Cytodifferentiation in *Tetrahymena vorax* is linked to glycosyl-phosphatidylinositol-anchored protein assembly

Xiangli YANG and Phillip E. RYALS*

Department of Biochemistry and Molecular Biology, Box 9650, Mississippi State University, MS 39762, U.S.A.

The role of glycosyl-PtdIns (GPI)-anchored proteins in the cytodifferentiation of *Tetrahymena vorax* was examined. Labelling of cells with [³H]myristate or [³H]palmitate followed by electrophoresis showed an array of proteins carrying covalently bound lipids. Electrophoresis of protein from cells labelled with the GPI-anchor components [³H]Ins and [¹⁴C]ethanolamine revealed three polypeptides on fluorograms which have apparent molecular masses of approx. 28, 50 and 82 kDa. Labelled lipid associated with these polypeptides was susceptible to release by *in vitro* exposure to *Bacillus thuringiensis* PtdIns-specific phospholipase C (PI-PLC). Using labelled fatty acids, cells induced to differentiate showed altered GPI-anchored protein-labelling patterns in comparison with undifferentiated control cells, with a heavily labelled 32 kDa band appearing upon differentiation.

Pre-incubation of cells in 10 mM D-mannosamine, an inhibitor of GPI incorporation into protein, resulted in a reduction of the incorporation of label into the three GPI-anchored proteins, nearly complete inhibition of differentiation and a reduction in the rate of digestive vacuole formation. A 50% inhibition of differentiation was obtained using 500 μ M mannosamine. The inhibitory impact of D-mannosamine on differentiation could be competitively and completely reversed by the inclusion of D-mannose, but not D-glucose. Neither glucosamine nor tunicamycin inhibited differentiation. Incubation of cells in PI-PLC (5 units/ml) plus the differentiation inducer resulted in an acceleration of differentiation and generally higher percentages of differentiated cells versus controls.

INTRODUCTION

Cell-surface proteins secured to the plasmalemma by structurally complex glycosyl-PtdIns anchors (GPI anchors) display broad functional diversity ranging from hydrolytic enzyme activities as in acetylcholinesterase to the various antigenic roles of surface antigens. It has been suggested that the GPI-anchor itself may exhibit functional properties in addition to its well documented and obvious role in tethering selected proteins to the exterior boundary of cells. For example, degradation of the anchor would result in the release of the affixed protein and, in certain cases, generate lipid catabolites (diacylglycerols and inositides) known to participate in signal-transduction cascades. However, elucidation of the functional significance of GPI anchors has lagged significantly in comparison with the detailed structural studies performed.

Recently, several papers describing the occurrence of GPI-anchored proteins in various species of the ciliated protozoan *Tetrahymena* have been published [1–4], including preliminary structural studies on the PI-glycan (where PI is PtdIns) precursor from *Tetrahymena mimbres* [3]. These studies have shown that the *T. mimbres* PI-glycan is structurally unique and represents a third, intermediate PI-glycan arrangement. It has also been reported that the immobilization antigens of *Tetrahymena thermophila* are GPI-anchored [4]. Working from this information and reports of the potential of GPI-anchored proteins to transduce signals across cellular membranes, we hypothesized that GPI-anchored proteins would be present in *Tetrahymena vorax* and were likely to be involved in the signal-mediated differentiation of these cells.

This paper presents data that demonstrate the presence of GPI-anchored proteins in *T. vorax*, a species capable of differentiating into an alternative phenotype in response to an external chemical signal. The differentiation involves massive reorgan-

ization of the cells' internal membranes and restructuring of their cytoskeletal architecture. In nature, the differentiation is triggered by the presence of other protozoa that are a potential food source for *T. vorax*. In the laboratory, differentiation can be induced by supplying *T. vorax* with a complex, water-soluble exudate obtained from the potential prey organisms [5]. This material, known as stomatin, has eluded attempts to fully characterize it, but is known to be composed largely of nucleic acid catabolites [5,6]. The mechanism of action of stomatin in the induction of differentiation in *T. vorax* is unknown; however, our data show a relationship between continuous GPI-anchored protein assembly and the ability of *T. vorax* to respond to the differentiation inducer.

MATERIALS AND METHODS

Cell culture

Cultures of *T. vorax* V₂S_(Subline3-C) were grown at 20 °C in 500 ml Erlenmeyer flasks containing 50 ml of autoclaved enriched proteose peptone medium [7,8]. Stocks were transferred to fresh medium every 72 h and cell densities were estimated by the method of Hall et al. [9].

Preparation of the differentiation inducer and induction of differentiation

The differentiation inducer, stomatin, was prepared from litre cultures of *Tetrahymena pyriformis* GL as described by Buhse [5]. Dry stomatin was solubilized in distilled, deionized water to a concentration of 15 mg/ml and stored at –20 °C in 1.5 ml filter-sterilized aliquots. Cells in late exponential phase (2.0×10^6 cells/ml) were manipulated as previously described to induce differentiation [5,10]. The percentage of differentiated cells was determined by direct microscopic count of glutaraldehyde-killed

cells using an Olympus BH-2 research-grade microscope equipped with phase-contrast optics and a Nikon camera attachment. Cells were photographed using Kodak T-Max 100 film.

***In vivo* radiolabelling**

Cellular lipids and proteins modified with lipid were radiolabelled *in vivo* by adding 250 μ Ci of [9,10- 3 H]palmitic acid (60 Ci/mmol; DuPont/NEN) in 20 μ l of ethanol to 15 ml of cell suspension. In some experiments, [9,10- 3 H]myristic acid (39.3 Ci/mmol; DuPont/NEN) was used. GPI-anchors were labelled with [1,2- 14 C]ethanolamine hydrochloride (100 mCi/mmol; ICN Radiochemicals) according to Ryals et al. [1] or with [1,2- 3 H]Ins (50.2 Ci/mmol, DuPont/NEN). Cellular proteins were labelled by the addition of 10 μ Ci of [3,4,5- 3 H]leucine (179.6 Ci/mmol; DuPont/NEN).

Extraction, delipidation, and assay of protein from labelled cells

Following incubation of the cells in the presence of radiolabel, the cells were pelleted by microcentrifugation. The supernatants were removed by aspiration and 100 μ l of Laemmli sample buffer [11] lacking reducing agents and tracking dye was added to each pellet. After rapid mixing, the samples were heated at 100 °C for 3 min and cooled to room temperature. Protein was precipitated by the addition of 5 vol. of -20 °C acetone followed by incubation on ice for at least 15 min. Protein was pelleted by microcentrifugation for 3 min. Remaining non-covalently associated labelled lipids were removed by washing the pellets five times in 1.0 ml volumes of chloroform/methanol (2:1, v/v). All supernatants from the organic solvent washes were saved and counted for radioactivity using a Packard Tri-Carb 2250CA liquid scintillation counter.

The delipidated protein pellets were dried under reduced pressure and solubilized in small volumes of appropriate buffer. Some samples were then extracted with chloroform/methanol/water (10:10:3, by vol.) to remove free PI-glycans [12]. Aliquots of protein and PI-glycan were removed for scintillation counting and protein assay using either the Bradford [13] or bicinchoninic acid [14] method, depending on buffer composition.

SDS/PAGE and fluorography

Total cellular proteins and molecular-mass standards (unlabelled and 14 C-labelled markers; Gibco/BRL) were electrophoresed (BRL Model V16) on 15% (w/v) polyacrylamide slab gels at a constant 150 V according to Laemmli [11]. Proteins were visualized by staining in Coomassie Brilliant Blue R-250. Gels were then either cut into 1 mm slices for scintillation counting or prepared for fluorography by soaking in Resolution (EM Corp.). Dry gels containing fluor were placed against Kodak X-Omat film that had been pre-flashed in order to increase its sensitivity and linearize the signal response. Exposures were carried out at -70 °C for 1-4 weeks.

PtdIns-specific phospholipase C incubations

Dry delipidated protein labelled with [3 H]fatty acid was solubilized in either 25 mM Hepes/NaOH buffer or 25 mM Tris/malate buffer, each pH 7.2, containing 0.1% (w/v) deoxycholate. Commercial PI-specific phospholipase C from *Bacillus thuringiensis* (American Radiolabeled Chemicals) was added to a concentration of 10 munits/ml for *in vitro* experiments (5 units/ml for *in vivo* experiments). After incubation at room temperature, aliquots were removed at various intervals to determine the time of incubation required for maximum lipid release. Released lipid was extracted in chloroform/methanol according to Bligh and

Dyer [15]. Aliquots of extracted lipid and treated protein were subjected to scintillation counting to determine the percentage of released radioactivity. Lipids were then separated by t.l.c.

Thin layer chromatographic analysis of released lipids

Lipids released from labelled protein by enzymic means or by deamination [16] were chromatographed on silica gel G plates in either hexane/diethyl ether/acetic acid (70:30:1, by vol.; solvent system I) to resolve neutral lipid components, or in chloroform/acetic acid/methanol/water (75:25:5:2.2, by vol.; solvent system II) to resolve polar lipid components. After briefly exposing the developed plates to I_2 vapour, areas of the plates corresponding to standards were marked then scraped into vials for scintillation counting after allowing residual I_2 to sublime completely. Material released from labelled protein by deamination was also chromatographed on potassium oxalate-impregnated silica gel G plates according to Jolles et al. [17] to verify the identity of the inositol-containing deamination products.

D-Mannosamine, D-mannose, D-glucose, tunicamycin, O₂ uptake and *in vivo* PI-PLC experiments

D-Mannosamine (2-amino-2-deoxy-D-mannose; ManN) is an inhibitor of GPI incorporation into GPI-anchored proteins [18,19]. The ability of ManN to inhibit incorporation of 3 H-labelled fatty acid into PI-glycans and GPI-anchored proteins of *T. vorax* was examined by exposing cells to 10 mM ManN for 2 h before addition of label. Label uptake and specificity of incorporation were determined by scintillation counting and fluorographic analysis of SDS/PAGE gels of delipidated protein.

The effect of ManN on *T. vorax* differentiation was examined by pre-incubating cells in various concentrations of the inhibitor for 30 min to 3 h before the addition of the differentiation inducer, stomatin. In other experiments, D-mannose and D-glucose were tested for their ability to inhibit competitively the effects of ManN. A second amino sugar, D-glucosamine, was also tested. The percentage of differentiated cells in treated samples was compared with that in control samples lacking ManN. Other experiments were performed to determine the rate of phagocytosis in the presence of ManN. Cells were pre-incubated for various times in 20 mM ManN. Pelikan India ink (2 μ l) was added to 1 ml of cell suspension and incubation was continued for precisely 5 min, at which time 20 μ l of 25% glutaraldehyde were added and the killed cells were observed using phase-contrast microscopy. The number of vacuoles containing ink/cell was determined and the number of vacuoles formed/cell per min was calculated.

ManN has been reported to affect N-glycosylation of proteins [20]. Cells were pre-incubated in the presence of various concentrations of tunicamycin before the induction of differentiation to determine any requirement for N-glycosylation.

To estimate the effect of ManN on the general metabolic state of cells, O₂ consumption rates were determined at 25 °C using an O₂ electrode (Banks Bros., Bottisham/Cambridge, U.K.). Data were standardized to reflect μ mol of O₂ consumed/min per 10⁶ cells.

The effect of PI-PLC on differentiation *in vivo* was examined by treating cells with 5 units/ml PI-PLC either in the absence or presence of the differentiation inducer. Percentage differentiation was determined as described above.

RESULTS

Identification of GPI-anchored proteins by *in vivo* radiolabelling

Cells in growth medium and cells in differentiation medium

Figure 1 Densitometric scan of [^3H]Ins-labelled proteins from *T. vorax*

Labelled, delipidated proteins were electrophoresed and the resulting fluorogram was subjected to densitometry. Four peaks of radioactivity were detected, corresponding to molecular masses of approx. 82, 50, 32 and 28 kDa (arrows). Labelled molecular mass standards were electrophoresed in parallel, analysed by densitometry and used to estimate the unknown molecular masses. A heavy band of radioactivity migrates as a smear near the buffer front of gels; this is extractable in chloroform/methanol/water (10:10:3, by vol.) and is composed of PI-linked glycans. See text and Figure 3 for additional details.

Figure 2 Densitometric scan of [^{14}C]ethanolamine-labelled proteins from *T. vorax*

Incubation of cells in the presence of [^{14}C]ethanolamine followed by electrophoresis of the delipidated protein, fluorography, and densitometry of the resulting X-ray film showed that label was incorporated into polypeptides having apparent molecular masses of 82, 50 and 28 kDa.

Figure 3 Fluorogram of [^3H]fatty acid-labelled proteins from *T. vorax*

(a) Time-course protein labelling using tritiated myristic acid ([^3H]C $_{14:0}$) of cells in growth medium (lanes 1, 4 and 7), buffer (lanes 2, 5 and 8), and buffer plus stomatin (lanes 3, 6 and 9). Cells were removed for protein analysis at 2 h (lanes 1–3), 4 h (lanes 4–6), and 6 h (lanes 7–9). Each lane contains approx. 1.5×10^5 d.p.m. Note the characteristic GPI-anchored protein-labelling pattern in the 6 h sample from fully differentiated cells (lane 9) versus the growth medium and buffer controls (lanes 7 and 8). (b) Cells in growth medium (lanes 1–3), buffer (lanes 4–6), and buffer plus stomatin (lanes 7–9) were labelled for 8 h in the presence of tritiated palmitic acid, [^3H]C $_{16:0}$. Labelled, delipidated protein samples were extracted in chloroform/methanol/water (10:10:3, by vol.). The residual protein remaining in the aqueous phases (lanes 2, 5 and 8) and the extracted PI-glycan fractions (lanes 3, 6 and 9) from each sample were also electrophoresed. A characteristic GPI-anchored protein labelling pattern (protein apparent molecular masses are shown on the right) is present in protein from fully differentiated cells (lane 7) compared with growth medium and buffer controls (lanes 1 and 4). Note the reverse density of labelling of the 28 and 32 kDa proteins when labelled with [^3H]C $_{14:0}$ [(a), lane 9] as opposed to [^3H]C $_{16:0}$ (b).

(either containing or lacking the differentiation inducer) were radiolabelled *in vivo* with a variety of GPI-anchored protein components, including fatty acid, Ins, ethanolamine and leucine. Aliquots of cells were removed for analysis at 2, 4 and 8 h following label addition.

Three bands migrating on polyacrylamide gels with apparent molecular masses of approx. 28, 50 and 82 kDa acquired label following incubation of cells with [^3H]Ins (Figure 1) or [^{14}C]ethanolamine (Figure 2). In each case, a portion of the radioactivity was found to migrate on the gels near the buffer front, indicating the presence of PI-glycan. A fourth polypeptide with an apparent molecular mass of 32 kDa was detectable on fluorograms of [^3H]Ins-labelled protein (Figure 1). A single

Table 1 *In vitro* release of radiolabelled lipid from GPI-anchored proteins by PI-PLC

Fatty acid-labelled proteins in 25 mM HEPES/NaOH buffer, pH 7.2, and containing 0.1% (w/v) deoxycholate were incubated for 120 min in the presence or absence of PI-PLC. Released lipids were extracted in chloroform/methanol. Data from separate experiments are presented. ND, not determined.

	Experiment	Radioactivity (d.p.m.)			Radioactivity released (%)	Radioactivity recovered (%)
		Starting protein	Organic phase	Aqueous phase		
(+)PI-PLC [^3H]C _{14:0} labelled substrate	1	4280	3186	ND	74	ND
	2	30786	17969	ND	58	ND
	3	274070	193570	36750	71	84
(–)PI-PLC [^3H]C _{14:0} labelled substrate	1	4280	204	ND	4	ND
	2	30786	4928	ND	16	ND
	3	274070	260	239100	< 1	87
(+)PI-PLC [^3H]C _{16:0} labelled substrate	1	30778	23290	ND	76	ND
	2	477000	214770	211939	50	89
(–)PI-PLC [^3H]C _{16:0} labelled substrate	1	30778	281	ND	1	ND
	2	477000	41570	260085	14	90

experiment utilizing [^3H]leucine resulted in the labelling of total cellular proteins, including four polypeptides which have mobilities on gels identical to those of the same proteins labelled by Ins and ethanolamine (results not shown).

Heavy labelling of the putative GPI-anchored proteins occurred after incubation of cells with tritiated myristate ([^3H]C_{14:0}) or tritiated palmitate ([^3H]C_{16:0}) (Figures 3a and 3b, respectively). Several bands in addition to those from the [^{14}C]ethanolamine and [^3H]Ins experiments were found to carry label and were found to represent acylated proteins known to be present in *Tetrahymena* [21]. Uptake and covalent attachment of [^3H]fatty acids to protein were more pronounced (as judged by specific radioactivity calculations) for cells labelled in the differentiation medium. Total protein from cells labelled in growth medium exhibited a 3–7-fold lower specific radioactivity (d.p.m./ μg of protein) than protein obtained from cells labelled in differentiation medium.

Little or no labelling of the 82 kDa GPI-anchored polypeptide occurred during incubations in growth medium, but the peptide was heavily labelled when cells were incubated with tritiated fatty acids under conditions of nutrient deprivation (Figure 3). Profuse and progressive incorporation of [^3H]C_{14:0} into the 28 and 32 kDa polypeptides over time was seen in samples from cells labelled in buffer containing the differentiation inducer, stomatin, with the 28 kDa polypeptide being the most heavily labelled (Figure 3a). In samples labelled in buffer without stomatin, however, the most prominent band was the 32 kDa protein. The [^3H]C_{16:0} labelling pattern was similar to that of [^3H]C_{14:0} with one pronounced exception. The density of labelling of the 28 and 32 kDa polypeptides was reversed, with the 32 kDa protein being more heavily labelled in samples from differentiated cells and the 28 kDa protein being more heavily labelled in samples from undifferentiated control cells in buffer (Figure 3b, lanes 4 and 7).

Incubation of [^3H]C_{16:0}-labelled protein with PI-PLC resulted in the release of approx. 70% of the protein-associated radioactivity into the organic solvent phase following chloroform/methanol extraction (Table 1). Sensitivity to PI-PLC is generally considered to be diagnostic for GPI-anchored proteins. The label was selectively lost from the three GPI-anchored proteins and from PI-glycan, as determined by electrophoresis of the enzymically treated fractions (Figure 4). T.l.c. analysis of the released lipid in solvent system I showed that approx. 72% of the radioactivity co-migrated with authentic monoacylglycerol

(monopalmitin)/glyceryl ether (octadecyl glycerol) standards. Of the radioactivity released by deamination of labelled protein using nitrous acid, 94% remained at the origin in solvent system I, indicating a highly polar lipid, 83% of the radioactivity from the same sample co-migrated with authentic PtdIns in solvent system II. When the same samples were chromatographed on potassium oxalate-impregnated silica gel using the solvent system of Jolles et al. [17], 74% of the radioactivity co-migrated with authentic PtdIns and 20% of the loaded radioactivity co-migrated with authentic lyso-PI.

Effect of mannosamine on label incorporation, differentiation, metabolic state, N-glycosylation and phagocytosis

Incubation of cells in 10 mM ManN/HCl (in differentiation buffer) for 2 h before the addition of [^3H]C_{16:0} inhibited incorporation of label into the 28, 50 and 82 kDa proteins. The incorporation of label on a d.p.m./ μg of protein basis was reduced by 71–86% in comparison with controls. When equivalent amounts of protein from (–)-ManN and (+)-ManN samples were electrophoresed and fluorographed, the reduction in label incorporation into protein was visible (results not shown).

The inclusion of 10 mM ManN in buffer containing the differentiation inducer blocked the ability of cells to differentiate (Table 2). Differentiation in control populations averaged $80.5 \pm 10.3\%$ (mean $\pm$ S.D.) whereas 10 mM ManN reduced differentiation to $3.1 \pm 2.5\%$. The effect was concentration-dependent, with 500 μM ManN yielding a 50% inhibition in differentiation. A second amino sugar, glucosamine, had no inhibitory effect on the ability of cells to differentiate even when used at twice the maximum concentration of ManN (Table 2).

The inhibitory effect of ManN on differentiation could be competitively reversed by supplying cells with 20 mM D-mannose 30 min after ManN addition (Table 2). The mean differentiation obtained when cells were supplied with both mannose and ManN was $72.3 \pm 10.3\%$, indicative of a near complete reversal of the inhibitory effect of ManN. The degree of competitive reversal was dependent on mannose concentration (results not shown).

To test the specificity of the mannose-induced reversal of ManN inhibition, experiments were conducted wherein the ManN-inhibited cells were supplied with 10 mM D-glucose (Table 2). Populations rescued from ManN inhibition by 10 mM D-

Figure 4 Susceptibility of *T. vorax* GPI-anchored proteins to PI-PLC

A sample of [^3H]C_{14:0}-labelled protein was divided into two equal aliquots. One aliquot served as a control (a) and was electrophoresed. The second aliquot was treated with PI-PLC and electrophoresed (b). The result demonstrates the susceptibility of the 82, 50 and 28 kDa GPI-anchored proteins to lipid release by PI-PLC. A substantial reduction in PI-glycan radioactivity was also apparent. Equivalent amounts of protein were loaded for the control and PI-PLC-treated samples. Shown is a densitometric scan of a fluorogram resulting from electrophoresis of the samples.

mannose, differentiated to at least 65 % of control values, whereas populations supplied with 10 mM Glu differentiated to only ~ 27 % of control values.

To determine whether the inhibitory effect of ManN on differentiation was attributable to an overall suppression of cellular metabolism, O₂ consumption by (–)-ManN and (+)-ManN cells was monitored in triplicate using an oxygen electrode. Control cultures in buffer lacking ManN showed a mean uptake of $0.039 \pm 0.003 \mu\text{mol of O}_2/10^6 \text{ cells per min}$ (S.D., $n = 3$). In the presence of 0.5 or 10 mM ManN, mean uptake was 0.030 ± 0.009 and $0.045 \pm 0.008 \mu\text{mol of O}_2/10^6 \text{ cells per min}$, respectively. It was also possible that ManN might be exerting an inhibitory effect on N-glycosylation [20]. Cultures exposed to $0.5 \mu\text{g}$ of tunicamycin/ml for 75 min before stomatin addition exhibited $73 \pm 2.1 \%$ differentiation versus $84 \pm 8.5 \%$ differentiation in

controls lacking tunicamycin. Some homologues of tunicamycin are known to also inhibit protein synthesis. As continuous protein synthesis is required for differentiation to proceed, [^3H]leucine incorporation was monitored. Even in the presence of $1 \mu\text{g}$ of tunicamycin/ml, differentiation remained three times higher than the degree of protein synthesis inhibition (approx. 21 %).

Because differentiation in *T. vorax* requires the disassembly and reallocation of digestive vacuole membrane into a new and discrete structure [22], the effect of ManN on phagocytosis was investigated. At each time point examined, the rate of vacuole formation was reduced by 50 % or more in cells exposed to 20 mM ManN. Interestingly, whereas ManN appears to be specific in its inhibition of differentiation, glucosamine addition also reduced phagocytosis rates, but to a lesser degree (results not shown).

In vivo PI-PLC treatment of *Tetrahymena vorax*

Results from differentiation experiments wherein cells were pre-incubated with PI-PLC (5 units/ml of cell suspension) are shown in Table 2. PI-PLC alone exhibited no capacity to induce differentiation above the background seen in buffer controls. When the differentiation inducer was included with PI-PLC, however, the differentiation process was accelerated. Maximum production of differentiated cells ($\bar{X}$ differentiation = $86.0 \pm 3.8 \%$, $n = 2$) consistently occurred 6 h after induction in the presence of PI-PLC. Controls lacking PI-PLC reached comparable maximum levels of differentiation 8 h after induction ($80.5 \pm 10.3 \%$, $n = 10$). No attempt was made in this study to recover GPI-anchored proteins released into the medium by *in vivo* PI-PLC treatment as these experiments were performed on a micro-scale (100 μl volumes) to conserve enzyme.

DISCUSSION

The data presented demonstrate that under standard culture conditions, *T. vorax* contains at least three discrete polypeptides (apparent molecular masses of 28, 50 and 82 kDa, respectively) that are anchored by GPI moieties. This conclusion is based on the fact that all three proteins could be labelled *in vivo* with a variety of radioactive components known to be present in GPI-anchored proteins, including fatty acid, Ins, ethanolamine and amino acids. Furthermore, *in vitro* experiments showed that radiolabelled lipids could be enzymically released from these proteins by PI-PLC, a result generally considered to be diagnostic for GPI-anchored proteins. Although mannose labelling was not attempted, it was possible to inhibit ^3H -labelled fatty acid incorporation into the GPI-anchors by pre-incubating the cells in ManN, an expected result if mannose is an integral part of the PI-glycan structure.

Fatty acid labelling experiments showed that the 32 kDa polypeptide incorporated more [^3H]C_{16:0} than the 28 kDa polypeptide in samples from differentiated cells, with the reverse pattern being observed in buffer controls. Conversely, [^3H]C_{14:0} labelling revealed that the 28 kDa polypeptide contained more label than the 32 kDa protein in samples from differentiated cells, with the reverse labelling pattern being observed in buffer controls. These results would seem to indicate that fatty acid retailing occurs on these GPI-anchors specifically in response to differentiation. Remodelling of GPI-fatty acid is known to occur during GPI-anchor assembly in trypanosomes [23], but not in response to a defined signal-mediated physiological process.

Results from the t.l.c. analyses of the lipid released from labelled protein by PI-PLC and by nitrous acid treatment

Table 2 Amino sugar and PI-PLC effects on *Tetrahymena vorax* differentiation

T. vorax cells were induced to differentiate under the indicated conditions. Data are presented as the means $\pm$ S.D.; the numbers in parentheses indicate the number of experiments performed. For data obtained from two experiments, the deviation is presented in place of the S.D. ND, not determined.

Condition	Mean cytodifferentiation (%)		
	4 h	6 h	8 h
Buffer	5.4 $\pm$ 6.4 (10)	7.1 $\pm$ 6.8 (10)	10.7 $\pm$ 11.7 (10)
Buffer + stomatin	22.8 $\pm$ 16.3 (10)	62.5 $\pm$ 20.7 (10)	80.5 $\pm$ 10.3 (10)
Buffer + stomatin + 10 mM ManN	3.4 $\pm$ 2.9 (10)	4.5 $\pm$ 3.5 (10)	3.1 $\pm$ 2.5 (10)
Buffer + stomatin + 10 mM ManN + 20 mM Man	20.8 $\pm$ 14.2 (4)	65.0 $\pm$ 9.4 (4)	72.3 $\pm$ 10.3 (4)
Buffer + stomatin + 10 mM ManN + 10 mM Man	10.0 (1)	32.0 $\pm$ 7.7 (2)	52.0 $\pm$ 9.8 (2)
Buffer + stomatin + 10 mM ManN + 10 mM Glu	3.0 (1)	15.0 $\pm$ 2.8 (2)	22.0 $\pm$ 1.4 (2)
Buffer + stomatin + 20 mM glucosamine	42.3 $\pm$ 17.2 (3)	76.0 $\pm$ 8.0 (3)	87.0 $\pm$ 3.6 (3)
Buffer + 5 units of PI-PLC	8.0 $\pm$ 1.0 (2)	15.0 $\pm$ 0.1 (2)	ND
Buffer + stomatin + 5 units of PI-PLC	48.0 $\pm$ 8.5 (2)	86.0 $\pm$ 3.8 (2)	ND

indicated that the PI associated with the anchors is likely to be heterogeneous and composed of alkylacyl-PI and lyso-PI. This result is in agreement with similar, more comprehensive analyses performed using *T. mimbres* [1–3]. No evidence that the *T. vorax* GPI-anchors contain ceramide lipid was obtained.

Of particular interest were the data indicating that the ability of populations of *T. vorax* to differentiate depends on continuous GPI-anchored protein assembly and that a new GPI-anchored protein appears during the differentiation event. The conclusion that GPI-anchored protein assembly is a probable requirement for differentiation comes primarily from the ManN inhibition experiments. Of critical importance is the observation that the reversal of ManN inhibition of differentiation was specific for mannose. This indicates that the reversal effect of mannose is not simply a matter of it serving as a carbon source for the nutrient-deprived cells. It might be argued that mannose was competing with ManN for uptake by the cells, but this is unlikely because ManN was administered to test cultures 30 min before the addition of mannose. The specificity of mannose reversal of ManN inhibition was demonstrated by the fact that glucose addition to inhibited cells yielded only a modest improvement in differentiation percentage, compared with the near complete reversal of inhibition elicited by mannose. These data, combined with the finding that heavy labelling of a typically minor 32 kDa GPI-linked polypeptide occurs during differentiation, strongly suggest a critical role for ongoing GPI-anchored protein assembly in differentiation. Previous work has demonstrated that *T. vorax* differentiation is dependent on continuous transcription [10,24], translation [10], and fatty acid biosynthesis [10], all of which must be functional for ongoing GPI-anchored protein synthesis and GPI-anchor assembly.

The hypothesis that ManN is specifically inhibiting differentiation by blocking GPI-anchor assembly is supported by the results that show that ManN, even at a relatively high concentration, does not significantly alter overall metabolism as judged by O₂ consumption. The inability of tunicamycin to substantially inhibit differentiation indicates that the target of

ManN inhibition is not N-glycosylation. These data strengthen our supposition that differentiation is linked to ongoing GPI-anchor formation.

Our observation that ManN depresses the rate of phagocytosis probably does not indicate a direct dependence of phagocytosis on GPI-anchored proteins or their continuous biosynthesis. This conclusion is based on the finding that glucosamine was nearly as effective as ManN in suppressing vacuole formation at later time points. This interpretation is made somewhat ambiguous in that at early time points (30 min) after amino sugar addition to cells, glucosamine had little effect on vacuole formation rate whereas ManN exhibited a pronounced inhibitory effect.

The finding that *T. vorax* differentiation is accelerated in the presence of exogenous PI-PLC is difficult to interpret at this time. A number of hypothetical mechanistic scenarios are conceivable. It is possible that the observed effect is coincidental and non-specific, or that PI-PLC is acting on the small amount of membrane PI present in these cells to introduce localized disruptions in membrane structure which might facilitate the entry of stomatin into cells.

That the PI-PLC effect on differentiation is seen only in the presence of stomatin shows that the release of cell-surface GPI-anchored proteins alone is insufficient to trigger differentiation. Pre-incubation of cells in PI-PLC before addition of stomatin should have prevented or slowed the induction of differentiation upon stomatin addition if a GPI-anchored protein was serving as a conventional receptor for a component of stomatin. It did not; differentiation was enhanced. A possible interpretation of these data would be that the GPI-anchored proteins are themselves a target of the differentiation process, and the presence of exogenous PI-PLC merely accelerates the normal sequence of events.

Although the data available are insufficient to explain the role of GPI-anchored proteins in *Tetrahymena* differentiation, an exciting possibility is that a transmembrane-signalling cascade is involved, perhaps one analogous to the caveolae-mediated potocytosis phenomenon recently reported for the transport of folate

[25]. This hypothesis could account for the observations made to date in terms of the binding of a component of stomatin to a GPI-anchored receptor followed by internalization of the receptor plus the bound ligand. Interestingly, *Tetrahymena* have surface-associated structures known as parasomal sacs [26]. Their function is unknown, but given their ultrastructural characteristics, they might function as primitive analogues of caveolae. Efforts are underway to purify the GPI-anchored proteins from *T. vorax* for detailed structural analysis, antibody production and gene cloning.

This work was supported by the Mississippi Agricultural and Forestry Experiment Station (Project No. MIS-6304) and a Mississippi State University Research Initiation Grant (0210115-990) to P.E.R. This is publication # J-8331 of the Mississippi Agricultural and Forestry Experiment Station. The authors are happy to acknowledge Mr. John F. Larkin for his able assistance in the laboratory.

REFERENCES

- Ryals, P. E., Pak, Y. and Thompson, G. A., Jr. (1991) *J. Biol. Chem.* **266**, 15048–15053
- Pak, Y., Ryals, P. E. and Thompson, G. A., Jr. (1991) *J. Biol. Chem.* **266**, 15054–15059
- Weinhardt, U., Thomas, J. R., Pak, Y., Thompson, G. A., Jr. and Ferguson, M. A. J. (1991) *Biochem. J.* **279**, 605–608
- Ko, Y. G. and Thompson, G. A., Jr. (1992) *J. Protozool.* **39**, 719–723
- Buhse, H. E., Jr. (1967) *J. Protozool.* **14**, 608–613
- Butzel, H. M. and Fischer, J. (1983) *J. Protozool.* **30**, 247–250
- Thompson, G. A., Jr. (1967) *Biochemistry* **6**, 2015–2022
- Nelsen, E. M., Frankel, J. and Martel, E. (1981) *Dev. Biol.* **88**, 27–38
- Hall, R. P., Johnson, D. F. and Loefer, J. B. (1935) *Trans. Am. Microsc. Soc.* **54**, 298–300
- Buhse, H. E., Jr., Nabazas, S., Buhrfeind, C. and Ryals, P. E. (1991) *J. Protozool.* **38**, 124–129
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Krakow, J. L., Hereld, D., Bangs, J. D., Hart, G. W. and Englund, P. T. (1986) *J. Biol. Chem.* **261**, 12147–12153
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 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
- Bligh, E. G. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- Orlandi, P. A., Jr. and Turco, S. J. (1987) *J. Biol. Chem.* **262**, 10384–10391
- Jolles, J., Wirtz, K. W. A., Schotman, P. and Gispen, W. H. (1979) *FEBS Lett.* **105**, 110–114
- Lisanti, M. P., Field, M. C., Caras, I. W., Menon, A. K. and Rodriguez-Boulton, E. (1991) *EMBO J.* **10**, 1969–1971
- Pan, Y.-T., Kamitani, T., Bhuvaneshwaran, C., Hallaq, Y., Warren, C. D., Yeh, E. T. H. and Elbein, A. D. (1992) *J. Biol. Chem.* **267**, 21250–21255
- Pan, Y.-T., De Gaspari, R., Warren, C. D. and Elbein, A. D. (1992) *J. Biol. Chem.* **267**, 8991–8999
- Ryals, P. E. and Thompson, G. A., Jr. (1988) *Arch. Biochem. Biophys.* **266**, 408–415
- Smith-Somerville, H. E., Verchot, M. E. and Ryals, P. E. (1986) *J. Protozool.* **33**, 261–266
- Masterson, W. J., Raper, J., Doering, T. L., Hart, G. W. and Englund, P. T. (1990) *Cell* **62**, 73–80
- Nicolette, J. A., Buhse, H. E., Jr. and Robin, M. S. (1971) *J. Protozool.* **18**, 87–90
- Anderson, R. G. W., Kamen, B. A., Rothberg, K. G. and Lacey, S. W. (1992) *Science* **255**, 410–411
- Elliott, A. M. and Kennedy, J. R. (1973) in *Biology of Tetrahymena* (Elliott, A. M., ed.), pp. 57–87, Dowden, Hutchinson and Ross, Inc., Stroudsburg