a Thermo Accela HPLC system (San Jose, CA) after 10 µl of each sample was injected into a Peeke Scientific Spectra C8 HPLC column (150 × 3 mm). A linear gradient was used for separation and buffers consisted of 2 mM of ammonium formate and 0.2% formic acid (buffer A) and 1 mM ammonium formate supplemented with 0.2% of formic acid in methanol (buffer B). The buffer A:buffer B ratios corresponded to

30:70 (v/v) for 5 min, 10:90 (v/v) for 9 min and finally 1:99 (v/v) for 9 min, under a flow rate of 500 $\mu\text{L}/\text{min}$. The HPLC system was attached to the HESI source of a Thermo TSQ Quantum Ultra triple quadrupole mass spectrometer (San Jose, CA). The operation settings of the mass spectrometer were positive ion mode with the high voltage 3.5 kV, auxiliary gas pressure at 15, sheath gas pressure at 60, vaporizer temperature at 400 °C and capillary temperature of 300 °C. For the collision cell, 1.5mTorr of argon was used. Each lipid transitions were checked at 50 or 100 ms upon the experiment timeline. Data were analyzed on Thermo Xcalibur 2.2 Quan Browser software and reported using Microsoft Excel files. Molecular classes detected in this analysis included sphingosines (sphingosine, sphingosine-1-phosphate, phytosphingosine, phytosphingosine-1-phosphate, dihydrosphingosine, dihydrosphingosine-1-phosphate), ceramides (C18, 08:1, C24, C24:1, C26, C26:1, C18-dihydroceramide, C18:1-dihydroceramide, C24-dihydroceramide, C24:1-dihydroceramide, C26-dihydroceramide, C26:1-dihydroceramide, dl8:2/ 08:0 h-ceramide, dl8:1/C18:0 h-ceramide, dl9:2/C18:0 h-ceramide), glucosylceramides (dl8:1/08:0 h, dl8:2/C18:0 h, dl9:2/C18:0 h, dl9:2/ C24:0 h, dl9:2/C26:0 h), phytoceramides (08, 08:1, C24, C24:1, C26, C26:1, C18-aOH, C18:1-aOH, C24-aOH, C24:1-aOH, C26-aOH, C26:1-aOH) and inositol phosphorylceramides (IPC-36:0;2, IPC-42:0;2, IPC-44:0;2, IPC-46:0;2, IPC-36:0;3, IPC-36:0;3-X, IPC-36:0;3-Y, IPC-42:0;3, IPC-44:0;3-A, IPC-44:0;3-B, IPC-46:0;3-A, IPC-46:0;3-B, IPC-36:0;4, IPC-42:0;4, IPC-44:0;4, IPC-46:0;4-A, IPC-46:0;4-B, IPC-36:0;5, IPC-42:0;5, IPC-44:0;5, IPC-46:0;5-A, IPC-46:0;5-B), and sterylglucoside. Since the general composition of *C. neoformans* sphingolipids and sterols has been recently described [43], results presented in this manuscript were normalized to inorganic phosphate content and included only those showing statistically significant differences between WT, mutant and complemented cells.

Analysis of phospholipids

Since APTs preferentially transfer PS and PE from the outer membrane leaflet to the inner leaflet [44], molecular classes of PS and PE were the targets of our investigation, which included detection of the 16:0/16:0, 16:0/16:1, 16:0/18:0, 16:0/18:1, 16:1/16:1, 16:1/18:0, 16:1/18:1, 18:0/18:0, 18:0/18:1, and 18:1/18:1 derivatives by mass spectrometry. Samples from total cell extracts were collected into 13 × 100 mm borosilicate tubes with a Teflon-lined cap (catalog #60827–153, VWR, West Chester, PA). Then 2 ml of methanol and 1 ml of chloroform were added along with the internal standard cocktail (500 pmol of each species dissolved in a final total volume of 20 μL of chloroform). The contents were dispersed using an ultra sonicator at room temperature for 30 s. This single-phase mixture was incubated at 48°C for 4 h. The extract was centrifuged using a table-top centrifuge (5000 *g* for 5 min), and the supernatant was removed by a Pasteur pipette and transferred to a new tube. The extract was vacuum-dried and the dried residue was reconstituted in 0.5 ml of the starting mobile phase solvent for LC-MS/MS analysis, sonicated for 15 s, then centrifuged for 5 min in a tabletop centrifuge before transfer of the clear supernatant to the autoinjector vial for analysis. PS and PE were separated by reverse phase LC using a Supelco 2.1 (i.d.) × 150 mm Ascends Express C18 column (Sigma, St. Louis, MO) and a binary solvent system at a flow rate of 0.4 ml/min with a column oven set to 45 °C. *prior* to injection of the sample, the column was equilibrated for 0.5 min with a solvent mixture of 55% Mobile phase A (methanol/water, 50/50, v/v/v, with 10 mM ammonium formate) a 0.5%

formic acid) and 45% Mobile phase B (isopropanol with 10 mM ammonium formate a 0.5% formic acid) and after sample injection (typically 20 μ l), the A/B ratio was maintained at 55/45 for 5 min, followed by a linear gradient to 90% B over 25 min. This was followed by a linear gradient to 100% B over 2 min which was held at 100% B for 1 min, followed by a 2-min gradient return to 55/45 A/B. The column was re-equilibrated with 55/45 A/B for 0.5 min before the next run. Each PE and PS was quantified during each run by MRM analysis, and in addition, the structure was confirmed *via* a MS2 scan within the linear ion trap during the elution of each peak. For LC-MS/MS analyses, a Shimadzu Nexera LC-30 CE binary pump system coupled to a SIL-30 AC autoinjector and DGU20A₅R degasser coupled to an AB Sciex 5500 quadrupole/linear ion trap (QTrap) (SCIEX Framingham, MA) operating in a triple quadrupole mode was used. Q1 and Q3 was set to pass molecularly distinctive precursor and product ions (or a scan across multiple *m/z* in Q1 or Q3), using N₂ to collisionally induce dissociations in Q2 (which was offset from Q1 by 30–120 eV); the ion source temperature set to 500 °C. Internal standards were 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine (17:0/17:0 PE) and 1,2-diheptadecanoyl-sn-glycero-3-phospho-L-serine (17:0/17:0 PS).

Statistical analysis

Image J software (NIH) was used to analyze TEM images. Statistical differences in grouped analysis were performed by One-way or Two-way analysis of variance (ANOVA), followed by Tukey's or, alternatively, Sidak's multiple comparisons tests. All statistical tests were performed with GraphPad Prism 6.0 software (GraphPad Software, Inc.).

Results

Lack of APT1 affected vacuolar morphology

Previous studies demonstrated that deletion of *APT1* in *C. neoformans* did not affect PS exposition and lipid translocation across the plasma membrane [26]. Instead, *apt1* cells showed an abnormal distribution of Golgi membranes and a defect in membrane trafficking during endocytosis [26,37], suggesting intracellular localization of the protein encoded by *APT1*. To determine whether organelle membranes were affected in *apt1* cells, ultrastructural aspects were analyzed by transmission electron microscopy (TEM) after cultivation of *C. neoformans* for 48 or 72 h. Compared to WT and complemented cells, the *apt1* mutant showed abnormal vacuolar morphology with irregular structural patterns and aberrant endomembrane folding after 48 h of growth (Fig. 1A-F) and formation of gigantic multivesicular body (MVB)-like compartments after 72 h (Fig. 1G-L).

The diversity in the membrane changes observed in the *apt1* mutant led us to classify these vacuoles into different morphological groups according to organelle morphology and electron density (Fig. 2). The main morphological groups in *C. neoformans* cells were generally classified into regular or irregular vacuoles. Regular compartments included ordinary vacuoles, which were either empty or contained amorphous material and/or few vesicular structures, and MVB-like structures, which manifested the typically numerous vesicles that characterize the luminal face of MVBs [10]. Irregular compartments included aberrant vacuoles, which presented distorted membrane folding, and oversized MVB-like

structures, which resembled MVBs but manifested increased dimensions. Vacuoles that did not fit into any of the above classifications were grouped as miscellaneous.

The WT strain had both regular and irregular vacuoles. Ordinary vacuoles predominated after both 48 and 72 h of growth (80 and 74%, respectively). Regular and oversized, MVB-like structures, as well as aberrant vacuoles, were observed in minor proportions after 48 h of growth. After 72 h of cultivation, the distribution of MVB-like and aberrant vacuoles slightly differed, but the oversized MVBs were no longer detected. Aberrant vacuoles (50%) predominated in the *aptl* mutant after 48 h of growth, but ordinary vacuoles (40%) were also abundantly observed. Oversized, MVB-like structures were detected at minor proportions (5%). After 72 h, ordinary vacuoles were observed in higher frequency (60%). Marked changes were observed in the frequency of detection of aberrant and oversized, MVB-like vacuoles. A 3.8-fold decrease in the frequency of detection was observed in aberrant vacuoles, while detection of the oversized compartments was increased 5-fold. Vacuole quantification in the complemented strain was more heterogeneous, but the morphological profile was similar to that observed in WT cells. These results are summarized in Fig. 2

Deletion of APT1 is associated with accumulation of intracellular vesicles

Since flippases are intimately associated with vesicular trafficking in *S. cerevisiae*, where they are essential for an efficient vesicle-mediated transportation of proteins from the trans-Golgi network to the plasma membrane [28], we sought to assess the role of flippase Apt1 in *C. neoformans* secretory trafficking. In comparison to the WT and complemented strains, *aptl* cells appeared to accumulate intracellular vesicles after both 48 and 72 h of growth (Fig. 3A). The increased number of vesicles in mutant cells was observed inside irregular vacuoles, where we counted as many as 26 vesicles within a single vacuole in 48 h and 37 intravacuolar vesicles after 72 h of growth. These measurements were also associated with an increased proportion of pigment-containing vesicles per vacuole in *aptl* cells (Fig. 3B). Together, these results suggest that lack of Apt1 profoundly affects the secretory apparatus of *C. neoformans*

Lack of APT1 is associated with reduced detection of intracellular GXM

Lipid flippases play important roles in polysaccharide synthesis and secretion in plants [45]. Our previous studies also demonstrated that *aptl* cells had defective GXM secretion during *in vitro* regular growth, in the presence of host cells and *in vivo*, which is in agreement with the observation of aberrant Golgi morphology [26]. Since GXM synthesis is Golgi-associated [19], we asked whether *APT1* deletion would impact intracellular GXM detection. Analyses of fungal sections obtained after 48 h growth and stained with mAb18B7 revealed a significant decrease in the intracellular GXM detection in *aptl* mutant cells, in comparison to WT cells (Fig. 4A). After 72 h of cultivation, however, the levels of GXM detection were similar in WT and mutant cells. Morphological analysis of the WT cells obtained in the above-mentioned conditions revealed intracellular and extracellular vesicle-associated GXM staining after 72 h of growth (Figs. 4A and B). Vesicular structures containing GXM were rarely observed in *aptl* cells (Fig. 4C).

AptI participates in lipid biosynthesis

Phospholipids in the outer membrane leaflet, preferentially PS and PE, are enzymatically transferred to the inner leaflet by APTs [44]. In this context, we asked whether deletion of *APT1* impacted phospholipid metabolism in *C. neoformans*. Mass spectrometry analysis revealed that, independently of the time of growth, the levels of PE were generally decreased in the *aptI* mutant in comparison to WT and complemented cells (Fig. 5). Although decreased PE detection was a general trend in the *aptI* mutant, statistical differences were observed only for 1-pal-mitoyl-2-palmitoleoyl-sn-glycero-3-PE (16:0/16:1) after 48 h of growth. PS analysis revealed a similar profile. The levels of this glycerophospholipid tended to be generally lower in the *aptI* mutant in comparison to WT and complemented cells. Significant differences, however, were detected only for PS containing C16 or C18 fatty acids after 72 h of cultivation.

The changes in phospholipid content and the aberrant morphological profile in the *C. neoformans aptI* mutant were indicative of a general defect in lipid metabolism. Therefore, we asked whether other key lipids would be affected by *APT1* deletion. To address this question, molecules related to the synthesis of glucosylceramide (GlcCer), inositolphosphoryl ceramides (IPCs) and sterylglucoside were chosen, since these three lipid classes are linked to cellular physiology and/or immunopathogenesis of cryptococcal infection [15,46–48]. Lipid extracts of WT, mutant and complemented cells were analyzed after cultivation of fungal cells for 48 or 72 h. Among the 60 lipids included in our analysis, some major lipid classes were changed in the flippase mutant exclusively after 48 h of cultivation. Specifically, two molecular species of IPCs (IPC-36:0;2 and IPC-36:0;4) were reduced in the *aptI* mutant after 48 h of growth in comparison to WT and complemented cells (Fig. 6A). These results are consistent with the decreased detection in the mutant of phytosphingosine, which is a building block for IPC biosynthesis [48]. Analysis of ceramides and related molecules also revealed a significant impact of *APT1* deletion. C24-ceramide was decreased in the *aptI* mutant (Fig. 6A). The virulence-associated 9-methylated cryptococcal GlcCer (dI9_2_C18_OH-Gluc-Cer) and its main precursor (dC19_2_C18-OH-Cer_9me) were also decreased in the mutant after 48 h of growth (Fig. 6A). However, detection of unmethylated, saturated GlcCer (dC18_1_C18-OH-Gluc-Cer) was increased in the *aptI* mutant after 48 and 72 h of growth (Fig. 6B). Together, these results suggest that the distorted pattern of membrane architecture observed in this strain resulted in altered GlcCer synthesis. Finally, the amounts of sterylglucoside were also increased in the mutant strain independently of the time of growth (Fig. 6B). This lipid was linked to the immunological control of animal cryptococcosis in a recent study [47].

Discussion

Lipid flippases participate in intracellular membrane organization and secretory pathways [33,45,49,50]. In *C. neoformans*, previous studies have indicated fundamental roles for putative flippases [26,37–39]; however, functional connections between the primary activity of flippases and their biological roles in fungi have remained unclear. For instance, AptI was not required for the global flippase activity of *C. neoformans* [26], thus raising the possibility that this protein is not an active enzyme. In this manuscript, we demonstrate that

APT1 deletion affects the primary functions of eukaryotic flippases in *C. neoformans*, since endomembrane architecture and lipid synthesis were profoundly affected in a cryptococcal *aptl* mutant. This observation agrees with results obtained in other models. For example, deletion of the flippase gene *DRS2* in *Saccharomyces cerevisiae* led to intracellular accumulation of abnormal membrane-bound structures [33]. In the same organism, a lack of the Neol flippase resulted in defective endocytic and secretory pathways associated with fragmented vacuoles and abnormal processes of membrane fusion [49]. In *Aspergillus nidulans*, the DnfA flippase is segregated within the Spitzenkorper, which concentrates secretory and endocytic vesicles [50]. Additionally, in *Arabidopsis thaliana*, deletion of the flippase-coding gene, *ALA3*, results in the appearance of abnormally large vacuole-like structures [45]. Altogether, these previous studies indicate an important role of lipid flippases in endomembrane organization. This hypothesis is supported by the currently observed phospholipid imbalance in the mutant, which agrees with the previous observation that this strain is hypersensitive to cinnamycin [37]. This molecule is known for this ability to induce transbilayer lipid movement leading to the exposure of PE to the outer leaflet of the plasma membrane.

Hankins and collaborators have demonstrated that the plasma membrane proteins Pmal and Can1 were missorted to vacuoles in *S. cerevisiae drs2A* cells [34], indicating that lipid flippases may play a role in sorting specific cargoes into vesicles. In our study, the morphological analysis of *aptl* cells by TEM demonstrates that lack of Aptl resulted in abnormal organization of endomembranes in *C. neoformans*. Ultra-structural differences between WT, *aptl* and complemented strains included increased amounts of irregular vacuoles and accumulation of intra-vacuolar vesicles in mutant cells. Another important phenotypic difference resulting from *APT1* deletion was the accumulation of vesicles containing pigments inside vacuoles. These results suggest that the putative flippase Aptl is involved in intracellular vesicle trafficking and presumably associated with the segregation of vesicle cargo in *C. neoformans*. Further experiments are necessary for a broader understanding of the routes of intracellular trafficking impacted by *APT1* deletion. On the basis of the multiple intracellular sites predicted for the Aptl flippase [51], it is in fact likely that multiple biological pathways might be affected in *aptl* cells.

Most glycosphingolipids are synthesized by the sequential addition of monosaccharides to GlcCer in the lumen of the Golgi apparatus. Similarly, glycosyltransferases responsible for transferring carbohydrate units to proteins or polysaccharide chains are Golgi-associated [52]. Our current and previous findings demonstrate that the lack of Aptl profoundly affects intracellular organelles, including the Golgi [26]. On the basis of the aberrant organelle morphology described in this study, we hypothesize that the mislocalization of enzymes and substrates involved in the synthesis of glycans and complex lipids is likely in the *aptl* mutant. Specifically, changes in membrane architecture can impact the interaction between lipid- and polysaccharide-synthesizing enzymes with their substrates, resulting in structurally altered biomolecules and/or imbalance of certain molecules inside the cell. Therefore, alterations in polysaccharide and lipid synthesis were anticipated in mutant. To illustrate this view, we highlight the observation of an increased detection of unmethylated, saturated GlcCer. Under regular conditions, fungal ceramides are 9-methylated and 8-unsaturated for final addition of glucose through the activity of GlcCer-synthase [48]. Mislocalization of this

enzyme could result in the preferential use of unmethylated, saturated ceramide as a substrate instead of its 9-methylated, 8-unsaturated form. Additional explanations for the altered lipid profile, however, cannot be ruled out. For instance, sterylglucoside is a component of *C. neoformans* membranes [47]. Therefore, increased detection of sterylglucoside in the *aptI* mutant may compensate for the decrease of specific GlcCer and IPC species, which was in fact observed in the present study.

Most *C. neoformans* virulence factors are extracellular including the polysaccharide GXM [7,20,53,54]. GXM secretion was suggested to require post-Golgi vesicular traffic [19,22], but it is now widely accepted that multiple pathways participate in GXM exportation [24–26,51]. Our previous results have shown that *APT1* deletion resulted in decreased extracellular vesicle-associated GXM exportation, indicating that AptI plays a role in unconventional secretion pathways [26]. It was speculated that absence of AptI could result in defective sorting of the polysaccharide inside secretory vesicles or in attenuated GXM synthesis, since *aptI* cells had altered Golgi morphology [26]. In the present work, we demonstrate a reduction in detection of intracellular GXM in *aptI* cells. This finding strengthens the supposition that an aberrant Golgi resulting from *APT1* deletion leads to defective GXM synthesis. Similarly, the flippase Ala3 has been localized to Golgi membranes in *Arabidopsis thaliana* and impacts the trans-Golgi traffic of polysaccharide-containing vesicles [45], which is in agreement with our hypothesis. Finally, although *aptI* cells are less efficient in synthesizing and secreting GXM [26], cell wall and capsule GXM are similar in WT and mutant cells [37]. This observation suggests the existence of two mechanisms of GXM traffic, one of them resulting in localization of GXM to the cell wall and a second one for extracellular export of this complex polysaccharide. The fact that capsular and extracellular GXM manifest structural differences [55] is consistent with and supportive of the existence of two mechanisms for GXM traffic.

Our results provide novel insights to the understanding of the role of fungal flippases in virulence. was required for survival during interactions with macrophages [37]. In addition, *aptI* mutants exhibited attenuated virulence in a mouse inhalation model of cryptococcosis and inefficacy in colonization of the brain [26,37]. In our study, the *aptI* mutant had decreased levels of main GlcCer-and IPC-related molecules. Previous studies demonstrated that down-regulation of IPC synthase, the enzyme responsible for IPC biosynthesis, resulted in a reduction in the pathogenicity of *C. neoformans* in a rabbit model of infection [46]. *C. neoformans* GlcCer was also characterized as a key virulence regulator of cryptococcal pathogenicity [56]. Importantly, the pathogenic role of GlcCer required unsaturation in C8 and methylation in C9 [57]. In our study, 9-methylated, 8-unsaturated GlcCer was reduced in the *aptI* mutant. On the other hand, GlcCer lacking C8 unsaturation and C9 methylation was more abundant in these cells. This unbalanced IPC and GlcCer distribution is consistent with the hypo-virulent phenotype of the *aptI* A mutant. Finally, a mutant strain lacking cryptococcal sterylglucosidase with consequent accumulation of sterylglucosides was non-pathogenic in a mouse model of cryptococcosis [47]. This mutant strain acted as a vaccine preparation that protected mice against cryptococcosis. In the current study, we observed increased levels of sterylglucoside in the *aptI* mutant. We speculate that, similar to the mutant lacking sterylglucosidase expression, accumulation of sterylglucosides can induce immune responses that might protect the host against *C.*

neoformans. Together, these results suggest that the hypovirulent phenotype of the apt1 mutant results from multiple defects related to intracellular organelles and a general effect on the synthesis of virulence-related lipids and polysaccharides.

Our current study suggests that Aptl plays fundamental roles in intracellular membrane organization, lipid distribution and GXM synthesis, which apparently impacts secretory mechanisms [26] and virulence [37]. The results herein described add relevant functions to the already known importance of fungal flippases in physiological mechanisms of *C. neoformans*. On the basis of these observations, ongoing studies in our laboratory aim to investigate the potential of Aptl as a target for the development of novel antifungals.

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Fig. 1.

Lack of Apt1 resulted in disorganization of vacuolar membranes after 48 (upper panels) or 72 h (lower panels) of cultivation of *C. neoformans*. Representative transmission electron microscopy (TEM) images of wild type (WT) cells are shown in A-B and G-H; mutant cells are illustrated in C-D and I-J; complemented cells are shown in E-F and K-L. Mutant cells showed abnormal vacuoles (pointed by arrows), suggesting defects in membrane folding and increased dimensions. Boxed areas illustrating vacuolar morphologies were magnified in images of all strains. Scale bars represent 1 μ m (panels A, C, E, G, I, K), 500 nm (panels B, D, F, H, J, L) and 250 nm (magnified fields).

Group of vacuolar morphology		Frequency of detection						
		48 h			72 h			
		WT	<i>apt1Δ</i>	<i>apt1Δ::APT1</i>	WT	<i>apt1Δ</i>	<i>apt1Δ::APT1</i>	
Regular vacuoles	Ordinary		80%	40%	64%	74%	60%	54%
	MVB-like		8%	0	0	4%	0	23%
	Aberrant		4%	50%	9%	18%	13%	8%
Irregular vacuoles	Oversized, MVB-like		8%	5%	9%	0	27%	0
	Miscellaneous		0	5%	18%	4%	0	15%

Fig. 2.

Morphological classification of vacuoles in wild type (WT), mutant (*apt1*^Δ) and complemented cells (*apt1*^Δ *::APT1*) cells. General morphological aspects of vacuoles in *C. neoformans* were analyzed after 48 and 72 h of growth and classified into four major different groups. Frequency of detection of each group in the three strains is described. Scale bars, 250 nm. Vacuoles that did not fit into any of the four major groups were classified as miscellaneous.

Fig. 3.

Deletion of *APT1* resulted in accumulation of intravacuolar vesicles in *C. neoformans*. A. Analysis by TEM of WT, *apt1* Δ and *apt1* Δ ::*APT1* cells cultivated for 48 or 72 h revealed numerous vesicle-containing vacuoles. Vesicular structures are highlighted with asterisks. Quantification of the structures illustrated by TEM demonstrated that mutant irregular vacuoles contained increased amounts of luminal vesicles. Error bars represent standard deviations. Statistical significance was tested by two-way ANOVA. Scale bars, 500 nm. Boxed areas were magnified in B, which shows TEM analysis of pigment-containing vesicles in WT, *apt1* Δ and *apt1* Δ ::*APT1* cells cultivated for 48 or 72 h. Pigment-containing

vesicles are highlighted with arrows. Quantification of the structures illustrated by TEM indicated and increased amount of pigment-containing vesicles in the *apt1A* mutant in both time points. Error bars represent standard deviations. Statistical analysis was performed with one-way ANOVA. Scale bars, 100 nm.

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Fig. 4.

Deletion of *APT1* resulted in the reduced detection of intracellular GXM in *C. neoformans*. A. Representative TEM images and corresponding quantitative analysis of immunogold labeling of WT and *apt1* cells, cultivated for 48 h and 72 h, with a monoclonal antibody to GXM (mAb 18B7). A reduced amount of intracellular GXM was detected in the mutant strain compared to WT cells after cultivation for 48 h. Similar levels of gold particles were observed in both strains after 72 h of growth. B. Illustrative images showing the association of GXM with vesicular structures in WT cells, which were virtually absent in the mutant strain (C). Right panels in B and C represent magnifications of the boxed areas in left panels.

Arrows indicate gold particle detection. CW: Cell wall, Cap: Capsule, ves: vesicle. Scale bars correspond to 500 and 100 nm in magnified panels. In panel A, error bars represent standard deviations. Statistical significance was evaluated with the two-way ANOVA test.

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Fig. 5.

Quantitative analysis of PE and PS in wild type (WT), mutant (*apt1*Δ) and complemented (*apt1*Δ::*APT1*) *C. neoformans* cells after 48 or 72 h of cultivation. A general trend of decreased detection of PE and PS in mutant cells was observed independently on the time of growth. Panels showing statistically significant ($P < 0.05$) reduction of phospholipid detection in mutant cells are marked with an asterisk. Statistical analysis was performed with one-way ANOVA.

Fig. 6.

Quantitative analysis of pathogenesis-related lipids in wild type (WT), mutant (*apt1*) and complemented (*apt1Δ::APT1*) cells after 48 or 72 h of cultivation of *C. neoformans*. Sixty molecular species were analyzed; results are shown for systems showing statistically significant alterations ($P < 0.05$) of lipid detection in mutant cells. A. Reduced lipid detection (phytosphingosine, ceramides, IPCs and GlcCer) in mutant cells. All differences were observed after 48 h of cultivation of *C. neoformans*. B. Increased lipid detection (sterylglucoside and C9-unmethylated, C8 saturated GlcCer) in mutant cells after 48 or 72 h of cultivation. Statistical significance was performed with one-way ANOVA.