Temperature-induced alteration of inositolphosphorylceramides in the putative glycosylated lipid precursors of *Tetrahymena mimbres* glycosylphosphatidylinositol-anchored proteins

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Tetrahymena species contain relatively prominent glycosylphosphatidylinositol (GPI)-anchored proteins as well as their putative precursor phosphatidylinositol (PI) glycans. We have characterized the lipid components of the two principal *T. mimbres* PI glycans. Following their purification by preparative TLC, the PI glycans were hydrolysed in methanolic HCl or NaOH, and resulting lipids were analysed by chromatography and mass spectrometry. The two PI glycans contained nearly identical lipid moieties having long-chain bases with N-linked fatty acids. The predominant long-chain base, 3-*O*-methylsphinganine, was first assumed to be *O*-methylated as an artifact of hydrolysis, but subsequently, on the basis of control experiments, it was shown to be naturally occurring. PI glycans from cells grown at 28 °C

contained primarily palmitic acid (79 %) and some stearic acid (11 %), whereas the principal PI glycan from 38 °C-grown *T. mimbres* contained 65 % stearic acid. In 15 °C-grown cells stearic acid accounted for only 2 % of ceramide-bound fatty acids and was almost totally replaced by palmitic acid (95 %). The distributions of fatty acids bound to *T. mimbres* GPI-anchored proteins [Ko, Hung and Thompson (1995) *Biochem. J.* 307, 115–121] were similar but not identical to those of the PI glycans described here. Temperature-induced specification of the lipid components of mature *T. mimbres* GPI-anchored proteins appears to be established both at the level of PI-glycan synthesis and the level of PI-glycan utilization for protein attachment.

INTRODUCTION

Phosphatidylinositol (PI) glycans linked covalently to the C-termini of certain proteins effectively anchor those proteins to the exterior surface of a cell's plasma membrane [1,2]. Experimental evidence has established that the PI glycans are pre-assembled in the endoplasmic reticulum (ER) and attached en bloc to the nascent proteins during or immediately following the translocation of the peptides across the ER membrane [3]. Several of these glycosylphosphatidylinositol (GPI)-anchored proteins and their PI-glycan precursors have now been well characterized [2]. In each case, the C-terminus of the protein is linked via phosphoethanolamine to a glycan having the common backbone sequence: Man-Man-Man-GlcN, with the GlcN being covalently bound to PI. Superimposed upon this recurring pattern are numerous species-specific variations. Thus the trypanosome variant surface glycoprotein (VSG) anchor has a galactose (Gal) side-chain [4], the procyclic acidic repetitive protein (PARP) anchor has a complex glycan side-chain containing Gal, GlcNAc and sialic acid [5], and GPI anchors of mammalian cells sometimes have additional ethanolamine-phosphates [6,7]. The functional significance of these variations remains to be elucidated.

The lipid moieties of GPI anchors can also vary in a species-specific or stage-specific manner. They are glycerophosphatides in VSG and PARP, or sphingosylphosphatides in GPI-anchored proteins of yeast [8] and slime moulds [9,10]. In some of these

anchors an additional fatty acid (palmitic acid) esterified to the inositol ring confers resistance to the lipid moiety against cleavage by PI-specific phospholipase C (PI-PLC) [6]. Inositol acylation may be under developmental control in *Trypanosoma cruzi*, since PI-PLC resistance of the IG7 antigen increases as the cells develop from a non-infectious to an infectious stage [11].

Tetrahymena mimbres and closely related *Tetrahymena* species are free living ciliates whose biology and biochemistry are well documented [12]. Membranes of *T. mimbres* contain both free PI glycans and GPI-anchored proteins [13,14]. The free PI glycans of *T. mimbres* were shown to have an unusual core structure, featuring certain atypical sugar linkages [15], but the lipid moieties have been only partly described. In this paper we further characterize two PI glycans which are candidate precursors of *T. mimbres* GPI-anchored proteins and show them to contain inositolphosphoryl-ceramides (InsPCers). The major *T. mimbres* GPI-anchored proteins are shown, in the accompanying report [16], to contain the same kind of lipid anchors. We also report for the first time that the chain length of a PI-glycan fatty acid is determined by the cell growth temperature.

EXPERIMENTAL

Cell culture and *in vivo* labelling

Small inocula of *T. mimbres* NT1 were grown in enriched proteose peptone medium to the mid-logarithmic phase

Abbreviations used: ER, endoplasmic reticulum; FAME, fatty acid methyl ester; GPI, glycosylphosphatidylinositol; InsPCer, inositolphosphoceramide; ceramide-AEP, ceramide-aminoethylphosphonate; LCB, long-chain base; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; PI-PLD, phosphatidylinositol-specific phospholipase D; pCMPSA, *p*-chloromercuriphenylsulphonic acid; PARP, procyclic acidic repetitive protein; VSG, variant surface glycoprotein; TMS, tetramethylsilyl.

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[(2–4) × 10⁶ cells/ml] at 15 °C, 28 °C, or 38 °C as described [17]. The cells were labelled *in vivo* by adding [9,10-³H]myristic acid (22.4 Ci/mmol, Amersham Corp., Arlington, IL, U.S.A.), [1-³H]ethan-1-ol-2-amine hydrochloride (29.5 Ci/mmol, Amersham), or D-[2-³H]mannose (23.0 Ci/mmol, New England Nuclear DuPont, Boston, MA, U.S.A.). Before labelling, cells were washed twice with inorganic medium (47 mM NaCl, 1 mM KH₂PO₄, 4 mM K₂HPO₄, 1 mM MgSO₄) by centrifugation for 5 min at 900 *g* and then resuspended in inorganic medium to give a density of 1 × 10⁶ cells/ml. Cell density was monitored using a Coulter counter model ZB. Aliquots (20 ml) of cells were pre-incubated for 15 min in 50-ml Erlenmeyer flasks before adding 300 µCi of [9,10-³H]myristic acid, 200 µCi of [1-³H]ethan-1-ol-2-amine hydrochloride, or 200 µCi of D-[2-³H]mannose. The cells were then incubated for 6 h at 28 °C.

Lipid extraction

The labelled cell cultures were rapidly chilled in a mixture of acetone and solid CO₂, and washed twice with cold inorganic medium containing 1 mM *p*-chloromercuriphenylsulphonic acid (pCMPSA). Cell pellets were resuspended in 0.5 ml of water containing 1 mM pCMPSA, immediately treated with an equal volume of hot SDS buffer (100 mM Tris, pH 6.8, 2 mM SDS, 1 mM pCMPSA), and boiled at 100 °C for 5 min. After the mixtures were chilled, 5 vol. of ice-cold acetone were added, and the tubes were sealed and kept at –20 °C for more than 2 h. The acetone mixtures were centrifuged for 10 min at 10000 *g*, and the pellets were resuspended with sonication in 1.8 ml of ice-cold chloroform/methanol (2:1, v/v) for each 10⁷ cells and stored for 1 h at –20 °C to solubilize most of the phospholipids and the less polar glycolipids. The chloroform/methanol (2:1) extraction was repeated three times. After the final centrifugation, the pellets were extracted three times with ice-cold chloroform/methanol/water (10:10:3, by vol.) (CMW 10:10:3) using 1.6 ml per 10⁷ cells each time. During each CMW 10:10:3 extraction, the samples were tightly capped and kept at –20 °C for 1–2 h. The mixtures were centrifuged, and the CMW 10:10:3 supernatants were pooled and concentrated to dryness in a rotary evaporator. The residues were dissolved in 0.5 ml of water-saturated butanol per 10⁷ cells. After two phase partitions against 0.25 ml of water, the upper butanol-rich phases were pooled, and the water phase was extracted again with butanol. The upper phases were combined and washed with water again. The final upper phase was dried under a nitrogen stream, dissolved in 200 µl of chloroform/methanol (2:1), and stored at –20 °C for further analysis. This was the final butanol extract.

TLC analysis

TLC was performed on plates of silica gel H with 2.5 % potassium oxalate using the following solvent systems for analysis of the butanol extract: A, chloroform/methanol/water (10:10:3, by vol.); B, chloroform/methanol/water (20:24:9, by vol.); C, chloroform/methanol/1 M ammonium hydroxide (10:10:3, by vol.); D, chloroform/methanol/acetic acid/water (25:15:4:2, by vol.). The plates were dried and sprayed with either α-naphthol/sulphuric acid to detect glycolipids, or ninhydrin reagent to detect lipids containing a free amino group. For detection of isotope-labelled components, plates were sprayed with EN³HANCE (New England Nuclear DuPont) and fluorographed on Kodak X-OMAT films at –70 °C for 7–15 days. Radioactivity was quantified by scraping 0.5 cm bands of silica gel into vials and counting by liquid-scintillation spectrometry.

To analyse long-chain bases (LCBs), the following solvent systems were used on silica-gel G plates: E, chloroform/

methanol/2 M ammonium hydroxide (40:10:1, by vol.); F, chloroform/methanol/water (50:21:3, by vol.).

TLC systems G [chloroform/methanol/4.2 M ammonium hydroxide (9:7:2, by vol.)] and H [chloroform/methanol/water (11:9:2, by vol.)] were utilized to analyse deamination products of PI glycans.

Fatty acid methyl esters (FAMES) were chromatographed on TLC system I [light petroleum/diethyl ether (17:3, v/v)].

Purification by TLC

To purify the PI glycans, the butanol extract was chromatographed on TLC system A or B. The TLC plates were pre-run in the same solvents to remove contaminants. After chromatography, the lipid spots were detected by spraying with primulin and viewing under UV light. Spots of interest were scraped off for further analysis.

PI-PLC and PI-specific phospholipase D (PI-PLD) treatments

The butanol extract of D-[2-³H]mannose-labelled cells was dried and dissolved in 100 µl of 25 mM Hepes buffer, pH 7.2, containing 0.1 % sodium deoxycholate. Then 2 µl (3.4 units) of PI-PLC from *Bacillus thuringiensis* (a generous gift from Dr. Martin G. Low, Columbia University) was added to the sample (or 2 µl of buffer to a control sample). The samples were incubated for 2 h at 37 °C. Then water-saturated butanol was added to induce a two-phase partition. Radioactivity in both phases was counted, and the butanol-rich phase was concentrated, dissolved in chloroform/methanol (1:1, v/v), and chromatographed on TLC system B. Standard lipids were chromatographed in parallel, and the sample lane on the plate was scraped and counted for radioactivity. The conditions for PI-PLD-containing rabbit serum digestion were the same as for PI-PLC treatment except that 100 mM Tris buffer, pH 7.4, containing 0.1 % sodium deoxycholate and 2.5 mM CaCl₂ was used. The PI-PLD activity in rabbit serum was pre-tested using PI as substrate.

For PI-PLC hydrolysis of GPI-anchored protein, a 1 mg sample of gpi 23 purified by preparative electrophoresis (see accompanying report [16]) was treated with 34 units of PI-PLC in the above-mentioned Hepes buffer for 48 h at 37 °C. Lipid products were extracted by adding 3 vol. of chloroform/methanol (2:1, v/v), then 1 vol. each of water and chloroform. The chloroform phase was concentrated and resolved by TLC. This involved development of the mixed lipids and detergent on a 0.25 % potassium oxalate silica gel H plate in chloroform/methanol/2 M NH₄OH (9:7:2, by vol.) to half plate height, followed by a second and full development of the dried plate in petroleum ether/ethyl ether/acetic acid (70:30:1, by vol.) to resolve detergent from the PI-PLC product. This yielded a single lipid band migrating in the same position as bovine brain ceramides, as visualized with primulin. The gpi 23-derived lipid was converted into an *O*-tetramethylsilyl derivative and analysed by chemical ionization MS.

Deamination treatment of InsPCers

Silica gel containing each TLC-purified, ³H-fatty acid-labelled PI glycan, namely InsPCer 1 and InsPCer 2, was scraped into screw-cap glass tubes. Sodium acetate buffer (200 µl; 200 mM sodium acetate, pH 3.7, 0.1 % Nonidet P-40) and then an equal volume of 0.5 M sodium nitrite were added to each sample. Then the samples were incubated for 3–5 h at 37 °C. After a butanol partition, the upper phase was concentrated, redissolved in chloroform/methanol (2:1, v/v), and chromatographed on TLC systems D, G and H.

Mild alkaline hydrolysis

To the butanol extract of cells, concentrated and dissolved in 100 μ l of CMW 10:10:3, were added 20 μ l of 0.6 M methanolic NaOH. After incubation for 30 min at 30 °C, 20 μ l of 1 M acetic acid were added to neutralize the mixture. After the sample was dried completely under a nitrogen stream, the mild alkali-resistant lipids were extracted by water–butanol partition. The butanol-soluble lipids were chromatographed on TLC system B.

Strong alkaline hydrolysis

The TLC-purified InsPCers 1 and 2 were each dissolved in 1 ml of 1 M KOH in 80% methanol and sealed tightly under N₂ in screw-cap tubes. The samples were hydrolysed at 70 °C for 18 h. After hydrolysis, 2 ml of water was added and the samples were concentrated to remove methanol. The released LCBs were recovered by three extractions with 2 ml of diethyl ether.

Strong acid hydrolysis

The TLC-purified InsPCers 1 and 2 were each subjected to strong acid hydrolysis for 18 h at 80 °C using 2 M methanolic HCl in sealed tubes. The released FAMES were extracted with hexane three times, and then the aqueous phase was adjusted to pH 11 with 1 M NaOH. The free LCBs were extracted with diethyl ether three times. The diethyl ether extracts were combined, dried and stored for further analysis. The effectiveness of the hydrolysis was proved using bovine brain cerebroside.

Strong acid hydrolysis in deuteriated methanol

The hydrolysis was performed as described above except that fully deuteriated methanol was used instead of methanol. The extracted LCBs were then heated with 2 M H₂SO₄ in methanol for 10 min at 50 °C to remove exchangeable deuterium, and the pH was adjusted to 11. The LCBs were extracted with diethyl ether three times.

Lipid analysis by GC

Capillary GC was carried out with a Varian model 3700 Gas Chromatograph using a SP2330 capillary column (0.25 mm internal diam. $\times$ 30 m). To analyse FAMES, column temperature was held at 170 °C, injector 230 °C, detector 250 °C, and carrier gas pressure at 16 p.s.i.

The LCBs were converted into *N*-acetyl-*O*-TMS derivatives [18]. Column temperature was held at 220 °C for 2 min, then increased to 240 °C at 5 °C/min, and finally held at 240 °C. The *N*-acetyl-*O*-TMS derivatives of sphingosine and LCBs from bovine brain cerebroside were used as standards.

MS analysis of LCBs and derivatives

MS analysis was carried out with a Finnigan MAT TSQ70 mass spectrometer. The compounds were ionized with methane (CI⁺ mode), and positive ions were detected.

GC–MS analysis

Capillary GC–MS was performed with a Varian model 3400 gas chromatograph using a DB-5 capillary column (0.2 mm internal diam. $\times$ 30 m). For analysis of *N*-acetyl-*O*-TMS derivatives of LCBs, a program of 170 °C to 280 °C (20 °C/min, then hold) was used. Compounds were characterized by electron-impact-ionization (70 eV) MS with a Finnigan MAT 700 spectrometer. The *m/z* was scanned from 60 to 600.

RESULTS

Isolation of putative PI-glycan precursors of GPI-anchored proteins

Cells growing at 28 °C were labelled with [³H]myristic acid, [³H]mannose, or [³H]ethanolamine in inorganic medium for 6 h. The cells were delipidated, and PI glycans were extracted as described in the Experimental section. Briefly, the CMW 10:10:3 extract was concentrated to dryness and partitioned between water and butanol. The butanol phases were collected, concentrated and analysed on TLC using system A or B. Four lipid spots in the butanol extract became labelled with [³H]mannose, and the two most hydrophilic spots also became labelled with [³H]myristic acid and [³H]ethanolamine (Figure 1). For easy reference, the more hydrophilic spot will be designated as InsPCer 1 (inositolphosphoceramide 1) and the other spot as InsPCer 2. These compounds corresponded precisely to two *T. mimbres* PI

Figure 1 Distribution of radioactivity on TLC plates after chromatographing butanol extracts of delipidated cells using solvent system B

(a) [³H]Mannose labelling; (b) [³H]myristate labelling; (c) [³H]ethanolamine labelling. Arrows indicate positions of ninhydrin- and α -naphthol-staining InsPCers.

Figure 2 Nitrous acid deamination of the InsPCers

Butanol extracts of the mixed [^3H]mannose-labelled PI glycans were treated with buffer alone, resulting in no change in TLC mobility of InsPCer 1 (a, left-hand arrow) or InsPCer 2 (a, right-hand arrow) in solvent system B, or with nitrous acid, resulting in the loss of both peaks. When InsPCer 1 was purified from this mixture and deaminated separately, a new pattern of butanol-soluble radioactive products was detected by TLC system H (b). InsPCer 2 deamination yielded an identical pattern.

glycans recently shown to incorporate [^3H]inositol and [^{14}C]glucosamine as well as [^3H]ethanolamine and [^3H]myristate [13]. Non-radioactive PI glycans were also extracted and analysed. Several lipid spots staining with ninhydrin and with α -naphthol, a reagent for detecting glycolipids, were found by TLC analysis, and two of them co-migrated with InsPCer 1 and InsPCer 2 (Figure 1). InsPCer 1 had an R_f value of 0.11–0.15 on TLC system B and accounted for 3.7% of the total radioactivity in the [^3H]myristate-labelled butanol extract, while InsPCer 2 had an R_f value of 0.25–0.30 and accounted for 2.2% of the total radioactivity. However, the butanol extract contained only 2% of the total incorporated radioactivity from [^3H]myristic acid, with the remainder being in the major cellular glycerolipids [19]. Thus InsPCer 1 and InsPCer 2 were minor lipid species of *T. mimbres*.

Sensitivity of InsPCer 1 and InsPCer 2 to PI-PLC and PI-PLD

To characterize further InsPCer 1 and InsPCer 2, the butanol extract of [^3H]mannose-labelled cells was digested with PI-PLC from *B. thuringiensis*, with rabbit serum (containing PI-PLD activity), or with buffer as a control. The PI-PLC-treated sample lost 84% of its original radioactivity into the water phase, and the rabbit serum-treated sample lost 86%. TLC analysis in solvent system B showed that the radiotracer-labelled peaks of InsPCer 1 and InsPCer 2 disappeared in the enzyme-treated samples. Clearly, InsPCers 1 and 2 were sensitive to PI-PLC and

Figure 3 Products of [^3H]myristate-labelled InsPCer hydrolysis in 2 M methanolic HCl

(a and c) Panels show the distribution of FAME radioactivity from hydrolysates of InsPCer 1 and InsPCer 2 respectively, as resolved by TLC solvent system I. (b and d) This shows the distribution of LCB radioactivity from hydrolysates of InsPCer 1 and InsPCer 2 respectively, as resolved by TLC system E. The O-Me-LCB label indicates the position of O-methyl-long chain bases.

PI-PLD. The sensitivity of the two compounds to PI-PLC suggested that neither was acylated on its inositol ring [20].

InsPCer 1 and InsPCer 2 are inositolphosphoceramides

Butanol extracts containing [^3H]mannose-labelled InsPCer 1 and InsPCer 2 were deaminated with nitrous acid, and the products were recovered by butanol–water partition. Radioactivity measurements showed that while the butanol phase from a control sample not containing nitrous acid retained 95% of the original radioactivity and retained the original TLC pattern (Figure 2a), the nitrous acid-treated sample lost more than 90% of its original radioactivity into the water phase. TLC analysis confirmed that InsPCers 1 and 2 were both sensitive to deamination treatment.

TLC-purified, [^3H]myristic acid-labelled InsPCers 1 and 2 were deaminated individually. In both cases the lipid products of this reaction co-migrated with PI on TLC system D (results not shown) but had slightly lower R_f values than that of PI on TLC system H, as illustrated for the products of InsPCer 1 in Figure 2(b). This suggested that the deamination products from InsPCers 1 and 2 were not PI.

Butanol extracts containing [^3H]mannose-labelled lipids were subjected to mild alkaline hydrolysis and the lipid products were re-extracted into butanol. TLC analysis of these products showed that InsPCer 1 and InsPCer 2 were unchanged and therefore resistant to the mild alkali. This result suggested that InsPCers 1 and 2 have sphingolipid rather than the more typical [2] glycerophospholipid anchoring moieties.

[^3H]Myristic acid-labelled InsPCers 1 and 2 were individually hydrolysed with 2 M methanolic HCl. TLC of the FAME-containing hexane extract (Figures 3a and 3c) showed that InsPCers 1 and 2 contained only normal fatty acids, lacking the

Figure 4 Mass spectra of the free LCB and its derivative

(a) CI mass spectrum of the free LCB recovered from the acid hydrolysate of InsPCer 1. (b) CI mass spectrum of the *N*-acetyl-*O*-TMS derivative of the LCB recovered from the acid hydrolysate of InsPCer 2. (c) EI mass spectrum obtained from the major GC peak of *N*-acetyl-*O*-TMS derivative of InsPCer 1. (d) EI mass spectrum of the *O*-TMS derivative of the gpi 23-derived ceramide.

α -hydroxy-fatty acids which were found in the ceramide-aminoethylphosphonates (ceramide-AEPs) of *T. mimbres* [18]. The residual aqueous phase was adjusted to pH 11 and extracted with diethyl ether to remove LCBs. This extract was chromatographed alongside sphingosine, sphinganine and LCB prepared from bovine brain cerebroside using TLC systems E and F. In the *T. mimbres* diethyl ether extract, there was only one ninhydrin-positive and [^3H]myristic acid-labelled spot, and that co-migrated with 3-*O*-methyl sphinganine on the TLC plate (Figures 3b and 3d).

Confirmation of the InsPCer 1 and InsPCer 2 fatty acid and LCB composition

The LCB preparation recovered after strong acid hydrolysis and *N*-acetyl-*O*-TMS derivatives prepared from those LCBs were analysed by MS using a chemical-ionization (CI)-positive mode. The major sphingoid base of the crude extract was a molecule of molecular mass [315 + H] (Figure 4a), as would be expected for C_{19} -sphinganine. However, the mass spectrum of the *N*-acetyl-*O*-TMS derivative (Figure 4b) showed a molecular ion of [429 + H], indicating that the major LCB only had one free hydroxy group [315 - 2H + CH_3CO + TMS + H]. This raised the possibility that the LCB was 3-*O*-methylsphinganine. When the *N*-acetyl-*O*-TMS derivative was further analysed by GC-MS, the major electron-impact-ionization fragments were 430 ($M + 1$), 414 ($M - 15$), 340 ($M - 89$), 326 ($M - 103$), and 174 [$^+\text{CHNH}(\text{CH}_3\text{CO})\text{CH}_2\text{OTMS}$] (Figure 4c), in agreement with the structure of 3-*O*-methylsphinganine.

3-*O*-Methylsphinganine has not, to our knowledge, been found in nature, but minor amounts of 3-*O*-methyl derivatives have been reported following strong acid or base hydrolysis [21]. To examine whether the 3-*O*-methylsphinganine was an artifact of our strong acid hydrolysis procedure, we hydrolysed InsPCer 1 and InsPCer 2 in fully deuteriated methanol and analysed the resulting LCB extract by MS (CI $^+$ mode). The molecular ion was the same as obtained after the acid hydrolysis in ordinary methanol, namely 316, and there was no indication of the 319 ion expected if a deuteriated methyl group had been added. In further tests we hydrolysed a purified sample (see accompanying report [16]) of the *T. mimbres* GPI-anchored protein gpi 23, which is anchored to the membrane by InsPCer, in ethanolic HCl instead of the usual methanolic HCl. Although the protein had never been exposed to methanol during purification or hydrolysis, it was shown by MS to contain a 3-*O*-methyl group rather than a 3-*O*-ethyl group. Fatty acids were recovered from the reaction entirely as ethyl esters. As a further test of the 3-*O*-methyl group's origin, purified gpi 23 from 28 $^{\circ}\text{C}$ -grown *T. mimbres* was hydrolysed by PI-PLC to yield ceramide, and its *O*-TMS derivative was analysed by CI $^+$ MS. The spectrum indicated the presence of the 1-*O*-TMS derivative of *N*-stearoyl-3-*O*-methylsphinganine ($[M + 1]^+ = 654$) plus some underivatized *N*-stearoyl-3-*O*-methylsphinganine ($[M + 1]^+ = 582$) (Figure 4d). The major ions observed in a spectrum of a standard TMS-derivatized *N*-palmitoylsphinganine from bovine brain were 684 and 612, representing the 1,3-bis-*O*-TMS and 1-*O*-TMS derivatives respectively (results not shown). Thus all tests point to the natural presence of a 3-*O*-methyl group on the LCB moieties of both the InsPCers and the GPI-anchored proteins.

Temperature-induced change in the InsPCers 1 and 2 lipid composition

Butanol extracts of cells growing at 15 $^{\circ}\text{C}$, 28 $^{\circ}\text{C}$, and 38 $^{\circ}\text{C}$ all contained InsPCers 1 and 2. The amide-linked fatty acid com-

Table 1 InsPCer fatty acid composition

Amide-linked fatty acids of *Tetrahymena* InsPCer 1 and InsPCer 2 from cells grown at the temperature shown. InsPCers were isolated and their FAMES were analysed by GC as described under the Experimental section. The values are expressed as average percentages of total peak area $\pm$ S.D. from three independent experiments.

Fatty acids	InsPCer 1 (%)			InsPCer 2 (%)		
	15 °C	28 °C	38 °C	15 °C	28 °C	38 °C
14:0	2.5 $\pm$ 1.7	0.4 $\pm$ 0.2	0.5 $\pm$ 0.1	3.5 $\pm$ 1.5	1.1 $\pm$ 0.6	1.2 $\pm$ 0.2
15:0	0.1 $\pm$ 0.0	0.9 $\pm$ 0.0	0.1 $\pm$ 0.1	0.4 $\pm$ 0.3	0.9 $\pm$ 0.4	0.4 $\pm$ 0.1
16:0	95.2 $\pm$ 2.2	78.9 $\pm$ 0.5	28.6 $\pm$ 3.1	92.6 $\pm$ 2.7	79.0 $\pm$ 3.1	31.4 $\pm$ 4.0
17:0	0.5 $\pm$ 0.2	7.3 $\pm$ 0.1	1.8 $\pm$ 0.2	0.7 $\pm$ 0.5	5.1 $\pm$ 1.5	2.3 $\pm$ 1.2
18:0	1.7 $\pm$ 0.8	11.3 $\pm$ 0.3	64.7 $\pm$ 2.2	3.0 $\pm$ 2.1	12.3 $\pm$ 2.3	61.1 $\pm$ 3.9
19:0	Trace	0.9 $\pm$ 0.0	2.4 $\pm$ 0.5	Trace	1.2 $\pm$ 0.8	2.1 $\pm$ 0.4
20:0	Trace	0.5 $\pm$ 0.1	2.0 $\pm$ 0.2	Trace	0.5 $\pm$ 0.0	1.9 $\pm$ 0.2

Trace indicates less than 0.1%.

positions of the two PI glycans are shown in Table 1. We confirmed that all the fatty acids were saturated by observing an identical GC pattern after catalytic hydrogenation of the FAMES. InsPCers 1 and 2 from the same cells always had the same fatty acid composition, with the major species being palmitic acid (16:0) and stearic acid (18:0). However, the ratios of the two fatty acids changed dramatically with growth temperature. The major fatty acid of both InsPCer 1 and InsPCer 2 from 15 °C-grown cells was palmitic acid (96%), but the major fatty acid from 38 °C-grown cells was stearic acid (64%). Thus *T. mimbres* produced protein anchor precursors with longer fatty acids after acclimatizing to high-temperature conditions. The analyses reported in Table 1 were done on material from growing cells. However, analyses were also performed on InsPCers from cells which had been starved for 24 h, thereby corresponding to conditions used in isolating GPI-anchored proteins (see [16]). There were no significant differences in InsPCers 1 and 2 fatty acid composition from growing cells and starved cells.

The major LCB of InsPCers 1 and 2 from cells grown at different temperatures was always 3-O-methylsphinganine plus very small amounts of other LCBs. The composition of the LCB did not change significantly with culture temperature.

DISCUSSION

Inositol-containing phosphoglycolipids have now been identified as trace components of many eukaryotic organisms [2]. Thus far, the most prevalent of these lipids have phosphoacylglycerols as their principal hydrophobic moiety, but there have been increasingly numerous reports of sphingolipid-based inositol phospholipids [22]. Representatives of the latter class, termed inositolphosphorylceramides (InsPCers), have been reported from animals as complex as *Aplysia* [23], but are more frequently encountered in fungi and protozoa [22]. Although there are exceptions [24,25], the fungal InsPCers usually contain phytosphingosine LCB derivatives while protozoa have sphingosine bases [22].

The InsPCers that we have described from *T. mimbres* are generally similar in their glycosyl moiety to a number of previously described precursors of GPI-anchored proteins, yet some features of the carbohydrate linkages are atypical. Thus the head group contains a phosphorylated Man α 1-2Man α 1-3Man α 1-4GlcN α 1- glycan attached to the inositol-containing

phospholipid [15]. The 3-substituted mannose differs from the 6-substituted mannose found typically in protein-linked GPI chains and their precursor glycolipids of *Trypanosoma* [26] but resembles that found in *Leishmania* glycolipids [27]. Conversely, the terminal Man α 1-2Man α 1- is common to the protein-linked GPI chains and the glycolipids of *Trypanosoma*.

In our previous work with the lipid moiety of *T. mimbres*, we measured the incorporation of radiolabelled myristate and inositol into material that could be extracted from delipidated proteins by chloroform/methanol/water (10:10:3) [13]. The InsPCer 1 and InsPCer 2 described in the present report correspond precisely to those previously described materials. The original partial characterization of these compounds was made by phospholipase C treatment and chemical degradation of total chloroform/methanol/water (10:10:3) extracts. Products identified as inositol, palmitic and stearic acids, and a non-polar lipid tentatively identified as alkyl glyceryl ether, were recovered. The additional analyses of these lipids reported here involved the individual InsPCer 1 and InsPCer 2 further purified by elution from TLC plates. This not only permitted us to compare the two components but also eliminated certain other polar lipids which were in some cases retained in the protein residue after delipidation and therefore included in chloroform/methanol/water (10:10:3) extracts. These latter impurities may have given rise to the alkyl glyceryl ethers reported earlier [13], but not confirmed here.

Chromatographic and mass spectral analyses establish that the lipid anchors of InsPCers 1 and 2 are ceramides. Determination of the LCB and fatty acid composition of the two InsPCers indicates that the lipid moieties of these compounds are nearly identical with respect to their hydrocarbon chain length distributions. Since the presence of inositol and phosphorus in each InsPCer was confirmed in our previous study [13], the complete structures of the anchoring lipids are established. At the present time we do not know how InsPCer 1 and InsPCer 2 differ from each other structurally. There may be additional attachments on the glycan chain which have not yet been identified.

In their detailed composition, the InsPCers are surprisingly distinct from the assemblage of ceramide-AEPs which co-exist with them in much greater abundance [18]. Although our analyses of cellular levels of the sphingolipid classes have not been strictly quantitative, rough estimates suggest that there are about 1 nmol of GPI-anchored proteins, approximately 0.03 nmol of InsPCers, and about 3 nmol of ceramide-AEP per 10⁶ cells. The ceramide-AEPs have non-O-methylated d16:1, d17:1 and d19:1 as their principal bases rather than the O-methylated d18:0 base predominating in the InsPCers. The ceramide-AEP fatty acids are mainly normal or hydroxy 16:0 and 17:0 in comparison with 16:0 and 18:0 in the InsPCers. Apart from the common feature of having an LCB component, there is no indication of a biosynthetic relationship between the two lipid classes.

3-O-Methyl-LCBs have been reported for years [28] and are generally assumed to arise entirely as minor artifacts of methanolic hydrolysis. It was therefore unexpected that the LCB derived from the InsPCers and the InsPCer-containing proteins [16] were all methylated at the 3 position. Initially we also assumed that this methyl group was an artifact, although hydrolysis of other sphingolipids such as brain cerebroside and ceramides and *T. mimbres* ceramide-AEPs yielded only traces of material having the same TLC mobility. The following data now strongly suggest that the 3-O-methyl group of the InsPCers exists as a natural constituent of this lipid: (1) TLC of InsPCer acid or base hydrolysates in different solvent systems showed the presence of the 3-O-methyl LCB and no trace of conventional LCBs; (2) acid hydrolysis of InsPCers using fully deuteriated methanol

as solvent did not yield LCB containing a deuterated 3-O-methyl substituent; (3) hydrolysis of the *T. mimbres* GPI-anchored proteins using ethanolic HCl yielded the same 3-O-methyl LCB as obtained before with no trace of 3-O-ethyl LCB; (4) CI-MS of the *O*-TMS derivative of ceramide obtained by PI-PLC cleavage of purified gpi 23 revealed the presence of a ceramide containing a 3-O-methyl LCB. Taken together, these analyses show conclusively that the precursor InsPCers and the completed GPI-anchored proteins contain this unique 3-O-methyl LCB.

The *O*-methylation of these LCBs (but not the other LCB-containing lipids of *T. mimbres*) may influence their metabolic fate. Normal LCB catabolism involves cleavage between positions 2 and 3 of the carbon chain to yield ethanolamine phosphate and a fatty aldehyde [29]. Methylation of the hydroxy group at position 3 could well interfere with the cleavage reaction.

The differences between ceramide-AEP and InsPCer metabolism are accentuated by their disparate responses to temperature change. *T. mimbres*, shifted from 15 °C to 39 °C, sustained a decrease in d16:1 and d17:1 and a rise in d19:1 LCB species in ceramide-AEP [18], while the InsPCers experienced no significant changes. The ceramide-AEP fatty acid chain length was not altered at the higher growth temperature, but the principal fatty acids (16:0 and 17:0) were largely converted into their α -hydroxy analogues. No hydroxy fatty acids were detected in 38 °C-grown *T. mimbres* InsPCers.

The metabolic pool which most resembles the InsPCer fatty acid composition is that containing the cell's free fatty acids. This pool amounts to less than 1 % of the cell's ester-bound fatty acids and is composed largely of 16:0 and 18:0 fatty acids [30]. Here, too, increasing growth temperature forced a drop in the ratio of palmitic acid/stearic acid from 2.2 in 15 °C-grown cells to 1.7 in 38 °C-grown cells. The remainder of the cellular lipids are predominantly glycerophospholipids, which are highly enriched in polyunsaturated fatty acids and respond very differently to temperature change [31].

The structure of the *T. mimbres* InsPCer lipid moiety and its environmentally induced changes closely resemble those described in the companion report concerning lipid anchors of *T. mimbres* GPI-anchored proteins [16]. Our previous ³H-precursor pulse-labelling studies [14] also indicated a metabolic connection. Thus the pattern of increased InsPCer labelling over a period of about 6 h was distinctive (membrane phospholipids reach constant specific radioactivity within minutes [32]) and was followed by a steady decline to barely measurable levels by 24 h. Radioactivity first appeared in the GPI-anchored proteins only during the period of InsPCer decline. Those findings, taken together with the relatively small InsPCer pool size, compared with that of glycoinositol phospholipids found in parasitic protozoa [33], and the close structural similarity between component lipids of these two classes, as reported here, strongly suggest that the *T. mimbres* InsPCers function mainly as precursors of GPI-anchored proteins. However, identification of the atypical [15] glycan linkage of the InsPCers in the mature GPI-anchored proteins will be necessary to confirm the precursor-product relationship. At the present time we cannot eliminate the possibility that some of the InsPCers are metabolic end-products

having non-precursor functions of their own. Such mixtures have been reported in *Leishmania* [34]. Meanwhile, further studies of regulatory mechanisms responsible for the observed specificity in sphingolipid synthesis, utilization and modification are in progress.

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