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Characterization of inositol phospho-sphingolipid-phospholipase C 1 (Isc1) in *Cryptococcus neoformans* reveals unique biochemical features

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Abstract

In this work, we biochemically characterized inositol phosphosphingolipid-phospholipase C (Isc1) from the pathogenic fungus *Cryptococcus neoformans*. Unlike Isc1 from other fungi and parasites which hydrolyze both fungal complex sphingolipids (IPC-PLC) and mammalian sphingomyelin (SM-PLC), *C.neoformans* Isc1 only exerts IPC-PLC activity. Genetic mutations thought to regulate substrate recognition in other Isc1 proteins do not restore SM-PLC activity of the cryptococcal enzyme. *C.neoformans* Isc1 regulates the level of complex sphingolipids and certain species of phytoceramide, especially when fungal cells are exposed to acidic stress. Since growth in acidic environments is required for *C.neoformans* to cause disease, this study has important implications for understanding of *C.neoformans* pathogenicity.

Keywords

Cryptococcus neoformans; phospholipase C; inositol sphingolipids; sphingomyelin; phytoceramide; plasma membrane ATPase

Introduction

In the past several years, it has become increasingly clear that sphingolipid metabolism is intimately linked with the virulence of the fungal pathogen *Cryptococcus neoformans* (*Cn*), a medically important microorganism [1]. *Cn* is a facultative intracellular pathogen and it can grow and replicate within the phagolysosome of phagocytic cells, such as alveolar

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macrophages (AMs), as well as in the extracellular spaces, such as in the alveoli or in the bloodstream [2-6].

Our laboratory has previously showed that cryptococcal sphingolipids regulates signaling events leading to the production of virulence factors [7,8] and to the regulation of phagocytosis [9,10]. Interestingly, sphingolipid not only regulate the internalization of *Cn* by macrophages but also promote fungal growth in these environments (intracellular and extracellular). Intracellular fungal growth is controlled by Ipc1 [11] and inositol phosphosphingolipid phospholipase C 1 (Isc1) [12] whereas extracellular growth is controlled by glucosylceramide synthase (Gcs1) [13,14]. The regulation of these processes significantly affects the interaction of *Cn* with macrophages, particularly in the lung environment with an important effect on the outcome of the disease.

In *Saccharomyces cerevisiae* (*Sc*) the enzyme which hydrolyzes complex sphingolipids, such as inositol phosphorylceramide (IPC), generating phytoceramide and inositol phosphate was identified as Isc1 by Sawai et al. [15]. *In vitro, Sc* Isc1 metabolizes not only IPC and its mannosylated derivatives mannosylinositol phosphorylceramide (MIPC) and mannosyldiinositol phosphorylceramide ($M(IP)_2C$), thus exerting IPC-PLC activity, but it also metabolizes the mammalian complex sphingolipid sphingomyelin (SM), thus exerting SM-PLC activity, in spite of the fact that fungal cells do not contain SM [15].

In the parasite *Leishmania major*, the Isc1 homolog (*ISCL*) does possess both IPC-PLC and SM-PLC activity, even though the parasite does not produce SM and, the breakdown of host SM is an essential process for *L. major* to cause disease [16].

In *Cn*, deletion of Isc1 generated a *Cn* mutant (*Δisc1*) that is hypersensitive *in vitro* to nitrogen oxide, hydrogen peroxide and acidic stresses and, thus, it cannot survive within the phagolysosome of macrophages. In this study, we examined the biochemical characteristics of the *Cn* Isc1 enzyme and analyzed the biochemical features of the *Cn* strain lacking Isc1 compared to the wild type (WT) and a reconstituted strain ($\Delta isc1^{REC}$) in acidic and neutral conditions.

Materials and Methods

Strains and growth media

Cn var. *grubii* serotype A strain H99 wild type (WT); *Cn* var. *neoformans* serotype D strain JEC21; *Cn* var. *gattii* serotype B (strain MMRL 1336) and serotype C (strain MMRL 1343) [the latter two strains were a kind gift from Wiley Schell, Duke University Medical Center, Durham, NC U.S.A.]; *Candida albicans* (*Ca*) wild type strain A39; *Schizosaccharomyces pombe* strains 972 (h-) and 975 (h+); *Saccharomyces cerevisiae* wild type strain (Jk9-3Dα); *Sc* Δ*isc1* strain [15]; *Cn* Isc1 deletion strain (*Cn* Δ*isc1*) and the reconstituted strain ($\Delta isc1^{REC}$) were used in this study. For in vitro growing condition, please see supplementary materials.

In vitro IPC and sphingomyelin SM phospholipase C (IPC-PLC and SM-PLC) activity

Phospholipase C activity against IPC (IPC-PLC) was performed as previously described [15]. Briefly, cell lysates containing 100 μg protein were incubated at 30°C for 30 min in 100 μl of buffer containing 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.1% Triton X-100, 10 nmoles of phosphatidylserine (Avanti Polar Lipids), 2 nmoles of unlabeled IPC, and 30,000 dpm of *myo*-[2-^{3}H]inositol –labeled IPC. After the incubation, 0.8 ml of chloroform, 0.4 ml of methanol, and 0.2 ml of 1% perchloric acid were added [17], and the radioactivity in a portion (300 μl) of the upper (aqueous) phase was measured by liquid scintillation counting. Phospholipase C activity against SM (SM-PLC) was tested in a

similar manner, except that 2 nmoles SM and 30,000 dpm of [choline-methyl-^{14}C]-labeled SM were used in place of unlabeled and labeled IPC, respectively. After incubation, 0.8 ml of chloroform, 0.4 ml of methanol, and 0.2 ml of distilled water were added and the radioactivity in a portion (300 μl) of the upper phase was measured by liquid scintillation counting. As a negative control, the *Sc Δisc1*+pYES empty strain was assayed for Isc1 activity and its value subtracted from values obtained with Isc1 wild-type or mutated forms. Experiments were repeated at least three times. Radiolabeled IPC was prepared by incubating WT cultures with [2-^{3}H] inositol for 4 hours and extracting sphingolipids as previously described. Non-radioactive labeled C26-IPC was custom-made by Avanti Polar Lipids, Alabaster, AL, U.S.A.

Mutagenesis Studies

The *Cn* Isc1 cDNA from wild-type of mutated forms were subcloned from pCR-TOPO-*Cn*-Isc1 [12] into the pYES vector (Invitrogen) and the Xpress tag was inserted at the 5′ of the *Cn* Isc1 gene to monitor protein expression in *Sc Δisc1* cells. Mutagenesis studies were performed as described in the supplementary materials and the primers used are illustrated in the Supplementary Table 1. For molecular docking studies, coordinates for phosphocholine were generated and docked manually into the *Bc Isc1* structure using the program O version 11 {Jones, 1991 #2404}.

In vivo labeling with ^{3}H-inositol or ^{3}H dihydrosphingosine

For quantitative measurements of IPC-B, IPC-C/D, MIPC and M(IP)2C in *Cn* wild-type, *Δisc1* and *Δisc1*^{REC} strain, cells were incubated for 30 minutes with 25 μCi of myo-[2-^{3}H] inositol (20 Ci/mmol), and the lipid were extracted and loaded onto a TLC on silica gel 60 plates (EM Science) using the solvent system chloroform/methanol/water (65:25:4). The separated radioactive lipids were scraped from the TLC and quantified using a liquid scintillation counter [15]. The mass of each species was normalized to phosphorus levels of each sample. Further details are illustrated in the Supplementay Materials.

Extraction and mass spectrometry analysis of yeast sphingolipids

Lipids for mass spectrometry analysis were extracted by standard methods (please see Supplementary Materials). An aliquot of the extraction (300 μl) was used for phosphorous determination. Internal standards were added to the remaining aliquots, and sphingolipids were extracted in a one-phase neutral organic solvent (propan-2-ol/water/ethyl acetate, 30:10:60, by vol.) Samples were then analyzed by a Surveyor/TSQ 7000 liquid chromatography–MS system.

Statistical analysis

The two-way analysis of variance (ANOVA) was used. For all statistical tests, *P*-values less than 0.05 were considered significant

Results and Discussion

Cn lacks sphingomyelinase activity

Our first interest was to investigate whether *Cn* cell protein lysates would hydrolyze the mammalian complex sphingolipid sphingomyelin (SM), as was observed for the *Sc* protein lysates. Unexpectedly, we found that *Cn* cannot metabolize SM (Figure 2), suggesting that the cryptococcal Isc1 does not have SM-PLC activity. In addition, we did not detect any SM-PLC activity in other serotypes (B, C and D) of *Cn*, nor in another pathogenic yeast, such as *Candida albicans* (*Ca*). As expected, we did detect SM-PLC activity in *Sc* and in *Saccharomyces pombe* protein lysates. We confirmed these results by *in vivo* labeling with

NBD-labeled C6-SM, and found that whereas *Sc* could generate NBD-labeled ceramide, *Cn* could not (data not shown). These results suggest that the cryptococcal Isc1 enzyme may possess different biochemical properties than those exerted by *Sc*.

Because we observed that *Cn* protein extracts do not possess SM-PLC activity (Figure 2), we wondered whether a cryptococcal factor(s) may inhibit such activity. To address this hypothesis, different concentrations of protein lysates isolated from *Cn Δisc1* mutant were mixed with protein lysates isolated from *Sc Δisc1* + pYES *Sc* Isc1, and IPC-PLC and SM-PLC activities were measured. We found that presence of *Cn* proteins did not inhibit SM-PLC or IPC-PLC activities of *Sc* Isc1 (Figure 3A). Next, we wondered whether a factor(s) from *Sc* would be necessary for SM-PLC activity. Thus, the *Cn* Isc1 cDNA was expressed into the *Sc Δisc1* strain and both IPC- and SM-PLC activity were measured. *Sc* Isc1 cloned into pYES was used as a positive control, whereas the pYES empty plasmid was used as a negative control. As additional controls for Isc1 protein expression, we also included the *Sc* wild-type JK-3Dα transformed with pYES empty vector. We found that IPC-PLC activity is promptly restored in the *Sc Δisc1* strain when over-expressing either *Sc* Isc1 or *Cn* Isc1 (Figure 3B). However, only the expression of *Sc* Isc1 and not *Cn* Isc1 restores SM-PLC activity in the *Sc Δisc1* strain (Figure 3C). These results suggest that *Cn* Isc1 is biochemically different from *Sc* Isc1 in terms of substrate specificity.

Functional analysis of Cn Isc1 by site-directed mutagenesis of the P-loop-like domain

Studies on the *Sc* Isc1 [18,19] and *Bacillus cereus* SMase [20] have identified distinct domains within the enzyme which are essential for substrate binding and hydrolysis. One such domain, the P-loop-like domain, shows homology among the Isc1 superfamily, including bacterial and human SMases. The *Cn* Isc1 P-loop domain is contained between G113 and A120 and is illustrated in Figure 4A. Okamoto *et al*. have shown that substituting the second glycine of the P-loop with an alanine decreased *Sc* Isc1 activity against SM nearly 100-fold, though activity against IPC was not assayed [19]. Of interest, *Cn* has a lysine instead of a glycine at this position and additional differences between the two Isc1 homolog exist (Figure 4A). Thus, we wondered whether the differences in amino acid residues found in the *Cn* Isc1 are responsible for the lack of SM-PLC activity. We expressed *Cn* Isc1 in *S.cerevisiae Δisc1* and assessed the role of the P-loop residues on both SM-PLC and IPC-PLC activities. The inset in Figure 4B is representative Western blot of the expressed proteins showing that the level of expression was found to be comparable. We found that D114A and K119A mutations lost IPC-PLC activity completely Figure 4B, suggesting that these two amino acids are essential for catalysis of *Cn* Isc1. In contrast to *Sc*, neither of the remaining mutated forms lost IPC-PLC activity nor they did restore SM-PLC activity, even when the *Cn* Isc1 P-loop was identical to the *Sc* Isc1 P-loop (Figure 4B) [19]. These results suggest that either the P-loop is not involved in the substrate recognition or that it is not sufficient to discriminate between IPC and SM.

We therefore performed docking studies of *Cn* Isc1 protein in the attempt to identify key residues that would interact with IPC and not with SM based on the available structure of *B. cereus* SMase [21] and found none. Amino acids predicted to interact with the choline phoshate of SM, such as D195, N197, W232, D295, W284 and F285, are either conserved in *Cn* Isc1 or absent in other eukaryotic SMase. Thus, which amino acid(s) are most likely to be responsible for substrate (IPC or SM) recognition or exclusion by *Cn* Isc1 is still unknown.

The observation that *Cn* Isc1 is not able to breakdown host SM as other microbes do is intriguing. It is possible that, since SM is an essential sphingolipid for mammalian cell viability [22,23], opportunistic pathogenic yeasts, such as *Cn*, may have lost SM-PLC activity to prevent mammalian cell death due to their close interaction with their host. this

hypothesis is supported by the observation that *Ca*, an opportunistic and commensal fungal pathogen, also does not diplay SM-PLC activity. In non-opportunistic pathogens, such as *L. major*, the ability to breakdown host SM is maintained by their Isc1 homolog *ISCL* and, importantly, the authors elegantly showed that this activity is required for virulence [16].

Isc1 regulates the level of complex sphingolipids and specific phytoceramide species

Since Isc1 generates phytoceramide during the hydrolysis of complex sphingolipids, we next investigated how the loss of Isc1 modulated the level of complex sphingolipids and phytoceramide. Complex sphingolipids were measured by pulse chase labeling using [^{3}H]-inositol because mass spectrometry analysis could not be performed due the lack of IPC, MIPC and M(IP)2C standards. *Cn* Δ*isc1* cells showed a significantly increased of IPC C/D and M(IP)2C compared to *Cn* WT or Δ*isc1*^{REC} strains when cells were grown at either pH 4.0 (Figure 5) or 7.0 (data not shown). Similar results were obtained when cultures were labeled with tritiated dihydrosphingosine.

Interestingly, deletion of Isc1 causes the accumulation of two complex sphingolipids more polar than M(IP)2C. In other fungi, such as *Ca*, a new inositol containing sphingolipid, dimmannose inositol phosphorylceramide (M2IPC), has been identified {Trinel, 2002 #1577}. In contrast to IPC, MIPC and M(IP)2C that are anchored to the plasma membrane, M2IPC can “diffuse” from the plasma membrane to the cell wall. In the dimorphic fungus *Histoplasma capsulatum*, in addition to M2IPC another sphingolipid has been identified as galactosyldimmannose inositolphosphorylceramide (GalM2IPC) which exists in two forms depending on the addition of a galactofuranose residue at the 6-position of mannose (yeast form specific) or the addition of galactopyranose residue at the 4-position of mannose (hyphal form specific) {Barr, 1984 #1578; Barr, 1984 #1579}. Although not described thus far, most likely these new complex sphingolipid species are also present in *C. neoformans*, and, since they are more polar than M(IP)2C, they would run below M(IP)2C on a TLC plate. Presumably, M2IPC and GalM2IPC are breakdown by Isc1 and, thus, they would accumulate in condition in which Isc1 is deleted. Further studies are clearly needed to investigate such hypotheses.

When we measured the level of phytoceramide using mass spectrometry, we found that, except at 12 hours of growth, at pH 7.0 the WT or Δ*isc1*^{REC} strain contained approximately the same level of total phytoceramide than Δ*isc1* mutant (Figure 6A) but not at pH 4.0, in which the level of phytoceramide at 12 and 24 hours of growth is significantly lower (Figure 6B). Interestingly, the Δ*isc1* strain grown at pH 7.0 recovered its total phytoceramide pools very quickly and by 24 hours its level is similar to the one measured in the WT or Δ*isc1*^{REC} strain. In contrast, the Isc1 mutant grown at pH 4.0 does recover its phytoceramide pool to a level similar to the WT strains at 48 hours of growth.

An advantage of mass spectrometry analysis is the ability to quantify the different species of lipids. Using this method, we found that the level of C26 phytoceramide in WT or Δ*isc1*^{REC} strain is significantly higher when the strains are grown at low compared to neutral pH (Figure 6C and 6D). Importantly, the Δ*isc1* strain displayed a significantly lower level of C26 phytoceramide compared to WT and Δ*isc1*^{REC} strains, especially at low pH (Figure 5D). This suggests that Isc1 may directly regulate the level of C26 phytoceramide in *Cn*. The lack of C26-phytoceramide in the Δ*isc1* mutant is intriguing, as it suggests a possible involvement of the plasma membrane ATPase (Pma1), a proton-extruding plasma membrane pump, in the regulation of *Cn* growth when cells are exposed to low pH [24], which has also been proposed in *Sc* [25-27].

Although the involvement of sphingolipids in the fungal stress response appears to be conserved, our study adds a new dimension because *Cn* is a facultative intracellular

pathogen. Clinically treating cryptococcal infections is often hampered by the persistence of fungal cells in infected organs. Similar to *Mycobacterium tuberculosis*, cryptococcal persistence may be a result of its intracellular ability to survive within macrophages [3]. This hypothesis is supported by studies suggesting that *Cn* may lay dormant in the lungs of individuals many years before disease occurs [28,29], and by studies in *M. tuberculosis* that, peculiarly, accumulates C26 triacylglycerides during hypoxia, an *in vitro* condition that mimics the *in vivo* dormancy prior reactivation [30]. Thus, through the production of C26-phytoceramide, *Cn* may be protected against acidic stresses so that it can survive within the hostile phagolysosome of macrophages.

In conclusion, we show that *Cn* Isc1 is biochemically different than *Sc* Isc1 and it synthesizes mainly C26 phytoceramide especially in acidic environments. Since the intracellular niche of host cells in which *Cn* grows is characteristically acidic, these results may have important implications for the interaction of *Cn* with the host.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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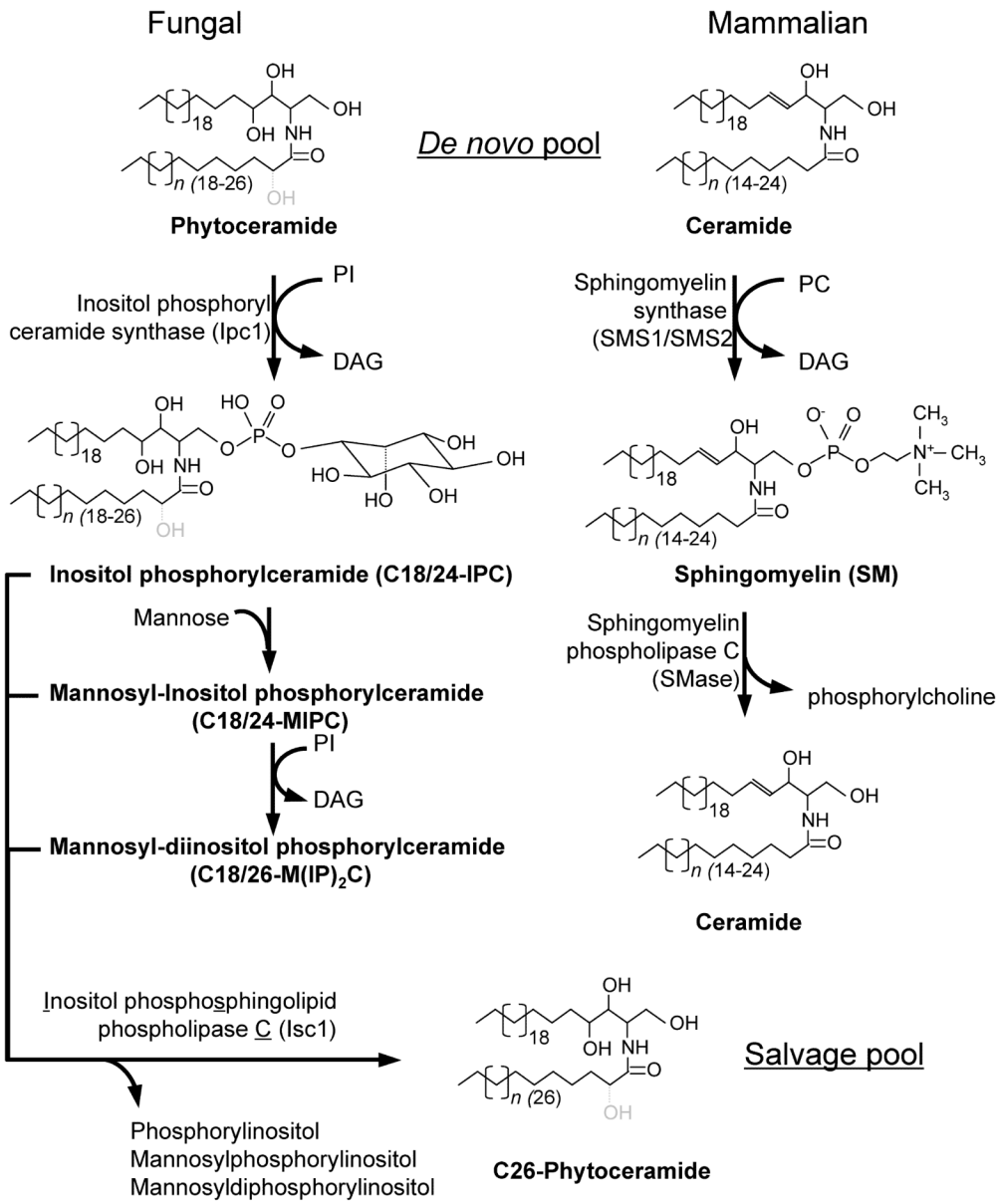

Figure 1. Ipc1 and Isc1 biosynthetic pathway

Ipc1 consumes phytoceramide and generates diacylglycerol (DAG) in the synthesis of IPC, which is subsequently transformed into MIPC and $M(IP)_2C$. Isc1 generates phytoceramide in the hydrolysis of IPC, MIPC or $M(IP)_2C$. The mammalian homologs, sphingomyelin synthase (SMS1 and SMS2) and sphingomyelinase (SMases), which consume and generate ceramide, respectively, are shown for comparison. In fungal cells, the fatty acid in the phytoceramide species can be hydroxylated (in gray) in position 2 (dotted line). Bracket's numbers represent the length of the sphingosine backbone and fatty acids. PI, phosphatidylinositol; DAG, diacylglycerol; PC, phosphatidylcholine.

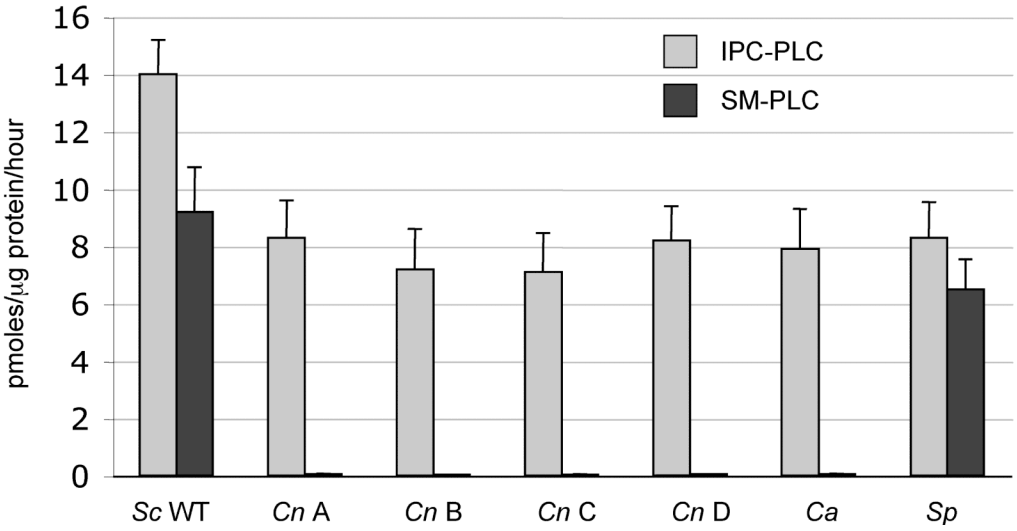

Figure 2. *neoformans (Cn)* and *C. albicans (Ca)* lack SM-PLC Activity

Crude cell lysates from fungi were assessed for neutral phospholipase C (PLC) activity against either inositol phosphorylceramide (IPC) or sphingomyelin (SM). The fungi tested included *Sc* (*Sc*), *Cn* var. *grubii* serotype A strain H99 (*Cn A*), *Cn* var. *gattii* serotype B (*Cn* B), *Cn* var. *gattii* serotype C (*Cn* C), *Cn* var. *neoformans* serotype D (*Cn D*), *Ca*, and *S. pombe* (*Sp*).

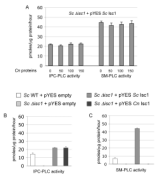

Figure 3. Expression of *C. neoformans (Cn)* Isc1 in *S. cerevisiae (Sc) Δisc1* does not restore SM-PLC activity

A) IPC-PLC and SM-PLC activity using protein lysates obtained from *ScΔisc1* + pYES *Sc* Isc1 mixed with 50, 100 or 150 μg of *Cn* protein lysates. B) IPC-PLC and C) SM-PLC activity of *Sc* wild-type (WT) + pYES empty, *ScΔisc1* + pYES empty, *ScΔisc1* + pYES *Sc* Isc1, and *ScΔisc1* + pYES *Cn* (*Cn*) Isc1. Data are average ± standard deviation of three separate experiments.

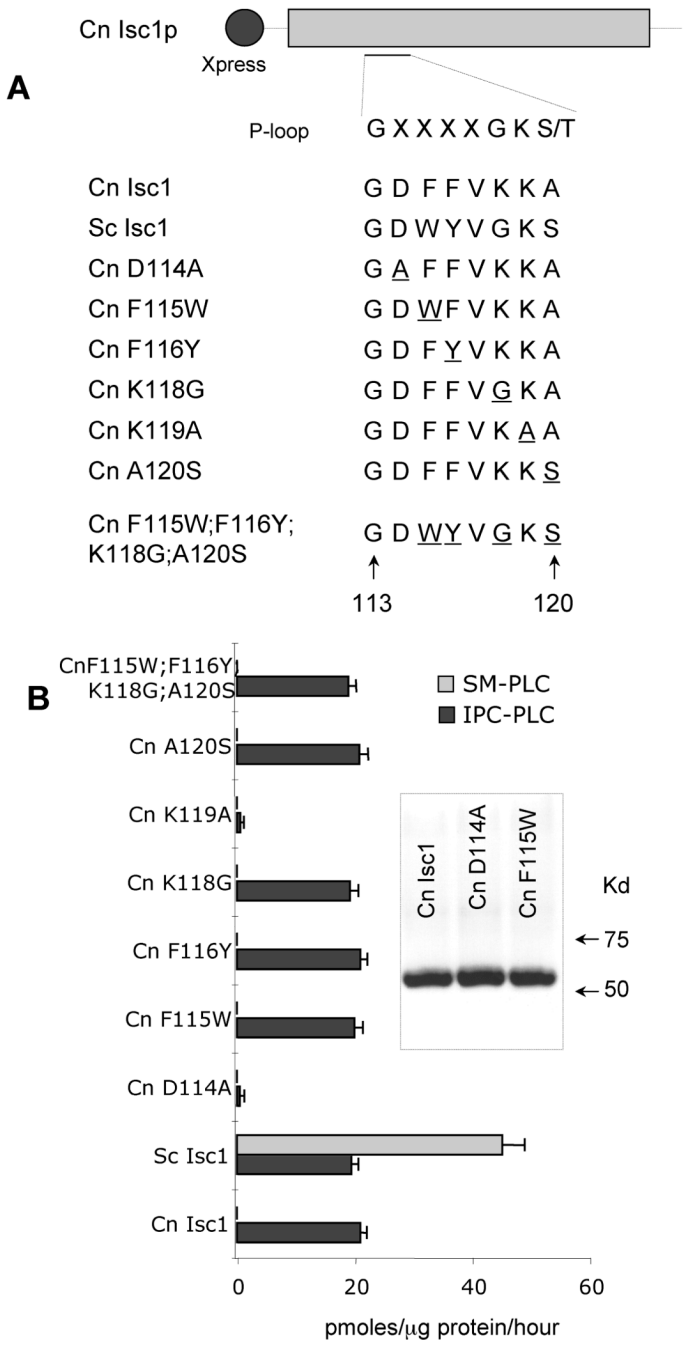

Figure 4. Effect of mutations of the *C. neoformans* Isc1 P-loop domain on IPC-PLC and SM-PLC activity

(A) Alignment of amino acid sequences of P-loop-like wild-type domain and mutant constructs. Amino acids sequences are given for residues 113-120 of *Cn* Isc1. The residue(s) mutated from the wild-type is underlined. **(B)** Effect of mutations in the P-loop on Isc1 activity. Isc1 activity was measured using SM (SM-PLC) or IPC (IPC-PLC) as a substrate, as described. Inset in B shows a representative Western blot of P-loop-like domain mutants of *Cn* Isc1 with anti-Xpress antibody. Shown are two different clones (*Cn* D114A) and *Cn* F115W) of each over expressed cells compared to *Cn* Isc1 wild-type.

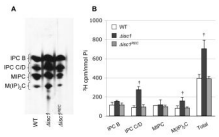

Figure 5. *In vivo* labeling of WT, Δ*isc1*, and Δ*isc1*^{REC} strains of *C. neoformans*

Cultures were labeled with ^{3}H-inositol for thirty minutes, and lipids were extracted and resolved in a TLC (**A**) as described in "Materials and Methods." Panel A is a representative of three separate experiments. **B**) Quantitative analysis of scraped lipids from the TLC normalized to phosphorus (Pi) content. † $P < 0.05$, Δ*isc1* versus WT or Δ*isc1*^{REC}. Data are average ± standard deviation of three separate experiments.

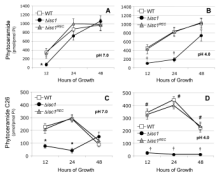

Figure 6. Isc1 regulates phytoceramide levels in *C. neoformans*

Mass spectrometry quantification of total phytoceramide pools (**A** and **B**) and very long chain (C26) phytoceramide (**C** and **D**) of WT, Δ*isc1*, and Δ*isc1*^{REC} grown at pH 7.0 (**A** and **C**) or pH 4.0 (**B** and **D**). * $P < 0.05$, Δ*isc1* versus WT or Δ*isc1*^{REC} at pH 7; † $P < 0.05$, Δ*isc1* versus WT or Δ*isc1*^{REC} at pH 4; # $P < 0.05$, WT at pH 4.0 versus pH 7.0. Data are average ± standard deviation of three separate experiments.