Temperature regulation of the *Tetrahymena mimbres* glycosylphosphatidylinositol-anchored protein lipid composition

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By incubating *Tetrahymena mimbres* cells with [³H]myristic acid, [³H]ethanolamine, [³H]inositol, and [³H]mannose, proteins having apparent molecular masses of 23 and 63 kDa were identified as the cells' principal glycosylphosphatidylinositol (GPI)-anchored proteins. These proteins accounted for as much as 2–5 % of the whole cell proteins, with the higher levels being recovered from non-growing cells. The two proteins, gpi 23 and gpi 63, were purified to near homogeneity through Triton X-114/water partitioning followed by preparative SDS/PAGE. The lipid components of the GPI anchors were determined by chemical and enzymic hydrolysis. Both proteins were anchored by ceramides, with the principal long-chain base being C₁₈ sphinganine containing an O-methyl group at the 3 position. O-Methylation was shown not to be an artifact of hydrolysis. When *T. mimbres* was cultured at 15 °C, the ceramide fatty acid

component of the GPI anchors was principally palmitic acid (75 % in gpi 23 and 76 % in gpi 63). GPI anchors from 28 °C-grown cells contained mainly stearic acid (79 % in gpi 23 and 70 % in gpi 63). Temperature change had little effect on the long-chain-base composition. The direction of temperature-induced lipid change in the protein-bound anchors was the same as found in the inositolphosphorylceramide putative precursors of the protein anchors described in the accompanying paper [Hung, Ko and Thompson (1995) *Biochem. J.* **307**, 107–113], but the detailed fatty acid compositions of the precursors and the protein-bound lipids were quite different. The precise metabolic regulation of anchor lipid chain length supports the concept that composition of the lipid anchor is important in the function and/or metabolism of the anchored protein.

INTRODUCTION

More than 100 species of eukaryotic cell proteins are known to contain glycosylphosphatidylinositol (GPI) anchors linking them to the external face of the plasma membrane [1]. The typical GPI-anchored protein is joined at its C-terminus to phosphoryl-ethanolamine, which is in turn linked through a chain of three mannose residues, glucosamine, and finally phosphatidylinositol (PI) to the plasma membrane. Just what functional advantages this non-covalent yet tenacious mechanism of membrane attachment may confer is a matter of considerable interest.

Many structural variations of the typical anchor structure have been reported. In some cells additional sugars or phospho-ethanolamine moieties are attached to the glycan as side branches, and the inositol ring may contain an esterified long-chain fatty acid [2]. The lipid terminating many GPI-anchors contains a diacylglycerol moiety, but it is not uncommon to find an alkylacyl glycerol instead [2], and in a few cases ceramide structures have been identified in this position [3,4]. It has been proposed that the precise nature of the terminal lipid portion of the GPI anchor may be critical in promoting lipid–lipid and lipid–protein interactions [5] and determining the glycan's susceptibility to cleavage by endogenous phospholipases [6], but the physiological significance of these alternative structural features is not clear.

Ryals et al. [7] identified GPI-anchored proteins in *Tetrahymena mimbres* on the basis of radiolabelled precursor incorporation studies. PI-glycan intermediates were isolated and partially characterized, and [³H]myristate pulse-labelled PI

glycans were shown to lose radioactivity with time as the radiolabel appeared in the GPI-anchored proteins [8]. The glycan structure was subsequently shown to contain features unique to *Tetrahymena* [9], but a detailed structure of the lipid anchor was not determined.

Recent studies [10] have established that GPI-anchored proteins of *Tetrahymena* belong to a much-studied family of surface glycoproteins designated as immobilization antigens [11]. These proteins vary in molecular mass from one cell strain to another [12]. Some resemble, and may be identical to, proteins involved in cell–cell interactions such as sexual conjugation [13]. In view of the potential importance of these surface proteins, we describe in this paper a more detailed analysis of the lipid moieties anchoring them to the membrane. We also report for the first time a significant difference in the composition of the terminal lipid anchor of these proteins isolated from cells grown at high and low temperature. In the accompanying paper [14] we describe complementary studies of the precursor PI glycans utilized by the cell in forming these GPI anchors.

EXPERIMENTAL

Materials

PI-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* was kindly provided by M. G. Low (Columbia University, New York, NY). [1-³H]Ethan-1-ol-2-amine hydrochloride (19.5 Ci/

Abbreviations used: GPI, glycosylphosphatidylinositol; pCMPSA, *p*-chloromercuriphenylsulphonic acid; FAME, fatty acid methyl ester; PI-PLC, phosphatidylinositol-specific phospholipase C; InsPCer, inositolphosphoceramide; AEP, aminoethylphosphonate; ceramide-AEP, ceramide-aminoethylphosphonate.

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mmol), [9,10-³H]myristic acid (22.4 Ci/mmol), *myo*-[2-³H]-inositol (17.7 Ci/mmol) and [³H]mannose (23.0 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL, U.S.A.).

Cell culture and harvesting

Cultures of *T. mimbres* were grown with shaking at 28 °C in 500-ml Erlenmeyer flasks containing 200 ml of sterile enriched proteose peptone medium [15]. Stocks were transferred to fresh medium every 48 h. For each experiment, mid-logarithmic-phase cells [(2–4) × 10⁵ cells/ml] or stationary-phase cells [(5–6) × 10⁵ cells/ml] were used from cultures grown at 15 °C, 28 °C, or 38 °C. Unless otherwise stated, the cells were washed twice with inorganic medium (47 mM NaCl, 1 mM KH₂PO₄, 4 mM K₂HPO₄, 1 mM MgSO₄), resuspended in inorganic medium to give a density of 8 × 10⁵ cells/ml, and incubated for 24 h on a rotary shaker before harvesting. Cell density was monitored using a Coulter counter model ZB. Cells were harvested by centrifugation for 5 min at 400 *g* after the cultures were rapidly chilled to 3–5 °C in a solid CO₂-acetone mixture.

In vivo labelling

T. mimbres was grown to a density of (2–4) × 10⁵ cells/ml at 28 °C in enriched medium. Before labelling cells with [³H]myristic acid, they were washed twice with inorganic medium at room temperature and then resuspended in inorganic medium to give a density of 4.5 × 10⁵ cells/ml. Aliquots (20 ml) were preincubated for 15 min in 50-ml Erlenmeyer flasks before adding 500 µCi of [9,10-³H]myristic acid in 20 µl of ethanol. The cells were then incubated at 28 °C for 24 h. For labelling with [1-³H]ethan-1-ol-2-amine hydrochloride, the density of the twice-washed cells was brought to 8 × 10⁵ cells/ml in inorganic medium, and 20 ml of cells were preincubated for 15 min in a scintillation vial at 28 °C. After adding 330 µCi of [³H]ethanolamine, the cells were incubated at 28 °C for 24 h. Aliquots of 1.6 × 10⁶ cells to be labelled with [³H]mannose or *myo*-[³H]inositol were washed twice with inorganic medium, resuspended in 2 ml of that medium and incubated with the radiotracer (40 µCi) for 24 h.

Protein extraction

A labelled cell culture (9 × 10⁶ cells) was rapidly chilled in a mixture of acetone and solid CO₂ and washed twice with cold inorganic medium by centrifugation for 5 min at 400 *g*. Cell pellets were immediately treated with 2 ml of hot SDS buffer [2 mM SDS, 22 % glycerol, 1 mM *p*-chloromercuriphenylsulphonic acid (pCMPSA), 100 mM Tris/HCl, pH 6.8] and held at 100 °C for 5 min. After chilling the sample, adding fresh pCMPSA to a final concentration of 2 mM, and centrifuging at 18000 *g* for 10 min, the clear supernatant was recovered, assayed for protein by the bicinchoninic acid method [16] and stored at –70 °C.

Triton X-114 partition and purification of GPI-anchored proteins

Four batches of cells (6.4 × 10⁸) incubated in inorganic medium for 24 h were harvested, incubated on a magnetic stirrer with 140 ml of lysis buffer (150 mM NaCl, 1 mM pCMPSA, 2 % Triton X-114, and 10 mM Tris/HCl, pH 7.4) at 0 °C for 1 h, and then centrifuged (8800 *g*, 0 °C, 10 min). An efficient purification of GPI-anchored proteins present in the 8800 *g* supernatant required that it be stored for at least 1 day at –20 °C [17]. Meanwhile, the pellet was resuspended in buffer A (150 mM

NaCl, 1 mM pCMPSA, 0.06 % Triton X-114, 10 mM Tris/HCl, pH 7.4) and recentrifuged (8800 *g*, 0 °C, 10 min). The final pellet was treated with hot SDS buffer containing 1 mM pCMPSA and stored at –70 °C.

After storage at –20 °C, the supernatant from the first centrifugation was used for Triton X-114 partitioning by the Hooper and Bashir method [18], as modified in our laboratory [17]. Briefly, in this modification the detergent phase obtained following two sequential partitions at 32 °C was chilled, incubated at 0 °C for 10 min, and then centrifuged at 18000 *g* for 10 min at 0 °C, yielding a sizeable pellet. The pellet was set aside and the GPI-anchored protein-containing supernatant was partitioned once again at 32 °C. Proteins were precipitated from the final detergent phase with cold acetone.

Removal of non-covalently-bound labelled lipid from the protein precipitated from the final detergent phase was achieved by washing it with chloroform/methanol (2:1, v/v), chloroform/methanol (1:1, v/v) and chloroform/methanol/water (10:10:3, by vol.). The proteins were resolubilized with SDS-buffer.

Delipidated proteins (6 mg) from the final detergent phase were loaded on to a Model 491 Prep-Cell (Bio-Rad, Hercules, CA, U.S.A.) containing a 12.2 % polyacrylamide gel in the case of gpi 23 and a 6.4 % polyacrylamide gel in the case of gpi 63. After elution as described elsewhere [17], proteins from the pooled fractions containing gpi 63 or gpi 23 were concentrated by Triton X-114 partitioning, after adding trichloroacetic acid equivalent to a final concentration of 10 %, and washing the precipitated proteins twice with cold acetone to remove Triton X-114.

Inositol analysis

[³H]Inositol-labelled and purified gpi 23 (10000 d.p.m.) was hydrolysed with 0.6 ml of 6 M HCl at 100 °C for 48 h. The hydrolysate was dried repeatedly under a stream of nitrogen gas to remove HCl, resuspended in 4 µl of water, and analysed by paper chromatography with the solvent system ethyl acetate/pyridine/water (5:2:5, by vol.) [19]. The paper (Whatman #1) was cut into 3 cm strips and counted in a Packard Tricarb Model 300 scintillation counter (Packard Inst. Co., Downers Grove, IL, U.S.A.).

Treatment of the GPI-anchored proteins with phospholipase C, nitrous acid, mild alkali and strong acid

Deamination of partially purified and delipidated GPI-anchored proteins (100 µg) was carried out in 200 µl of 0.25 M sodium acetate/0.25 M NaNO₂, pH 4.0. Control samples were incubated in 0.25 M sodium acetate/0.25 M NaCl, pH 4.0. The samples were mixed vigorously and kept at room temperature for 4 h. Triton X-114 was added to the reaction vials in a final concentration of 2 % and a single Triton X-114 partition was carried out as described above. The proteins from both the aqueous and the detergent phases were collected with cold acetone.

For PI-PLC treatment, partially purified and delipidated GPI-anchored proteins (100 µg) were treated with 3.4 units of PI-PLC in 100 µl of 25 mM Hepes, pH 7.2, 0.1 % sodium deoxycholate at 37 °C for 3 h. A single Triton X-114 partition and a protein recovery were carried out as described above.

For mild base treatment, [³H]myristic acid-labelled and purified gpi 23 or gpi 63 (50 µg) was completely dried and then resuspended by sonication in 500 µl of 30 % NaOH/methanol (1:1, v/v) or in 500 µl of water/methanol (1:1, v/v) as a control. The samples were incubated at 37 °C for 4 h and then adjusted

to a pH of 7.4 with acetic acid. Hexane extraction was carried out three times.

For strong acid treatment of gpi 63 (3 mg) or gpi 23 (1.5 mg), the proteins were completely dried in a Speed-Vac and sonicated after adding 1 ml of 2 M HCl in methanol. The sample was incubated at 75 °C for 18 h. Fatty acid methyl esters (FAMES) were extracted three times with 1 ml of hexane. The pH of the final lower phase, after extracting FAMES, was increased to 11.0 with NaOH, and long-chain bases (LCBs) were extracted three times with 1 ml of diethyl ether. FAMES and LCBs were analysed by TLC with hexane/ether (17:2, v/v) or chloroform/methanol/2 M NH_4OH (40:10:1, by vol.) as solvent systems. Radioactivity was quantified by scraping 0.5 cm bands of silica gel into vials and counting by scintillation counter.

Gas-liquid chromatography (GLC), gas chromatography-mass spectrometry (GC-MS), and chemical-ionization mass spectrometry (CI-MS)

FAME and LCB prepared from gpi 63 and gpi 23 were analysed by GC and MS as described in the companion paper [14].

Electrophoresis, gel slicing and autoradiography

Samples containing 100 μg of protein were electrophoresed alongside authentic molecular-mass standards (Bio-Rad, Richmond, CA, U.S.A.) on a 12% polyacrylamide discontinuous slab gel (160 mm $\times$ 130 mm $\times$ 1.5 mm) at a constant 30 mA current according to Laemmli [20]. Following electrophoresis, gels were stained with 0.05% Coomassie Brilliant Blue R-250 in methanol/acetic acid/water (5:1:4, by vol.). After destaining, selected lanes of the Coomassie Blue-stained gel were excised, frozen with solid CO_2 and cut transversely into 1-mm-thick slices with a gel slicer (Joyce Loeb, Burlington, MA, U.S.A.). Each slice was incubated in a scintillation vial with 0.3 ml of 30% hydrogen peroxide at 50 °C for 24 h before adding 10 ml of scintillation cocktail (Bio Safe II, Research Product International Corp., Mt. Prospect, IL, U.S.A.) and analysing in a Packard Tricarb Model 300 scintillation counter (Packard Inst. Co., Downers Grove, IL, U.S.A.). Alternatively, the destained gel was treated with autoradiography enhancer (Resolution, EM Corp, Chestnut Hill, MA, U.S.A.) according to the manufacturer's instructions, dried, and placed next to hypersensitized Kodak X-Omat AR X-ray film at -70 °C for up to 20 days in order to prepare fluorograms.

RESULTS

Two proteins were strongly labelled with [^3H]myristic acid, [^3H]ethanolamine, *myo*-[^3H]inositol and [^3H]mannose

In order to identify all putative GPI-anchored proteins of *T. mimbres*, cells in inorganic medium were incubated in the presence of [^3H]myristic acid, [^3H]ethanolamine, *myo*-[^3H]inositol, or [^3H]mannose for 24 h. SDS/PAGE gels containing whole cell proteins extracted with SDS buffer (100 mM Tris/HCl, pH 6.8, 2 mM SDS, 22% glycerol, 1 mM pCMPSA) revealed that only two proteins of molecular masses 63 kDa and 23 kDa were labelled by all three radiolabelled tracers (Figure 1). Proteins of similar molecular masses were also labelled with [^{14}C]glucosamine in previous studies [8]. These labelling patterns confirmed the previous identification of a 23 kDa GPI-anchored protein made on the basis of shorter-term labelling experiments [8] and also directed our attention to the 63 kDa protein.

Acid hydrolysis of [^{14}C]glucosamine- and [^3H]mannose-labelled *Tetrahymena* PI glycans previously showed that radio-

Figure 1 Distribution of radioactivity in 1-mm slices of SDS/PAGE gels containing *T. mimbres* proteins labelled *in vivo* with (a) [^3H]ethanolamine, (b) [^3H]myristic acid, (c) *myo*-[^3H]inositol and (d) [^3H]mannose and then solubilized with SDS buffer

Each gel lane contained 100 μg of protein.

activity was recovered only in glucosamine and mannose respectively [7]. To confirm that the key precursor [^3H]inositol was incorporated intact into the GPI-anchored proteins, [^3H]inositol-labelled (10000 d.p.m.) gpi 23 purified by preparative electrophoresis (see below) was hydrolysed with 6 M HCl, and the aqueous products were analysed by paper chromatography. Radioactivity was present only in the same position occupied by an authentic *myo*-inositol standard.

Triton X-114 partitioning of two GPI-anchored proteins

Some GPI-anchored proteins selectively enter the Triton-enriched phase during Triton X-114/water partitioning [18]. Partial purification of GPI-anchored proteins from crude *Tetrahymena* proteins was achieved by Triton X-114 partitioning as modified [17] from Hooper and Bashir [18] (see the Experimental section for details). When [^3H]myristic acid-labelled *Tetrahymena* proteins were extracted with lysis buffer, partitioned between Triton X-114 and water, and resolved by SDS/PAGE, radioactivity corresponding with 63 kDa and 23 kDa proteins was found almost exclusively in the detergent-rich supernatant, indicating that these two proteins were solubilized by Triton X-114 (Figure 2). In order to achieve the best resolution of

Figure 2 Distribution of radioactivity in fractions recovered during gpi 23 and gpi 63 purification from [^3H]myristic acid-labelled cells

(a) Insoluble fraction after initial Triton X-114 extraction (100 μg); (b) aqueous and detergent phases after initial Triton X-114/water partition of Triton X-114-solubilized proteins (100 μg , respectively); (c) pellet (100 μg) and supernatant (30 μg) following 18000 g centrifugation; (d) purified gpi 23 (10 μg); (e) purified gpi 63 (10 μg).

GPI-anchored proteins, it was important to store the original Triton X-114-solubilized protein mixture at -20°C for 1 day before carrying out the phase partitioning [17]. Figure 3 (lanes 1–4), shows an SDS/PAGE resolution of the fractions.

From the final detergent-phase supernatant, gpi 63 and gpi 23 were further purified by preparative electrophoresis after delipidation with chloroform/methanol (2:1, v/v), chloroform/methanol (1:1, v/v) and then chloroform/methanol/water (10:10:3, by vol.). The SDS/PAGE patterns of protein and radioactivity distribution are illustrated in Figures 2 and 3.

Figure 3 SDS/PAGE gel illustrating fractions obtained during gpi 23 and gpi 63 purification

Lane 1, insoluble fraction after initial Triton X-114 extraction (100 μg); lane 2, aqueous phase after initial Triton X-114/water partition of soluble proteins (100 μg); lane 3, pellet that was discarded following 18000 g centrifugation of detergent Triton X-114 phase (100 μg); lane 4, GPI-anchored protein mixture in final detergent phase (100 μg); lane 5, gpi 23 purified by preparative gel electrophoresis (10 μg); lane 6, gpi 63 purified by preparative gel electrophoresis (10 μg). Molecular masses (M) are shown.

Quiescent cells contain more GPI-anchored proteins than do proliferating cells

Cells incubated in enriched medium, or kept for 24 h in inorganic medium, were lysed in lysis buffer and submitted to Triton X-114 extraction and purification of GPI-anchored proteins in order to determine the absolute level of GPI-anchored proteins present. Interestingly, 5% of proteins from nutrient-depleted cells and 2% of proteins from proliferating cells were recovered in the form of gpi 23 plus gpi 63. There were no electrophoretic differences between the protein patterns in the final detergent phase of proliferating and quiescent cells. Therefore, the non-dividing cells contained almost three times more of the same GPI-anchored proteins than did rapidly proliferating cells. The content of GPI-anchored proteins was also dependent upon the phase of cell growth. Preparations taken from logarithmic-phase cells had approximately twice as much gpi 23 as gpi 63, but in the higher density stationary-phase cells the gpi 63 content had increased to approximately the same level as gpi 23.

Release of the lipid anchor from gpi 63 and gpi 23

When GPI-anchored proteins were partially purified from whole cell extracts by Triton X-114 partitioning and were delipidated, treated with PI-PLC, and again partitioned between Triton X-114 and water, most of the proteins previously associated with Triton X-114 were now recovered in the aqueous phase (Figure 4). These included the 63 kDa protein and a protein of apparent molecular mass 25 kDa derived from gpi 23. In addition, less abundant proteins of 26 and 44 kDa, as well as the added PI-PLC (33 kDa), were also detected in the aqueous phase. The minor components were clearly not GPI-anchored proteins because they were not labelled by radioactive precursors of the GPI anchor (Figure 1). However, these proteins may normally be

Figure 4 Coomassie Blue-stained SDS/PAGE gel of aqueous-phase and detergent-phase proteins following Triton X-114/water partition of control (left side) and PI-PLC-treated (right side) *T. mimbres* GPI-anchored proteins which had been partially purified and delipidated

The band at approximately 33 kDa is PI-PLC. The very slightly higher mobility of the 63, 44, and 26 kDa bands in the control sample versus the PI-PLC sample is an artifact of loading. Molecular masses (*M*) are shown.

Figure 5 Radioactive products from methanolic HCl hydrolysis of *T. mimbres* GPI-anchored proteins

Distribution of LCB (a) and FAME (b) derived from gpi 23, and LCB (c) and FAME (d) derived from gpi 63.

associated with gpi 23 and 63 as a high-molecular-mass complex because they, and the two GPI-anchored proteins, were all eluted in the void volume when the Triton X-114-purified product

Figure 6 EI mass spectrum obtained from major GC peak of *N*-acetyl-*O*-TMS derivative of gpi 23 LCB moiety

mixture was passed through a gel-filtration column having an exclusion limit of 1500 kDa (results not shown).

These 63 kDa and 25 kDa proteins also appeared in the aqueous phase after treating partially purified protein with nitrous acid (results not shown). On the other hand, when [^3H]myristic acid-labelled gpi 63 and gpi 23 were individually purified and treated with mild alkali, no radiolabelled material appeared in a hexane extract. The stability of the lipid anchor to mild base hydrolysis suggested the absence of fatty acyl esters in the lipid anchor.

The two GPI-anchored proteins are anchored by ceramide derivatives

In order to characterize further the lipid moieties of gpi 63 and gpi 23, the purified [^3H]myristic acid-labelled proteins were individually hydrolysed with 2 M methanolic HCl. TLC analysis of material extracted from the acidic hydrolysates with hexane revealed the presence of radioactive FAME (Figures 5b and 5d). Re-extraction of the hydrolysate with diethyl ether after adjusting its pH to 11 with NaOH yielded LCBs migrating during TLC at a position slightly higher than d18, 4-sphinganine (Figures 5a and 5c).

GC-MS analysis of lipids from non-radioactive gpi 23 and gpi 63 acid hydrolysates (Figure 6), yielded a fragmentation pattern essentially identical to that obtained for the PI glycans as described in the companion paper [14], and identified the major LCB as 3-*O*-methylsphinganine. The 3-*O*-methyl group from sphinganine was not an artifact of methanolic hydrolysis because GC-MS analysis of LCBs from gpi 23 and gpi 63 showed the same fragmentation pattern after ethanolic hydrolysis. The fatty acids were almost exclusively palmitic and stearic acids. The relative proportions of these fatty acid components were variable, depending upon the growth temperature of the cells, as described below.

Effects of growth temperature on the gpi 23 and gpi 63 anchor lipid composition

Cells were cultured at 15 °C, 28 °C and 38 °C, and gpi 23 was isolated in pure form from each culture. There were no significant

Table 1 Amide-linked fatty acids of *Tetrahymena* gpi 23 and gpi 63 from cells at various growth temperatures

GPI-anchored proteins were purified and their fatty acid methyl esters (FAMES) were analysed by GC as described in the Experimental section. The FAME analysis was carried out twice and the two data sets showed few differences. The data are the averages of two experiments. Abbreviation: nd, not detected.

Fatty acid	gpi 23			gpi 63	
	15 °C	28 °C	38 °C	15 °C	28 °C
16:0	74.5	7.7	6.8	76.2	16.3
17:0	2.1	1.2	nd	2.6	3.6
18:0	19.7	79.4	77.1	21.2	70.0
18:1	2.0	2.5	2.2	nd	nd
19:0	nd	4.6	6.6	nd	1.4
20:0	1.9	4.7	7.4	nd	4.6

differences in the yield of gpi 23 from the three temperature regimes. The fatty acid compositions are shown in Table 1. In agreement with the dramatic temperature-induced changes in inositolphosphoceramide (InsPCer) fatty acid composition [14], gpi 23 contained palmitic acid almost exclusively (75 %) at 15 °C and stearic acid (77 %) at 38 °C. Changes in LCB were minimal (data not shown).

A minimal number of lipid analyses have been carried out on gpi 63. While this protein was the most prominent GPI-anchored protein of *T. mimbres* grown at 15 °C and 28 °C, only traces of it were present in 38 °C-grown cells. At 15 °C, gpi 63 contained palmitic acid almost exclusively (76 %), while at 28 °C the palmitic acid level had declined to 16 % in favour of stearic acid (Table 1). Although the directions of change in gpi 23 and gpi 63 were similar, the degrees of change were significantly different.

DISCUSSION

In the work reported here we have extended the characterization of *T. mimbres* GPI-anchored proteins begun by Ryals et al. [7] and Pak et al. [8]. The earlier characterization relied heavily on the incorporation of radioactive precursors into the lipid-anchored glycan moiety of an approximately 23 kDa protein. By employing longer periods of radiolabelling, we have identified a second major GPI-anchored protein of 63 kDa. Through the use of a modified Triton X-114 partitioning procedure to partially purify the GPI-anchored proteins, followed by preparative gel electrophoresis to resolve the individual GPI-anchored protein species, we were able to purify the two major components, gpi 23 and gpi 63 (Figures 2 and 3). Together these two proteins accounted for as much as 5 % of the cellular protein in cells that had been starved for 24 h and about 2 % in non-starved cells. Preliminary cell fractionation studies indicate that not all of the proteins were on the cell surface at any one time (C.-Y. Hung and A. G. Thompson, Jr., unpublished work).

Detailed chromatographic and MS analysis of the lipids recovered from gpi 23 and gpi 63 hydrolysates revealed that both proteins were attached to the membrane by ceramides. The detailed makeup of the ceramides was strongly affected by the cells' growth temperature. While only one LCB was present in all situations, the fatty acid composition showed significant differences (Table 1). In cells grown at 28 °C, gpi 23 contained predominantly stearic acid (79 %) with small amounts of palmitic acid (8 %). Under the same growth conditions the larger protein, gpi 63, contained a significantly larger proportion of palmitic acid (16 %).

In addition to the compositional differences between protein species co-existing within the same cell, there were also striking across-the-board effects of temperature on all the anchor lipids. In gpi 23 there was a total reversal of fatty acid distribution from having predominantly (75 %) palmitic acid at 15 °C to having an overwhelming abundance (79 %) of stearic acid at 28 °C (Table 1). In contrast, cells grown at 38 °C exhibited only a slight further increase in the average ceramide-bound fatty acid chain length.

A fairly similar pattern of fatty acid change was found in the larger gpi 63 protein when the growth temperature was lowered from 28 °C to 15 °C (Table 1). No comparison was possible using 38 °C-grown cells since gpi 63 was not expressed at that temperature.

The data in Table 1 show clearly that the most pronounced GPI-anchored protein fatty acid changes occurred between 15 °C and 28 °C. It would be interesting to know whether the alteration in ceramide fatty acid chain length is induced uniformly over that temperature range or whether changes would be more rapid as the growth temperature exceeded a certain threshold. While no systematic study of this point has begun, a single analysis of gpi 63 from cells grown at room temperature (approximately 24 °C) may be worth mentioning. The ceramide fatty acid composition (palmitic acid = 49 %, stearic acid = 48 %) was still considerably different from that determined at 28 °C, suggesting that the rate of change in lipid composition with temperature is more pronounced in the upper part of the 15 °C–28 °C range.

This is the first clear example of environmentally induced change in the lipid composition of a GPI-anchored protein. The data confirm and extend the previous demonstration by Ryals and Thompson [21] of temperature-induced fatty acid changes in uncharacterized lipid structures linked to *T. mimbres* proteins.

As yet there are no data bearing on the physical consequences that the altered fatty acid composition may impart to the GPI-anchored proteins. However, the very fact that *T. mimbres* exerts such precise control of its GPI-anchored protein lipid composition implies that there is some physiological value in doing so. There is currently much interest in learning what special advantages having a membrane anchor of this type confers upon a protein. GPI-anchored proteins would appear to have a lateral mobility that is only about 2-fold higher than that of proteins possessing transmembrane domains [22], and it is unlikely that changing lipid chain length would significantly affect this parameter [22,23]. In fact, those molecules of gpi 23 and gpi 63 located on the cell surface may be effectively immobilized by association with clusters of ceramide-AEP lipids concentrated there [23].

There is a different physical property which may be more strongly influenced by changes in lipid chain length. Peitzsch and McLaughlin [24] have found that the membrane-binding energy of a model lipid-anchored peptide increases by 0.8 kcal/mol for each carbon atom added to the lipid chain. Thus, a simple replacement of stearic acid (C₁₈) for palmitic acid (C₁₆) in a lipid anchor would add 1.6 kcal/mol to the binding energy, thereby significantly lessening the chances for desorption of that protein. At the present time there is no convincing evidence for the desorption of intact GPI-anchored proteins, but detection of such a movement would be difficult, particularly if it involved a cell-to-cell transfer. It is intriguing that gpi 63, the largest GPI-anchored protein of *T. mimbres*, disappeared from cells raised to 38 °C. Model studies have indicated that as the molecular mass of a lipid-anchored protein increases, its attachment to the membrane becomes more tenuous [25].

A possibility that seems more likely is that the strength of the anchor lipid association with the membrane lipid bilayer may

determine the susceptibility of the lipid moiety to cleavage by a phospholipase. Increased sensitivity of the GPI-anchored protein CD14 to PI-PLC was observed in Chinese hamster ovary cell mutants deficient in membrane bilayer sphingolipids [26]. A change in the anchored protein's own lipid composition might equally well result in a weaker anchorage and a greater exposure to phospholipase action.

Because of the extensive literature on responses of *Tetrahymena* lipid metabolism to environmental stress [27], *T. mimbres* is a useful model for exploring those factors regulating the lipid composition of GPI-anchored proteins and relating them to the different but very substantial changes in membrane lipid composition. Likewise, previous widespread studies of *Tetrahymena* cell-cell interactions [13] qualify the system well for the analysis of GPI-anchored protein function and ultimate disposition.

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