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

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## Supplementary Materials

### *In vitro* growing conditions

The construction of the *C. neoformans* Δ*isc1* and Δ*isc1*$^{REC}$ strains are discussed elsewhere [1]. All strains were routinely grown on yeast extract peptone dextrose (YPD) medium from Difco buffered to pH 7.0 with 25 mM HEPES (from Sigma), unless otherwise noted. Synthetic minimum medium with either 2% glucose or 2% galactose and Ura dropout supplement was purchased from Clontech.

From overnight YPD broth cultures of *C. neoformans* WT, Δ*isc1*, and Δ*isc1*$^{REC}$ strains, cells were washed twice in sterile deionized water, resuspended, and diluted to a final density of $10^4$ cells/mL into 40 mL fresh YPD broth with 25 mM HEPES at a pH of 7.0 or 4.0. Aliquots of media were sampled during growth to ensure the media pH was stable throughout the experiments. The cultures were incubated at 250 rpm in a shaker incubator at 30°C. Cells were taken from the cultures after 24 hours of growth (Log phase), washed in phosphate buffered saline (PBS), counted and used for the experiments illustrated below.

For IPC and sphingomyelin SM phospholipase C (IPC-PLC and SM-PLC) activity, *C. neoformans* strains were grown as in 10 mL YPD for 24 hours at 30°C at 250 rpm, whereas *S. cerevisiae* strains were grown in 10 mL minimum Ura drop-out medium containing 2% galactose for 24 hours at 250 rpm. Cells were harvested by centrifugation, washed with PBS, and the pellets were resuspended in lysis buffer [25 mM Tris pH 7.4, 5 mM EDTA, 1 mM phenylmethylsulphonylfluoride (PMSF, Sigma), and 10 μg/mL each Chymostatin (Sigma), Leupeptin (Sigma), Antipain (Sigma), and Pepstatin A (Sigma)]. Then, acid-washed glass beads (for a volume equal to 3/4 of the cell suspension) were added, and cells were homogenized three

times for 45 sec using the Mini-BeadBeader-8 (Bio-Spec). After centrifugation at 2500 x *g* for 10 min at 4°C, supernatant (~100 µL) was transferred to a sterile 1.5-mL microcentrifuge tube for protein quantification by the Bradford method [2]. These protein lysates were used to assess IPC-PLC and SM-PLC activities.

**Mutagenesis**

The *C. neoformans* Isc1 cDNA from H99 strain was subcloned from pCR-TOPO-*C. neoformans*-Isc1 [1] into the pYES vector (Invitrogen) and the Xpress tag was inserted at the 5' of the *C. neoformans* Isc1 gene to monitor protein expression in *S. cerevisiae Δisc1* cells. The resulting plasmid was called pYES *C. neoformans*-Isc1. Similarly, the *S. cerevisiae* Isc1 gene was subcloned from the pYES2/FLAG-ISC1 [3] into a pYES vector in which the Xpress tag was placed at the 5' of the *S. cerevisiae* Isc1 gene. This plasmid was named pYES *S. cerevisiae*-Isc1. Point mutations were introduced into the pYES *C. neoformans*-Isc1 using the Gene-Tailor site-directed mutagenesis kit (Invitrogen). The nucleotides used for mutagenesis are listed in Table 1. All constructs were subsequently sequenced to verify that the desired mutations had been introduced into the sequence. All of the constructs were individually introduced into the *S. cerevisiae Δisc1* by lithium acetate transformation and gene expression was induced by incubating cells in synthetic medium lacking uracil plus 2% galactose. Western blot analysis using anti-Xpress antibody (Invitrogen) was performed in all protein lysates to assess the level of expression of Isc1 WT and mutated proteins.

***In vivo* labeling**

Radiolabeled cryptococcal complex inositol sphingolipids were extracted and purified from 5 x $10^7$ cells incubated for 30 minutes with 25 μCi of myo-[2-$^3$H] inositol (20 Ci/mmol) (American Radiolabeled Chemicals, St. Louis, MO) or 5 μCi of $^3$H dihydrosphingosine (Dr. Alicja Bielawska, Synthetic Lipidomic Core, Medical University of South Carolina, Charleston, SC) using a solvent containing ethanol, water, diethyl ether, pyridine, and concentrated ammonia (15:15:5:1:0.018) [4]. Extracted lipids were base-hydrolyzed with monomethylamine reagent, dried down, dissolved in chloroform/methanol/water (2:2:0.6), and separated by TLC with a solvent containing chloroform, methanol, and 4.2 N ammonium hydroxide (9:7:2). *S. cerevisiae* MIPC synthase deletion mutant (*SUR1*) [5] and WT JK9-3dα were used for the preparation of an IPC standard.

**Extraction and mass spectrometry analysis of yeast sphingolipids**

*C. neoformans* strains were grown in YPD pH 7.0 for 24 hours, washed twice with PBS, and were inoculated into fresh YPD pH 7.0 or pH 4.0 media. At appropriate time points, cells were collected by centrifugation at 3000 rpm for 10 minutes, washed twice with PBS, and the pellets were stored at -80°C. Neutral lipids were extracted by the method described by Bligh and Dyer [6]. An aliquot of the extraction (300 μl) was used for phosphorous determination [7]. 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. Lipids were qualitatively defined by parent-ion scanning for known fragments characteristic for a specific sphingolipid class, including sphingoid bases, ceramides, phytoceramides or for glycerolipid class, such as diacylglycerol. Samples were quantitatively analyzed on the basis of calibration

curves generated with synthetic standards. The mass of each species was normalized to phosphorous levels of each sample.

**References**


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**Supplementary Table 1**. List of different *C. neoformans* Isc1 constructs used in this study[a]

| Name | PCR Primers | Fusion Tag |
|---|---|---|
| D114A | F: TCAAGCTTTTGCAGGGG**C**CTTTTTCGTTAAGA<br>R: CCCCTGCAAAAGCTTGAGCCGGCGAAC<br>Full-length *C. neoformans* Isc1 with point mutation (D114A) | Xpress |
| F115W | F: TCAAGCTTTTGCAGGGGACT**GG**TTCGTTAAGA<br>R: AGTCCCCTGCAAAAGCTTGAGCCGGCG<br>Full-length *C. neoformans* Isc1 with point mutation (F115W) | Xpress |
| F116Y | F: AGCTTTTGCAGGGGACTTTT**A**CGTTAAGAAG<br>R:AAAAGTCCCCTGCAAAAGCTTGAGCCGGCGA<br>Full-length *C. neoformans* Isc1 with point mutation (F116Y) | Xpress |
| K118G | F: TTGCAGGGGACTTTTTCGTT**GG**GAAGGCTGCT<br>R:AACGAAAAAGTCCCCTGCAAAAGCTTGAGC<br>Full-length *C. neoformans* Isc1 with point mutation (K118G) | Xpress |
| K119A | F: CAGGGGACTTTTTCGTTAAG**GC**GGCTGCTGC<br>R: CTTAACGAAAAAGTCCCCTGCAAAAGC<br>Full-length *C. neoformans* Isc1 with point mutation (K119A) | Xpress |
| A120S | F: GGGACTTTTTCGTTAAGAAG**T**CTGCTGCCAA<br>R: CTTCTTAACGAAAAAGTCCCCTGCAAAAGC<br>Full-length *C. neoformans* Isc1 with point mutation (A120S) | Xpress |
| F115W;<br>F116Y; | F:GCTCAAGCTTTTGCAGGGGACT**GG**T**A**CGTT**GG**GAAG**T**CTGCT<br>GCCAATG | Xpress |

| K118G; A120S | R: GTCCCCTGCAAAAGCTTGAGCCGGCGAACCAGA<br>Full-length *C. neoformans* Isc1 with point mutations (F115W;F116Y;K118G;A120S) | |
|---|---|---|

[a] The nomenclature of various recombinant *C. neoformans* Isc1 proteins, the PCR primers utilized to create the proteins, and the fusion tags present at the 5' are shown. F and R refer to the forward and reverse PCR primers, respectively. All primer sequences are oriented 5' to 3'.