An apparent association between glycosylphosphatidylinositol-anchored proteins and a sphingolipid in *Tetrahymena mimbres*

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Sphingolipids are thought to stabilize glycosylphosphatidylinositol (GPI)-anchored protein-rich membrane domains of yeast and polarized higher animal cells during the processing and targeting of these proteins to the plasma membrane. A widely used criterion for identifying the stable sphingolipid- and GPI-anchored protein-enriched membrane domains is the resistance of these lipid-modified proteins to solubilization by the detergent Triton X-100 (TX-100) at low temperature. Surprisingly, there have been no reports of sphingolipid/GPI-anchored protein association in protozoans, despite the fact that these cells contain considerably higher levels of GPI-anchored proteins than does any other organism. We report here the presence in *Tetrahymena mimbres* of a significant pool of GPI-anchored proteins which resisted extraction by 1 % TX-100 at 4 °C but not at 37 °C. Of the total cellular complement of GPI-anchored proteins, which

together accounted for more than 2 % of whole-cell protein and were especially enriched in surface membranes, 10 % of the major 63 kDa component (gpi63) and 23 % of a somewhat less abundant component (gpi23) were insoluble in TX-100 at 4 °C. A substantial proportion of the cell's only abundant sphingolipid, ceramideaminoethylphosphonate (CAEP), was also insoluble in 1 % TX-100 at 4 °C. Radiolabelling studies involving [³H]leucine incorporation into proteins and [³H]palmitic acid incorporation into lipids revealed that the TX-100-resistant gpi63, gpi23 and CAEP molecules were all metabolically distinct from their TX-100-soluble counterparts in other compartments of the cell. The presence of detergent-resistant sphingolipid/GPI-anchored protein domains in non-polarized ciliate and trypanosomatid cells was probably obscured in previous studies by the profusion of accompanying detergent-soluble molecules.

INTRODUCTION

Well over 100 membrane proteins, including receptors, hydrolytic enzymes and complement factors, are attached to the ectoplasmic leaflet of the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor [1,2]. The carbohydrate and lipid structures of GPI anchors in several diverse cell types are now well understood, and it appears that the basic structure is the same in all organisms [3–6]. However, the functions of the GPI anchor, which in most cases provides the only attachment of these proteins to the cell membrane, are still uncertain and under intense study [7–9].

In polarized epithelial cells, GPI-anchored proteins are preferentially targeted to the apical plasma membrane [10,11]. Since the intracellular sorting of a GPI-anchored protein is not mediated by an amino acid motif within the polypeptide, it has been suggested that the GPI anchor itself is the sorting signal [11,12]. Sphingolipids are also transported preferentially to the cell surface, notably to the apical plasma membrane of polarized epithelial cells [13,14]. One of the striking characteristics of sphingolipids is that they are able to self-associate to form Triton X-100 (TX-100)-insoluble microdomains in the lipid bilayer. These findings led [15] to the postulation that nascent GPI-anchored proteins are incorporated into sphingolipid 'rafts' in the Golgi apparatus through an interaction between the GPI anchor and the sphingolipids. The lipid-protein rafts would then be sorted into vesicles destined for the apical plasma membrane.

These hypotheses have since been supported by observations that TX-100-insoluble membrane fragments of cells disrupted at 4 °C are highly enriched in GPI-anchored proteins and in sphingolipids [15–17]. TX-100 insolubility is now accepted by many investigators as a standard criterion for establishing the presence of tightly associated GPI-anchored protein-sphingolipid aggregates being processed for intracellular dissemination.

These hypotheses have received additional credence from studies using inhibitors of sphingolipid synthesis. When the synthesis of sphingolipids was blocked, the intracellular transport of GPI-anchored proteins was in some cases misdirected [18] and in others dramatically slowed or totally abolished, whereas the transport of secretory and transmembrane proteins was unaffected [19,20].

Although GPI-anchored proteins exist in all animal cells examined, the most abundant are those expressed on the cell surface of protozoans [21,22]. Surprisingly, the TX-100-insoluble GPI-anchored proteins-sphingolipid complex has not been reported from protozoans, and it has been assumed to be absent there [5]. However, a careful reading of the reports describing GPI-anchored proteins in protozoans [23–26] revealed that the detergent-solubility criteria necessary to test for microdomain association were not employed.

The ciliated protozoan *Tetrahymena mimbres* contains two GPI-anchored proteins accounting for 2–5 % of whole-cell protein [22]. It also contains large amounts of a single class of

Abbreviations used: AcP, acid phosphatase; GPI, glycosylphosphatidylinositol; CAEP, ceramide-2-aminoethylphosphonate; HCAEP, α -hydroxy fatty acid-containing ceramide-2-aminoethylphosphonate; LCB, long-chain base; NCAEP, non-hydroxy fatty acid-containing ceramide-2-aminoethylphosphonate; pCMPSA, *p*-chloromercuriphenylsulphonic acid; T100B, TX-100 extraction buffer; TFB, *Tetrahymena* fractionation buffer; TX-100, Triton X-100; TX-114, Triton X-114.

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sphingolipid, ceramideaminoethylphosphonate (CAEP) [27–29]. Much of the two GPI-anchored proteins and the CAEP is expressed on the cell surface [27]; X. Zhang, C.-Y. Hung and G. A. Thompson, Jr., unpublished work). The proteins appear to be members of a family of environmentally variable GPI-anchored ‘immobilization antigens’ [30], which participate in a poorly understood way in the *Tetrahymena* mating process [31]. As the first step in studying the intracellular transport of these GPI-anchored proteins and sphingolipids in *Tetrahymena*, we have searched for GPI-anchored protein–sphingolipid complexes. The data presented in this report indicate that such complexes do exist and that the targeting of the GPI-anchored proteins and sphingolipids in *Tetrahymena* has much in common with that in mammalian and yeast cells.

EXPERIMENTAL

Cell culture and harvesting

T. mimbres was cultured at 28 °C with constant rotary shaking (100 rev./min) in 500 ml Erlenmeyer flasks containing 200 ml of sterile enriched proteose peptone medium [32]. The cell density was monitored with a Coulter counter model ZB. Unless otherwise indicated, cultures in the mid-exponential growth phase [(2–4) × 10⁵ cells/ml] were used, and cells were harvested by centrifugation at 400 *g* for 5 min at room temperature.

TX-100 extraction

After centrifugation of 50 ml of cell culture (4 × 10⁵ cells/ml) (approx 24 mg of total protein), the cell pellet was set on ice and immediately resuspended to a final volume of 3 ml in ice-cold 1 % TX-100 extraction buffer (T100B) composed of 1 % TX-100, 150 mM NaCl, 5 mM EDTA, 2 mM PMSF, 2 mM *p*-chloromercuriphenylsulphonic acid (pCMPSA) and 25 mM Tris/HCl, pH 7.5. The lysed cell preparation was vortex-mixed for 5 s and set on ice for 10 min with occasional inversions. Then the 3 ml mixture was distributed equally into three Microfuge tubes and centrifuged at 15000 *g* at 4 °C for 2 min in an IECentra-M microcentrifuge (International Equipment Co., Needham Hts, MA, U.S.A.). From each tube 0.9 ml of supernatant (SUP1) was removed and combined for analysis. The loosely packed pellets (PEL1) were resuspended in the same volume of ice-cold T100B and set on ice for 5 min with occasional inversions. The suspensions were then re-centrifuged as above. The supernatants (SUP2) were combined in a separate tube, and the pellets (PEL2) were resuspended in the same volume of cold T100B and then set at 37 °C for 5 min with occasional inversions. After the 37 °C incubation, the tubes were set on ice for 2 min before being centrifuged as above. The final supernatants (SUP3) were combined, and the pellets (PEL3) were resuspended in the same volume of cold T100B. The fractions were analysed for their protein and lipid compositions. To test whether chilling cells before TX-100 extraction could cause a difference in extraction results, cells were quickly chilled to 4 °C in a solidified CO₂/acetone bath before harvesting, and processed as described above.

Electrophoresis, silver staining, Western blotting and densitometry

Electrophoresis was carried out with a Mini-PROTEAN II Electrophoresis System (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The proteins were electrophoresed in SDS/polyacrylamide (12%) gels at a constant 40 mA current as described by Laemmli [33].

To study the protein composition of cells and TX-100 extracts, gels were silver-stained. Unless otherwise indicated, the volume

of solutions mentioned below was 100 ml per gel and all of the following steps were carried out with gentle shaking. After electrophoresis, gels were sequentially soaked in water/methanol/acetic acid (63:30:7, by vol), water/methanol/acetic acid (88:5:7, by vol.) and then aq. 10 % glutaraldehyde solution, each for 30 min. Then the gels were washed with distilled water for 1.5 h with frequent water changes. The gels were then soaked in freshly prepared DL-dithiothreitol solution (0.5 mg of dithiothreitol/100 ml of distilled water) for 30 min. After this solution had been drained off, the gels were soaked in aq. 0.1 % silver nitrate for 30 min. The gels were then quickly rinsed with distilled water twice, and with developing solution (3 % Na₂CO₃ and 0.0185 % formaldehyde) twice, each time with 25 ml. After quick removal of the developing solution, the gels were placed in 50 ml of fresh developing solution until protein band intensities were appropriate. Then 2.5 ml of 2.3 M sodium citrate was added to stop the colour development.

For immunoblotting, the proteins were transferred to nitrocellulose membranes with a SemiPhor Semi-Dry transfer unit (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). The nitrocellulose membranes were then incubated with 3 % non-fat milk in PBS overnight at 4 °C. After blocking, the membranes were incubated with the appropriate mouse polyclonal antiserum in PBS with 0.1 % BSA (dilution 1:3000 for anti-gpi63 and 1:4000 for anti-gpi23) for 2 h at room temperature. After being washed with 0.05 % Tween 20 (3 × 10 min), the nitrocellulose membranes were incubated with the secondary antibody, alkaline phosphatase-conjugated goat anti-mouse IgG (whole molecule) (Sigma, St. Louis, MO, U.S.A.; dilution 1:1000 in PBS) for 1 h at room temperature. The nitrocellulose membranes were then washed with 0.05 % Tween 20 (3 × 10 min). Alkaline phosphatase activity was detected with the phosphatase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD, U.S.A.), and the reaction was stopped by rinsing the membrane with a large volume of distilled water. The band intensities were quantified with a scanner (Real-Tech 800) controlled by NIH IMAGE software. On the basis of tests with different known amounts of purified gpi63 compared with increasing increments of a gpi63-rich *Tetrahymena* protein extract, it was established that quantification by Western blotting was linear over a range of 15–80 ng of gpi63. Because no highly purified gpi23 was available, only relative measurements were possible, but these gave linear readings over a 5-fold range of phosphatase-staining intensity.

In vivo protein labelling, TX-114 partitioning and gel slicing

The cell pellet from 400 ml of cell culture was washed with 200 ml of inorganic medium composed of 47 mM NaCl, 4 mM K₂HPO₄, 1 mM KH₂PO₄ and 1 mM MgSO₄ [34] and resuspended in 100 ml of inorganic medium. After 20 min of incubation, 2 mCi of L-[4,5-³H]leucine (173 Ci/mmol; Amersham Corp., Arlington Heights, IL, U.S.A.) was added to the cell resuspension. The cells were incubated for 10 min with the radiotracer before the addition of non-radioactive L-leucine (Sigma) in organic medium to give a final concentration of 1 mM. The cells were then centrifuged, and the cell pellet was resuspended in 200 ml of the growth medium containing 1 mM unlabelled L-leucine. The culture was then evenly divided into two 200 ml Erlenmeyer flasks and cultured for an additional 0 min and 50 min respectively. After each chase period, 5 ml of the culture was centrifuged, and the pellet was treated with 200 µl of 2 % TX-114 extraction buffer (2 % TX-114, 150 mM NaCl, 2 mM PMSF, 2 mM pCMPSA and 10 mM Tris/HCl, pH 7.5) for 30 min with frequent vortex-mixing. The homogenate was then

centrifuged at 15000 *g* for 2 min at 4 °C. The supernatant was saved into a microcentrifuge tube and stored at -20 °C overnight.

The 95 ml of cells remaining from each labelled culture were extracted twice, each time with about 2 ml of 1 % TX-100 at 4 °C (see under TX-100 extractions for details). The surface of the centrifuged PEL2 pellet was carefully rinsed with 200 μ l of 25 mM Tris/HCl, pH 7.5, to remove residual TX-110, and the pellet was resuspended in 700 μ l of 1 % TX-114 extraction buffer (1 % TX-114, 150 mM NaCl, 2 mM PMSF, 2 mM pCMPSA and 10 mM Tris/HCl, pH 7.5). The mixture was incubated at 37 °C for 15 min and then set on ice for 2 min before being centrifuged at 15000 *g* for 2 min at 4 °C. The supernatant was recovered in a microcentrifuge tube. After the concentration of TX-114 had been adjusted to 2 %, the tube was stored at -20 °C overnight.

The supernatants from PEL2 and from whole cells were partitioned as described by Ko and Thompson [22]. The TX-114-phase proteins were subjected to SDS/PAGE, stained with 0.05 % Coomassie Brilliant Blue R-250 in methanol/acetic acid/water (5:1:4, by vol.), and destained with water/methanol/acetic acid (63:30:7, by vol.). The selected gel lanes were excised, frozen with solidified CO₂, and cut transversely into 1 mm-wide slices with a gel slicer (Joyce-Loebl, Burlington, MA, U.S.A.). Each slice was incubated in a scintillation vial with 0.3 ml of 30 % H₂O₂ at 50 °C for 24 h before the addition of 10 ml of scintillation cocktail (Econo-safe, Research Products International Corp., Mount Prospect, IL, U.S.A.), and analysed in a Packard Tricarb model 300 scintillation counter (Packard, Downers Grove, IL, U.S.A.).

In vivo lipid labelling and lipid quantification

[9,10-³H]Palmitic acid (15 μ Ci of a 30–60 Ci/mmol solution; American Radiolabeled Chemicals, St. Louis, MO, U.S.A.) in 15 μ l of 100 % ethanol was added to 7.5 ml of cell culture having a density of 2.5×10^5 cells/ml. The culture was then incubated for 10 min before being diluted with growth medium to 50 ml. To achieve uniform labelling of lipids, the cells were further cultured at 28 °C for 14 h (approx. four cell generations). That this time was sufficient to achieve uniform radiolabelling was established by exposing cells to [³H]palmitic acid for time periods ranging from 4 to 24 h. After lipid extraction, the distribution of radioactivity among phospholipid classes was measured on TLC plates of silica gel H made with 2.5 % potassium oxalate, using the solvent system chloroform/acetic acid/methanol/water (75:25:5:2.2, by vol.) (solvent system A). Bands identified on the plates by exposure to I₂ vapour were scraped off and assayed for radioactivity by scintillation counting. After several hours of rapidly changing ³H distribution among the polar lipid classes, the labelling pattern stabilized and remained constant from 14 h to at least 24 h, except for a slight continued hydroxylation of CAEP fatty acids (see below). This steady-state pattern matched the distribution of phospholipid mass, as estimated by densitometry of TLC plates sprayed with 50 % H₂SO₄ and charred, and independently by inspection of TLC plates stained for lipid phosphorus. The pattern was similar to that determined previously by quantitative phosphorus analysis [35].

Lipid extraction and analysis

Lipids in the TX-100 extracts and cell fractions were extracted as described by Bligh and Dyer [36], in most cases immediately after the samples were prepared. The lipids were dissolved and stored in 100 μ l of chloroform/methanol (6:1 v/v). In general, 10 μ l of the lipid extracts was used to quantify total radioactivity of the

labelled samples and the remaining 90 μ l was used to study the lipid composition by TLC.

To study possible lipolysis during fraction isolation, aliquots of SUP1 were incubated at 4 °C for 0 or 30 min, or for 30 min at 4 °C followed by a 5 min incubation at 37 °C before lipid extraction. A possible phospholipase-induced increase in non-esterified fatty acid radioactivity during SUP1 incubation was tested by TLC using the solvent system petroleum ether/ethyl ether/acetic acid (70:30:1, by vol.) (solvent system B).

To carry out strong alkaline hydrolysis, appropriate amounts of lipids were first placed in a side-arm tube and dried under a stream of N₂. The lipids were then dissolved in 1 ml of 1 M KOH in 95 % ethanol and refluxed at 60 °C for 2 h. After hydrolysis, the lipids were extracted three times with 2 ml of ethyl ether. The ethyl ether extracts were combined and washed twice with 2 ml of water. It was then evaporated under a stream of N₂. The hydrolysed lipids were dissolved in a minimum amount of chloroform and run on TLC plates alongside authentic fatty acid standards in the solvent system B. Lipids on the TLC plates were quantified as above.

In order to remove glycerophospholipids and retain CAEP, total lipids were subjected to mild alkaline hydrolysis. Total lipids in a side-arm tube were dissolved in 2.5 ml of chloroform/methanol (1:4 v/v) and then mixed with 0.75 ml of 2 M NaOH. The mixture was set at room temperature for 1 h before its pH was adjusted to 4 by the addition of 1 M HCl. Then chloroform was added to cause biphasic partition. The lower phase was recovered, washed with 2 vol. of methanol/water (1:1, v/v) and solvents were evaporated under a stream of N₂. The hydrolysed lipids were dissolved in a minimum amount of chloroform and run on TLC plates in solvent system A.

Cell fractionation

T. mimbres culture (400 ml) was quickly chilled to 4 °C in an acetone/solidified CO₂ bath. All of the following procedures were carried out at 4 °C, unless otherwise indicated. The cells were collected by centrifugation and washed with 100 ml of cold *Tetrahymena* fractionation buffer (TFB): 0.2 M potassium phosphate, 0.1 M NaCl and 3 mM EDTA, pH 7.2. The cell suspension was centrifuged at 300 *g* for 5 min, and the cell pellet was resuspended in 10 ml of cold TFB. The cells were deciliated by drawing them 12 times through a 16-gauge needle [37]. The deciliated cells were spun down by centrifugation at 1500 *g* for 5 min. The supernatant was further spun at 15000 *g* for 5 min to bring down the cilia. The cilia were resuspended in 10 ml of cold TFB and recovered by centrifugation at 15000 *g* for 5 min. The purified cilia were then extracted with 1 % TX-100 extraction buffer.

The deciliated cells were resuspended in 15 ml of TFB and further processed to prepare microsomes and cytosol. After disruption of the deciliated cells with a glass homogenizer, the homogenate was diluted to 20 ml with TFB and spun at 20000 *g* for 10 min. The supernatant was carefully removed into new tubes and centrifuged at 100000 *g* for 60 min to yield the microsomal pellet [38]. The supernatant from this centrifugation was designated cytosol.

Miscellaneous

Proteins were quantified by the bicinchoninic acid method [39] with reagents from Pierce (Rockford, IL, U.S.A.). Acid phosphatase (AcP) assays were carried out essentially as described by Banno and Nozawa [40]. All experiments described below were performed at least twice with similar results.

RESULTS

A unique group of *Tetrahymena* proteins resisted extraction by TX-100 at low temperature

To test the detergent solubility of *Tetrahymena* proteins, cells were subjected to a sequence of extractions with 1 % TX-100 (Figure 1). In a typical experiment, the supernatant (SUP1) from the first extraction, made at 4 °C, contained approx. 80 % of the whole-cell protein (Table 1). The residual flocculent pellet (PEL1) was re-extracted with 1 % TX-100. This re-extraction yielded a supernatant (SUP2) containing 8–9 % of the whole cell protein, all of which could be accounted for by the 10 % of SUP1 that had

Figure 1 Flow chart of the TX-100 extraction scheme
See the Experimental section for details. The numbers in parentheses show the yield of protein as percentage of total cellular protein.

Table 1 gpi63 content of cells and TX-100 fractions

The enrichment is ng of gpi63/ μ g of fraction protein divided by ng of gpi63/ μ g of whole-cell protein.

Fraction	Protein (μ g)	Yield (% of whole-cell protein)	gpi63 (μ g)	Yield (% of total gpi63)	ng of gpi63/ μ g of protein	Enrichment
Whole cell	24335	100.0	725	100.0	30	—
SUP1	19944	82.0	582	80.3	29	1.0
SUP2	2171	8.9	72	9.9	33	1.1
PEL2	2220	9.1	71	9.8	30	1.0
SUP3	608	2.5	68	9.4	113	3.8
PEL3	1612	6.6	3	0.4	2	0.1

Figure 2 SDS/PAGE of proteins from *Tetrahymena* whole cells and TX-100 fractions

Lanes 1–6 are whole cells, SUP1, SUP2, SUP3, PEL2 and PEL3 respectively. Each lane contains 1 μ g of protein. Lane 7 shows molecular mass standards. The gel was silver-stained. Arrowheads by lane 4 indicate enriched proteins.

not been decanted from PEL1 after the first extraction (see the Experimental section).

In a control experiment to test for completeness of extraction, the insoluble pellet from the second TX-100 extraction at 4 °C (PEL2) was extracted a third time at 4 °C. Almost all (> 93 %) of the total PEL2 protein remained insoluble after this third exposure to detergent. Since the SDS/PAGE pattern of the supernatant from the third extraction resembled that of SUP1 and SUP2, it was considered to contain a negligible remnant of soluble proteins. The third extraction was therefore not routinely included in the extraction scheme.

In the standard protocol the twice-extracted insoluble residue (PEL2) (9 % of whole-cell protein) was then extracted with 1 % TX-100 at 37 °C, causing additional protein (2.5 % of whole-cell protein) to be solubilized into SUP3 and leaving behind in PEL3 an insoluble 6.6 % of whole-cell protein.

The SDS/PAGE patterns of proteins from SUP1, the 37 °C TX-100 extract (SUP3) and the pellet (PEL3) remaining after the 37 °C extraction were all different from each other (Figure 2), indicating that individual protein species were selectively solubilized by TX-100 under different conditions. An enrichment of gpi63, gpi23 and an unidentified protein of approx. 26 kDa in size was always noted in SUP3 (see the arrowheads in Figure 2, lane 4). The SUP2 proteins resembled those in SUP1, as expected.

Table 2 gpi23 contents of cells and TX-100 fractions

The enrichment is units of gpi23/ μ g of fraction protein divided by units of gpi23/ μ g of whole-cell protein

Fraction	Units of gpi23/ μ g of protein	Yield (% of total gpi23)	Enrichment
Whole cell	21.0	100.0	—
SUP1	17.7	68.0	0.8
SUP2	21.4	9.0	1.0
PEL2	55.8	23.0	2.7
SUP3	188.6	22.2	9.0
PEL3	2.6	0.8	0.1

Table 3 AcP activity in cells and TX-100 fractions

Fraction	Yield (% of whole cell protein)	Yield (% of total AcP activity)	Units of AcP activity/ μ g of protein
Whole cell	100.0	100.0	0.06
SUP1	82.0	88.2	0.06
SUP2	8.9	8.0	0.05
PEL2	9.1	3.9	0.02
SUP3	2.5	2.7	0.06
PEL3	6.6	1.2	0.01

Centrifugation of SUP1 at a higher force, 150000 *g* for 20 min, did not sediment additional proteins.

Two GPI-anchored proteins were enriched in the TX-100 extract at 37 °C

The various extracts and pellets were analysed further for the presence of GPI-anchored proteins by immunoblotting and densitometry. Approx. 90% of the cells' major GPI-anchored protein species, gpi63, was recovered in SUP1 plus SUP2 (Table 1). However, the relatively small quantity of protein recovered in SUP3 (the 37 °C TX-100 extract) included significant amounts of gpi63 that were not extractable at 4 °C. In SUP3, gpi63 was enriched 3.8-fold over its concentration in whole cells. In contrast, the insoluble residue (PEL3) was essentially free of gpi63.

A similar measurement was made of the less abundant GPI-anchored protein, gpi23, in fractions from the same experiment (Table 2), except that the mass of the gpi23 standard used for densitometry was not accurately known, making absolute quantification impossible. Nevertheless, by comparing arbitrary absorbance units, a significant enrichment of gpi23 in SUP3 relative to whole cells was obvious. The presence in SUP3 of 22% of the cells' gpi23 in only 2.5% of the cellular protein (9-fold enrichment) was particularly striking. No gpi23 was detected in PEL3.

The subset of GPI-anchored proteins that resists solubilization by TX-100 at 4 °C in many cell types has been found to be readily solubilized at 4 °C by the detergent octyl glucopyranoside [11]. Both gpi63 and gpi23 were also completely solubilized by the latter detergent at 4 °C (results not shown), further suggesting their involvement in tightly associating domains thought to exist in mammalian cells.

In comparison with the GPI-anchored proteins, a *Tetrahymena* transmembrane protein also under study, AcP [41], was almost entirely (96%) extracted by TX-100 at 4 °C (Table 3). Only

Figure 3 Distribution of radioactivity in proteins extractable by TX-100/TX-114 (TX-114 proteins) after pulse–chase labelling with [³H]leucine

(a) Distribution of radioactivity in 1 mm SDS/polyacrylamide gel slices of TX-114 proteins (0 min chase) and TX-114 proteins (50 min chase). (b) Protein gel pattern of TX-114 proteins after Coomassie Blue staining.

2.7% of AcP activity was carried through into SUP3, and there was no enrichment over the whole cell level.

GPI-anchored proteins solubilized by TX-100 at different temperatures represent metabolically distinct pools

As GPI-anchored proteins of mammalian cells move into their site of synthesis in the endoplasmic reticulum through the Golgi apparatus and on to secretory sites at the plasma membrane, their recruitment into TX-100 resistant microdomains takes place only after they reach the sphingolipid-enriched Golgi cisternae [42]. In our initial step towards determining whether the microdomain assembly of nascent *Tetrahymena* GPI-anchored proteins is also preceded by their movement into the secretory system, pulse–chase experiments were conducted using [³H]leucine. Cells labelled for 10 min and chased for an additional 0 or 50 min were extracted by a protocol identical in its first steps with that described above. However, because quantitative specific radioactivity measurements of the GPI-anchored proteins were needed, the last part of the extraction protein was modified as follows to obtain gel bands containing pure gpi23 and pure gpi63.

PEL2, the pellet of proteins remaining insoluble after TX-100 extraction at 4 °C, was washed free of TX-100 and then re-extracted with TX-114 buffer at 37 °C, a temperature that induces phase partitioning of TX-114 and water (see the Experimental section). We previously showed that the TX-114 phase recovered after partitioning *Tetrahymena* whole-cell proteins contains essentially only gpi63, gpi23 and two relatively minor proteins, all of which were well separated by SDS/PAGE [22]. Other hydrophobic proteins had been removed by a novel low-temperature precipitation step inserted into this protocol.

In the present studies, Coomassie Blue-stained gels of the detergent phase from the TX-100-extracted and then TX-114-partitioned cells did indeed reveal a simple gel pattern, which included gpi63, gpi23, and three less abundant and well-separated bands (Figure 3b). After [³H]leucine labelling, radioactivity appeared on the gels in discrete peaks coinciding with the Coomassie Blue-stained bands (Figure 3a).

Bands representing gpi63 and gpi23 were quantified by densitometry of Western blots. Identical lanes from a separate gel were sliced and compared by scintillation counting with equivalent preparations obtained after TX-114 partitioning of whole

Table 4 Pulse-chase radiolabelling of GPI-anchored proteins

Chase time (min)...	0		50	
Fraction ...	Whole cell	SUP3	Whole cell	SUP3
d.p.m./ μ g of gpi63	2070	1461	1914	4033
d.p.m./unit of gpi23	1061	900	1216	1825

cells (without prior TX-100 treatment, see the Experimental section) set aside in the same pulse-chase labelling experiments. The estimated specific radioactivities of the proteins are shown in Table 4.

The 0 min specific radioactivities of the whole-cell gpi63 and the TX-100-insoluble TX-114-soluble gpi63 were appreciably different from each other, and the ratio of these differences changed over the time period examined (Table 4). Whole-cell gpi63 was more radioactive than TX-100-insoluble TX-114-soluble gpi63 initially, but after a 50 min chase the latter was twice that measured for whole cells. Smaller differences in specific radioactivity but a similar trend of change was noted with gpi23 (Table 4). This pattern of labelling, which was confirmed in a second independent experiment, indicates that the GPI-anchored proteins in the TX-100-insoluble TX-114 soluble phase come from a metabolic pool that must be distinct from certain other pools contributing heavily to the average specific radioactivity measured in whole cells. These clear intracellular differences in GPI-anchored protein specific radioactivity also establish that the GPI-anchored proteins that resist TX-100 extraction at 4 °C were not simply gpi63 and gpi23 molecules drawn randomly from a common whole-cell pool and non-specifically adsorbed to the other insoluble proteins as a detergent-induced artifact.

Sphingolipids were the most abundant lipids in the TX-100 extract at 37 °C

Certain lipids were also resistant to extraction by TX-100 at 4 °C. Using cells exposed to [3 H]palmitate for 14 h, a time sufficient to give uniform labelling of lipids (see the Experimental section), TX-100 extractions were made as described above, and lipids were purified from the extracts. TLC showed that, although most polar lipid species were preferentially extracted at 4 °C (phosphatidylcholine and phosphatidylethanolamine are given as examples), some CAEPs, the ceramide lipids previously identified in *Tetrahymena* [28], resisted extraction until the temperature was raised to 37 °C (Table 5). Then CAEPs were readily extracted,

Figure 4 Temporal changes in the NCAEP/HCAEP radioactivity ratio in whole cells (●) and SUP (◇) after pulse-labelling with [3 H]palmitate

yielding a SUP3 that was significantly enriched in CAEPs relative to whole-cell levels. On the basis of replicate experiments, from 14 to 19 % of whole cell CAEPs was determined to be insoluble in TX-100 at 4 °C. Of the two ceramides, the normal fatty acid-containing CAEP (NCAEP) was much more highly enriched in SUP3 than the chromatographically separable hydroxy fatty acid-containing CAEP (HCAEP) (Table 5). PEL3 contained only 0.1 % of the cells' polar lipids, and accurate quantification was not possible. However, CAEPs seemed to account for less than 10 % of the radioactivity recovered in PEL3.

Very non-polar lipids were also highly enriched in SUP3, accounting for as much as 30 % of the total radioactivity at short labelling times. The identity of these radioactive components as triacylglycerols was confirmed by TLC of the parent lipids and by showing, also by TLC, that the only radioactive products of base hydrolysis were fatty acids. The virtual absence of non-esterified fatty acids in the TX-100 extracts indicated that no significant lipolysis had occurred during the extraction procedures.

The low-temperature and high-temperature TX-100-extractable CAEPs represent distinct intracellular pools

It is known from previous work [29] that NCAEP is rapidly hydroxylated to HCAEP during the first few hours after its synthesis. The changing ratio of radioactivity between the two ceramide lipid subclasses in pulse-labelled whole cells provides a

Table 5 Polar lipid composition of cells and TX-100 fractions

Data are expressed as % of total polar lipids extracted from the indicated fraction, as described in the Experimental section. The values in parentheses after the fraction represent the percentage of whole cell polar lipid radioactivity recovered in that fraction. Enrichment for NCAEP was calculated as % of total fraction d.p.m. as NCAEP/% of total whole-cell d.p.m. as NCAEP. Enrichment for HCAEP was calculated as % of total fraction d.p.m. as HCAEP/% of total whole-cell d.p.m. as HCAEP.

Fraction	Phosphatidylcholine	Phosphatidylethanolamine	NCAEP + HCAEP	NCAEP (enrichment)	HCAEP (enrichment)
Whole cell (100)	15.1	39.1	17.9	8.0 —	9.9 —
SUP1 (86.2)	16.1	42.5	14.4	5.3 (0.66)	9.1 (0.92)
SUP2 (8.7)	14.1	34.8	21.3	11.1 (1.39)	10.2 (1.03)
PEL2 (5.2)	5.4	8.5	49.7	31.7 (3.96)	18.0 (1.82)
SUP3 (5.1)	5.3	8.2	50.8	32.8 (4.10)	18.0 (1.82)
PEL3 (0.1)	Trace	Trace	Trace	Trace	Trace

Table 6 Enrichment of gpi63 and gpi23 in whole cilia and TX-100 fractions

Enrichment of gpi63 over whole cell value is expressed as ng of gpi63/ μ g of fraction protein divided by 30 ng of gpi63/ μ g of whole cell protein (see Table 1). Enrichment of gpi23 over whole cell value is expressed in units of gpi23/ μ g of fraction protein divided by 21 units of gpi23/ μ g of whole-cell protein (see Table 2).

Fraction	Yield (% of total ciliary protein)	Yield (% of total ciliary gpi63)	ng of gpi63/ μ g of protein	gpi63 enrichment	Yield (% of total ciliary gpi23)	Units of gpi23/ μ g of protein	gpi23 enrichment
Whole cilia	100.0	100.0	162	5.4	100.0	195	9.3
SUP1	43.6	50.0	186	6.2	47.1	210	10.0
SUP2	12.5	9.4	120	4.0	11.9	185	8.8
SUP3	22.0	33.1	243	8.1	35.7	315	15.0
PEL3	21.9	7.5	54	1.8	5.3	46	2.2

sensitive measure of this conversion ([29] and Figure 4). Figure 4 also shows that the ratio of NCAEP to HCAEP radioactivities at various times after a pulse of [3 H]palmitate was not the same in all parts of the cell. The initial d.p.m. of NCAEP/d.p.m. of HCAEP ratio was relatively high in whole cells, in agreement with our earlier findings [29], and then declined at a time when the ratio in the SUP3 lipids was rising sharply (the very high 4 h SUP3 point is erratic, as judged from the lower 3 h time point measured in a later experiment). This suggests that the NCAEP that found its way into a TX-100-resistant compartment was protected against rapid hydroxylation or, alternatively, that CAEP molecules that did become hydroxylated were promptly shed from the insoluble domains. There was no net migration of labelled molecules from one pool to the other, since the combined CAEP radioactivity recovered in SUP3 accounted for approx. 25 % of the whole-cell CAEP radioactivity at all times.

It was important to confirm that the distinctiveness of the SUP3 ceramide lipid pool was genuine and that the association of GPI-anchored proteins with CAEPs was not simply an artifact of the extraction conditions. One important factor that has been shown to alter the physical relationships between proteins and lipids in intact cells, including *Tetrahymena* [43], is temperature. Our standard extraction procedure involved an initial treatment with cold TX-100 of cells that had not been chilled below their growth temperature of 28 °C. As a control, some cultures were chilled from 28 to 4 °C over a period of 2 min before extraction to determine whether changes *in vivo* in protein–lipid associations induced by chilling [43] might be detectable. The results with respect to GPI-anchored protein and lipid dissemination to the various detergent extracts were the same as those obtained using unchilled cells (Tables 1, 2 and 5).

A different kind of control was designed to ascertain whether the observed protein–lipid association was induced by low temperature after the addition of TX-100. To investigate this

point, PEL2 was extracted by TX-100 at 37 °C, and then part of the resulting SUP3 was cooled very slowly over a period of 40 min to 4 °C and re-centrifuged. Comparison of the lipids and GPI-anchored proteins indicated that gpi63 and gpi23 and the CAEPs remained in the supernatant under these conditions, just as they did after more rapid cooling. Therefore, once these components were extracted from accompanying proteins at 37 °C, the association could not be re-formed by a slow annealing process.

The two GPI-anchored proteins were highly enriched in the plasma membrane and even more enriched in 37 °C TX-100 extracts of the plasma membrane

The amounts of gpi63 and gpi23 in detached cilia were 5.4 times and 9.3 times higher respectively than in whole cells on a μ g/mg of total protein basis (Table 6). Microsomes were also slightly enriched in gpi63 and gpi23, with values of 1.4 and 2.8 times that in whole cells respectively (Table 7). Cytoplasm, which in *Tetrahymena* accounts for approx. 40 % of whole-cell proteins, contained no measurable GPI-anchored proteins (results not shown).

When purified cilia were subjected to the TX-100 extraction procedure used with whole cells, the 37 °C extract (SUP3) was also highly enriched in both GPI-anchored proteins (Table 6). Thus gpi63 was enriched in SUP3 to about 8.1 times more than the whole-cell level and gpi23 to about 15.0 times.

Some 16 % of ciliary proteins were calculated to be gpi63, and 33 % of these were in SUP3, i.e. insoluble in TX-100 at 4 °C. This insoluble fraction represented a very sizeable proportion (23 %) of the whole-cell 4 °C-TX-100-insoluble gpi63, and would undoubtedly account for an even higher percentage if the entire complement of cilia could be recovered. It is clear from this that

Table 7 Enrichment of gpi63 and gpi23 in microsomes (MIC) and TX-100 fractions

Enrichment of gpi63 over whole cell value is expressed as ng of gpi63/ μ g of fraction protein divided by 30 ng of gpi63/ μ g of whole cell protein (see Table 1). Enrichment of gpi23 over whole cell value is expressed in units of gpi23/ μ g of fraction protein divided by 21 units of gpi23/ μ g of whole-cell protein (see Table 2).

Fraction	Yield (% of total MIC protein)	Yield (% of total MIC gpi63)	ng of gpi63/ μ g of protein	gpi63 enrichment	Yield (% of total MIC gpi23)	Units of gpi23/ μ g of protein	gpi23 enrichment
MIC	100.0	100.0	41	1.4	100.0	59	2.8
SUP1	80.8	90.4	45	1.5	90.4	67	3.2
SUP2	6.5	7.4	45	1.5	7.2	65	3.1
SUP3	1.6	2.2	57	1.9	2.4	90	4.3
PEL3	11.2	0.0	0	0.0	0.0	0	0.0

much of the 4 °C-TX-100-insoluble gpi63 was situated on the cell surface. However, in these highly purified surface membranes there was still much (50 % for gpi63 and also for gpi23) GPI-anchored protein that could be solubilized in TX-100 at 4 °C.

The SUP3 prepared from microsomes was much less enriched in gpi63 and gpi23 than was that from cilia (Table 7). This fraction was not completely homogeneous and possibly contained, in addition to endoplasmic reticulum, fragments of plasma membrane dislodged from the ruptured pellicle [38].

Ceramide lipids were also especially enriched in the plasma membrane

Lipid extraction of cells and cilia uniformly labelled with [³H]palmitic acid showed that 32 % of the ciliary polar lipids were CAEP, and this was further enriched to 56 % in ciliary SUP3 lipids. In comparison, 17.9 % of the whole cell polar lipids were CAEP, and CAEP levels in SUP3 from whole cells increased to 51 % (Table 5).

After construction of a balance sheet, the total ciliary CAEP insoluble in TX-100 at 4 °C was estimated to account for 15 % of the whole-cell 4 °C-TX-100-insoluble CAEP, a sizeable enrichment considering that cilia constitute only about 2 % of whole-cell protein. Another complement of plasma membrane roughly equal to that encompassing the cilia encloses the body cell itself [38] but cannot be efficiently purified.

DISCUSSION

Much effort is currently being directed towards understanding the mechanisms for GPI-anchored protein processing and targeting to the plasma membrane [20]. The most instructive work has involved mammalian epithelial cells, where GPI-anchored proteins are directed to the apical plasma membrane in preference to the basolateral plasma membrane. A variety of evidence [16,19,42] strongly suggests that a tight association of the GPI-anchored proteins with certain lipids, especially sphingolipids, is necessary for efficient targeting. However, the precise function of the sphingolipids is unclear. In a recent study [45], exogenous ceramide lipids and synthetic analogues were utilized by cells differently depending on whether they contained normal or 2-OH fatty acids. Glycosylceramides of Caco-2 cells were targeted to the apical plasma membrane (as were the GPI-anchored proteins) if the fatty acid moiety was normal and to the basolateral plasma membrane if it was 2-OH substituted. In other situations the sugar substituent of the glycosylceramide, generally either glucose or galactose, appeared to determine the cellular destination. Because of the large variety of sphingolipids present and the somewhat unnatural manner of sphingolipid precursor administration, a complete evaluation of the factors most important in stabilizing lipid-lipid and lipid-protein associations in these cultured cells was not possible. It was clear, however, that the structure of the free lipids greatly influences the targeting process. Less is known about the impact on targeting of differences in the GPI-anchored protein's own covalently bound lipid anchor.

The data presented in this report indicate that the targeting of GPI-anchored proteins and lipids in the more primitive protozoan cell has much in common with that in metazoan cells. *Tetrahymena* is a simpler and experimentally more amenable system in several respects. Its plasma membrane does not have separate apical and basolateral domains, and therefore there is no need for specialized pathways of membrane translocation to functionally distinct regions of the cell surface. It contains two major GPI-anchored proteins, both present in quantities that are

easily measurable in whole cells or cell fractions. It contains only one sphingolipid class of any abundance, CAEP, and this exists as two chromatographically separable subclasses, one having a saturated unsubstituted fatty acid and the other having a 2-OH substituent. The relative proportions of both the GPI-anchored proteins [46] and the sphingolipid subclasses [28] can be altered experimentally by changing the growing temperature.

We show here that metabolically distinct subpopulations of both the sphingolipids (Table 5) and the GPI-anchored proteins (Tables 1 and 2) are resistant to the solubilizing effects of TX-100 at 4 °C but not at 37 °C. This solubility property is the key criterion established by many groups [15–17] to indicate involvement in a tight lipid-protein molecular association undergoing processing for dissemination to the mammalian cell plasma membrane. Our findings of a similar pattern in *Tetrahymena* suggest that this special type of association is of ancient evolutionary origin. Bearing in mind the sensitivity of the GPI-anchored protein domain targeting process to structural modifications in the associated free lipids, as described above for mammalian cells [45], it is remarkable that the equivalent phenomenon in the protozoan cell succeeds using different sphingolipids and a fundamentally different lipid entity bound covalently to the protein anchor. Indeed, this is the first demonstration that proteins containing a ceramide anchor can form the type of tight lipid associations reported for glycerophosphatidylinositol-anchored proteins.

In cultured animal cells GPI-anchored proteins do not become TX-100 resistant until they move into the Golgi complex for processing [42]. Radiolabelling studies indicate that newly synthesized *Tetrahymena* GPI-anchored proteins and sphingolipids also enter this detergent-resistant domain after a delay, in this case of less than an hour. Some fatty acids become hydroxylated at the 2 position after their incorporation into the nascent sphingolipid [29], and these products are much less likely to enter the complex than are the sphingolipids containing normal fatty acids (Table 5).

Surface membranes, represented here by isolated and highly purified cilia, are greatly enriched in both TX-100-resistant and TX-100-soluble gpi63 and gpi23 (the two GPI-anchored proteins) and also in both TX-100-resistant and TX-100-soluble forms of sphingolipids. This raises the possibility, previously suggested for trypanosomes [47], that some elements of the tightly associated domains are dissembled once the domains reach the cell surface. These unencumbered components may then be recycled back into the cell for use elsewhere.

Certain aspects of the *Tetrahymena* system are unique. Both gpi63 and gpi23 are themselves anchored to the cell surface by an inositol-containing sphingolipid in which mainly palmitate and stearate are amide-linked to the previously undescribed long-chain base (LCB) 3-*O*-methylsphingosine [46]. *O*-Methylation of the LCB 3-hydroxyl group may have a significant effect on hydrogen-bonding between the anchor lipid and free lipids adjacent to it [48]. Surprisingly, there is no LCB *O*-methylation in the free sphingolipids, namely, the CAEP. These lipids all contain 2-aminoethylphosphonate as the polar head group [28]. The major LCB species are *n*-hexadeca-4-sphinganine and *n*-nonadeca-4-sphinganine, and the fatty acids are saturated C₁₄ to C₁₈ normal or iso-branched species, with a significant percentage having the 2-OH modification [28]. Depending on the nature of the sphingolipid polar head group, fatty acid hydroxylation can have a marked effect on the lipid-phase transition, depending on the lipid species involved. Fatty acid hydroxylation can lower the phase-transition temperature of cerebrosides by 10 °C, but hydroxylation has no effect on the cerebroside sulphate transition temperature [48]. It has been suggested that fatty acid hy-

droxylation may be more important in stabilizing the liquid crystalline phase than it is in disrupting the gel phase [48].

Interestingly, the extent of hydroxylation of the CAEP fatty acids is determined by the cells' growing temperature. In the present work, *Tetrahymena* grown at 28 °C had nearly equal proportions of HCAEP and NCAEP (Table 5), but cells grown at 15 °C had mainly NCAEP and those grown at 39 °C mainly HCAEP [28]. When chilled, the *Tetrahymena* surface membranes are very susceptible to temperature-induced lipid-phase separation [43], and the initial separation was observed at a temperature more than 20 °C lower in 15 °C-grown cells than in 39 °C-grown cells. We postulate that the physical properties of the membranes, including those domains containing GPI-anchored proteins, are modulated in part by the proportion of HCAEP present. Experimentation is now under way to repeat some of the analyses described here using high- and low-temperature-grown *Tetrahymena*.

By quantitative Western blotting in conjunction with direct protein assay, it was possible to quantify not only the cellular content of gpi63, the major *Tetrahymena* GPI-anchored protein, but also the proportion resistant to low-temperature TX-100 extraction. Thus of the gpi63 molecules, which account for a large proportion (2 % or more) of whole-cell protein, 9.4 % are insoluble in TX-100 at 4 °C. The TX-100-insoluble gpi63 therefore constitutes 0.2 % of whole-cell protein. It is difficult to compare this level with raft-associated GPI-anchored proteins in other cells because those components have rarely been determined as a percentage of whole-cell protein. Nevertheless, it is unlikely from recent reports (e.g. [16,49,50]) that TX-100-resistant proteins of mammalian cells exceed the 0.2 % measured in *Tetrahymena*.

The quantitative estimates made here and in our earlier studies [22] give a range of values for gpi63 of 0.29–0.43 nmol/10⁶ cells. Likewise, estimates of CAEP levels range from 13 nmol of CAEP/10⁶ cells in the present study to approx. 1.3 nmol of CAEP/10⁶ cells made some years ago by a different method [35]. Using these values to calculate a lipid/protein molar ratio gives a minimum value of 3.0 nmol of CAEP/nmol of gpi63 and a maximum value of 45 nmol of CAEP/nmol of gpi63 for whole cells.

Using these calculations plus the additional findings that 9.4 % of cellular gpi63 and approx. 16 % of cellular CAEP were resistant to TX-100 at 4 °C, it is possible to estimate the ratios within the detergent-resistant complexes. Based on the maximum value for whole cells given above, the ratio in the detergent-resistant compartment is approx. 60 nmol of CAEP/nmol of gpi63.

The lack of a pure gpi23 standard prevented us from measuring its absolute level. However, analysis of its relative distribution in different cell fractions confirmed that its behaviour was generally similar to that of gpi63. Gpi23 was approximately twice as highly enriched as gpi63 in the low-temperature TX-100-resistant fractions of whole cells (compare Tables 1 and 2), cilia (Table 6) and microsomes (Table 7). Because the lipid anchors are essentially identical [46], the greater retention of gpi23 must be due to either its significantly smaller size or structural differences in the protein or carbohydrate moieties.

This is the first example of tightly associated GPI-anchored protein-ceramide lipid rafts in protozoan cells. It has been assumed [5] that protozoan cells lack this type of specific association because of the large quantities of GPI-anchored proteins reported to be freely soluble in TX-100 at low temperature [23–25,28]. However, to our knowledge there has until the present study not been a careful search for GPI-anchored proteins remaining insoluble after a TX-100 extraction at 4 °C.

Therefore, if *Tetrahymena* is typical of protozoa, there is, in addition to the large population of freely TX-100-soluble GPI-anchored proteins, a subpopulation of detergent-resistant GPI-anchored proteins in these cells comparing favourably in abundance with that found in mammalian cells. Hence the failure to recognize the presence of detergent-resistant GPI-anchored proteins in protozoa does not reflect a low absolute abundance. It is more likely that the unusually high level of readily soluble GPI-anchored proteins in these cells has masked the relatively much smaller insoluble pool.

In some studies, e.g. that involving Madin-Darby canine kidney epithelial cells [51], physical separation of ceramide lipid/sterol/GPI-anchored protein-rich membrane fragments was possible by taking advantage of their relatively low density in sucrose gradients. We did not detect material of this sort in the TX-100 extracts in *Tetrahymena*. If present, large membrane fragments of this nature may well have been trapped inside the detergent-permeabilized pellicle (the proteinaceous cell envelope), which could be seen by phase-contrast microscopy to remain intact after low-temperature TX-100 extraction (X. Zhang and G. A. Thompson, Jr., unpublished work).

We conclude that processing and trafficking of newly synthesized GPI-anchored proteins involve a tight association of GPI-anchored proteins and ceramide lipids in protozoa just as they do in mammalian cells. Other lipids such as the cholesterol analogue, tetrahymanol, may also be involved, but our analysis did not evaluate that point.

The presence of freely soluble GPI-anchored proteins in surface membranes as well as other cellular compartments suggests that the tight association may be transient, as it also is in mammals. *Tetrahymena* lacks the well-developed Golgi apparatus typical of most eukaryotic cells and has instead 300–400 diminutive dictyosomes of one or two cisternae localized near the cell surface [52]. Some parasitic protozoa [53] also lack a morphologically identifiable Golgi. Further study is needed to clarify the dynamics of the flux through the protozoan trafficking apparatus and the extent of tight lipid-protein association at each site.

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REFERENCES

- Low, M. G. (1989) *Biochim. Biophys. Acta* **988**, 427–454
- Udenfriend, S. and Kodukula, K. (1995) *Annu. Rev. Biochem.* **64**, 563–591
- Cross, G. A. M. (1990) *Annu. Rev. Cell. Biol.* **6**, 1–34
- Englund, P. T. (1993) *Annu. Rev. Biochem.* **62**, 121–138
- McConville, M. J. and Ferguson, M. A. J. (1993) *Biochem. J.* **294**, 305–324
- Stevens, V. L. (1995) *Biochem. J.* **310**, 361–370
- Bülow, R., Overath, P. and Davoust, J. (1988) *Biochemistry* **27**, 2384–2388
- Huang, J. H., Getty, R. R., Chisar, F. V., Fowler, P., Greenspan, N. S. and Tykocinski, M. L. (1994) *Immunity* **1**, 607–613
- Incardona, J. P. and Rosenberry, T. L. (1996) *Mol. Biol. Cell* **7**, 613–630
- Lisanti, M. P. and Rodriguez-Boulton, E. (1991) *Cell Biol. Int. Rep.* **15**, 1024–1047
- Brown, D. A., Crise, B. and Rose, J. K. (1989) *Science* **245**, 1499–1501
- Chen, R., Udenfriend, S., Prince, G. M., Maxwell, S. E., Ramalingam, S., Gerber, L. D., Knez, J. and Medof, M. E. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 2280–2284
- Thompson, T. E. and Tillack, T. W. (1985) *Annu. Rev. Biophys. Chem.* **14**, 361–386
- Rodriguez-Boulton, E. and Nelson, W. J. (1989) *Science* **245**, 718–725
- Brown, D. A. and Rose, J. K. (1992) *Cell* **68**, 533–544
- Zurzola, C., van't Hof, W., van Meer, G. and Rodriguez-Boulton, E. (1994) *EMBO J.* **13**, 42–53

- 17 Hanada, K., Nishijima, M., Akamatsu, Y. and Pagano, R. (1995) *J. Biol. Chem.* **270**, 6254–6260
- 18 Mays, R. W., Siemers, K. A., Fritz, B. A., Lowe, A. W., van Meer, G. and Nelson, W. J. (1995) *J. Cell. Biol.* **130**, 1105–1116
- 19 Horvath, A., Sütterlin, C., Manning-Krieg, U., Rao Movva, N. and Riezman, H. (1994) *EMBO J.* **13**, 3687–3695
- 20 Futerman, A. H. (1995) *Trends Cell Biol.* **5**, 377–380
- 21 Ferguson, M. A. J., Low, M. G. and Cross, G. A. M. (1985) *J. Biol. Chem.* **260**, 14547–14555
- 22 Ko, Y.-G. and Thompson, Jr., G. A. (1995) *Anal. Biochem.* **224**, 166–172
- 23 Bouvier, J., Etges, R. J. and Bordier, C. (1985) *J. Biol. Chem.* **260**, 15504–15509
- 24 Schell, D., Evers, R., Pries, D., Ziegelbauer, K., Kiefer, H., Lottspeich, F., Cornelissen, A. W. C. A. and Overath, P. (1991) *EMBO J.* **10**, 1061–1066
- 25 Das, S., Traynor-Kaplan, A., Reiner, D. S., Meng, T. C. and Gillin, F. D. (1991) *J. Biol. Chem.* **266**, 21318–21325
- 26 Fankhauser, C., Homans, S. W., Thomas-Oates, J. E., McConville, M. J., Desponds, C., Conzelmann, A. and Ferguson, M. A. J. (1993) *J. Biol. Chem.* **268**, 26365–26374
- 27 Ramesha, C. S. and Thompson, G. A. (1982) *Biochemistry* **21**, 3612–3617
- 28 Kaya, K., Ramesha, C. S. and Thompson, Jr., G. A. (1984) *J. Biol. Chem.* **259**, 3548–3553
- 29 Kaya, K., Ramesha, C. S. and Thompson, Jr., G. A. (1984) *J. Lipid Res.* **25**, 68–74
- 30 Ko, Y.-G. and Thompson, Jr., G. A. (1992) *J. Protozool.* **39**, 719–723
- 31 Doerder, F. P., Arslanyolu, M., Saad, Y., Kaczmarek, M., Mendoza, M. and Mita, B. (1996) *J. Eukaryotic Microbiol.* **43**, 95–199
- 32 Thompson, Jr., G. A. (1967) *Biochemistry* **6**, 2015–2022
- 33 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 34 Hamburger, K. and Zeuthen, E. (1957) *Exp. Cell Res.* **13**, 443–453
- 35 Fukushima, H., Martin, C. E., Iida, H., Kitajima, Y., Thompson, Jr., G. A. and Nozawa, Y. (1976) *Biochim. Biophys. Acta* **431**, 165–179
- 36 Bligh, E. G. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- 37 Subbaiah, P. V. and Thompson, Jr., G. A. (1974) *J. Biol. Chem.* **249**, 1302–1310
- 38 Nozawa, Y. and Thompson, Jr., G. A. (1971) *J. Cell Biol.* **49**, 712–721
- 39 Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76–85
- 40 Banno, Y. and Nozawa, Y. (1984) *Biochim. Biophys. Acta* **799**, 20–28
- 41 Williams, J. T. and Juo, P. S. (1976) *Biochim. Biophys. Acta* **422**, 120–126
- 42 Cerneus, D. P., Ueffing, E., Posthuma, G., Strous, G. J. and van der Ende, A. (1993) *J. Biol. Chem.* **268**, 3150–3155
- 43 Kitajima, Y. and Thompson, Jr., G. A. (1977) *J. Cell Biol.* **72**, 744–755
- 44 Brown, D. A. (1992) *Trends Cell Biol.* **2**, 338–343
- 45 van der Bijl, P., Lopes-Cardozo, M. and van Meer, G. (1996) *J. Cell Biol.* **132**, 813–821
- 46 Ko, Y.-G., Hung, C.-Y. and Thompson, Jr., G. A. (1995) *Biochem. J.* **307**, 115–121
- 47 Steverding, D., Stierhof, Y. D., Fuchs, H., Tauber, R. and Overath, P. (1995) *J. Cell Biol.* **131**, 1173–1182
- 48 Boggs, J. M. (1987) *Biochim. Biophys. Acta* **906**, 353–404
- 49 Fukuoka, S.-I., Freedman, S. D., Yu, H., Sukhatme, V. P. and Scheele, G. A. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1189–1193
- 50 Rosen, C. L., Lisanti, M. P. and Salzer, J. L. (1992) *J. Cell Biol.* **117**, 617–627
- 51 Melkonian, K. A., Chu, T., Tortorella, L. B. and Brown, D. A. (1995) *Biochemistry* **34**, 16161–16170
- 52 Kurz, S. and Tiedke, A. (1993) *J. Protozool.* **40**, 10–13
- 53 Banting, G., Bunting, J. and Lingelbach, K. (1995) *Trends Cell Biol.* **5**, 340–343