Accumulation of glucosaminyl(acyl)phosphatidylinositol in an S3 HeLa subline expressing normal dolicholphosphomannose synthase activity

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Glucosaminyl(acyl)phosphatidylinositol [GlcN(acyl)PI], the third intermediate in the mammalian glycosylphosphatidylinositol (GPI) anchor pathway, is undetectable in most cells. This intermediate was previously shown to accumulate, however, in murine lymphoma mutant E and in yeast mutant *dpm1*, both of which lack dolicholphosphomannose synthase activity. Here we report that a mammalian HeLa S3 subline, denoted D, produces large amounts of GlcN(acyl)PI. The level of GlcN(acyl)PI in this subline is twice that in the murine lymphoma mutant E and 4 times that in the parental S3 line. This HeLa D

subline differs from the previously reported mutants that accumulate GlcN(acyl)PI because no defects in the synthesis or utilization of dolicholphosphomannose were found. Kinetic analysis indicated that in this HeLa subline there is an increased rate of synthesis of GlcN(acyl)PI, whereas the rate of metabolism for this GPI is comparable to that in wild-type cells. Furthermore, HeLa D cells accumulate GlcN(acyl)PI without a block in the synthesis of the downstream mannosylated GPI anchor precursors and GPI-anchored proteins. These findings might be relevant for understanding the regulation of the GPI pathway.

INTRODUCTION

Membrane-bound proteins can be post-translationally modified by fatty acids (myristic and palmitic), isoprenoids (farnesyl and geranylgeranyl) or glycosylphosphatidylinositol (GPI) [1]. Modification of cell surface proteins by GPI serves as a ubiquitous anchoring mechanism that is found in parasites, yeast and mammals [2]. The glycan core of GPI-anchored proteins consists of EthN-P-Man α 1-2Man α 1-6Man α 1-4GlcN α 1-6-Ins (where EthN-P represents ethanolamine phosphate) and is conserved among all species. However, different substituent groups that branch from this core, including EthN-P, GalNAc, sialic acid, Gal and Man, cause the heterogeneity seen in the glycan part of the anchor. Although most of the branching substituents are found in several species, an EthN-P attached to the Man residue adjacent to inositol is present only in mammalian cells. The lipid part of the GPI anchor generally involves alkylacylglycerols or diacylglycerols, but in yeast and the slime mould *Dictyostelium discoideum* ceramide-based GPIs are also found [2].

Glucosaminyl(acyl)phosphatidylinositol [GlcN(acyl)PI] is the third intermediate in the proposed mammalian pathway for GPI anchor biosynthesis [3]. The steps leading to GlcN(acyl)PI formation are: (1) modification of phosphatidylinositol (PI) by *N*-acetylglucosamine to produce GlcNAcPI; (2) subsequent deacetylation of this intermediate to GlcNPI [4,5]; and (3) acylation of GlcNPI (probably by palmitic acid) [6,7]. The pathway continues after GlcN(acyl)PI by the sequential addition of Man residues and EthN-P groups to yield Man₃GPIs: ManMan(EthN-P →)ManGPI (H6), EtN-P-ManMan(EtN-P →)ManGPI (H7) and EtN-P-Man(EthN-P →)Man(EtN-P →)ManGPI (H8) [3,8,9]. H7 and H8 are the most likely GPI anchor precursor candidates, but the presence of a fatty acid on the inositol in H7 and H8 distinguishes these

structures from the ones seen attached to most mammalian GPI-anchored proteins [10]. This structural discrepancy can be reconciled by the postulation of a deacylase activity that cleaves the fatty acid of the inositol before or soon after addition to the polypeptide.

H6, H7 and H8 seem to be in excess of what is needed to anchor GPI-anchored proteins [9]. Furthermore, kinetic analysis of the synthesis and metabolism of H6, H7 and H8 argues for roles in addition to that of precursors of protein anchors [10]. This hypothesis was further supported by the presence of these GPIs outside the endoplasmic reticulum (ER), including in the plasma membrane [10–12].

GlcN(acyl)PI is present at very low levels in wild-type cells. It can be detected in the murine lymphoma mutant E [7] and the yeast mutant *dpm1* [13] because both mutants are deficient in dolicholphosphomannose (Dol-P-Man) synthase, the enzyme responsible for the synthesis of Dol-P-Man. Dol-P-Man is the donor of the mannosyl residues in GPIs as well as of four mannosyl residues in the lipid-linked oligosaccharide precursor of asparagine-linked glycan chains, Glu₃Man₆GlcNAc₂-PP-Dol. Lec35, a Chinese hamster ovary (CHO) mutant with normal levels of Dol-P-Man but with a mutation affecting its utilization, was also suggested to produce high amounts of GlcN(acyl)PI and to give inefficient GPI mannosylation [14].

We recently reported the isolation and chemical characterization of GlcN(acyl)PI [6] in an S3 HeLa subline. In this study we compare the levels of GlcN(acyl)PI in four HeLa cell lines: CCL-2, S3 (a clonal derivative from the parental CCL-2), D (a S3-derived cell line) and D3 (a clone from D). Mutants with lesions in the GPI anchor pathway characteristically accumulate the GPI intermediate preceding the site of the lesion and synthesize decreased amounts of downstream intermediates or GPI-anchored proteins. In contrast, this HeLa S3 subline

Abbreviations used: CHO, Chinese hamster ovary; DAF, decay accelerating factor; DMEM, Dulbecco's modified Eagle's medium; Dol-P-Man, dolicholphosphomannose; EthN-P, ethanolamine phosphate; ER, endoplasmic reticulum; GlcN(acyl)PI, glucosaminyl(acyl)phosphatidylinositol; GPI, glycosylphosphatidylinositol; PI, phosphatidylinositol; PI-PLC, PI-phospholipase C; PIP, phosphatidylinositol phosphate; lysoPI, 2-lysophosphatidylinositol.

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accumulates GlcN(acyl)PI without significantly affecting the levels of downstream intermediates or GPI-anchored proteins.

EXPERIMENTAL

Materials

Dulbecco's modified Eagle's medium (DMEM) as well as inositol-free and glucose-free DMEM were obtained from Gibco. Heat-inactivated newborn calf serum was from Sigma. PI-phospholipase C (PI-PLC) was purified from the medium of a *Bacillus subtilis* strain that overexpresses PI-PLC of *B. thuringiensis* [15]. *myo*-[2-³H]inositol (20 Ci/mmol) was from American Radiolabeled Chemicals and [2-³H]Man (20–30 Ci/mmol) from ICN.

Cells and culture conditions

Two HeLa cell lines, CCL-2 and S3, were obtained from the American Type Culture Collection. S3 is a clone of CCL-2 that readily grows in suspension culture. Two additional lines, D and D3, were isolated in our laboratory. HeLa D was derived from S3 by continuous passage of HeLa S3 cells with a rubber 'policeman' or a bent Pasteur pipette to wash off the cells from the flask walls, whereas CCL-2 and S3 cells were routinely passed with trypsin. HeLa D3 is a clone of D obtained by limiting dilution. HeLa cells were maintained in DMEM supplemented with 10% (v/v) newborn calf serum, 100 i.u./ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine in an atmosphere of 5% CO₂ in air at 37 °C. Karyotype analysis of D cells showed the same modal chromosome count (65–67) and the same copy numbers of HeLa markers (one of M1, one of M2, two of M3, one of M4) reported for HeLa S3.

Labelling and extraction of glycolipids

Cells (10⁶) were plated in 75 cm² flasks and incubated with 10 µCi of [³H]inositol for 3 days in 10 ml of inositol-free DMEM supplemented as described above. For labelling with [³H]Man, 2 × 10⁶ cells were preincubated for 1 h in 2 ml of glucose-free DMEM supplemented with 100 i.u./ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 100 µg/ml glucose and 10 µg/ml tunicamycin and then for a further 1 h in the same medium supplemented with 100 µCi of [³H]Man. After the incubations, the cells were detached with a cell scraper, washed with 20 mM PBS and extracted twice with 4 ml of chloroform/methanol/water (10:10:3, by vol.). The dried lipid extract was partitioned between water-saturated n-butanol and n-butanol-saturated water as previously described [16]. Lipids labelled with [³H]inositol or [³H]Man were partitioned into the butanol phase and were analysed by TLC developed with chloroform/methanol/water (10:10:3, by vol.). The distribution of the radioactivity on the plates was determined by a Bioscan System 200 Imaging Scanner or by fluorography after spraying the plates with En³Hance (DuPont).

Dol-P-Man synthase activity

Dol-P-Man was assayed as described elsewhere [17] with some modifications. Cells (2 × 10⁷) were washed with PBS and resuspended in 1.6 ml of ice-cold PBS containing 0.02 mM MgCl₂ and 1 mM MnCl₂ (buffer A). The cells were subjected to six probe sonication pulses (10 s per pulse at maximum probe intensity), and the lysate was sedimented at 120 000 g for 1 h at 4 °C. The pellet was resuspended in 1 ml of buffer A containing

0.2% Triton X-100 and homogenized with a Teflon pestle homogenizer after transfer to an Eppendorf tube. To membranes containing 200 µg of protein were added 400 µg of Dol-P and 1 µCi of GDP-[³H]Man and the volume was brought to 300 µl with buffer A without detergent and incubated for 30 min at 37 °C. The [³H]Man-labelled lipids were extracted with 700 µl of chloroform/methanol (2:1, v/v). The aqueous phase was removed after centrifugation and was back-extracted with fresh organic phase pre-equilibrated with aqueous phase. The combined organic phases were dried and resuspended in chloroform/methanol (2:1, v/v) for analysis by TLC. The plate was developed in chloroform/methanol/water (10:10:3, by vol.). A main peak co-migrating with the standard was revealed after fluorography, and this silica band was scraped and subjected to scintillation counting.

Analysis of lipid-linked oligosaccharides by size-exclusion chromatography

Cells (2 × 10⁶) were incubated with 1 mCi of [³H]Man in 1 ml of glucose-free DMEM supplemented with 10% (v/v) newborn calf serum/100 µg/ml glucose for 20 min at 37 °C. Cells were then washed with PBS and resuspended in PBS/newborn calf serum (1:1, v/v), and chloroform/methanol (3:2, v/v) was added to give chloroform/methanol/water (3:2:1, by vol.). The organic phase was back-extracted with aqueous phase pre-equilibrated with organic phase, dried and incubated in 500 µl of n-propanol and 1 ml of 10 mM HCl for 20 min at 100 °C. Hydrolysed samples were partitioned between chloroform and water, and 3 × 10⁵ c.p.m. of the water phase was subjected to chromatography on a P4 column. The relative elution constant (K_d) of an oligosaccharide peak was calculated from the formula $K_d = (V_x - V_e)/(V_i - V_e)$, where V_x is the elution volume (fraction number) of the oligosaccharide of interest, V_e is the exclusion volume (determined with albumin) and V_i is the inclusion volume (determined with [³H]Man).

SDS/PAGE

GPI-anchored proteins were detected after extraction of the cells with Triton X-114 and treatment of the extracts with PI-PLC [18]. Cells labelled with 100 µCi of [³H]inositol for 3 days were extracted by incubation with 700 µl of 20 mM phosphate buffer, pH 6.9, containing 5 mM EDTA, 1 mM EGTA, 150 mM NaCl, 2% (w/v) Triton X-114, 1 µg/ml leupeptin and 1 µg/ml pepstatin for 30 min on ice. The extract was centrifuged for 10 min at 15 000 g, the pellet was re-extracted with 300 µl of the same buffer and the supernatants were combined. After phase separation at 37 °C [19] and centrifugation, the aqueous phase was removed and the detergent phase was washed with 300 µl of fresh aqueous phase. The pooled aqueous phase was then back-extracted with fresh condensed detergent phase, divided into two, diluted in 20 mM phosphate buffer and incubated overnight with PI-PLC (10 µg/ml) at 4 °C. At the end of the incubation period, the samples were warmed at 37 °C for phase separation. The detergent and the aqueous phases were washed by a second cycle of phase partitioning before mixing with 0.5 vol. of trichloroacetic acid (1 g/ml) containing 1 mg/ml sodium deoxycholate. After incubation overnight at 4 °C, the precipitates were collected by centrifugation at 14 000 g for 15 min and washed twice with cold acetone. The pellets were resuspended in loading buffer and resolved by SDS/10% PAGE [20]. The gel was soaked in Amplify (Amersham) and subjected to fluorography.

Two-site immunoradiometric assay

Cells (2×10^6) were plated in 100 mm dishes the day before the experiment. On the next day the medium was removed and the resulting subconfluent cells (4×10^6) were washed once with DMEM and then incubated in 3 ml of DMEM containing 10 μ g/ml PI-PLC for 30 min at 37 °C. A 1 ml portion was removed and centrifuged for 10 min at 15000 *g*, and the immunoradiometric assay was performed on the supernatant as previously described [21]. Briefly, microtitre plate wells were coated with anti-DAF monoclonal IA10 (capturing antibody), and blocked with 1 % (w/v) BSA in PBS, after which 25 μ l of sample was added. After incubation for 2 h at 20 °C, 125 I-labelled anti-DAF monoclonal I1H6 (revealing antibody, directed against a different DAF epitope) was added. Wells were then washed and the radioactivity remaining in the cut-out wells was measured in a γ -counter.

Modelling of radiolabelled inositol incorporation and decay

A simple kinetic model for inositol incorporation into the GPI biosynthetic pathway is shown in Scheme 1. In this simplified model, inositol (A) is incorporated into PI (B) with a rate constant k_1 . PI is modified further into GlcN(acyl)PI (C) with a rate constant k_2 , and GlcN(acyl)PI is converted into a mannosylated GPI (D) with a rate constant k_3 . The rate constants for degradation of each species are k_A , k_B and k_C respectively. The amounts of each species at the start are $[A]_0$, $[B]_0$ and $[C]_0$ respectively.

Scheme 1

In this single one-compartment model, each species is assumed to be kinetically homogeneous. The rate equations that result can be solved to predict the concentration of each species with time. If we define the rate constants for metabolism of the respective species as $\alpha = k_1 + k_A$, $\beta = k_2 + k_B$, $\gamma = k_3 + k_C$, then the concentrations are:

$$[A] = [A]_0 e^{-\alpha t} \quad (1)$$

$$[B] = k_1 [A]_0 (e^{-\alpha t} - e^{-\beta t}) / (\beta - \alpha) + [B]_0 e^{-\beta t} \quad (2)$$

$$\begin{aligned}
 [C] = k_1 k_2 [A]_0 \left[\frac{e^{-\alpha t}}{(\beta - \alpha)(\gamma - \alpha)} + \frac{e^{-\beta t}}{(\alpha - \beta)(\gamma - \beta)} + \frac{e^{-\gamma t}}{(\alpha - \gamma)(\beta - \gamma)} \right] \\
 + \frac{k_2 [B]_0 (e^{-\beta t} - e^{-\gamma t})}{(\gamma - \beta)} + [C]_0 e^{-\gamma t} \quad (3)
 \end{aligned}$$

Experimental data were fitted to eqns. (1)–(3) by nonlinear regression analysis with Fig.P (version 6.0), and parameter values that fitted both the pulse and chase initial conditions were estimated.

RESULTS

Comparative analysis of GlcN(acyl)PI in HeLa cell lines

In a previous study we reported on a HeLa S3 subline, denoted HeLa D, with unusually high levels of GlcN(acyl)PI (approx. 10^7

CCL-2

S3

D

Figure 1 Phase-contrast microscopy of CCL-2, S3 and D HeLa cell lines

Cells were plated into 175 cm² flasks for 3 days and photographed with phase-contrast optics. Magnifications: $\times 40$ for the CCL-2 cells and $\times 20$ for S3 and D cells.

molecules per cell), that allowed us to perform compositional analyses of this GPI intermediate [6]. In the present study we analysed four HeLa cell lines to determine whether a high level of GlcN(acyl)PI is a common feature of HeLa cells or is a characteristic unique to the HeLa D subline. These cell lines were CCL-2, the original HeLa line; S3, a clone of CCL-2; D, derived from S3 [6]; and D3, a clone from D obtained by limiting dilution. The morphologies of CCL-2, S3 and D are quite different and might reflect their different sensitivity to growth inhibition by contact. Cells from the CCL-2 line appeared elongated, whereas HeLa D cells were rounded and did not form monolayers but rather grew in clusters (Figure 1). S3 cells showed a heterogeneous population of cells with both characteristics and clone D3 had an appearance similar to D.

The four HeLa cell lines were labelled for 3 days with

Figure 2 GlcN(acyl)PI accumulates in HeLa D cells

Cells (10^6) from each HeLa cell line were incubated with $10 \mu\text{Ci}$ of [^3H]inositol for 3 days, the lipids extracted in chloroform/methanol/water (10:10:3, by vol.), dried and partitioned between butanol and water as outlined in the Experimental section. The butanol phases, containing approx. 500 000 c.p.m., were run on TLC plates developed in chloroform/methanol/water (10:10:3, by vol.). The distribution of radioactivity shown was obtained by radioscanning the TLC plate.

Figure 3 Comparative analysis of [^3H]inositol-labelled lipids from HeLa D and mutant E cells

HeLa D and lymphoma mutant E cells (10^6) were labelled with $10 \mu\text{Ci}$ of [^3H]inositol for 3 days. Labelled lipids were extracted from HeLa D and mutant E cells and samples containing equal amounts of protein (with approx. 500 000 and approx. 300 000 c.p.m. respectively) were analysed as described in the legend to Figure 2.

[^3H]inositol, and the extracted [^3H]inositol-labelled lipids were analysed by TLC (Figure 2). Although the total amount of cell-associated radiolabel was similar for all the cell lines (approx.

50%), radioscanning of the TLC plates revealed quantitative and qualitative differences in the [^3H]inositol-labelled lipids in each cell line. The distribution of the radioactivity in three predominant peaks, phosphatidylinositol phosphate (PIP), 2-lysophosphatidylinositol (lysoPI) and PI in the CCL-2 cell line represents a pattern typical of most wild-type cell lines [22]. In contrast, in the S3 cell line, a small peak more apolar than PI was apparent. This peak was identified as GlcN(acyl)PI by a combination of enzymic and chromatographic analyses [6]. Quantification of GlcN(acyl)PI in HeLa cell lines by scraping of the bands and scintillation counting revealed that HeLa D and clone D3 cells produced approx. 6 times more GlcN(acyl)PI (47 700 and 43 200 c.p.m. respectively) than the S3 cells (7000 c.p.m.) and two orders of magnitude more than the CCL-2 cells (371 c.p.m.).

Because CCL-2 cells were passaged with trypsin whereas D cells were scraped off the flasks, the level of GlcN(acyl)PI in HeLa D was reinvestigated after 19 passages of these cells with trypsin. No change was observed in cell morphology or in the GlcN(acyl)PI level (results not shown), indicating that the difference in the amounts of this GPI between these two HeLa cell lines is not due to any effect of trypsin.

HeLa D cells produce higher GlcN(acyl)PI levels than mutant E

Lymphoma mutant E cells harbour a defect in Dol-P-Man synthase and as a consequence accumulate GlcN(acyl)PI [7]. We compared mutant E and HeLa D cells for their abilities to accumulate GlcN(acyl)PI. After [^3H]inositol labelling to steady state, GlcN(acyl)PI was detected in E cells (Figure 3). After normalization for cell number, the smaller lymphoma cells incorporated only 10% of the added [^3H]inositol label, in comparison with 50% in HeLa D. [^3H]inositol-labelled lipids from equivalent amounts of membranes from both cell lines were compared on TLC (Figure 3). HeLa D cells produced 4 times

Table 1 Dol-P-Man synthase activity in HeLa cell lines and mutant E

Microsomal fractions from cell lysates were assayed as described in the Experimental section. Membranes (approx. 200 µg of protein) in buffer A supplemented with 2 mg of Dol-P were incubated with 1 µCi of [³H]GDP-Man for 30 min at 37 °C. The reaction was stopped by the addition of chloroform/methanol (2:1, v/v) and the organic phase was analysed by TLC. The TLC band comigrating with Dol-P-Man was scraped from the plate and the radioactivity was determined by scintillation counting.

Cell line	Dol-P-Man (c.p.m./µg of protein)
CCL-2	34
S3	19
D	27
D3	20
E	0.2

more GlcN(acyl)PI than lymphoma E cells, and the ratio of GlcN(acyl)PI to PI in HeLa D cells was twice that in the mutant E cells.

GlcN(acyl)PI is the substrate for the first mannosyltransferase, which uses Dol-P-Man as the mannose donor. Two types of defect affecting Dol-P-Man can disrupt the normal mannosylation of GPIs and result in the potential for GlcN(acyl)PI accumulation: (1) total lack of Dol-P-Man synthase activity (as found in mutant E cells) or partial lack of Dol-P-Man synthase activity (as represented in CHO cell [17] and T-cell hybridoma mutants) [23], and (2) impaired Dol-P-Man utilization (as represented in Lec 35). In view of the above findings that HeLa D cells are the highest producers of GlcN(acyl)PI reported, the following experiments were designed to answer whether the defect in these cells involves Dol-P-Man synthesis or utilization.

Dol-P-Man synthase activity is normal in HeLa D cells

To compare Dol-P-Man synthase activity between HeLa cell lines, the synthesis of [³H]Dol-P-Man from [³H]GDP-Man and Dol-P was assayed, with mutant E as a negative control. As shown in Table 1, similar levels of Dol-P-Man synthase activity were observed in the HeLa cell lines and contrasted with the absence of activity in lymphoma mutant E. This result ruled out the possibility that HeLa D cells and the mutant E share the same defect. It is, however, noteworthy that these synthase activity measurements do not reflect the steady-state levels of Dol-P-Man or utilization of the Dol-P-Man pool.

HeLa D cells can synthesize mannosylated-GPIs in the presence or absence of tunicamycin

Cell mutants that are partly deficient in Dol-P-Man produce smaller amounts of mannosylated-GPIs and GPI-anchored proteins [17,23]. In these leaky Dol-P-Man synthase mutants, the levels of mannosylated-GPIs and GPI-anchored proteins can be boosted by the addition of tunicamycin [17,23]. Tunicamycin inhibits the synthesis of Glu₃Man₉GlcNAc₂PPDol, the precursor of N-glycans on proteins, and Dol-P-Man is then used only in the GPI pathway. For this reason mannose labelling of GPIs is routinely performed in the presence of tunicamycin.

We first investigated the levels of the mature mannosylated-GPIs (H6, H7 and H8) and Dol-P-Man in HeLa D cells and in the other HeLa cell lines in the presence of tunicamycin: after 1 h of [³H]Man labelling, H6, H7 and H8 were detected in HeLa D and D3 cells but the levels of ³H in the two latter intermediates and Dol-P-Man were lower than in the CCL-2 cells

Table 2 Quantification of Man₃GPIs and Dol-P-Man in HeLa cell lines

HeLa cells were preincubated in glucose-free DMEM with or without tunicamycin (Tu) (10 µg/ml) for 1 h at 37 °C. After the addition of 100 µCi of [³H]Man, the incubation was continued for another 45 min. The [³H]Man-labelled lipids were extracted and analysed by TLC as described in the legend to Figure 2. The bands co-migrating with standards were identified on a fluorogram and were scraped and quantified by scintillation counting.

Cell line	Tu ...	Radioactivity (c.p.m.)							
		H6		H7		H8		Dol-P-Man	
		–	+	–	+	–	+	–	+
CCL-2		2060	640	840	600	8020	6880	960	3960
S3		1140	620	280	210	2230	1600	960	890
D		2370	1980	490	250	1390	1060	1620	1330
D3		1110	1860	300	250	1120	2090	950	1330

(Table 2). To rule out the possibility that HeLa D is a leaky Dol-P-Man synthase mutant, the amounts of endogenous Dol-P-Man available for GPI-mannosylation were decreased by omitting tunicamycin from the medium. In this condition, as expected, Dol-P-Man levels were decreased by 70 % in CCL-2 cells but surprisingly were unchanged in S3 and S3-derived cells (Table 2). Furthermore, removal of tunicamycin caused no significant decrease in H6, H7 and H8 in HeLa D and D3 cells, indicating that in these cells Dol-P-Man was not limiting (Table 2). The relative levels of these Man₃GPIs differed between the cell lines. H8 was the major [³H]Man-labelled Man₃GPI species in CCL-2 cells, whereas similar levels of H6 and H8 were observed in the S3 and S3-derived cell lines (D and D3) (Table 2). Moreover, incorporation of [³H]Man into H7, H8 and Dol-P-Man was 2–4 times higher in CCL-2 cells than in the other cell lines (Table 2), although steady-state levels of H8 in HeLa D and CCL-2 measured by [³H]inositol labelling were comparable within the different cell lines (results not shown). The lack of an effect of tunicamycin on HeLa D GPIs together with the normal levels of Dol-P-Man synthase activity (Table 1) in these cells clearly indicates that they are not deficient in Dol-P-Man.

HeLa D cells synthesize a Man₉ lipid-linked oligosaccharide

Lec 35 is a CHO mutant that expresses normal levels of Dol-P-Man but is unable to use this carrier as a mannose donor for the synthesis of lipid-linked oligosaccharides and presumably mannosylated GPIs [14]. This CHO mutant produces low levels of GPI-anchored proteins and apparently no mannosylated GPIs [14]. Because of an inactive Dol-P-Man pool, Lec 35 cells do not synthesize mature dolichol-linked oligosaccharides containing nine mannosyl residues but, as in the lymphoma mutant E cells [24], accumulate lipid-linked oligosaccharides having only five mannosyl residues [14]. One possible explanation for the fact that levels of Dol-P-Man did not rise in S3 and S3-derived cells (D and D3) when tunicamycin was added to the incubation medium (Table 2) is that in these cell lines Dol-P-Man is not used for the synthesis of lipid-linked oligosaccharides. To test this possibility, size analysis of the [³H]Man-labelled glycans released from dolichol-linked oligosaccharides from CCL-2 and D cells was performed by P4 exclusion chromatography. Glycans from both lines eluted with similar relative elution constants (*K_d* values of 0.24 and 0.23 respectively) (Figure 4), very close to the values reported for Glc₃Man₉GlcNAc₂ derived from many different cell types [25] but lower than those for the truncated species from mutant E cells ([25], and results not shown). The shoulder eluting

Figure 4 Analysis of lipid-linked oligosaccharides by size-exclusion chromatography

HeLa cells were labelled with 1 mCi of [3 H]Man for 20 min at 37 °C and extracted with chloroform/methanol/water (3:2:1, by vol.). After phase separation, the organic phase was dried, oligosaccharides were released with 10 mM HCl and an aliquot (300 000 c.p.m.) was run on a P4 column as outlined in the Experimental section. The exclusion and inclusion volumes were at fractions 59 and 166 respectively for the sample from CCL-2 cells and 58 and 158 respectively for the sample from HeLa D cells. Fractions of 0.45 ml were collected and taken for scintillation counting.

ahead of the Glc₃Man₉GlcNAc₂ peak from HeLa D cells (Figure 4) has been observed previously [25]. It was suggested to be Glc₃Man₉GlcNAc₂-X, where X is an unidentified moiety attached to the terminal GlcNAc residue [25]. These findings thus indicate that Dol-P Man in HeLa D cells is used in both the mannosylation of GPIs (Table 2) and the formation of normal lipid-linked oligosaccharides (Figure 4), distinguishing this S3 subline from Lec 35 cells. The fact that S3 and S3-derived cells make Glc₃Man₉GlcNAc₂-PP-Dol leaves us without a logical explanation for the puzzling observation that tunicamycin fails to increase Dol-P-Man levels in S3 and S3-derived cell lines.

HeLa D cells express GPI-anchored proteins

The mammalian cell mutants noted above that are defective in the synthesis of mannosylated GPIs expressed decreased amounts of GPI-anchored proteins. To compare the profiles of GPI-anchored proteins in the four HeLa cell lines, we labelled the cells to a steady state with [3 H]inositol and extracted the labelled proteins with Triton X-114. SDS/PAGE profiles from the four cell lines showed [3 H]inositol-labelled proteins at 70, 40 and 20 kDa (Figure 5). PI-PLC treatment of the extracts shifted most of the radiolabel from the detergent phase to the aqueous phase, indicating that only GPI-anchored proteins are labelled with [3 H]inositol. Residual label in the 70 kDa band after treatment with PI-PLC (approx. 15%) was probably due to fatty acid acylation of the inositol [26,27]. It is noteworthy, however, that the 70 kDa band from the S3 and the S3-derived cells was heterogeneous and ran consistently more slowly than the 70 kDa band from CCL-2 (Figure 5). This apparent higher molecular mass did not seem to be caused by partial N-glycosylation because HeLa D cells synthesized a normal precursor of N-glycosylation (Figure 4), although it is possible that the glycan

Figure 5 SDS/PAGE of [3 H]inositol-labelled proteins

HeLa cells were incubated with 100 μ Ci of [3 H]inositol for 3 days at 37 °C. The cells were extracted in buffer containing 2% (v/v) Triton X-114. After phase separation at 37 °C, the detergent phases were incubated overnight at 4 °C with or without PI-PLC. At the end of the incubation period, the samples were phase partitioned again and the detergent phases were precipitated with trichloroacetic acid. The pellets were resuspended in loading buffer and run on an SDS/10% polyacrylamide gel. The gel was soaked in Amplify and submitted to fluorography.

Table 3 Two-site immunoradiometric assay of GPI-DAF

HeLa cells were incubated with PI-PLC for 30 min at 37 °C and aliquots of the supernatant were assayed. Plates were coated with anti-DAF monoclonal IA10, blocked with 1% (w/v) BSA in PBS and incubated with 25 μ l of sample. After incubation for 2 h at 20 °C, [125 I]-labelled anti-DAF monoclonal IH6 was added and the radioactivity was measured in a γ -counter. The results are means of duplicate analyses.

Cell line	DAF (ng/mg of protein)
CCL-2	151 $\pm$ 56
S3	124 $\pm$ 10
D	108 $\pm$ 29
D3	126 $\pm$ 19

transfer to proteins is slow in these cells. To quantify this more rigorously, we focused on one GPI-anchored protein, decay accelerating factor (DAF), which is probably the most abundant GPI-anchored protein in these cells [28]. Both cell-surface DAF and total cell-associated DAF proteins were determined by immunoradiometric assay. When the amount of DAF in the supernatants was normalized to total cellular protein, no differences were found between the four HeLa cell lines in either cell-surface DAF (Table 3) or total DAF (results not shown).

Comparative pulse-chase of [3 H]inositol in HeLa D and CCL-2 cells

To explore the mechanistic basis for the accumulation of GlcN(acyl)PI in HeLa D cells, we compared the rates of synthesis and metabolism of this species in pulse-chase experiments with [3 H]inositol. For simplicity, results were interpreted in the context of a one-compartment model [Scheme 1 and eqns. (1)–(3)] that permitted rate constants to be estimated. The results shown in Figure 6 indicate that the rate constants for synthesis and metabolism of PI are similar for both cell lines. In contrast, the rate constant k_2 for GlcN(acyl)PI formation is approx. 40 times higher in HeLa D cells than in CCL-2, whereas no significant

Figure 6 Rates of approach to the steady state for metabolic labelling of PI and GlcN(acyl)PI with [³H]inositol

HeLa D cells (**a**) or CCL-2 cells (**c**) (10^6 cells) in 1 ml of inositol-free medium were incubated with 1–10 μ Ci/ml [³H]inositol for the indicated times before extraction and analysis by TLC as outlined in the legend to figure 2. Total radioactivity in lipids was determined by scintillation counting of the butanol phase and the distribution of lipid species was determined by radioscanning of the TLC plates and scintillation counting of the silica beads. After 70 h of incubation with [³H]inositol, the HeLa D cells (**b**) or CCL-2 (**d**) were washed to remove radiolabel in the medium, returned to 10 ml of complete medium and extracted at the indicated times and analysed as in (**a**) and (**c**). The observed amounts of radioactivity in PI (B, $\blacktriangle$) and GlcN(acyl)PI (C, $\square$) relative to the initial radioactivity of added inositol ($[A]_0$) are plotted against time and the time courses for both panels with each line were fitted to eqns. (2) and (3) derived from the one-compartment model. Data for HeLa D cells were fitted with the following values: $k_A = 0$, $k_1 = 0.012 \text{ h}^{-1}$, $k_B = 0.017 \text{ h}^{-1}$, $k_2 = 0.0031 \text{ h}^{-1}$ and $k_C + k_3 = 0.022 \text{ h}^{-1}$. Data for CCL-2 cells were fitted with the following values: $k_A = 0$, $k_1 = 0.012 \text{ h}^{-1}$, $k_B = 0.010 \text{ h}^{-1}$, $k_2 = 8 \times 10^{-5} \text{ h}^{-1}$ and $k_C + k_3 = 0.030 \text{ h}^{-1}$.

difference in the rate constants of metabolism in these cell lines was observed.

DISCUSSION

In this paper we describe a HeLa S3 cell subline designated D that accumulates GlcN(acyl)PI while still producing mannosylated GPIs and GPI-anchored proteins. The HeLa D cells comprise a less adherent subpopulation of cells within the parental S3 line. We observed an inverse correlation between HeLa cell adherence and GlcN(acyl)PI synthesis. Adherence decreased progressively for the CCL-2, S3 and D lines. Reciprocally, D cells produced 4 times more GlcN(acyl)PI than S3 and almost 40 times more than CCL-2. It is noteworthy that HeLa D cells produce twice the levels of GlcN(acyl)PI observed in lymphoma mutant E, the mammalian cell line first reported to accumulate GlcN(acyl)PI (Figure 3).

This D cell line is unique because it does not fall in the same group of Dol-P-Man-deficient mutants previously found to accumulate GlcN(acyl)PI [7,13]. Dol-P-Man synthase activity and Dol-P-Man levels are normal in HeLa D cells (Table 1 and

Table 2), and these characteristics clearly distinguish this cell line from the other mutants. A normal level of Dol-P-Man does not necessarily imply its normal utilization, as exemplified by Lec 35 cells [14], but HeLa D cells seem to use Dol-P-Man as a mannosyl donor in the synthesis of mannosylated-GPIs (Table 2) and lipid-linked oligosaccharides (Figure 4). Furthermore the levels of Man₃GPIs in D cells were not affected by tunicamycin (Table 2), indicating that the amount of Dol-P-Man is not limiting the mannosylation of GlcN(acyl)PI. These results provide further evidence that HeLa D cells are not a leaky Dol-P-Man mutant with low levels of Dol-P-Man.

The broader band of [³H]inositol-labelled GPI-anchored proteins at 70 kDa in all S3-derived cells (Figure 5), combined with the lack of effect of tunicamycin on Dol-P-Man levels in the S3 and S3-derived cell lines, raised the possibility that these cells had a glycosylation defect. Recent results on the topology of mannosylated-GPI biosynthesis have suggested that these reactions take place primarily on the cytoplasmic side of the ER [29]. In contrast, mannosylation of lipid-linked oligosaccharides is believed to involve Dol-P-Man on the luminal side of the ER [30]. If these topologies are assumed, the possible glycosylation

defect could involve a faulty flippase that produces a normal GPI-mannosylation and defective N-glycan synthesis. This hypothesis was discarded because lipid-linked oligosaccharides in HeLa D cells contained the full nine-mannosyl glycan core (Figure 4). Normal utilization of Dol-P-Man in the GPI pathway (Table 2) and in the synthesis of this precursor of N-glycosylation of proteins (Figure 4) distinguishes HeLa D from Lec 35 cells.

HeLa D cells produce at least as much GPI-anchored protein as the cell lines from which they were derived (Figure 5 and Table 3). Yeast mutants that accumulate GPI intermediates without decreasing the levels of GPI-anchored proteins were also recently reported by Conzelmann's group. Some of these mutants accumulated GPI anchor intermediates without effect on the levels of GPI-anchored proteins, whereas other mutants with close to normal amounts of GPI anchor intermediates showed low levels of GPI-anchored proteins [31].

The HeLa D cell phenotype could be explained if the cell population included two types of cells, in principle, one producing high levels of GlcN(acyl)PI and without mannosylated GPIs or GPI-anchored proteins and the other with undetectable GlcN(acyl)PI and normal amounts of Man₃GPIs and GPI-anchored proteins. Clone D3 ruled out this possibility because it showed the same profiles of mannosylated GPIs and GPI-anchored proteins as in the uncloned D cell line (Table 2 and Figure 5). Moreover, cell-surface DAF detected immunochemically with monoclonal anti-DAF antibodies and revealed by a secondary fluorescent antibody showed that all HeLa D cells were uniformly stained and that the numbers of cells in the bright and fluorescent fields were the same (results not shown).

The high levels of GlcN(acyl)PI in HeLa D cells could result from an increased rate of its synthesis, a decreased rate of its metabolism or a combination of both effects. Because there is no increase in the levels of downstream intermediates, any increased synthesis cannot be accompanied by an increase in mannosylation, either because the mannosyltransferase is saturated or because GlcN(acyl)PI accumulates in a compartment inside or outside the ER where it cannot undergo rapid mannosylation. Kinetic modelling of the rates of approach to the steady state during pulse-chase experiments with [³H]inositol in CCL-2 and D cells indicated that the apparent rate constant for the conversion of PI to GlcN(acyl)PI in HeLa D cells was almost 40 times higher than that in CCL-2. In contrast, the combined rate constants for decay of GlcN(acyl)PI are comparable in both cell lines. A higher rate of synthesis of GlcN(acyl)PI in HeLa D cells must arise from an increase in the rate constant for the rate-limiting step preceding GlcN(acyl)PI formation. This could be GlcNAc addition to PI, deacetylation of GlcNAcPI or acylation of GlcNPI. However, this step also could be the exit of GlcN(acyl)PI or an earlier precursor from the ER compartment where mannosylation is relatively rapid to an inert compartment where mannosylation is either slow or non-existent. Recent evidence supports such an exit to an inert compartment (A. Wonkanjorslip and T. Rosenberry, unpublished work). Although not yet identified, this compartment could be the plasma membrane.

Whatever the cause for the accumulation of GlcN(acyl)PI, the HeLa D subline has proved to be a useful tool by providing

sufficient material for compositional analysis of GlcN(acyl)PI [6] and for allowing the demonstration that exogenous PI added to the cells can be metabolized to GlcN(acyl)PI [32]. We also believe that this HeLa subline will be useful for studies of the GlcN(acyl)PI mannosyltransferase and of the regulation of the GPI pathway.

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REFERENCES

- 1 Chow, M., Der, C. J. and Buss, J. E. (1992) *Curr. Opin. Cell Biol.* **4**, 629–636
- 2 McConville, M. J. and Ferguson, M. A. J. (1993) *Biochem. J.* **294**, 305–324
- 3 Hirose, S., Prince, G. M., Sevlever, D., Ravi, L., Rosenberry, T. L., Ueda, E. and Medof, M. E. (1992) *J. Biol. Chem.* **267**, 16968–16974
- 4 Hirose, S., Ravi, L., Hazra, S. V. and Medof, M. E. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3762–3766
- 5 Stevens, V. L. and Raetz, C. R. H. (1991) *J. Biol. Chem.* **266**, 10039–10042
- 6 Sevlever, D., Humphrey, D. R. and Rosenberry, T. L. (1995) *Eur. J. Biochem.* **233**, 384–394
- 7 Urakaze, M., Kamitani, T., DeGasperi, R., Sugiyama, E., Chang, H.-M., Warren, C. D. and Yeh, E. T. H. (1992) *J. Biol. Chem.* **267**, 6459–6462
- 8 Ueda, E., Sevlever, D., Prince, G. M., Rosenberry, T. L., Hirose, S. and Medof, M. E. (1993) *J. Biol. Chem.* **268**, 9998–10002
- 9 Puoti, A. and Conzelmann, A. (1992) *J. Biol. Chem.* **267**, 22673–22680
- 10 Rosenberry, T. L., Sevlever, D. and Medof, M. E. (1994) *Braz. J. Med. Biol. Res.* **27**, 151–159
- 11 van't Hof, W., Rodriguez-Boulan, E. and Menon, A. K. (1995) *J. Biol. Chem.* **270**, 24150–24155
- 12 Singh, N., Liang, L.-N., Tyckocinski, M. L. and Tartakoff, A. M. (1996) *J. Biol. Chem.* **271**, 12879–12884
- 13 Costello, L. C. and Orlean, P. (1992) *J. Biol. Chem.* **267**, 8599–8603
- 14 Camp, L. A., Chauhan, P., Farrar, J. D. and Lehrman, M. A. (1993) *J. Biol. Chem.* **268**, 6721–6728
- 15 Henner, D. J., Yang, M., Chen, W., Helmiss, R., Rodriguez, H. and Low, M. G. (1988) *Nucleic Acids Res.* **16**, 10383
- 16 Sevlever, D. and Rosenberry, T. L. (1992) *J. Biol. Chem.* **268**, 10938–10945
- 17 Singh, N. and Tartakoff, A. L. M. (1991) *Mol. Cell Biol.* **11**, 391–400
- 18 Bordier, C. (1981) *J. Biol. Chem.* **256**, 1604–1607
- 19 Lisanti, M. P., Sargiacomo, M., Graeve, L., Saltiel, A. R. and Rodriguez-Boulan, E. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9557–9561
- 20 Lee, H.-C., Shoda, R., Krall, J. A., Foster, J. D., Selhub, J. and Rosenberry, T. L. (1992) *Biochemistry* **31**, 3236–3243
- 21 Kinoshita, T., Medof, M. E., Silber, R. and Nussenzweig, V. (1985) *J. Exp. Med.* **162**, 75–92
- 22 Moheny, R. P., Knez, J. J., Ravi, L., Sevlever, D., Rosenberry, T. L., Hirose, S. and Medof, M. E. (1994) *J. Biol. Chem.* **269**, 6536–6542
- 23 Thomas, L. J., Urakaze, M., DeGasperi, R., Kamitani, T., Sugiyama, E., Chang, H.-M., Warren, C. D. and Yeh, E. T. H. (1992) *J. Clin. Invest.* **89**, 1172–1177
- 24 Chapman, A., Li, E. and Kornfeld, S. (1979) *Cell* **17**, 509–515
- 25 Hubbar, S. C. and Robbins, P. W. (1980) *J. Biol. Chem.* **255**, 11782–11793
- 26 Roberts, W. L., Myher, J. J., Kuskis, A., Low, M. G. and Rosenberry, T. L. (1988) *J. Biol. Chem.* **263**, 18766–18775
- 27 Walters, E. I., Roberts, W. L., Rosenberry, T. L., Ratnoff, W. D. and Medof, M. E. (1990) *J. Immunol.* **144**, 1030–1036
- 28 Shenoy-Scaria, A. M., Timson Gauen, L. K., Kwong, J., Shaw, A. S. and Lublin, D. M. (1993) *Mol. Cell Biol.* **13**, 6385–6392
- 29 Vidugirene, Y. and Menon, A. (1994) *J. Cell Biol.* **127**, 333–341
- 30 Snider, M. and Rogers, O. C. (1984) *Cell* **36**, 753–761
- 31 Benghezal, M., Lipke, P. N. and Conzelmann, A. (1995) *J. Cell Biol.* **130**, 1333–1344
- 32 Wonkanjorslip, A. and Rosenberry, T. L. (1995) *J. Biol. Chem.* **270**, 9147–9153