Metabolism of exogenous *sn*-1-alkyl-*sn*-2-lyso-glucosaminyl-phosphatidylinositol in HeLa D cells: accumulation of glucosaminyl(acyl)phosphatidylinositol in a metabolically inert compartment

Adisak WONGKAJORNILP*, Daniel SEVLEVER†¹ and Terrone L. ROSENBERRY†^{1,2}

*Department of Pharmacology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand, and †Department of Pharmacology, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, U.S.A.

The somatic genetic defect in paroxysmal nocturnal haemoglobinuria (PNH) involves a block in the transfer of GlcNAc from UDP-GlcNAc to phosphatidylinositol (PI), the first step in the biosynthetic pathway for glycosylphosphatidylinositols (GPIs). We asked whether an exogenous lipid corresponding to an early intermediate in this pathway can be taken up by cells in culture and proceed through the GPI pathway. This approach could offer a strategy to bypass the block in PNH. To address this question we incubated HeLa D cells with *sn*-1-alkyl-*sn*-2-lyso-GlcN-³HPI (*lyso*-alkyl-GlcN-³HPI) for 24 h and analysed the cellular lipids. We found three lipid products: unaltered *lyso*-alkyl-GlcN-³HPI, GlcN-³HPI and GlcN(acyl)³HPI (GlcN-PI with a fatty acid acyl group on inositol). Since the latter two lipids are intermediates in the GPI biosynthetic pathway, this observation demonstrates that an exogenous lipid can enter and proceed partially through this pathway. However, the conversion of GlcN(acyl)PI to downstream mannosylated GPI intermediates

in the GPI pathway was inefficient, both for GlcN(acyl)PI produced from the exogenous lipid as well as from that obtained by metabolic labelling with [³H]inositol. We investigated this poor conversion by examining whether GlcN(acyl)PI, radioactively labelled sequentially by [¹⁴C]inositol and [³H]inositol, resided in one compartment and could be readily metabolized to downstream intermediates. Isotope ratios indicated that the turnover of GlcN(acyl)PI was slower than those of either downstream mannosylated GPIs or even GPI anchors on proteins, the final products of GPI pathway. This result is incompatible with the one-compartment model and indicates that GlcN(acyl)PI in HeLa D cells accumulates largely in a compartment that is inert to subsequent mannosylation.

Key words: glycosylphosphatidylinositols, glycolipid uptake, paroxysmal nocturnal haemoglobinuria.

INTRODUCTION

A biosynthetic pathway for glycosylphosphatidylinositols (GPIs) in mammalian cells has been deduced from analysis of several mutant cell lines that are defective in the expression of GPI-anchored proteins [1–4]. The pathway involves the stepwise addition of monosaccharides to phosphatidylinositol (PI) to form glycolipids with the core glycan structure of EthN-*P*-6Man₁-2Man₁-6Man₁-4GlcN attached to an inositol phospholipid (Scheme 1; reviewed in [5–8]). The affected cells of patients with paroxysmal nocturnal haemoglobinuria (PNH) acquire a somatic mutation in the first step of GPI biosynthesis, the transfer of GlcNAc from UDP-GlcNAc to PI. This mutation is equivalent to complementation class A derived previously in murine lymphoma cell lines [9–12]. PNH cells are susceptible to autologous complement-mediated lysis since DAF (decay-accelerating factor) and CD59, the GPI-anchored proteins responsible for preventing this autolysis, are not expressed on the cell surface [13]. Although the class A cDNA (PIG-A) responsible for this mutation has been cloned, gene therapy for treatment of PNH requires further development. A possible alternative therapeutic approach is to provide PNH cells with an exogenous lipid corresponding to a GPI intermediate downstream from the block

in PNH. The structure of such a lipid would have to include a GlcN in an α -1,6 linkage to inositol (Scheme 1).

To explore the ability of cells to take up and metabolize exogenous inositol phospholipids, we first studied exogenous *sn*-1-acyl-2-lyso-PI (*lyso*-PI) and PI [14]. We selected a HeLa S3 subline (subline D) as an attractive experimental system because these cells accumulate GlcN(acyl)PI [glucosaminyl(acyl)PI, which is glucosaminyl-PI (GlcN-PI) with a fatty acid acyl group on the inositol], the fourth intermediate in the proposed GPI biosynthetic pathway (Scheme 1), during metabolic labelling with [³H]inositol [15]. We found that both *lyso*-³HPI and [³H]PI were substantially taken up by HeLa D cells [14]. *lyso*-³HPI showed a greater extent of uptake and substantial cellular *sn*-2 acylation to [³H]PI. Furthermore, the incorporated *lyso*-³HPI and [³H]PI were translocated inside the cell and effectively modified into and accumulated as GlcN(acyl)³HPI, indicating that an exogenous lipid can proceed at least midway through the GPI pathway.

To continue our studies we then asked whether an exogenous lipid corresponding to an early intermediate in the GPI biosynthetic pathway could be incorporated extensively into cultured cells, and whether the incorporated lipid could be effectively metabolized through the GPI pathway bypassing the block in

Abbreviations used: PI, phosphatidylinositol; *lyso*-PI, *sn*-1-acyl-2-lyso-PI; GlcN-PI, glucosaminyl-PI; *lyso*-alkyl-GlcN-PI, *sn*-1-alkyl-*sn*-2-lyso-GlcN-PI; GlcN(acyl)PI, glucosaminyl(acyl)PI; PI-PLC, PI-specific phospholipase C; GPI, glycosylphosphatidylinositol; PNH, paroxysmal nocturnal haemoglobinuria; DMEM, Dulbecco's modified Eagle's medium.

¹ Present address: Research Department, Mayo Clinic Jacksonville, 4500 San Pablo Road, Jacksonville, FL 32224, U.S.A.

² To whom correspondence should be addressed (e-mail rosenberry@mayo.edu).

PNH. *sn*-1-Alkyl-*sn*-2-lyso-GlcN-PI (*lyso*-alkyl-GlcN-PI) is a logical choice for this uptake study because (i) similar to *lyso*-PI, it contains only one hydrophobic chain, allowing the lipid easily to diffuse as a monomer through the aqueous medium and insert into the outer leaflet of the plasma membrane, (ii) its structure is related to GlcN-PI, the third intermediate in the GPI pathway and thus downstream from the block in PNH (see Scheme 1) and (iii) it can be readily produced by alkaline hydrolysis of purified GlcN(acyl)PI. We showed that exogenous *lyso*-alkyl-GlcN-PI is substantially incorporated into cells and metabolized into GlcN-PI and GlcN(acyl)PI. However, further metabolism of the accumulated GlcN(acyl)PI to mannosylated GPIs could not be detected. We investigated the basis of this poor conversion by metabolic labelling with [14 C]- and [3 H]inositol.

Preparation of ³H-labelled inositol phospholipids

lys-alkyl-GlcN-[^3H]PI was prepared by base methanolysis of GlcN(acyl)[^3H]PI that had been purified from lipid extracts of HeLa D cells following metabolic labelling with [^3H]inositol [15]. Briefly, HeLa D cells (1×10^6 cells) on a 100 mm-diameter tissue-culture dish were incubated for 3 days with $100 \mu\text{Ci}$ of [^3H]inositol (American Radiolabelled Chemicals; 20 Ci/mmol) in medium 1 [inositol-free Dulbecco's modified Eagle's medium (DMEM), 10% dialysed horse serum, 50 units/ml penicillin, $50 \mu\text{g/ml}$ streptomycin and 5% CO_2] at 37°C . GlcN(acyl)[^3H]PI was purified from other ^3H -labelled endogenous lipids by chromatography on octyl-Sepharose with a linear gradient of 5–60% *n*-propyl alcohol in 100 mM ammonium acetate. Fractions containing GlcN(acyl)[^3H]PI ($\approx 1 \text{ Ci/mmol}$ based on inositol determination [16]) were pooled and concentrated in a SpeedVac (Savant), and GlcN(acyl)PI was extracted from the pooled fraction by partitioning with *n*-butanol (the overall radiochemical yield was 2–3% based on initial [^3H]inositol). For base methano-

lysis, 1.5–2 μCi of GlcN(acyl)[^3H]PI was dried and incubated with 50 μl of 0.2 M KOH in methanol for 30 min in a bath sonicator at room temperature. The reaction was stopped by adding 2 μl of glacial acetic acid, and the mixture was dried in a SpeedVac. After partitioning with *n*-butanol, the yield of alkaline-resistant lipid (*lyso*-alkyl-GlcN-[^3H]PI) was $\approx$ 8–20 % of the initial GlcN(acyl)[^3H]PI. The purity of *lyso*-alkyl-GlcN-[^3H]PI was determined to be at least 85–90 % by TLC, and no other discrete bands were detected. [^3H]PI and *lyso*-[^3H]PI (both at $\approx$ 1 Ci/mmol) were prepared as described previously [14], and ^3H -labelled H6, H7 and H8 lipid standards (Scheme 1) were prepared by metabolic labelling with [^3H]Man [17,18].

Incubation of HeLa D cells with *lyso*-alkyl-GlcN-[³H]PI

HeLa D cells [15] were maintained in 3 ml of medium 2 (DMEM, 10 % fetal bovine serum, 50 units/ml penicillin, 50 $\mu\text{g/ml}$ streptomycin and 5 % CO_2) at 37 °C and replated 24 h before each experiment. For *lyso*-alkyl-GlcN-[^3H]PI uptake, the lipid was dried, dissolved in ethanol and added to DMEM or DMEM plus 10 mM EGTA (the final concentration of ethanol was less than 1 %). Cells (5×10^6) on a 60 mm-diameter dish were washed twice with 3 ml of serum-free DMEM prior to addition of 3 ml of the lipid-containing medium. After incubation for 24 h in 5 % CO_2 at 37 °C, the cells and medium were removed and the pelleted cells were washed once with 3 ml of prechilled serum-free DMEM and incubated in 1 ml of serum-free DMEM for 1 h at 4 °C with or without 0.5 μg of purified *Bacillus thuringiensis* PI-specific phospholipase C (PI-PLC) [16]. The treated cells were washed twice with 3 ml of prechilled serum-free DMEM and lysed by adding 1 ml of chloroform/methanol (1:1, v/v) to a final mixture of chloroform/methanol/water (10:10:3, by vol.). The remaining pellet was re-extracted twice with 1 ml of chloroform/methanol/water (10:10:3, by vol.). The extract supernatants were combined, dried and partitioned by resuspending in 400 μl of water-saturated *n*-butanol and 200 μl of *n*-butanol-saturated water. The aqueous phase was removed

and extracted with 200 μ l of water-saturated *n*-butanol. The butanol phases were combined and re-extracted with fresh 200 μ l of *n*-butanol saturated water. The aqueous phases were also combined. Both pools were dried for analysis by TLC. Except where noted, the TLC plate was developed with a neutral solvent system of chloroform/methanol/water (10:10:3, by vol.); in some experiments a basic solvent system (chloroform/methanol/1 M NH_4OH , 10:10:3, by vol.) was also employed. The mobility of radiolabelled lipids on TLC was observed by scanning with a Bioscan System 200 imaging scanner and by fluorography after spraying the plate with EN^3HANCE (Dupont, Boston, MA, U.S.A.).

Enzymic and chemical treatments of lipids extracted from cells labelled with *lyso*-alkyl-GlcN-[^3H]PI

Lipids in cell extracts were cleaved with PI-PLC by drying an aliquot of the extract butanol phase, resuspending in 100 μ l of 50 mM Tris acetate (pH 5.5) containing 70 $\mu\text{g}/\text{ml}$ PI-PLC, and incubating overnight at room temperature to ensure complete cleavage of susceptible lipids. PI-PLC-resistant lipids were partitioned with *n*-butanol, dried and resuspended in 25 μ l of sodium acetate (pH 5) with or without Jack bean α -mannosidase (4 mg/ml; Sigma, St. Louis, MO, U.S.A.). After incubation at 37 °C for 8 h, a second 25 μ l aliquot of the same buffer with or without α -mannosidase was added. The incubation was continued for an additional 16 h at 37 °C, and the treated lipids recovered after partitioning with *n*-butanol were taken for TLC analysis.

Sequential double labelling of HeLa D cell lipids with [^{14}C]inositol and [^3H]inositol

HeLa D cells (1×10^6 cells) on a 100 mm-diameter dish were washed twice with 5 ml of inositol-free DMEM and incubated with 2 μCi of [^{14}C]inositol (American Radiolabelled Chemicals; 0.3 Ci/mmol) in 8 ml of medium 1. After incubation for 60 h in 5% CO_2 at 37 °C, the conditioned medium was removed and the cells were washed once with 5 ml of inositol-free DMEM. Cells were then incubated with 7 $\mu\text{g}/\text{ml}$ PI-PLC in 3 ml of inositol-free DMEM in 5% CO_2 at 37 °C for 1 h to remove cell-surface GPI-anchored proteins and washed three times with 5 ml of inositol-free DMEM. The incubation was then continued with 50 μCi of [^3H]inositol (20 Ci/mmol) in 8 ml of medium 1 for an additional 6–12 h. Cells were removed from the dish by scraping in the conditioned medium on ice, washed once with 3 ml of inositol-free DMEM and treated with 7 $\mu\text{g}/\text{ml}$ PI-PLC in 1 ml of inositol-free DMEM for 1 h in 5% CO_2 at 37 °C to remove GPI-anchored proteins recently transferred to the cell surface. The treated cells were washed three times with 5 ml of prechilled inositol-free DMEM, lysed by extraction with chloroform/methanol/water (10:10:3, by vol.), and partitioned with *n*-butanol as described above. The *n*-butanol phase of the cell extract was dried and taken for TLC analysis.

The lipid extract from the labelled cells was spotted with 50 μ l of water-saturated *n*-butanol as a 4 cm-long band on to a silica gel 60 TLC plate and developed with a solvent system of chloroform/methanol/water (10:10:3, by vol.). The mobilities of radiolabelled lipids were observed following fluorography after spraying the plate with EN^3HANCE . Bands corresponding to radiolabelled lipids detected by fluorography were scraped from the TLC plate and transferred to scintillation vials containing 0.5 ml of methanol, and 5 ml of scintillation fluid cocktail (Formula 963, NEN) was added for liquid scintillation counting. Corrections were applied for 31.25% of the ^{14}C counts that

crossed into the ^3H channel (none of the ^3H counts crossed into the ^{14}C channel).

Incorporation of the radiolabel into GPI anchors was estimated by measuring the radioactivity of proteins released from cells by PI-PLC treatments. The medium containing PI-PLC and released GPI-anchored proteins was dried, resuspended in 100 μ l of water, and mixed with 50 μ l of 1 g/ml trichloroacetic acid/4 mg/ml deoxycholic acid. This suspension was kept at 4 °C overnight, centrifuged at 14000 *g* at 4 °C for 15 min, washed twice with 500 μ l of cold acetone and centrifuged again under the same conditions. The pellet was resuspended by adding 40 μ l of SDS gel-loading buffer containing 50 mM dithiothreitol, heated to 65 °C for 5 min and loaded on to a SDS/polyacrylamide (10%) gel [19]. The developed gel was fixed in 100 mg/ml acetic acid/100 mg/ml glycerol for at least 15 min, and lanes containing the labelled GPI-anchored proteins were removed from the gel and cut transversely at 5 mm intervals with a gel slicer. Each gel slice was transferred to a scintillation vial, hydrated with 0.5 ml of water, hydrolysed with 0.5 ml of SolvableTM at 50 °C for at least 3 h to release the radiolabel from the gel, neutralized with 40 μ l of HCl and supplemented with 10 ml of scintillation fluid cocktail. The samples were left at room temperature for 2 days to equilibrate the released radiolabel with the cocktail prior to counting on a liquid scintillation counter. The extent of crossover between counts in the ^{14}C and ^3H channels was the same as that in lipid bands scraped from TLC plates.

RESULTS

Uptake of *lyso*-alkyl-GlcN-[^3H]PI by HeLa D cells

We first asked whether an exogenous lipid corresponding to an early intermediate in the GPI biosynthetic pathway (Scheme 1) could enter and proceed through this pathway. A convenient exogenous lipid was *lyso*-alkyl-GlcN-[^3H]PI, which we prepared by alkaline methanolysis of purified GlcN(acyl)PI derived from metabolic labelling of HeLa D cells with [^3H]inositol. For comparison, exogenous [^3H]PI and *lyso*-[^3H]PI at the same specific radioactivity were sometimes examined in parallel experiments. We assessed (i) the level of exogenous lipid uptake, (ii) its extent of localization on the cell surface and (iii) its metabolism to downstream GPI intermediates. HeLa D cells were incubated with the exogenous lipid for 24 h and then washed to remove residual label in the medium. After brief treatment with PI-PLC at 4 °C to remove susceptible labelled inositol phospholipids from the cell surface, the cells were scraped from the dish and extracted for determination of cellular lipids.

In initial experiments the efficiency of *lyso*-alkyl-GlcN-[^3H]PI uptake was low, corresponding to only 23% of the total label after a 24 h incubation, and most of this label was recovered in intracellular lipids (see Table 1). Degradation products obtained as hydrophilic metabolites in the medium were very abundant and constituted 67% of the total label. We reasoned that this degradation might result from the action of extracellular Ca^{2+} -dependent phospholipase(s), so we examined the effect of EGTA, a Ca^{2+} -chelating agent, on the uptake of *lyso*-alkyl-GlcN-[^3H]PI as well as of *lyso*-[^3H]PI and [^3H]PI. Addition of 10 mM EGTA to the medium during the 24 h incubation had only one obvious effect on the appearance of the HeLa D cells, namely that most cells were no longer attached to the plate at the end of the incubation period. More importantly, EGTA addition doubled the uptake of *lyso*-alkyl-GlcN-[^3H]PI and *lyso*-[^3H]PI into the cells and decreased the formation of hydrophilic metabolites in the medium from both *lyso* lipids by a factor of 2–3 (Table 1). The protective effect of EGTA was greater on these *lyso* lipids than on PI. EGTA addition did not increase [^3H]PI uptake and

Table 1 Distribution of radiolabel in HeLa D cells and in medium after incubation with 0.08–0.2 μ Ci of exogenous tritiated *lyso*-alkyl-GlcN-PI, *lyso*-PI or PI for 24 h

Incubations were conducted with or without addition of 10 mM EGTA to the medium. Cell-surface lipid was determined as radioactive label released from washed intact cells by treatment with PI-PLC for 1 h at 4 °C. Cells were then extracted with chloroform/methanol/water (10:10:3, by vol.), and both the cell extract and medium were partitioned with *n*-butanol into organic (intracellular lipids) and aqueous (hydrophilic metabolites) phases as outlined in the Experimental section. Amounts were calculated as percentages of the total labelled lipid added to a tissue-culture dish. Values shown are means $\pm$ S.E.M. for the number of experiments indicated in parentheses. ND, not determined

Compartment	Exogenous lipid ...	Distribution of radiolabel (%)					
		<i>lyso</i> -alkyl-GlcN-PI		<i>lyso</i> -PI		PI	
		– EGTA	+ EGTA	– EGTA*	+ EGTA	– EGTA*	+ EGTA
Cell							
Surface lipid, PI-PLC-susceptible		2	20 $\pm$ 1 (5)	2 $\pm$ 1 (2)	16	ND	ND
Intracellular lipids		15	25 $\pm$ 2 (5)	26 $\pm$ 1 (2)	54	31 $\pm$ 3 (4)	29
Hydrophilic metabolites		4	4 $\pm$ 1 (5)	7 $\pm$ 1 (2)	3	8 $\pm$ 2 (4)	3
Total		23 $\pm$ 2 (2)	50 $\pm$ 2 (5)	34 $\pm$ 1 (5)	68 $\pm$ 2 (2)	39 $\pm$ 2 (2)	32
Medium							
Lipid		11 $\pm$ 1 (2)	30 $\pm$ 4 (5)	2 $\pm$ 1 (5)	3 $\pm$ 0.1 (2)	27 $\pm$ 3 (6)	46
Hydrophilic metabolites		67 $\pm$ 1 (2)	20 $\pm$ 4 (5)	61 $\pm$ 3 (5)	29 $\pm$ 2 (2)	37 $\pm$ 4 (6)	23
Total		77 $\pm$ 2 (2)	50 $\pm$ 2 (5)	63 $\pm$ 2 (5)	32 $\pm$ 2 (2)	64 $\pm$ 2 (6)	68

* From data in [14].

Table 2 Distribution of intracellular lipid products derived from incubation of HeLa D cells with exogenous tritiated *lyso*-alkyl-GlcN-PI or *lyso*-PI for 24 h

Washed cells were treated with PI-PLC for 1 h at 4 °C and intracellular lipids were isolated as in Table 1. Amounts were calculated as percentages of the total labelled lipid added to a tissue-culture dish. Values are means $\pm$ S.E.M. for the number of experiments indicated in parentheses.

Intracellular lipid product	Exogenous lipid ...	Distribution of radiolabel (%)			
		<i>lyso</i> -alkyl-GlcN-PI		<i>lyso</i> -PI	
		– EGTA	+ EGTA	– EGTA	+ EGTA
<i>lyso</i> -alkyl-GlcN-PI	4		7.5 $\pm$ 1.6 (3)		
GlcN-PI	5		9.4 $\pm$ 1.8 (3)		
<i>lyso</i> -PI				1.9 $\pm$ 0.6 (2)	3
PI				18.3 $\pm$ 0.5 (2)	37
GlcN(acyl)PI	1.5 $\pm$ 0.1 (2)		4.0 $\pm$ 0.2 (6)	1.5 $\pm$ 0.2 (5)	2.5 $\pm$ 0.2 (2)

decreased the conversion of [3 H]PI to hydrophilic metabolites in the medium by only about 40%. Subsequent experiments included EGTA to increase incorporation of exogenous *lyso*-alkyl-GlcN-[3 H]PI into cellular lipids. After 24 h incubation, 30% of the total label remained as lipid in the medium, mostly in the form of unaltered *lyso*-alkyl-GlcN-[3 H]PI. Treatment of the incubated cells with PI-PLC at 4 °C removed 20% of the total label from the cell surface and left 30% inside the cells, mostly as intracellular lipids (see Table 1). Compared with *lyso*-alkyl-GlcN-[3 H]PI, *lyso*-[3 H]PI was taken up more quantitatively by cells. After *lyso*-[3 H]PI incubation in the presence of EGTA, only 3% of the total label remained as labelled lipids in the medium. PI-PLC removed 16% of the total label from the cell surface and left 57% inside the cells, mostly as intracellular lipids.

Cellular lipids derived from exogenous *lyso*-alkyl-GlcN-[3 H]PI were distributed in three major radiolabelled species both in the absence and presence of EGTA (Table 2). These species were identified as unaltered *lyso*-alkyl-GlcN-[3 H]PI and its fatty acylated derivatives GlcN-[3 H]PI and GlcN(acyl)[3 H]PI by their mobility relative to standard lipids on TLC (in both the neutral and basic solvent systems noted in the Experimental section). The assigned lipid structures also were consistent with their susceptibility or resistance to the bacterial PI-PLC used in this study. This PI-PLC cannot cleave polyphosphoinositides (e.g. PI

4-phosphate and PI 4,5-bisphosphate [20]) or GPIs containing an acylated inositol [21] but can act on inositol phospholipids and GPIs that do not contain these substituents. After resuspension of extracted intracellular lipids, the precursor *lyso*-alkyl-GlcN-[3 H]PI and its initial acylation product GlcN-[3 H]PI were completely susceptible to PI-PLC while GlcN(acyl)[3 H]PI was unaffected by PI-PLC treatment (Figure 1). PI-PLC treatment of the extracted lipids also revealed that a small amount of the exogenous *lyso*-alkyl-GlcN-[3 H]PI was incorporated and modified to more polar lipids. We sometimes observed a minor PI-PLC-resistant lipid product which migrated on TLC to a position slightly more polar than that of *lyso*-alkyl-GlcN-[3 H]PI and less polar than that of H6 (Figure 1, lanes 3 and 4). To examine whether this was a mannosylated GPI intermediate downstream from GlcN(acyl)PI, we treated the PI-PLC-resistant lipids with Jack-bean α -mannosidase, an enzyme that cleaves unsubstituted mannose residue(s) from GPIs and subsequently renders the cleaved GPIs less polar. However, treatment with α -mannosidase did not result in a clear shift in the mobility of the observed lipid (results not shown), and thus its nature remains unclear.

To assess the extent to which *lyso*-alkyl-GlcN-[3 H]PI and GlcN-[3 H]PI obtained after the 24 h incubation were exposed on the cell surface, lipids extracted from intact cells treated with or

Figure 1 PI-PLC susceptibility of cellular lipids produced from the uptake of *lyso*-alkyl-GlcN-[3 H]PI by HeLa cells

HeLa D cells (6×10^6 cells) were incubated in serum-free medium with *lyso*-alkyl-GlcN-[3 H]PI for 24 h, and the intact cells were treated with 0.5 μ g/ml PI-PLC for 1 h at 4 °C before extraction with chloroform/methanol/water (10:10:3, by vol.) and TLC analysis of the radiolabelled lipids (lanes 1 and 2, duplicate samples). An equal portion of dried extract was resuspended and treated with a high concentration of PI-PLC (50–70 μ g/ml) at room temperature overnight to ensure complete cleavage of susceptible lipids before TLC analysis (lane 3 and 4, duplicate samples). The developed TLC plate was sprayed with EN 3 HANCE and subjected to fluorography with an exposure period of 6 weeks. The mobility of standard lipids on the same TLC plate and the origin are indicated. The mobility of *lyso*-alkyl-GlcN-[3 H]PI is marked by the arrowhead. Man $_2$ GPI, GPI containing two mannose residues.

without PI-PLC were compared. As shown in Figure 2, about 40% of the cellular *lyso*-alkyl-GlcN-[3 H]PI and 50% of the cellular GlcN-[3 H]PI derived from exogenous *lyso*-alkyl-GlcN-[3 H]PI were removed by this PI-PLC treatment. These percentages are somewhat higher than those obtained for cellular lipids labelled by a comparable 24 h incubation with exogenous *lyso*-[3 H]PI, where only 15% of the cellular *lyso*-[3 H]PI and 20% of the cellular [3 H]PI were removed (A. Wongkajornsilp and T. L. Rosenberry, unpublished work). However, despite its lower uptake and internalization, *lyso*-alkyl-GlcN-[3 H]PI was more effectively converted to GlcN(acyl)[3 H]PI (4.0%) than was *lyso*-[3 H]PI (2.5%) in the presence of EGTA (Table 2).

To conclude that exogenous *lyso*-alkyl-GlcN-[3 H]PI was metabolically converted directly to other radiolabelled lipids, it was important to demonstrate that exogenous *lyso*-alkyl-GlcN-[3 H]PI was not degraded to [3 H]inositol and in turn utilized in *de novo* synthesis of radiolabelled lipid products. Addition of a large excess of 10 mM inositol [14] to HeLa cells during 72 h of metabolic labelling with [3 H]inositol resulted in a marked reduction of cellular radiolabelled lipid products from 63% to less than 0.2% of total label. In contrast, addition of 10 mM inositol to the medium during 24 h labelling with *lyso*-alkyl-GlcN-[3 H]PI changed neither the uptake level nor the distribution of label in the three major radiolabelled lipid products (results not shown). Thus it is unlikely that radiolabelled lipid products were synthesized *de novo* from [3 H]inositol derived from degraded

Figure 2 Cell-surface accessibility of cellular lipids produced from the uptake of *lyso*-alkyl-GlcN-[3 H]PI by HeLa D cells

HeLa D cells in paired dishes (6×10^6 cells) were incubated with *lyso*-alkyl-GlcN-[3 H]PI in serum-free medium for 24 h and treated with (dotted line) or without (solid line) 0.5 μ g/ml PI-PLC for 1 h at 4 °C. Cellular lipids were extracted and analysed by radioscaning after TLC as outlined in the Experimental section. Mobilities of standard H6, *lyso*-PI, *lyso*-alkyl-GlcN-PI, PI, GlcN-PI and GlcN(acyl)PI developed on adjacent TLC lanes are indicated. For clarity the dotted line is offset 0.2 cm to the right.

Scheme 2 One-compartment model

Inositol (A) is incorporated into PI (B) with rate constant k_1 . PI is modified into GlcN(acyl)PI (C; rate constant k_2) and GlcN(acyl)PI is converted into a mannosylated GPI (D; rate constant k_3). The rate constants for degradation or other metabolism of each species are k_a – k_d .

exogenous lipid, and this conclusion was supported by the fact that we did not observe formation of [3 H]PI during incubation with *lyso*-alkyl-GlcN-[3 H]PI. [3 H]PI is by far the most abundant lipid synthesized from [3 H]inositol.

GlcN(acyl)[3 H]PI accumulates in a relatively inert compartment in HeLa D cells

No detectable downstream mannosylated GPI intermediates were produced by labelling HeLa D cells with exogenous *lyso*-alkyl-GlcN-[3 H]PI (as noted above with Figure 1) or with [3 H]PI or *lyso*-[3 H]PI [14], even though GlcN(acyl)PI was effectively labelled and accumulated in a relatively large amount. To investigate the difficulty in labelling mannosylated GPIs in these cells, we asked whether the large amount of GlcN(acyl)[3 H]PI that accumulated during *de novo* synthesis from [3 H]inositol was confined to a single pool or compartment that could be readily metabolized to downstream intermediates. Previous data suggest that this is not the case. The turnover of GlcN(acyl)[3 H]PI in HeLa D cells is very slow, and its decay half-time of > 100 h following removal of [3 H]inositol from the medium is more than three times longer than that of [3 H]PI [22].

To examine this question in a more quantitative manner, we considered the one-compartment model in Scheme 2. In this

Table 3 $^3\text{H}/^{14}\text{C}$ ratios of inositol phospholipids and GPIs in HeLa D cells after sequential incubation with [^{14}C]inositol for 60 h followed by [^3H]inositol for 6 or 12 h

The cells were treated with PI-PLC for 1 h at 37 °C after both the first and the second incubations. Cellular lipids were extracted after the second incubation and analysed by TLC, and bands of radiolabelled lipids localized by fluorography were scraped from TLC plates and scintillation counted (see the Experimental section). GPIs are arranged in their order in the proposed biosynthetic pathway, and $^3\text{H}/^{14}\text{C}$ ratio data are the means ($\pm$ S.E.M.) from four measurements (two independent experiments in duplicate). Values for GPI anchors were obtained from GPI-anchored proteins released by the second PI-PLC treatment, precipitated with trichloroacetic acid and purified by SDS/PAGE. Ratios were determined by dual-channel scintillation counting of gel slices and are the means $\pm$ S.E.M. of two–four experiments. Absolute recoveries of ^{14}C ranged from 400 c.p.m. (H7) to 300 000 c.p.m. (PI). PIP, PI 4-phosphate; PIP₂, PI 4,5-bisphosphate.

Sample	Duration of [^3H]inositol labelling ...			
	6 h	12 h		
	$^3\text{H}/^{14}\text{C}$ ratio	Normalized ratio	$^3\text{H}/^{14}\text{C}$ ratio	Normalized ratio
PI	4.5 $\pm$ 0.1	1.0 $\pm$ 0.03	10.2 $\pm$ 1.0	1.0 $\pm$ 0.1
<i>lyso</i> -PI	3.7 $\pm$ 0.2	0.83 $\pm$ 0.04	7.0 $\pm$ 1.1	0.69 $\pm$ 0.11
PIP	3.9 $\pm$ 0.1	0.88 $\pm$ 0.03	8.6 $\pm$ 1.1	0.85 $\pm$ 0.1
PIP ₂	4.2 $\pm$ 0.1	0.93 $\pm$ 0.03	9.2 $\pm$ 1.0	0.9 $\pm$ 0.1
GlcN-PI	2.9 $\pm$ 1.0	0.64 $\pm$ 0.23	2.5 $\pm$ 0.7	0.25 $\pm$ 0.06
GlcN(acyl)PI	0.8 $\pm$ 0.0	0.18 $\pm$ 0.01	2.4 $\pm$ 0.2	0.24 $\pm$ 0.02
Lipid b	0.5 $\pm$ 0.1	0.1 $\pm$ 0.02	1.0 $\pm$ 0.1	0.09 $\pm$ 0.01
H6	2.1 $\pm$ 0.2	0.46 $\pm$ 0.05	4.6 $\pm$ 0.4	0.46 $\pm$ 0.04
H7	2.5 $\pm$ 0.2	0.55 $\pm$ 0.05	7.1 $\pm$ 1.1	0.7 $\pm$ 0.11
H8	1.8 $\pm$ 0.1	0.4 $\pm$ 0.02	4.3 $\pm$ 1.2	0.43 $\pm$ 0.12
GPI anchors	1.2 $\pm$ 0.1	0.26 $\pm$ 0.02	4.5 $\pm$ 0.1	0.45 $\pm$ 0.01

simple 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 or other metabolism of each species are k_a , k_b , k_c and k_d . The rate equations that result from Scheme 2 have been solved, and the relative time courses of [^3H]PI and GlcN(acyl)[^3H]PI accumulation in HeLa D cells during continuous labelling with [^3H]inositol and of decay when [^3H]inositol is removed from the medium are in accord with this model [22]. In particular, the one-compartment model requires that an upstream intermediate like PI always reaches the steady state more rapidly than a downstream intermediate like GlcN(acyl)PI. This feature suggested a double-labelling experiment with [^{14}C] and [^3H]inositol to provide a broader test of the one compartment model. In this experiment, HeLa D cells were first labelled to near steady state with [^{14}C]inositol for 60 h and then washed and treated with PI-PLC to remove existing cell-surface [^{14}C]GPI-anchored proteins. The cells were then incubated with the second label ([^3H]inositol) for an additional period of 6 or 12 h, a time too short to reach steady state for the ^3H -labelled species, and a second PI-PLC treatment of the intact cells was conducted to release freshly synthesized GPI-anchored proteins on the cell surface. During the 6–12 h pulse with [^3H]inositol there was also a concomitant 6–12 h chase of lipids derived from [^{14}C]inositol. However, the levels of [^{14}C]PI and GlcN(acyl)[^{14}C]PI change only slightly during a 6–12 h chase [22] so that ratios of [^3H]PI/[^{14}C]PI and GlcN(acyl)[^3H]PI/GlcN(acyl)[^{14}C]PI primarily reflect differences in the rate of synthesis of the ^3H -labelled lipids.

TLC analysis of the labelled cellular lipids obtained from the double-labelling experiments resolved a number of species in addition to PI and GlcN(acyl)PI, and each species was assigned by its mobility relative to standards. The silica band corresponding to each lipid was then scraped from the TLC plate, and its $^3\text{H}/^{14}\text{C}$ ratio was determined by dual-channel scintillation counting. The $^3\text{H}/^{14}\text{C}$ ratios of all the resolved lipids are shown in Table 3. To compare results obtained under different conditions, the observed $^3\text{H}/^{14}\text{C}$ ratio for each species was normalized to the $^3\text{H}/^{14}\text{C}$ ratio for PI in each experiment. As predicted by the one-compartment model, the normalized ratio

of 1.0 for the first intermediate PI was the highest normalized ratio among all labelled lipids. Extension of this model to the additional components of the GPI pathway in Table 3 predicts that the normalized $^3\text{H}/^{14}\text{C}$ ratio for other GPI intermediates should reflect their linear order in the GPI biosynthetic pathway: each downstream intermediate should have an equal or lower ratio than its predecessor in the pathway. An intermediate with a lower ratio than its successor deviates from the single compartment model and indicates an additional compartment with slower turnover for that intermediate. Ratios of just slightly less than 1.0 for PI 4-phosphate, PI 4,5-bisphosphate and *lyso*-PI in Table 3 revealed rapid turnovers and rates of approach to the steady state for these lipids. Furthermore, a lower $^3\text{H}/^{14}\text{C}$ ratio for GlcN(acyl)PI (0.18–0.24) than for PI initially is consistent with the prediction of the one-compartment model. However, Table 3 revealed a discordance relative to the ratios of the mannosylated GPIs H6, H7 and H8 that are downstream from GlcN(acyl)PI. Following 12 h labelling with [^3H]inositol, the ratios of $^3\text{H}/^{14}\text{C}$ in H6 (0.46), H7 (0.7) and H8 (0.43) were much higher than that of the upstream GlcN(acyl)PI (0.24). The low ratio for GlcN(acyl)PI indicated that this lipid is distributed between more than one pool, thus violating the one-compartment model, and demonstrated that some GlcN(acyl)PI has a distinctly slower turnover rate than H6, H7 or H8. Similar conclusions were evident from the relative ratios in the 6 h labelling with [^3H]inositol in Table 3.

We also measured the $^3\text{H}/^{14}\text{C}$ ratio of GPI-anchored proteins released by the second cell-surface PI-PLC treatment, since these released proteins consisted both of [^{14}C]GPI-anchored proteins that were initially immature during the first PI-PLC treatment and freshly synthesized [^3H]/[^{14}C]GPI-anchored proteins. The GPI-anchored proteins were precipitated from PI-PLC-treated medium and analysed by SDS/PAGE (Figure 3). Lanes containing GPI-anchored proteins were sliced at 5 mm intervals and scintillation counted to determine the $^3\text{H}/^{14}\text{C}$ ratio of the eluted radioactivity. Ratios of $^3\text{H}/^{14}\text{C}$ were roughly constant for two major inositol-labelled bands and corresponded to 0.45 for the 12 h labelling and 0.26 for the 6 h labelling. These ratios also were higher than those for GlcN(acyl)PI, indicating that even GPI anchors, the final product of the GPI pathway, have faster turnover rates than GlcN(acyl)PI.

Figure 3 SDS/PAGE analysis of [^3H]/[^{14}C]GPI-anchored proteins released by PI-PLC from the HeLa D cell surface

HeLa D cells (10^6) were labelled metabolically with [^{14}C]inositol and [^3H]inositol as outlined in Table 3 and the Experimental section. The medium taken from the second PI-PLC treatment was treated with trichloroacetic acid, and the precipitate was analysed by SDS/PAGE as outlined in the Experimental section. Individual gel lanes were sliced, and ^3H and ^{14}C c.p.m. were determined by scintillation counting.

DISCUSSION

In these experiments, we observed cellular uptake of exogenous *lyso*-alkyl-GlcN-PI by HeLa D cells. This uptake was improved by adding EGTA to reduce lipid degradation by extracellular Ca^{2+} -dependent phospholipase(s). Instead of simply remaining on the cell surface, the incorporated lipid entered the cells, as evidenced by its intracellular metabolism (e.g. inositol acylation) and its partial resistance to a membrane-impermeable probe (PI-PLC). This study demonstrates for the first time that an exogenous GPI can reach a GPI biosynthetic compartment in cultured cells and proceed at least part of the way along the pathway, as shown by its metabolism into GlcN(acyl)PI. This processing of exogenous *lyso*-alkyl-GlcN-PI supports the potential use of exogenous GPIs as therapeutic agents for PNH.

Exogenous *lyso*-alkyl-GlcN-PI as well as two other exogenous lipids (PI and *lyso*-PI) can be modified into GlcN(acyl)PI, and this GPI intermediate also accumulated to high levels in HeLa D cells after [^3H]inositol metabolic labelling [15]. The uptake of exogenous *lyso*-alkyl-GlcN-[^3H]PI and exogenous *lyso*-[^3H]PI in HeLa D cells shared some similarities: (i) the extents of their uptake were increased by EGTA, (ii) they were translocated inside the cell and (iii) they were susceptible to *sn*-2 acylation. However, *lyso*-alkyl-GlcN-[^3H]PI was taken up from the medium to a lesser extent than exogenous *lyso*-[^3H]PI. Exogenous *lyso*-alkyl-GlcN-[^3H]PI also was less efficiently translocated inside the cell than was exogenous *lyso*-[^3H]PI, as shown by the fact that PI-PLC treatment released more cell-surface lipid derived from *lyso*-alkyl-GlcN-[^3H]PI than from *lyso*-[^3H]PI. Acylation at the *sn*-2 position of exogenous *lyso*-alkyl-GlcN-[^3H]PI to give GlcN-[^3H]PI also was less efficient than acylation of exogenous *lyso*-[^3H]PI to [^3H]PI, since the percentage of unaltered cellular *lyso*-alkyl-GlcN-[^3H]PI was higher than that of unaltered cell-

ular *lyso*-[^3H]PI (Table 2). Despite these disadvantages in lower uptake, translocation and *sn*-2 acylation, exogenous *lyso*-alkyl-GlcN-[^3H]PI still was converted to GlcN(acyl)[^3H]PI in a greater percentage than exogenous *lyso*-[^3H]PI. The metabolic advantage of *lyso*-alkyl-GlcN-PI over PI and *lyso*-PI can be explained by its attachment of a GlcN residue to the inositol headgroup, thus bypassing the slow metabolic transfer of GlcNAc to PI. This slow transfer, known to be defective in PNH, is usually rate determining for the GPI pathway, and most exogenous *lyso*-[^3H]PI accumulates as [^3H]PI.

HeLa D cells are unusual in accumulating large amounts of GlcN(acyl)PI, and we view this as a decided advantage in our studies of *lyso*-alkyl-GlcN-PI as a precursor of GPI anchors. GlcN(acyl)PI was almost undetectable in other cell types, including HeLa CCL-2 cells, Madin-Darby canine kidney cells, insect S2 cells, rat hepatocytes, S49 class A lymphoma mutants, K562 cells and K562 class K mutants (A. Wongkajornsilp and T. L. Rosenberry, unpublished work). Furthermore, incubation of the class A and class K cells with the amounts of *lyso*-alkyl-GlcN-[^3H]PI used here failed to generate detectable amounts of either GlcN(acyl)[^3H]PI or its mannosylated derivatives (A. Wongkajornsilp and T. L. Rosenberry, unpublished work). The possibility that GlcN(acyl)PI mannosylation is defective in HeLa D cells has been ruled out [22]. While these cells accumulated unusual amounts of GlcN(acyl)PI (10^7 molecules/cell determined by metabolic labelling with [^3H]inositol [15]), mannosylated GPIs were observed in amounts typical of other cell lines. Therefore, the metabolism of GlcN(acyl)PI by the first mannosyl transferase in HeLa D cells was not blocked, but the GlcN(acyl)PI mannosyl transferase activity in these cells appeared not to exceed that in other cells despite the high level of GlcN(acyl)PI.

Current study of *lyso*-alkyl-GlcN-PI as a precursor is limited by the small amount of exogenous lipid radiolabel available and particularly by its low specific radioactivity. At a specific radioactivity of ≈ 1 Ci/mmol, the exogenous labelled lipids introduced in the experiments here (0.15 – 0.2 μCi) corresponded to about 1–2 % of the total cell-surface phospholipid. Addition of significantly larger amounts of exogenous *lyso* phospholipids could damage the cells. The production of *lyso*-alkyl-GlcN-[^3H]PI by chemical cleavage of GlcN(acyl)[^3H]PI obtained from metabolic labelling of HeLa D cells with [^3H]inositol at 20 Ci/mmol is inefficient and results in an overall radiochemical yield of only 0.2 %. With 0.2 μCi of exogenous *lyso*-alkyl-GlcN-[^3H]PI available and a 4 % efficiency of conversion to GlcN(acyl)[^3H]PI in HeLa D cells (Table 2), it would be difficult to detect further mannosylation of this GlcN(acyl)[^3H]PI even if it proceeded at the same rate at which GlcN(acyl)[^3H]PI synthesized *de novo* from [^3H]inositol is mannosylated. Mannosylation is inefficient with GlcN(acyl)PI produced endogenously from [^3H]inositol. The mannosylation efficiency, defined as the ratio of H6+H7+H8 to GlcN(acyl)PI, was only 0.097 ± 0.004 for the ^{14}C -labelled lipids in Table 3. A similar mannosylation efficiency for GlcN(acyl)[^3H]PI produced from 0.2 μCi of exogenous *lyso*-alkyl-GlcN-[^3H]PI would result in less than 1000 c.p.m. of H6, H7 and H8 in total, an amount barely detectable with our methods. One possible explanation for the failure to see increased mannosylation despite the high level of GlcN(acyl)PI in HeLa D cells is that the first mannosyl transferase is saturated at the low level of GlcN(acyl)PI typical of most cells. This would result in a constant rate of mannosylation regardless of the cellular GlcN(acyl)PI concentration. An alternative possible explanation is that GlcN(acyl)PI in HeLa D cells largely accumulates in an inactive compartment devoid of mannosyl transferase where an increased concentration of GlcN(acyl)PI would have no effect on the rate of mannosylation.

To investigate these two explanations for the poor efficiency of GlcN(acyl)PI mannosylation, we examined whether GlcN(acyl)PI synthesized *de novo* from radiolabelled inositol was in a single pool or compartment. We tested this single-compartment model with an experiment in which HeLa D cells were first labelled with [^{14}C]inositol for 60 h and then labelled with [^3H]inositol for 6 or 12 h. Ratios of $^3\text{H}/^{14}\text{C}$ incorporation provided information about several lipid species (Table 3). The one-compartment model in Scheme 2 predicts that the $^3\text{H}/^{14}\text{C}$ ratio in mannosylated GPIs will be lower than or equal to that in upstream GlcN(acyl)PI. Our data lead us to reject a one-compartment hypothesis for GlcN(acyl)PI, because the $^3\text{H}/^{14}\text{C}$ ratios in mannosylated GPIs (H6, H7 and H8) were higher than that of GlcN(acyl)PI. The $^3\text{H}/^{14}\text{C}$ ratio in the last component of the biosynthetic pathway, GPI-anchored proteins, is clearly of interest because it sets a lower limit for the ratio of any intermediate that is confined to only a single biosynthetic compartment. In an effort to improve the accuracy of the estimate of this ratio, HeLa D cells were treated with PI-PLC at 37 °C after the initial 60 h labelling with [^{14}C]inositol to clear as much pre-existing [^{14}C]GPI-anchored proteins as possible. At 4 °C, cell-surface GPI-anchored proteins were cleaved more slowly than cell-surface free inositol phospholipids by PI-PLC treatment, whereas both proteins and lipids were readily removed by PI-PLC treatment at 37 °C. Clearing from the cell surface was relatively effective, as a second treatment with PI-PLC 30 min later released only about 15% of the ^{14}C -labelled proteins cleaved in the initial PI-PLC treatment (A. Wongkajornsilp and T. L. Rosenberry, unpublished work). However, some intracellular ^{14}C -labelled GPI-anchored proteins still remained following this procedure. These proteins along with GPI-anchored proteins newly synthesized from the free GPI pools continued to move to the cell surface during the subsequent labelling with [^3H]inositol for an additional 6–12 h, and they lowered the $^3\text{H}/^{14}\text{C}$ ratio of the newly synthesized proteins released by another PI-PLC treatment. Nevertheless, the $^3\text{H}/^{14}\text{C}$ ratio for the released GPI-anchored proteins exceeded those for GlcN(acyl)PI at both the 6 and 12 h time points. The $^3\text{H}/^{14}\text{C}$ ratio obtained from 12 h labelling (0.45) is higher and more accurate than that from 6 h labelling (0.26) since the pre-existing pool of ^{14}C -labelled GPI-anchored proteins make a smaller contribution after 12 h. We conclude that [^3H]inositol was effectively incorporated into mannosylated GPIs and GPI anchors largely by bypassing the pool of accumulated GlcN(acyl)PI.

We suggest a two-compartment model to explain the heterogeneous GlcN(acyl)PI pool. The two separate compartments are defined on the basis of the turnover of GlcN(acyl)PI. The relatively low $^3\text{H}/^{14}\text{C}$ ratio in GlcN(acyl)PI indicated that freshly synthesized GlcN(acyl)[^3H]PI was more readily used for further biosynthesis of mannosylated GPIs than was the pre-existing GlcN(acyl)[^{14}C]PI. Therefore, freshly synthesized GlcN(acyl)[^3H]PI and pre-existing GlcN(acyl)[^{14}C]PI are not homogeneously mixed. Most pre-existing GlcN(acyl)[^{14}C]PI must accumulate in a separate compartment in which it is not actively utilized for further GPI biosynthesis. Some GlcN(acyl)PI in HeLa D cells is deposited in the plasma-membrane fraction (D. Sevillever, unpublished work) and the majority (70%) is present in Triton-insoluble microdomains [23]. These pools partially overlap, and one or both may correspond to GlcN(acyl)PI that is physically isolated from the corresponding first mannosyl transferase in the endoplasmic reticulum. The indication that most GlcN(acyl)PI resides in a compartment in which it undergoes mannosylation slowly if at all may be relevant to the success of exogenous lipids like *lyso*-alkyl-GlcN-

PI as GPI anchor precursors. If GlcN(acyl)PI generated from these exogenous lipids fails to enter compartment(s) active in GPI mannosylation and remains confined to an inert compartment, the exogenous lipids will be ineffective precursors. Further experiments with larger quantities and higher specific activities of *lyso*-alkyl-GlcN-[^3H]PI will be necessary to resolve this point for HeLa D cells as well as for other cells. Our results suggest that it may be difficult to utilize *lyso*-alkyl-GlcN-PI as an exogenous precursor to bypass the defect in PNH cells. However, the strategy of using exogenous glycolipids to correct this defect remains important and largely ignored. The possibility remains that other exogenous precursors, like a *lyso*-alkyl mannosylated (GlcN)PI, may be converted more efficiently into intermediates that do proceed through the GPI pathway.

Table 3 reveals that other GPI intermediates also distribute at least partially in an inert compartment. The lowest observed $^3\text{H}/^{14}\text{C}$ ratio belonged to lipid b, a lipid with a mobility between PI and GlcN(acyl)PI on TLC. Lipid b was previously implicated as a degradation product of GlcN(acyl)PI [15], and its very low $^3\text{H}/^{14}\text{C}$ ratio is consistent with its formation in the inert compartment which contains GlcN(acyl)PI. The $^3\text{H}/^{14}\text{C}$ ratio for GlcN-PI at 12 h of [^3H]inositol labelling (0.25) appeared to be as low as that of GlcN(acyl)PI. The GlcN-PI band on TLC was very close to the PI band and hence any contamination with the PI band during scraping could make the observed $^3\text{H}/^{14}\text{C}$ ratio for GlcN-PI artificially high, perhaps accounting for a higher ratio (0.64) at 6 h of [^3H]inositol labelling. Nevertheless, these ratios suggest that GlcN(acyl)PI may be converted from GlcN-PI in the inert compartment, in parallel with the same conversion in the more active GPI biosynthetic compartment. The $^3\text{H}/^{14}\text{C}$ ratio for H7 was higher than that for H6, particularly with the 12 h [^3H]inositol labelling, indicating that freshly synthesized H6 was more readily used for further biosynthesis than pre-existing H6. There is evidence for the distribution of H6 in multiple compartments, as H6, H7 and H8 have been reported in the plasma membranes of several cell lines, including K562, Madin-Darby canine kidney, HeLa and lymphoma (EL-4) [24–27]. Furthermore, inert compartments may exist even within the endoplasmic reticulum, as some steps of the mammalian GPI anchor pathway have been suggested to occur in different endoplasmic reticulum regions [23,28] and in endoplasmic reticulum-associated membranes [29]. The relatively high $^3\text{H}/^{14}\text{C}$ ratio for H7 suggests that H7 is rapidly metabolized, possibly into GPI anchors on proteins. Rapid transfer of H7 to protein acceptors would be compatible with the observation that the predominant structure in GPI anchors corresponds to that of H7 (e.g. rat brain Thy-1 [30], human erythrocyte acetylcholinesterase [31], hamster brain scrapie prion protein [32], human placental alkaline phosphatase [33] and CD52 [34]).

We thank Dr M. Edward Medof, Dr M. E. Maguire and Dr G. Dubyak for reviewing the manuscript. This work was supported by grant DK38181 (to T.L.R.) and DK55002 (to D.S.) from the National Institutes of Health.

REFERENCES

- 1 Lemansky, P., Gupta, D. K., Tucker, M. G. and Tartakoff, A. M. (1991) Atypical mannolipids characterize Thy-1-negative lymphoma mutants. *Mol. Cell. Biol.* **11**, 3879–3885
- 2 Puoti, A. and Conzelmann, A. (1993) Characterization of abnormal free glycosylphosphatidylinositols accumulating in mutant lymphoma cells of classes B, E, F, and H. *J. Biol. Chem.* **268**, 7215–7224
- 3 Hirose, S., Mohny, R. P., Mutka, S. C., Ravi, L., Singleton, D. R., Perry, G., Tartakoff, A. M. and Medof, M. E. (1992) Derivation and characterization of glycosylphospholipid anchor-defective human K562 cell clones. *J. Biol. Chem.* **267**, 5272–5278

- 4 Kamitani, T., Menon, A. K., Hallaq, Y., Warren, C. D. and Yeh, E. T. H. (1992) Complexity of ethanolamine phosphate addition in the biosynthesis of glycosylphosphatidylinositol anchors in mammalian cells. *J. Biol. Chem.* **267**, 24611–24619
- 5 Low, M. G. and Saltiel, A. R. (1988) Structural and functional roles of glycosylphosphatidylinositol in membranes. *Science* **239**, 268–275
- 6 Menon, A. K. (1991) Biosynthesis of glycosyl-phosphatidylinositol. *Cell Biol. Int. Rep.* **15**, 1007–1021
- 7 Englund, P. T. (1993) The structure and biosynthesis of glycosyl phosphatidylinositol protein anchors. *Ann. Rev. Biochem.* **62**, 121–138
- 8 Stevens, V. (1995) Biosynthesis of glycosylphosphatidylinositol membrane anchors. *Biochem. J.* **310**, 361–370
- 9 Armstrong, C., Schubert, J., Ueda, E., Knez, J. J., Gelperin, D., Hirose, S., Silber, R., Hollan, S., Schmidt, R. and Medof, M. E. (1992) Affected paroxysmal nocturnal hemoglobinuria T lymphocytes harbor a common defect in assembly of N-acetyl-D-glucosamine inositol phospholipid corresponding to that in class A Thy-1 murine lymphoma mutants. *J. Biol. Chem.* **267**, 25347–25351
- 10 Takahashi, M., Takeda, J., Hirose, S., Hyman, R., Inoue, N., Miyata, T., Ueda, E., Kitani, T., Medof, M. E. and Kinoshita, T. (1993) Deficient biosynthesis of N-acetylglucosaminyl-phosphatidylinositol, the first intermediate of glycosyl phosphatidylinositol anchor biosynthesis, in cell lines established from patients with paroxysmal nocturnal hemoglobinuria. *J. Exp. Med.* **177**, 517–521
- 11 Hidaka, M., Nagakura, S., Horikawa, T., Kawaguchi, T., Iwamoto, N., Kagimoto, T., Takasaki, K. and Nakakuma, H. (1993) Impaired glycosylation of glycosylphosphatidylinositol-anchor synthesis in paroxysmal nocturnal hemoglobinuria leukocytes. *Biochem. Biophys. Res. Commun.* **191**, 571–579
- 12 Hillman, P., Bessler, M., Mason, P. J., Watkins, W. M. and Luzzatto, L. (1993) Specific defect in N-acetylglucosamine incorporation in the biosynthesis of the glycosylphosphatidylinositol anchor in cloned cell lines from patients with paroxysmal nocturnal hemoglobinuria. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5272–5276
- 13 Yamashina, M., Ueda, E., Kinoshita, T., Takami, T., Ojima, A., Ono, H., Tanaka, H., Kondo, N., Orii, T., Okada, N. et al. (1990) Inherited complete deficiency of 20-kilodalton homologous restriction factor (CD59) as a cause of paroxysmal nocturnal hemoglobinuria. *N. Engl. J. Med.* **323**, 1184–1189
- 14 Wongkajornsilp, A. and Rosenberry, T. L. (1995) Uptake of exogenous *sn*-1-acyl-2-*lyso*-phosphatidylinositol into HeLa S3 cells; reacylation on the cell surface and metabolism to glucosaminyl(acyl)phosphatidylinositol. *J. Biol. Chem.* **270**, 9147–9153
- 15 Seveler, D., Humphrey, D. R. and Rosenberry, T. L. (1995) Compositional analysis of glucosaminyl(acyl)phosphatidylinositol accumulated in HeLa S3 cells. *Eur. J. Biochem.* **233**, 384–394
- 16 Deeg, M. A., Humphrey, D. R., Yang, S. H., Ferguson, T. R., Reinhold, V. N. and Rosenberry, T. L. (1992) Glycan components in the glycoinositol phospholipid anchor of human erythrocyte acetylcholinesterase. Novel fragments produced by trifluoroacetic acid. *J. Biol. Chem.* **267**, 18573–18580
- 17 Hirose, S., Prince, G. M., Seveler, D., Ravi, L., Rosenberry, T. L., Ueda, E. and Medof, M. E. (1992) Characterization of putative glycoinositol phospholipid anchor precursors in mammalian cells: localization of phosphoethanolamine. *J. Biol. Chem.* **267**, 16968–16974
- 18 Seveler, D. and Rosenberry, T. L. (1993) Mannosamine inhibits the synthesis of putative glycoinositol phospholipid anchor precursors in mammalian cells without incorporating into an accumulated intermediate. *J. Biol. Chem.* **268**, 10938–10945
- 19 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- 20 Griffith, O. H., Volwerk, J. J. and Kuppe, A. (1991) Phosphatidylinositol-specific phospholipase C from *Bacillus cereus* and *Bacillus thuringiensis*. *Methods Enzymol.* **197**, 493–502
- 21 Roberts, W. L., Myher, J. J., Kuksis, A., Low, M. G. and Rosenberry, T. L. (1988) Lipid analysis of the glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholine-esterase. Palmitoylation of inositol results in resistance to phosphatidylinositol-specific phospholipase C. *J. Biol. Chem.* **263**, 18766–18775
- 22 Seveler, D., Schiemann, D., Guidubaldi, J., Medof, M. E. and Rosenberry, T. L. (1997) Accumulation of glucosaminyl(acyl)phosphatidylinositol in an S3 HeLa subline expressing normal dolicholphosphomannose synthase activity. *Biochem. J.* **321**, 837–844
- 23 Seveler, D., Pickett, S., Mann, K. J., Sambamurti, K., Medof, M. E. and Rosenberry, T. L. (1999) Glycosylphosphatidylinositol anchor intermediates associate with Triton insoluble membranes in subcellular compartments that include the endoplasmic reticulum. *Biochem. J.* **343**, 627–635
- 24 Rosenberry, T. L., Seveler, D. and Medof, M. E. (1994) Metabolism of GPIs in mammalian cells. *Braz. J. Med. Biol. Res.* **27**, 151–159
- 25 van't Hof, W., Rodriguez-Boulant, E. and Menon, A. K. (1995) Nonpolarized distribution of glycosylphosphatidylinositols in the plasma membrane of polarized Madin-Darby canine kidney cells. *J. Biol. Chem.* **270**, 24150–24155
- 26 Singh, N., Liang, L.-N., Tykocinski, M. L. and Tartakoff, A. M. (1996) A novel class of cell surface glycolipids of mammalian cells. Free glycosyl phosphatidylinositols. *J. Biol. Chem.* **271**, 12879–12884
- 27 Baumann, N. A., Vidugiriene, J., Machamer, C. E. and Menon, A. K. (2000) Cell surface display and intracellular trafficking of free glycosylphosphatidylinositols in mammalian cells. *J. Biol. Chem.* **275**, 7378–7389
- 28 Stevens, V. L., Zhang, H. and Kristyenne, E. S. (1999) Stimulation of glycosylphosphatidylinositol biosynthesis in mammalian cell-free systems by GTP hydrolysis: evidence for the involvement of membrane fusion. *Biochem. J.* **341**, 577–584
- 29 Vidugiriene, J., Sharma, D. K., Smith, T. K. and Menon, A. K. (1999) Segregation of glycosyl-phosphatidylinositol biosynthetic reactions in a subcompartment of the endoplasmic reticulum. *J. Biol. Chem.* **274**, 15203–15212
- 30 Homans, S. W., Ferguson, M. A. J., Dwek, R. A., Rademacher, T. W., Anand, R. and Williams, A. F. (1988) Complete structure of the glycosyl phosphatidylinositol membrane anchor of rat brain Thy-1 glycoprotein. *Nature (London)* **333**, 269–272
- 31 Roberts, W. L., Santikarn, S., Reinhold, V. N. and Rosenberry, T. L. (1988) Structural characterization of the glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase by fast atom bombardment mass spectrometry. *J. Biol. Chem.* **263**, 18776–18784
- 32 Stahl, N., Baldwin, M. A., Hecker, R., Pan, K. M., Burlingame, A. L. and Prusiner, S. B. (1992) Glycosylphospholipid anchors of scrapie and cellular prion proteins contain sialic acid. *Biochemistry* **31**, 5043–5053
- 33 Redman, C. A., Thomas-Oates, J. E., Ogata, S., Ikehara, Y. and Ferguson, M. A. (1994) Structure of the glycosylphosphatidylinositol membrane anchor of human placental alkaline phosphatase. *Biochem. J.* **302**, 861–865
- 34 Treumann, A., Lifely, M. R., Schneider, P. and Ferguson, M. A. (1995) Primary structure of CD52. *J. Biol. Chem.* **270**, 6088–6099

Received 8 March 2001/18 June 2001; accepted 27 July 2001