

Inositol acylation of glycosylphosphatidylinositols in the pathogenic fungus *Cryptococcus neoformans* and the model yeast *Saccharomyces cerevisiae*

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Cryptococcus neoformans, an opportunistic fungus responsible for life-threatening infection in immunocompromised patients, is able to synthesize glycosylphosphatidylinositol (GPI) structures. Radiolabelling experiments *in vitro* with the use of a cryptococcal cell-free system showed that the pathway begins as in other eukaryotes, with the addition of *N*-acetylglucosamine to phosphatidylinositol, followed by deacetylation of the sugar residue. The third step, acylation of the inositol ring, seemed to involve a fatty acid other than palmitate, in contrast with previous findings in *Saccharomyces cerevisiae* and mammalian GPI pathways. A systematic study of inositol acylation in *C. neoformans* and *S. cerevisiae* showed that both organisms used a

variety of fatty acids in this step; these were transferred directly from acyl-CoA to inositol without modification. However, the specificity of fatty acid utilization was quite distinct in the two fungi, with the pathogen being substantially more restrictive. In mammalian cells fatty acids added exogenously as acyl-CoAs are not transferred directly to inositol. These results suggest significant differences in the GPI biosynthetic pathway between mammalian and *C. neoformans* cells that could represent targets for anti-cryptococcal therapy.

Key words: cryptococcal and mammalian glycosylphosphatidylinositols (GPIs), fatty acids, GPI anchors, GPI biosynthesis.

INTRODUCTION

Glycosylphosphatidylinositols (GPIs) are complex glycolipids that anchor proteins to the extracellular face of the plasma membrane (reviewed in [1–3]). The core GPI anchor structure is precisely conserved from protozoans to mammals and consists of an inositol phospholipid linked to a glycan containing glucosamine, mannose and phosphoethanolamine. In all species studied so far, the GPI biosynthetic pathway begins with the transfer of GlcNAc from UDP-GlcNAc to endogenous phosphatidylinositol (PI). The resulting GlcNAc-PI is deacetylated to generate glucosaminyl-PI (GlcN-PI); in the simplest case this is subsequently elongated by the sequential addition of three mannose residues and phosphoethanolamine. The resulting complete GPI precursor is then amide-linked to the C-terminus of the protein to be anchored.

Because the core structure of GPIs is conserved, the basic pathway occurs in all organisms so far examined. However, this structure can be modified by the addition of sugars, lipids or phosphoethanolamine, depending on the organism, the cell type and the anchored protein [2]. One modification is fatty acyl group addition at the 2-position of the inositol ring. This acylation, first described by Roberts et al. [4] on the anchor of human erythrocyte acetylcholinesterase, renders the GPI resistant to cleavage by phosphatidylinositol-specific phospholipase C (PI-PLC). Inositol-acylated GPIs have been found in protozoan parasites [5–8], *Saccharomyces cerevisiae* [9] and mammalian cell lines [10–12]. In yeast and mammals, acylation of inositol precedes mannosylation of GPI intermediates, whereas in trypanosomes it occurs farther downstream in biosynthesis and is required for the addition of phosphoethanolamine [2,3,9,13].

The literature on GPI suggests that the acyl group attached to the inositol ring is primarily palmitate (reviewed in [2,3]) but

biochemical analysis has shown this is not always so. Although mammalian GPIs that have been examined in detail are modified with palmitate [4,12], studies of acylated GPIs in trypanosomes demonstrate a mixture of fatty acids on inositol [8,14], and protein-bound GPI anchors of *Plasmodium falciparum* contain myristate in that position [7]. Further, there is considerable controversy about the precise source of the acyl group on inositol. It has been suggested that, in *S. cerevisiae*, palmitate and myristate might be transferred directly from the appropriate CoA derivative; however, other fatty acids were not tested [9]. In mammalian systems a study with exogenous short chain GlcN-PI as acceptor indicated the direct transfer of fatty acids from fatty acyl-CoA to GPI intermediates [15]. In contrast, earlier work examining endogenously synthesized GPIs suggested that the acyl group on inositol originates from membrane lipids and that the process utilized only the CoA portion of exogenous acyl-CoA [16].

We are interested in GPI anchor biosynthesis in the encapsulated pathogenic fungus *Cryptococcus neoformans*. *C. neoformans* is an opportunistic yeast responsible for life-threatening meningoencephalitis in immunocompromised individuals, especially those with AIDS [17,18]. The prevalence in the latter group is approx. 6–8.5% [19]. Current treatment alternatives for *C. neoformans* infections are inadequate and patients require lifelong suppressive therapy to prevent relapse [17,20].

As part of our studies of GPI biosynthesis in *C. neoformans* we discovered a unique feature of inositol acylation. An initial observation suggesting that *C. neoformans* utilizes a fatty acid other than palmitate for this modification prompted us to investigate this step in detail. Our studies on the source and nature of the acyl group show that inositol acylation in the pathogenic fungus is much more specific than in the model yeast *S. cerevisiae*.

Abbreviations used: CHO, Chinese hamster ovary; GPI, glycosylphosphatidylinositol; GPI-PLD, GPI-specific phospholipase D; PI, phosphatidylinositol; PI-PLC, PI-specific phospholipase C.

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MATERIALS AND METHODS

Materials

[glucosamine-6-³H]Uridine diphosphate-*N*-acetyl-D-glucosamine (34.8 Ci/mmol) and En³Hance were purchased from Dupont–New England Nuclear (Boston, MA, U.S.A.). [9,10-³H]Myristic acid and [9,10-³H]stearic acid (each 60 Ci/mmol) were from American Radiolabelled Chemicals (St. Louis, MO, U.S.A.) and medium components for fungal growth were from Difco Laboratories (Detroit, MI, U.S.A.). Unless indicated, all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Preparation of yeast membranes

S. cerevisiae strain RSY607 (*MATα* *ura3-52 leu2-3,112 PEP4::URA3*) was obtained from Dr. Randy Schekman (University of California at Berkeley, Berkeley, CA, U.S.A.). *C. neoformans* strain American Type Culture Collection 24067 [21] and the acapsular mutant strain Cap67 [22] were obtained from Dr. A. Casadevall (Albert Einstein College of Medicine, Bronx, NY, U.S.A.). All cultures were grown in YPD medium [1% (w/v) Bacto Yeast Extract/2% (w/v) peptone/2% (w/v) dextrose] at 30 °C with continuous shaking; growth was monitored by measuring D_{600} . A D_{600} of 1.0 in a 1 cm cuvette corresponds to a cell density of approx. 10^7 cells/ml.

Cells were grown overnight to D_{600} values of 1–2 (early exponential phase) and collected by centrifugation (3000 g, 15 min, 4 °C). The cell pellet was washed twice with cold TM buffer [50 mM Tris/HCl (pH 7.5)/2.0 mM MgCl₂] supplemented with 0.1% (v/v) 2-mercaptoethanol; the washed pellet was then resuspended at 500 *D* units/ml in TM buffer. Glass beads (0.5 mm; Biospec Products, Bartlesville, OK, U.S.A.) were added to the level of the meniscus and cells were lysed by vigorous vortex-mixing of the suspension for five periods of 1 min, alternating with equal durations on ice. Lysis was checked by phase-contrast microscopy (generally reaching approx. 90%) and the lysate was transferred to a fresh tube. Beads were rinsed in TM buffer and the rinse was pooled with the lysate. Unbroken cells and debris were removed from this pool by low-speed centrifugation (1000 g, 5 min, 4 °C), and the supernatant fraction was centrifuged at 100000 g (45 min, 4 °C). The resulting crude membrane pellet was washed twice in TM buffer with resedimentation under the same conditions. After the final centrifugation, the membrane pellet was resuspended in TM buffer and total protein was assayed (Bio-Rad Protein Assay; Bio-Rad Laboratories, Hercules, CA, U.S.A.). Glycerol was then added to a final concentration of 10% (v/v); 100 μl aliquots (approx. 1 mg of total protein) were flash-frozen in liquid nitrogen and stored at –80 °C.

Biosynthesis of GPI precursors *in vitro*

Frozen membranes were thawed on ice and combined with other components on ice. Standard assays (final volume 100 μl) contained 20 μg/ml tunicamycin, 0.5–2.0 μCi of UDP-[³H]GlcNAc and 0.25–0.50 mg of membrane protein in TM buffer. To examine inositol acylation, this reaction mixture was first incubated for 15 min at 30 °C, then supplemented with a 1000-fold excess of unlabelled UDP-GlcNAc (0.5 mM) and divided into tubes containing ATP (1 mM) or CoA (0.25 mM) or both, or fatty acyl-CoA (0.15 mM) as indicated. The incubation then continued for 30 min before being stopped by the addition of 660 μl of chloroform/methanol (1:1, v/v) to yield a final proportion of chloroform/methanol/water of 10:10:3 (by vol.). Radiolabelled lipids were vortex-mixed and then extracted for 30 min at room

temperature while being rotated end over end (Labquake Tube Rotator; VWR Scientific Products, NY, U.S.A.); insoluble components were removed by centrifugation. The organic extract was dried, resuspended in 200 μl of butan-1-ol and partitioned against water. The aqueous phase was re-extracted with butan-1-ol; the butanol phases were then pooled and washed once with water. The final organic extract was dried under a stream of nitrogen gas and resuspended in chloroform/methanol (2:1, v/v) for application to TLC plates (Kiesegel 60; Merck). Plates were developed in chloroform/methanol/water in a final ratio of 10:10:3 (by vol.) (solvent A) or 65:25:4 (by vol.) (solvent B). Radioactive products were localized by ³H scanning (Bioscan System 200A Imaging Scanner; Bioscan, Washington, DC, U.S.A.) or by autoradiography after being sprayed with En³Hance.

Chemical and enzymic treatments of radiolabelled lipids

Radiolabelled lipids in the dried butan-1-ol phase were subjected to chemical and enzymic treatments as described previously [23]. For treatment with GPI-specific phospholipase D (GPI-PLD), lipids were dissolved in 100 μl of 100 mM Tris/HCl (pH 7.4)/2.5 mM CaCl₂/0.1% sodium deoxycholate and incubated overnight at 37 °C in the presence of 10 μl of whole human serum as a source of enzyme. For treatment with mild base, lipids were dissolved in 200 μl of methanolic NH₃ and incubated overnight at 37 °C. After incubation, treated and control samples were partitioned into butan-1-ol as described above, then analysed by TLC.

Preparation and product analysis of GlcN-(acyl)PI containing [³H]myristate or [³H]stearate

[³H]Myristoyl-CoA was synthesized in a reaction (60 μl) containing 1 mCi of [³H]myristate, 1.0 mM dithiothreitol, 5 mM ATP, 1 mM CoA, 0.1 mM EGTA, 12 mM MgCl₂, 120 mM KCl, 80 mM Tris/HCl, pH 8.0, and 1 unit of *Pseudomonas* acyl-CoA synthetase. After 60 min at 37 °C, chloroform/methanol (2:1, v/v) was added to yield chloroform/methanol/water (8:4:3, by vol.); the mixture was mixed vigorously and centrifuged briefly to separate the two phases. The organic (lower) phase was then removed and washed with an aqueous upper phase from a mock (8:4:3, by vol.) partition [24]. [³H]Myristoyl-CoA (approx. 50% of the input radioactivity) in the washed aqueous phase was stored at –20 °C. The same procedure was used to prepare [³H]stearoyl-CoA from [³H]stearate.

Large-scale assays were prepared by scaling up (5-fold) the standard biosynthesis assay *in vitro* described above, except that UDP-[³H]GlcNAc was replaced by 0.5 mM unlabelled UDP-GlcNAc, and [³H]myristoyl-CoA or [³H]stearoyl-CoA (2×10^6 d.p.m. total) was included instead of unlabelled acyl donors. Reactions were incubated at 30 °C for 1 h, then processed as described above for UDP-[³H]GlcNAc labelling. Parallel assays done for comparison included controls containing radiolabelled fatty acyl-CoA without unlabelled UDP-GlcNAc, as well as standard reactions with UDP-[³H]GlcNAc and unlabelled fatty acyl-CoA.

Radiolabelled lipid products were digested with GPI-PLD to generate GlcN-([³H]acyl)inositol, then analysed by TLC. The GlcN-([³H]acyl)inositol was located by ³H scanning of the TLC plate with comparison with the standard assay, then recovered by scraping the appropriate area. This glycolipid was extracted from the silica by repeated washing with a total of 2 ml of chloroform/methanol/water (10:10:3, by vol.) and the pooled extracts were dried. The purified GlcN-([³H]acyl)inositol was treated overnight with mild base to release the [³H]fatty acid,

which was then converted to a fatty acid methyl ester as described [25] for analysis by argentation and reverse-phase TLC. Argentation TLC was performed on Silica Gel GHL plates impregnated with 5% (w/v) AgNO_3 (Analtech) developed in hexane:ether:acetic acid (95:5:1, by vol.) for 1 h at 4 °C. Reverse-phase TLC was done at room temperature on Analtech high-performance-TLC RP18F plates with the use of chloroform/methanol/water (15:45:3, by vol.) as solvent. Non-radioactive and radioactive fatty acid methyl ester standards were included in both TLC systems and detected as described previously [25].

RESULTS

Characterization of early GPI intermediates in *C. neoformans*

We first examined the production of early intermediates in GPI biosynthesis. We used membranes from an acapsular strain because cells were easier to lyse, although qualitatively identical results were obtained with wild-type cells. When cryptococcal membranes were incubated with UDP- ^3H GlcNAc, two major radiolabelled lipids were produced (Figure 1, left lane), which were characterized by chemical and enzymic treatments (results not shown). Both compounds were completely sensitive to PI-specific phospholipase C (PI-PLC), suggesting the presence of GPI structures without additional acyl groups esterified to inositol. Treatment with mild base released all of the radiolabel in hydrophilic form, supporting the presence of acyl-linked fatty acids in the phospholipid portion. The compound with higher mobility on TLC was degraded by deamination with nitrous acid, which is typical of GPI intermediates containing non-acetylated glucosamine, whereas the other compound was resistant to this treatment. Taken together, these results are consistent with the notion that the first two precursors in the

cryptococcal GPI biosynthetic pathway are GlcNAc-PI and GlcN-PI.

The addition of ATP and CoA or palmitoyl-CoA to the reaction allowed the formation of a more hydrophobic product (Figure 1, middle and right lanes). An analysis of this product (results not shown) showed that it was completely susceptible to degradation with nitrous acid and to mild base hydrolysis but resistant to digestion with PI-PLC. These results and the TLC migration were consistent with the presence of an additional acyl group on inositol, so the product was designated GlcN-(acyl)PI, the third intermediate in the cryptococcal GPI pathway. Surprisingly, the acylated GPI intermediate generated in the presence of ATP and CoA (allowing the formation of acyl-CoA from endogenous fatty acids) migrated differently from that made in the presence of palmitoyl-CoA (Figure 1). This result suggested that *C. neoformans* ordinarily uses a fatty acid other than palmitate in the inositol acylation step, which is of interest because acylation is generally attributed to the addition of palmitate [2]. For example, GlcN-(acyl)PI made by *S. cerevisiae* membranes in the presence of palmitoyl-CoA co-migrates with that made in the presence of CoA and ATP [9]. This observation led us to make a further investigation of inositol acylation in the pathogenic yeast *C. neoformans*, and to compare it with the analogous reactions in the model yeast *S. cerevisiae* and in mammalian cells.

Inositol acylation of GlcN-PI in *S. cerevisiae* and *C. neoformans* membranes involves direct transfer from acyl-CoA

To examine the inositol acylation step, the ability of acyl-CoAs of various chain lengths to stimulate this reaction was assessed in membranes from *C. neoformans* and *S. cerevisiae*. Membranes from each cell type were first incubated in the presence of UDP- ^3H GlcNAc to form the first two biosynthetic precursors. After a 15 min incubation the reaction was supplemented with an excess of unlabelled UDP-GlcNAc and either ATP and CoA or the fatty acyl-CoA being tested. This pulse-chase protocol was designed to minimize effects on earlier GPI synthetic steps that could be caused by the exogenous fatty acyl-CoAs. As shown in Figure 2, both *S. cerevisiae* and *C. neoformans* produce GlcN-(acyl)PI on the addition of a range of fatty acyl-CoAs. Differences in efficiency of stimulation of inositol acylation by individual acyl-CoA species will be addressed below. Because PMSF inhibits inositol acylation in trypanosomes [26], we tested the effect of this compound. PMSF (final concentration 1 mM) did not inhibit GlcN-(acyl)PI formation in either *C. neoformans* or *S. cerevisiae* membranes (results not shown). GTP is absolutely required for inositol acylation in mammalian systems ([16], and results not shown). In contrast, GTP (1 mM) did not affect this step in either fungal system and it was not included in standard reactions.

The experiments in Figure 2 showed that various acyl-CoAs stimulated the production of acylated products with slightly different TLC mobilities. In addition, these mobilities seemed to correspond to the physical properties of each acyl-CoA added to the reaction, suggesting the direct incorporation of the fatty acid. However, because GlcN-(acyl)PI contains fatty acids in three positions (two as part of the phospholipid moiety and one attached to inositol), it was important to examine whether the different mobilities were due to inositol acylation or to alteration of the phospholipid moiety. To address this question, standard assays were performed as shown in Figure 2 and the resulting radiolabelled lipids were treated with GPI-PLD. Digestion with this enzyme releases phosphatidic acid from GPI moieties to generate GlcN-(acyl)inositol, in which the only fatty acid present is on inositol. The ^3H GlcN-(acyl)inositol fragments generated

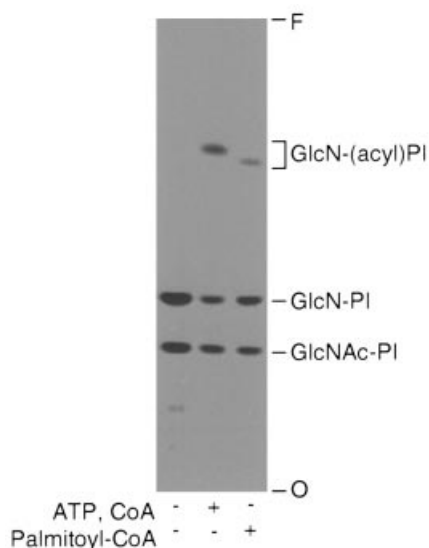


Figure 1 Early GPI intermediates are radiolabelled with UDP- ^3H GlcNAc in membranes from *C. neoformans*

Membranes from *C. neoformans* were incubated with UDP- ^3H GlcNAc and tunicamycin for 30 min with no addition, with ATP and CoA, or with palmitoyl-CoA. Assay was as described in the Materials and methods section except that all components were present throughout the period. Radiolabelled lipids were extracted and separated by TLC (solvent B); an autoradiograph of the TLC plate is shown. Abbreviations: O, origin; F, front.

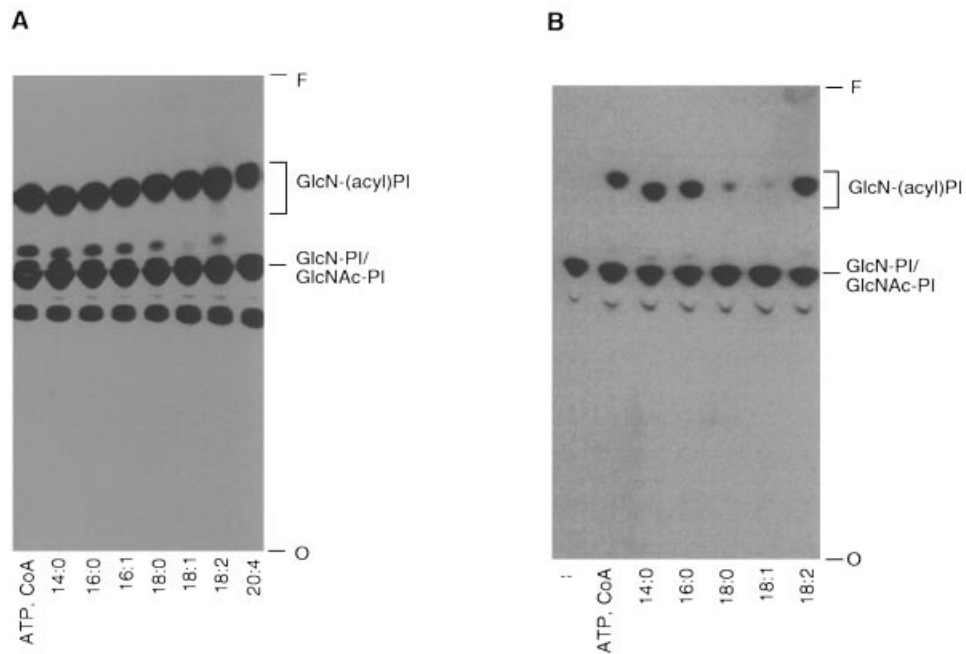


Figure 2 Acyl-CoAs stimulate GlcN-(acyl)PI formation in *S. cerevisiae* and *C. neoformans* membranes

Membranes from *S. cerevisiae* (A) or *C. neoformans* (B) were labelled with UDP-[^3H]GlcNAc for 15 min and chased with unlabelled UDP-GlcNAc for 30 min in the presence of ATP and CoA or acyl-CoA as indicated. Radiolabelled lipids were extracted and analysed by TLC (solvent A); an autoradiograph of each TLC plate is shown. This solvent system did not resolve GlcN-PI and GlcNAc-PI. The lane indicated by (-) contained no additions. Acyl-CoA addition is denoted by the characteristics of the fatty acid component (chain length: number of double bonds). The nature of labelled species not specifically identified in the figures is addressed in the Discussion. Abbreviations: O, origin; F, front.

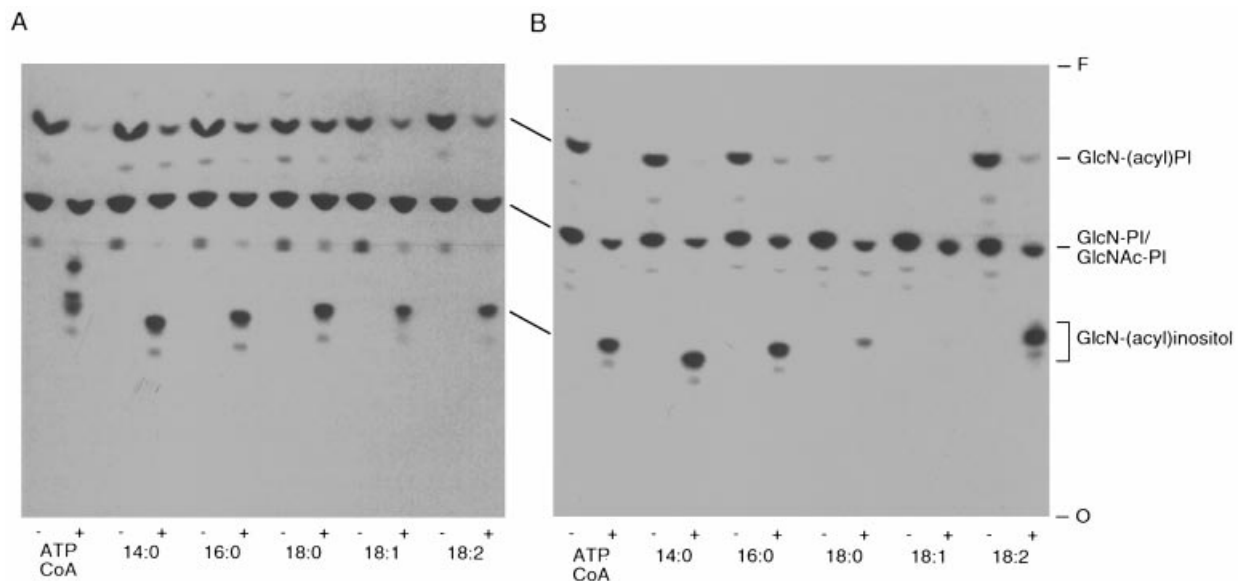


Figure 3 Exogenously added fatty acyl-CoAs directly influence inositol acylation in *S. cerevisiae* and *C. neoformans* membranes

Membranes from *S. cerevisiae* (A) or *C. neoformans* (B) were assayed as described in the legend to Figure 2 and the radiolabelled lipids were treated overnight with (+) or without (-) whole serum as a source of GPI-PLD. The products of enzymic digestion were recovered and analysed by TLC (solvent A) and autoradiography. The notation under each pair of lanes indicates the addition to the original labelling reaction (see legend to Figure 2). Because GlcN-PI and GlcNAc-PI co-migrated in this TLC system, but only GlcN-PI was cleaved by GPI-PLD, this band was removed only partly by enzyme treatment. Abbreviations: O, origin; F, front.

from GPIs made in the presence of different acyl-CoAs had a range of chromatographic mobilities (Figure 3, bracketed bands). This variation must have been due to the acyl group on inositol

because the remainder of this fragment was identical. Furthermore, the relative mobility of each [^3H]GlcN-(acyl)inositol corresponded to the physical properties of the fatty acid on the

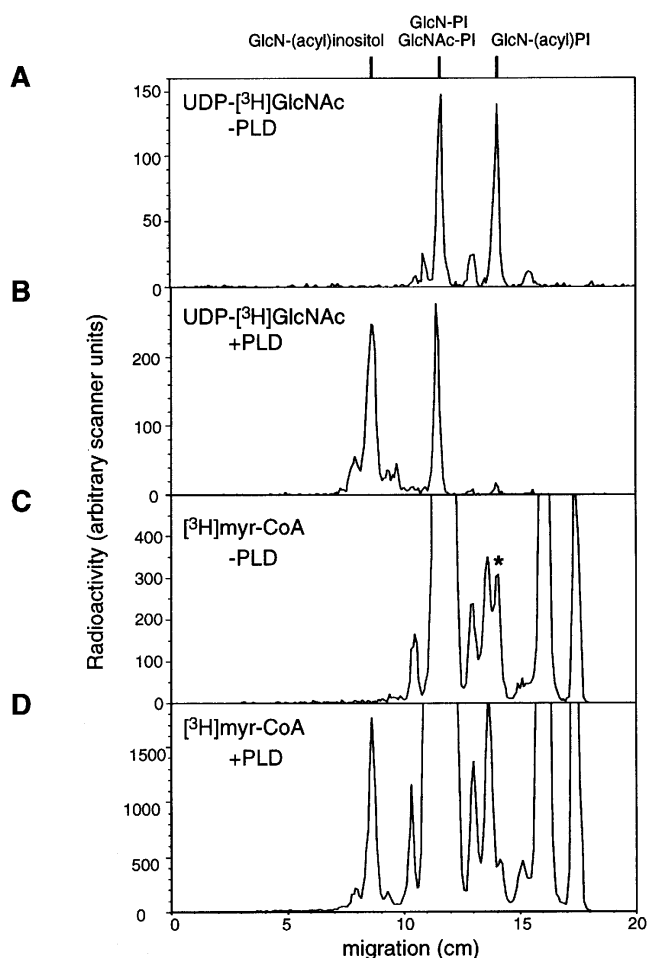


Figure 4 Transfer of [³H]myristate from [³H]myristoyl-CoA to GlcN-PI in *S. cerevisiae* membranes

Assays were performed in the presence of either UDP-[³H]GlcNAc and unlabelled myristoyl-CoA (A, B) or unlabelled UDP-GlcNAc and [³H]myristoyl-CoA (C, D), as described in the Materials and methods section. Lipids were then subjected to either GPI-PLD treatment (B, D) or mock incubation (A, C) and resolved by TLC (solvent A). Scans of the TLC plate, plotted in arbitrary scanner units, are shown. The identities of the GPI intermediates are indicated above the top panel; the radiolabel and treatment used are indicated on each panel. Note that peak heights between panels should not be compared, as preparative reactions (+PLD) were larger.

acyl-CoA included in the original reaction, with products from longer-chain acyl-CoAs migrating farther in this non-polar solvent system. These results strongly suggest that fatty acids are directly transferred from exogenously added fatty acyl-CoAs to GlcN-PI in both *S. cerevisiae* and *C. neoformans* membranes. Interestingly, *S. cerevisiae* glycolipids radiolabelled in the presence of ATP and CoA yielded a heterogeneous group of GPI-PLD products (Figure 3A). This might reflect the use of a mixture of endogenous fatty acids to synthesize acyl-CoA when none is provided.

A question still remained of whether the acyl group transferred to inositol remained unaltered or was modified in the course of the reaction. To answer this question we directly compared [³H]fatty acids recovered from the inositol moiety of GlcN-([³H]acyl)PI to the [³H]fatty acids of acyl-CoA donors. Preparative-scale assays were performed in the presence of unlabelled UDP-GlcNAc as the sugar donor and [³H]myristoyl-CoA or [³H]stearoyl-CoA as potential donors of the acyl group

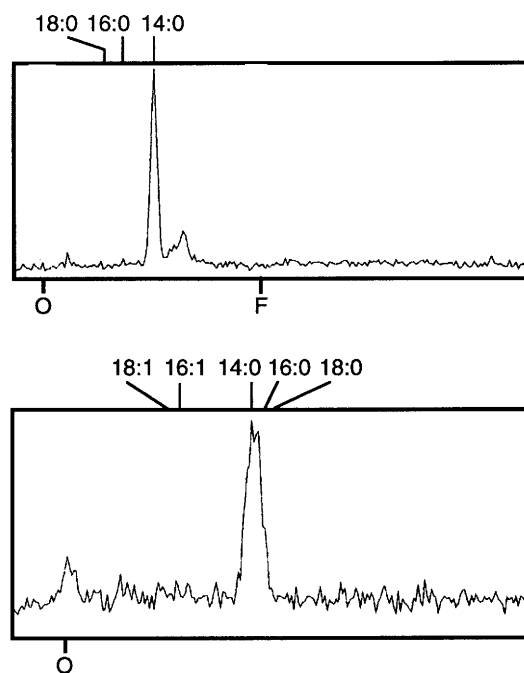


Figure 5 The fatty acid on GlcN-(acyl)inositol is not altered during transfer

The spot corresponding to GlcN-([³H]acyl)inositol [Figure 4(D), 7.5–8.5 cm] was scraped from the TLC plate and deacylated by treatment with mild base to generate [³H]fatty acid; the product was then converted to a fatty acid methyl ester. The final product was analysed by reverse-phase TLC (upper panel) and argentation TLC (lower panel) as described in the Materials and methods section, and identified by co-migration with fatty acid methyl esters of standards (indicated above each panel in the form chain length: number of double bonds). Abbreviations: O, origin; F, front.

on inositol. Parallel reactions containing UDP-[³H]GlcNAc and unlabelled myristoyl-CoA or unlabelled stearoyl-CoA were performed as controls. As shown in Figure 4, a radiolabelled compound was formed in the reaction containing [³H]myristoyl-CoA and unlabelled UDP-GlcNAc (Figure 4C, asterisk), which co-migrated with the [³H]GlcN-(acyl)PI formed in a standard reaction containing UDP-[³H]GlcNAc and unlabelled myristoyl-CoA (Figure 4A). This material was not formed if UDP-GlcNAc was omitted from the reaction (results not shown). On treatment with GPI-PLD the compound was almost totally converted to a more polar species that migrated closer to the origin [at approx. 8 cm (Figure 4D)]. This product exactly co-migrated with [³H]GlcN-(acyl)inositol generated by treatment of the standard reaction with GPI-PLD (Figure 4B), confirming that the original material was GlcN-([³H]acyl)PI.

The area of the TLC plate corresponding to GlcN-([³H]acyl)inositol was scraped and the lipid was recovered from the silica as described in the Materials and methods section. The material was then treated with mild base to release any [³H]acyl group on inositol and the labelled fatty acid was converted to a fatty acid methyl ester. The fatty acid methyl ester reaction product was analysed by reverse-phase TLC, which separates on the basis of chain length, and by argentation TLC, which separates mainly on the basis of degree of saturation (Figure 5). Comparison with standards demonstrated that the fatty acid recovered was indeed [³H]myristate. These results indicate that the [³H]myristate added to the reaction in the form of [³H]myristoyl-CoA was directly incorporated during the

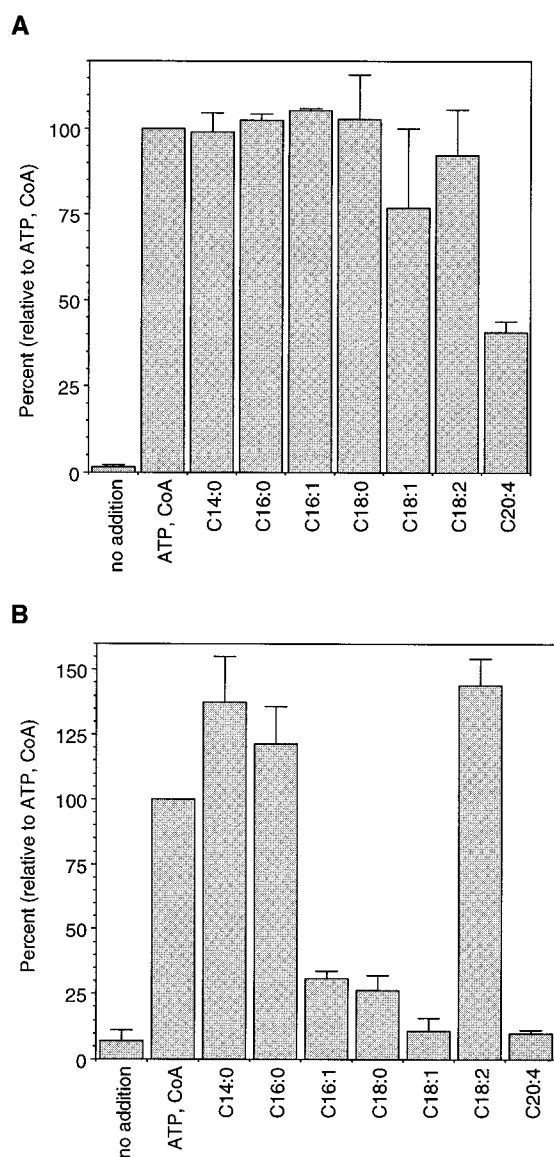


Figure 6 *C. neoformans* and *S. cerevisiae* differ in their efficiencies of incorporation of various fatty acyl donors in the inositol acylation step

Membranes from *S. cerevisiae* (A) or *C. neoformans* (B) were assayed as described in the legend to Figure 2 in four independent experiments. The TLC plates were scanned and the peak areas corresponding to GlcN-(acyl)PI were normalized to the amount produced in the presence of ATP and CoA, then plotted. Results are means \pm S.D. ($n = 4$). Pairwise comparisons of the amount of [3 H]GlcN-(acyl)PI produced in the presence of various acyl-CoAs were performed and statistical significance was evaluated by one-way analysis of variance followed by Bonferroni t test (using SIGMA-STAT 2.0 software). $P < 0.05$ was considered to be significant.

acylation step, and that it remained unchanged throughout the reaction. A similar analysis with [3 H]stearoyl-CoA yielded the same results (results not shown).

The experiment in Figure 2 showed striking differences in the efficiencies of utilization of various fatty acyl donors in *C. neoformans*, with less effect in *S. cerevisiae*. To assess this result we quantified the [3 H]GlcN-(acyl)PI produced in the presence of different fatty acyl-CoAs. Means and S.D. of [3 H]GlcN-(acyl)PI production in four experiments are shown in Figure 6, with all values normalized to the amount of product made in the presence

of ATP and CoA (the condition under which endogenous fatty acids are incorporated). The results indicate that the relative efficiency of incorporation of fatty acids differs substantially between the two fungi. While *S. cerevisiae* is promiscuous in acyl choice (Figure 6A), *C. neoformans* is much more selective, with several fatty acyl-CoA species supporting inositol acylation only poorly (e.g. palmitoleoyl-CoA and oleoyl-CoA) (Figure 6B). For *S. cerevisiae*, statistical analysis showed no significant differences between the amounts of GlcN-(acyl)PI formed on addition of the various acyl-CoAs, with the exception of arachidonoyl-CoA ($C_{20:4}$) ($P < 0.05$). For *C. neoformans*, myristoyl-CoA ($C_{14:0}$), palmitoyl-CoA ($C_{16:0}$) and linoleoyl-CoA ($C_{18:2}$) supported GlcN-(acyl)PI production to similar extents ($P > 0.05$); these were significantly better than palmitoleoyl-CoA ($C_{16:1}$), stearoyl-CoA ($C_{18:0}$), oleoyl-CoA ($C_{18:1}$) and arachidonoyl-CoA ($C_{20:4}$) ($P < 0.05$).

The results presented above suggested that there are *Cryptococcus*-specific aspects of inositol acylation. Because of the importance of identifying targets for antifungal drug discovery, we compared these results with inositol acylation in a mammalian system. Our work with Chinese hamster ovary (CHO) cell membranes (results not shown) confirmed studies in murine lymphoma cells by Stevens and Zhang [16]: the presence of different fatty acyl-CoAs did not alter the migration of the GlcN-(acyl)PI synthesized. We also treated the assay products with GPI-PLD and found that all of the GlcN-(acyl)inositol species co-migrated (results not shown). This suggests that the exogenous fatty acid was not directly transferred to inositol. We extended the set of acyl-CoAs tested by Stevens and Zhang [16] by examining CoA derivatives of myristic ($C_{14:0}$), palmitoleic ($C_{16:1}$), stearic ($C_{18:0}$), linoleic ($C_{18:2}$) and arachidonic ($C_{20:4}$) acids. All of these acyl-CoAs, with the exception of arachidonoyl-CoA ($P < 0.05$), supported GlcN-(acyl)PI synthesis to similar extents (results not shown). Arachidonoyl-CoA was less effective in all three systems tested, which could be due to difficulties in the proper localization of this long-chain compound.

DISCUSSION

The pathogenic fungus *C. neoformans* constructs several unique carbohydrate structures, such as the elaborate mannans that comprise its polysaccharide capsule (reviewed in [27]). Although these structures have been quite well studied, virtually nothing is known about other cryptococcal glycans. As part of our studies of glycosylation in this organism we have examined GPI biosynthesis. Our goal is to obtain a better understanding of the biochemical characteristics of this organism, focusing attention on unique features that could be explored as potential targets for anticryptococcal therapy.

Radiolabelling experiments *in vitro* allowed us to identify the early intermediates of the *C. neoformans* GPI biosynthetic pathway as GlcNAc-PI and GlcN-PI (Figure 1), the same as in all other systems studied so far [2]. On addition of ATP and CoA or palmitoyl-CoA to the assay, GlcN-PI became acylated on inositol to form GlcN-(acyl)PI, the third GPI intermediate of the cryptococcal pathway. We were interested to observe that the acylated intermediate differed in migration on TLC when it was generated in the presence of palmitoyl-CoA compared with CoA and ATP (Figure 1). These results suggested that in conditions under which endogenous fatty acids are used for inositol modification *C. neoformans* chooses species longer than palmitate, in contrast with what has been proposed for *S. cerevisiae* [9].

In the course of examining inositol acylation we observed the formation of several additional radiolabelled species [above and below GlcN-PI in Figure 2(A)]. The material that migrates just

above GlcN-PI is an inositol-acylated GPI (results not shown), probably a *lyso* form of GlcN-(acyl)PI. The inositol acylation is consistent with its altered migration depending on the acyl-CoA present. Another prominent radiolabelled species [below GlcN-PI in Figure 2(A)] is also a GPI, but is not inositol-acylated (results not shown) and does not vary with added acyl-CoA. This material might be *lyso*-GlcN-PI. Neither of these species is a later GPI intermediate containing mannose (results not shown). The presence of *lyso* forms of early GPI intermediates suggests that acyl chain remodelling (deacylation and reacylation) might occur at this point in the pathway, in addition to previously described remodelling steps late in GPI synthesis [28]. This possibility is supported by the incorporation of [³H]myristate into the diglyceride portion of GlcN-(acyl)PI during fatty acid labelling experiments (results not shown). A full characterization of these remodelling steps will require further investigation.

To pursue our observation that inositol acylation in *C. neoformans* differed from that in other organisms, we characterized the source and nature of the acyl group on inositol in the pathogenic fungus, and compared the results with similar investigations in *S. cerevisiae* and mammalian cells. Studies *in vitro* in *S. cerevisiae* showed that this organism uses a broad range of fatty acids for GPI inositol acylation (Figure 2A). Treatment of GPI intermediates with GPI-PLD suggested that the various fatty acids were transferred directly from acyl-CoAs to inositol (Figure 3A), and product analysis with the use of radiolabelled acyl donors indicated that the fatty acid incorporated remained unchanged, with no alteration in chain length or degree of saturation (Figures 4 and 5). *C. neoformans* also incorporated several fatty acids directly from acyl-CoA to inositol (Figures 2B and 3B). However, the pathogenic fungus was substantially more restrictive than *S. cerevisiae* in terms of acyl-CoA utilization (Figure 6). The explanation for the pattern of fatty acid preference is not obvious. It has no clear correlation with chain length because the reaction was supported by palmitate (C_{16:0}) but not by palmitoleic acid (C_{16:1}). The degree of saturation is also not the key parameter, as demonstrated by the optimal use of both myristic acid (C_{14:0}) and linoleic acid (C_{18:2}) but very poor incorporation of oleic acid (C_{18:1}). It might be that the observed specificity in *C. neoformans* is associated with some less obvious physical characteristic of how the fatty acid interacts with the acylation apparatus. For example, studies of protein N-myristoylation with the use of fatty acid analogues have suggested that fatty acids interact with the transferase in a bent conformation and that specific distances between double bonds and the carboxy group are crucial (reviewed in [29]). It is possible that similar parameters are important in GPI inositol acylation.

We also considered the possibility that the choice of acyl moiety in *C. neoformans* and *S. cerevisiae* could be related to membrane fatty acid compositions, which are quite different in the two fungi [30,31]. However, we could not establish a clear correlation between the abundance of a specific fatty acid and its use in the acylation of inositol. For example, oleic acid (C_{18:1}) is the most abundant fatty acid in *C. neoformans* (44% of total fatty acids), yet it supports acylation in this organism only poorly. Linoleic acid (C_{18:2}) is the next most abundant (28%) and works quite efficiently for this reaction. Similarly, in *S. cerevisiae* both stearic acid (C_{18:0}; less than 1% of total fatty acids) and palmitoleic acid (C_{16:1}; 59% of total fatty acids) support inositol acylation efficiently. Understanding the chemical basis for fatty acid choice in *C. neoformans* GPI inositol acylation will require further study, perhaps with the use of fatty acid analogues or by specifically investigating the fatty acid composition of membrane compartments where the reaction occurs.

To investigate inositol acylation as a potential target for drug

development, it was crucial to compare this process in the pathogen with the same reaction in mammalian cells. The source of the acyl group on inositol in mammalian cells is controversial, with one study suggesting a mechanism in which the modification is acyl-CoA independent (requiring only CoA) [16], and another hypothesizing the direct transfer of fatty acids from acyl-CoA [15]. In our studies of CHO cell membranes *in vitro*, the fatty acids were not transferred directly from acyl-CoA (results not shown), suggesting an activity similar to that proposed by Stevens and Zhang [16]. Consistently with this suggestion, different acyl-CoAs resulted in equal stimulation of the activity ([16], and results not shown). There are several possible reasons for the contrast between our results in CHO cell membranes and those of Doerrler et al. [15] in the same cell type. The latter study used a short-chain synthetic GlcN-PI analogue (GlcN α -PI[C₈]) as an acceptor for GPI biosynthetic enzymes, in contrast with our experiments, which followed GPI synthesis *de novo*. It is possible that the synthetic substrate was not readily accessible to, or not well recognized by, the enzymes involved in the acyl-CoA-independent activity. It is also possible that both acyl-CoA-dependent and acyl-CoA-independent activities occur in mammalian membranes, for example in different compartments, but only one was detected under the experimental conditions used in each laboratory [15].

A striking difference in the inositol acylation mechanism between yeasts and mammalian cells was the absolute requirement for GTP in the mammalian system. Neither *S. cerevisiae* or *C. neoformans* required this nucleotide. Stevens and Zhang [16] suggested that GTP could be involved in making GlcN-PI available for acylation, perhaps by playing a role in the transmembrane 'flip-flop' of this glycolipid from the cytoplasmic to the luminal face of the ER membrane. Menon et al. [32] have suggested that inositol acylation and earlier steps in GPI biosynthesis occur in separate but functionally associated membrane domains. In either case, the requirement for physical relocation, the topology of GPI biosynthesis or the nature of specialized membrane domains might differ between fungal and mammalian systems.

Our results indicate that model and pathogenic yeasts differ in the selectivity of the inositol-acylating activity for specific fatty acids. Mammalian cells differ from fungal organisms in a more profound way, using an apparently distinct source of acyl group and requiring GTP for the reaction. Thus, although the acylation of inositol is an obligatory step in both the yeast and mammalian GPI pathways, occurring before anchor mannosylation, it seems that this process is fundamentally different in the two systems. This observation suggests inositol acylation in *C. neoformans* as a potential target for the development of selectively toxic inhibitors of the GPI pathway.

GPI biosynthesis is essential for viability of *S. cerevisiae* [33]; we are currently assessing whether this is true of *C. neoformans* as well. In African trypanosomes, which selectively incorporate myristate into their GPI species, myristic acid analogues were found to be toxic at levels that did not injure mammalian cells in culture [34]. By analogy, fatty acid analogues might work to inhibit inositol acylation in *C. neoformans*. One such analogue, 4-oxatetradecanoic acid, is toxic to *Cryptococcus* in culture, although the mechanism of toxicity has not been established [35]. The specificity of inositol acylation in *C. neoformans* was unexpected. Further investigations of the cryptococcal GPI pathway to characterize later precursors might lead to the discovery of additional novel features of this organism.

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