Synthesis of mannosylglucosaminylinositol phospholipids in normal but not paroxysmal nocturnal hemoglobinuria cells

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ABSTRACT To identify mannosyl (Man)-containing intermediates of the human glycosinositol phosphopholipid (GPI) anchor pathway and examine their expression in paroxysmal nocturnal hemoglobinuria (PNH), mannolipid products derived from in vitro guanosine diphosphate [3H]Man labeling of HeLa cell microsomes were characterized. The defined GPI species were correlated with products derived from in vivo [3H]Man labeling of normal and (GPI-anchor defective) affected leukocytes. In vitro analyses in HeLa cells showed dolichol-phosphoryl (Dol-P)-[3H]Man and a spectrum of [3H]Man lipids exhibiting TLC mobilities approximating those of Trypanosoma brucei (Tryp) GPI precursors. Iatrobead HPLC separations and partial characterizations of the major isolated [3H]Man species (designated H1–H8) showed that all but H1 (Dol-P-Man) were sensitive to HNO3 deamination and serum GPI-specific phospholipase D digestion but were resistant to phosphatidylinositol-specific phospholipase D digestion unless previously deacylated with mild alkali. [3H]Man label in H3, H4, and H6 but not in H5 or H7 was efficiently released into the aqueous phase by jack bean α-mannosidase digestion. Bio-Gel P-4 and AX-5 sizing of the dephosphorylated core glycan fragments of H6 and H7 gave values that coincided precisely with the corresponding glycan fragments from the fully assembled Tryp anchor donor A' (P2). Affected leukocytes from four patients with PNH supported formation of GlcNAc- and GlcN-PI but all failed to express H6 and H7 as well as H8 and two showed complete absence of earlier Man-containing intermediates. These findings argue that human intracellular GPI mannolipids are built on acylated inositol phospholipids, that H6 and H7 contain differentially phosphoethanolamine-substituted Man-GlcN-inositol cores, and that PNH cells are defective in conversion of GlcN-PI into these more mature mannolipid structures.

Cell-surface proteins that are membrane anchored by covalently attached glycosinositol phosphopholipid (GPI) structures are widely distributed on eukaryotic cells. Experimental evidence to date (reviewed in ref. 1) indicates that, despite individual variations, the GPI-anchoring units of these proteins share a common core structure (Fig. 1) consisting of a phosphoethanolamine (P-Etn), three mannosyl (Man) residues, and a nonacetylated GlcN linked to an inositol phospholipid. This inositol phospholipid is characteristically phosphatidylinositol-specific phospholipase C (PI-PLC) sensitive but it may be PI-PLC resistant due to inositol acylation. In all cases studied, GPI-anchoring moieties appear to be preassembled in the endoplasmic reticulum (ER) and, once synthesized, transferred en bloc to primary translation products immediately upon their emergence from ribosomes. Although more than 50 proteins containing these structures have been identified, information concerning the biochemical pathway(s) responsible for their intracellular GPI-anchor assembly and protein incorporation is only beginning to emerge.

Among human GPI-anchored proteins are the decay-accelerating factor (DAF) and CD59, membrane-associated regulators of the complement cascade which protect host tissues from autologous complement attack (reviewed in refs. 2–4). Deficient expression of these proteins underlies an acquired hemolytic disorder termed paroxysmal nocturnal hemoglobinuria (PNH), and the lesion responsible for the deficit has been traced to defective GPI-anchor pathway function in a bone marrow stem cell(s) (5). The precise site(s) of interruption in the pathway, however, remains unidentified.

In a previous communication (6), we reported on the use of an in vitro experimental system employing hypotonic cell lysates to study mammalian GPI-anchor biosynthesis. The experimental system parallels that used for analyses of GPI-anchor structures synthesized in Trypanosoma brucei (Tryps) (7, 8). We previously demonstrated that mammalian cells transfer GlcNAc from UDP to a PI acceptor and rapidly deacetylate the GlcNAc-PI product to generate GlcN-PI, two species (designated GPI-A and -B) that correspond to the two initial intermediates in the Tryp GPI-anchor pathway (7, 8). We were further able to show that cell lysates of GPI-anchor defective leukocytes from two PNH patients were able to support these biochemical conversions with normal efficiencies.

In the present communication, we report on the extension of this experimental system to characterize further steps in the human GPI-anchor pathway. We also report on the partial localization within these further steps of the block(s) in GPI-anchor synthesis that undermines expression of DAF, CD59, and other GPI-anchored proteins in affected PNH cells.

EXPERIMENTAL PROCEDURES

Reagents. GDP-[3H]Man (24.3 Ci/mmol; 1Ci = 37 GBq). UDP-[3H]GlcNAc (26.8 Ci/mmol), d-[2-3H]Man (15 Ci/mmol), and ENHANCE spray were obtained from DuPont/New England Nuclear. [3H]Glucose oligomer standards were provided by J. Baenziger (Washington University, St. Louis, MO). Tunicamycin, ATP, sphingomyelinase, and all protease

Abbreviations: GPI, glycosinositol phosphopholipid; P-Etn, phosphoethanolamine; ER, endoplasmic reticulum; DAF, decay-accelerating factor; PNH, paroxysmal nocturnal hemoglobinuria; Tryps, Trypanosoma brucei; PI-PLC, phosphatidylinositol-specific phospholipase C; GPI-PLD, GPI-specific phospholipase D; PMN, polymorphonuclear leukocyte; Chl, chlorof orm; Dol-P, dolichol-phosphoryl. □To whom correspondence should be addressed: Institute of Pathology, Case Western Reserve University, 2085 Adelbert Road, Cleveland, OH 44106.
Fig. 1. A human GPI-anchor core structure characterized by an acylated inositol phospholipid is boxed in the center. Structures and partitioning behaviors of predicted products of various treatments are indicated. * Effective only in the absence of P-En (E-P).

**Mannolipid Purifications and Glycan Analyses.** [\(^3\)H]Man-labeled lipid products were separated on a 6RSP-8010 (4.6 × 250 mm) Iatrobed HPLC column (Iatron Laboratories, Tokyo) in a Dionex DX-300 apparatus using a linear gradient starting with Chl/methanol (80:20) extending to Chl/methanol (55:45) followed by water addition to Chl/methanol/water (41:50:9). One-milliliter fractions were collected. Reductions of HNO\(_2\) deamination fragments and dephosphorylations with HF were performed as described (6). P-4 (7) and AX-5 (12) chromatography were performed as described.

**RESULTS**

**Comparison of Mannolipids Synthesized in Vitro by HeLa Cells and Tryps.** In initial analyses, HeLa cell lysates were labeled with GDP-[\(^3\)H]Man. Products were then compared by TLC to those generated by Tryps. As shown in Fig. 2B, a spectrum of HeLa cell-derived [\(^3\)H]Man-labeled lipids was identified, the first corresponding to human dolichol-phosphoryl (Dol-P)-Man, and the others coinciding roughly in mobility (Fig. 2A) with Tryp GPI intermediates \(\bar{\gamma}, \bar{\delta}, \bar{\zeta}, A', \) and \(\bar{\theta}\) (see legend for structures).

Next, the HeLa-derived products were tested for properties of GPI mannolipids. First, the presence in the products of GlcN as well as Man was assessed by labeling with UDP-[\(^3\)H]GlcNAc [in the presence of excess (0.1 mM) unlabeled GDP-Man]. The formation of polar [\(^3\)H]GlcN-labeled products was markedly less efficient than in Tryps. In most experiments, no incorporation of label into these species occurred, but in some, [\(^3\)H]GlcN-labeled species co-migrating with several of the [\(^3\)H]Man-labeled species were observed. Next, labeling was repeated by substituting purified HeLa rough microsomes to verify that the products do not derive from other components in cell lysates. The same spectrum of labeled species was recovered (Fig. 2C). The yield increased as a function of microsome dose with 360 \(\mu\)g giving radioactivity approximately equal to that using 3 \(\times\) 10\(^5\) cell equivalents (56 \(\mu\)g) of Tryp lysate. Kinetic analyses showed that the HeLa species formed less rapidly than the Tryps species, with the most polar species requiring 30–60 min. Finally, the dependence of formation of the HeLa [\(^3\)H]Man-labeled products on added UDP-GlcNAc was assessed. In contrast to findings with Tryps (7) where UDP-[\(^3\)H]GlcNAc was essential, synthesis of the HeLa cell products was enhanced (Fig. 2C (Inset)) but not dependent on UDP-GlcNAc supplementation.

**Partial Structural Characterization of HeLa Cell Mannolipid Products.** Initially, the putative GPI species were analyzed unseparated with products obtained from labeling HeLa microsomes (360 \(\mu\)g) for 90 min. Similar to Tryp \(\gamma, \delta, \zeta, \) and \(A'\), HNO\(_2\) deamination (see Fig. 1) quantitatively released [\(^3\)H]Man label from all species except Dol-P-Man into the aqueous phase.

To permit further structural analyses, 25-fold increased amounts of the [\(^3\)H]Man-labeled products were prepared and products were separated by Iatrobed HPLC. The gradient (Fig. 3A) resolved Dol-P-Man (H1) and seven major species designated H2–H8, which were individually collected. After verification of homogeneties by TLC, aliquots (2000–5000

inhibitors were purchased from Sigma. * Bacillus thuringiensis PI-PLC was provided by T. Rosenberry (Case Western Reserve University, Cleveland, OH). GPI-specific phospholipase D (GPI-PLD) was obtained from K.-S. Huang (Hoffmann-La Roche). Jack bean \(\alpha\)-mannosidase was purchased from Oxford.

Murine anti-human DAF monoclonal antibody (mAb) IA10 (IgG2a) was described as prepared (9) and rat anti-human CD59 mAb YTH53.1 (IgG2a) was provided by P. Lachmann (Cambridge University, Cambridge, England). Appropriate murine and rat nonrelevant mAbs and fluorescein isothiocyanate-conjugated sheep anti-murine and goat anti-rat immunoglobulins were purchased from Organon Teknika–Cappel.

**Cell Preparations.** Tryps were expanded, human cells were cultured, and hypotonic lysates of each were prepared as described (6). Subcellular fractionation of HeLa cells was conducted as described (10). The rough microsome fraction was recovered at the 1.55/1.8 M sucrose interface. FNH cells stained with IA10 and YTH53.1 were analyzed on a Becton Dickinson FACStarPLUS flow cytometer. In patients (M.W. and S.T.) with DAF/CD59-positive polymorphonuclear leukocytes (PMNs), affected cell subsets were enriched by anti-DAF cell-affinity chromatography (5).

**Biobiosynthetic Labeling.** Human cell lysates were centrifuged and particulates were washed as described (6). Tryp lysates and purified microsomes were used unwashed with the buffer adjusted to contain the same constituents. Cell preparations were added to 10 \(\mu\)Ci of either GDP-[\(^3\)H]Man or UDP-[\(^3\)H]GlcNAc in a final vol of 200 \(\mu\)l containing 5 mM MnCl\(_2\), 1 mM ATP, 0.5 mM dithiothreitol, and tunicamycin (0.2 \(\mu\)g/ml). Intact cells (2 \(\times\) 10\(^5\)) were preincubated for 60 min in 10 ml of glucose-free RPMI medium containing 10% dialyzed fetal bovine serum, glucose (10 \(\mu\)g/ml), and tunicamycin (10 \(\mu\)g/ml) and then labeled for 45 min with 250 \(\mu\)Ci of [\(^3\)H]Man. Reaction mixtures were extracted with chloroform (Chl)/methanol, and dried extracts were partitioned in butanol/water (6).

**Enzymatic and Chemical Treatments.** Sphingomyelinase digestions were performed as described (11) using enzyme pretested for activity with sphingomyelin. HNO\(_2\) deaminations and GPI-PLD and PI-PLC digestions were performed as described (6) except that with PI-PLC buffer containing 2 mM deheptanyel lecithin instead of 0.2% Triton X-100 was used. For \(\alpha\)-mannosidase digestions, dried lipids were incubated at 37°C for 18 hr with 10 units of enzyme per ml in 10 \(\mu\)l of the manufacturer’s buffer supplemented with 0.2% Triton X-100. With all analyses, control samples subjected to identical conditions in the absence of a modifying agent were included. Reactions were stopped by extracting lipids with water-saturated 1-butanol and derivatives recovered in the 1-butanol and/or water phase were analyzed.

**TLC Analyses.** Radioactive species were chromatographed on Silica gel 60 TLC plates developed, for products of GDP-[\(^3\)H]Man labeling, with Chl/methanol/water (10:10:3) and, for products of UDP-[\(^3\)H]GlcNAc labeling, with Chl/methanol/1 M NH\(_3\) (10:10:3). TLC plates were analyzed directly on a Berthold LB 285 scanner, or after spraying with EN\(^3\)HANCE they were examined by fluorography.
cpm) of each species were analyzed. Treatment with sphingomyelinase (which cleaves phosphorylated head groups from ceramide in sphingomyelin glycolipids) had no effect on any of the species. In contrast, as shown in Fig. 3B for H2 and H6 (and as found for each of the other species), incubation with GPI-PLD [which cleaves phosphatidic acid from inositol (see Fig. 1) irrespective of inositol acylation] generated butanol phase-retained products with mobilities characteristic of acyl inositol glycan (see Fig. 1) derived from GPI-PLD-depleted HeLa lysate.

In accordance with this interpretation, mild alkali treatment [which cleaves acyl linkages (see Fig. 1)] released 88–96% of the label to the aqueous phase from each of the GPI-PLD-treated species as compared to 76% from the treated HeLa lysate. In further accordance, incubation of untreated H2–H8 with PI-PLC [which cleaves diradylglycerol from inositol monophosphate (see Fig. 1) but is constrained by inositol acylation] released <20% of the [3H]Man label into the aqueous phase. However, incubation of untreated H2–H8 with mild alkali (at 37°C for 30 min) released variable proportions of the [3H]Man label from each species, and each of the species exhibited augmented aqueous phase [3H]Man release upon PI-PLC exposure when examined in conjunction with alkali pretreatment. To clarify this observation, [3H]Man products remaining in the butanol phase after limited alkali treatment (20°C for 1 hr) of H2, H6, and H7 were analyzed. As shown in Fig. 3C for Tryp ζ, H2, and H6, incubation with PI-PLC had no effect on the residual unaltered substrates but differentially cleaved their partially deacylated products. Moreover, GPI-PLD induced comparable release (25% of counts). To exclude heterogeneity of glycans within individual species, the aqueous phase-released and butanol phase-retained products of alkali treatment of H6 were HNO2 deaminated. Analyses of the reduced and dephosphorylated [3H]Man glycan derivatives on P-4 columns gave identical elution times.

FIG. 2. (A and B) Tryp (6 x 10^11 cell equivalents) or HeLa cell (1 x 10^11 cell equivalents) lysates were labeled at 37°C for 30 min with GDP-[3H]Man in the presence of 2.0 mM unlabeled UDP-GlcNAc. (C) HeLa rough microsomes (360 µg) were substituted for HeLa lysate and the effect of labeling 90 µg in the presence (lane 1) or absence (lane 2) of excess unlabeled UDP-GlcNAc (Inset) was assessed. Lipids were separated on TLC developed in chloroform/methanol/water (10:10:3). γδ, Manα2,3Gal β,6, Moná3-P-GlcN-PI; βζ, Manα1,2Gal β,6, Moná3-P-GlcN-acyl-PI; A'αζ, P-Etn-Manα3-GlcN-PI/lipso-PI (7). Circles, Dol-P-Man; O and F, origin and front, respectively.

FIG. 3. (A) Lipids deriving from 9 mg of HeLa rough microsomes and 250 µCi of GDP-[3H]Man were separated by lathrobean HPLC with a linear Chl/methanol/water program. The fractions pooled from each of the peaks H1–H8 for chemical analyses are indicated. (B) Tryp ζ, H2, and H6 were treated with GPI-PLD or buffer control (BUF), and butanol partitions were compared. (C) Products (minor products designated by * of Tryp ζ, H2, and H6 remaining in the butanol phase after treatment with alkali at 20°C for 1 hr were treated with PI-PLC (1.6 µg/ml at 37°C for 16 hr) or buffer control, and butanol partitions were compared.
Block in GPI Assembly in Affected PNH Leukocytes. As a prerequisite to studies of PNH leukocytes, normal PMNs were labeled with $[^3H]$Man and the in vivo products correlated with H2–H8 deriving from GDP-$[^3H]$Man labeling of HeLa cell microsomes. In studies of six different donors, prominent products (Fig. 5B) aligning precisely with H6, H7, and H8 (Fig. 5A) were uniformly analyzed. Based on these findings, affected PMNs from two patients (J.V. and L.D.) with >95% DAF- and CD59-deficient PMNs were similarly analyzed. As shown in Fig. 5C and D, the cells from both patients synthesized Dol-P-Man but failed to support assembly of H6–H8. No accumulation of H5 or less polar species was observed, although J.V. showed minor products corresponding to H2 and H4. Affected PMNs from two other patients (M.W. and S.T.) next were analyzed for the same or different defects. While studies of three additional controls showed prominent H6–H8 peaks paralleling those of the other normal subjects, studies of both patients showed the complete absence of all three products, with that of M.W. exhibiting minor products corresponding to H2 and H4 as seen in J.V. UDP-$[^3H]$GlcNAc labeling demonstrated that as reported for J.V. and P.K. (6), patients L.D., M.W., and S.T. all synthesized GlcNAc- and GlcN-PI (GPI-A and -B) with normal efficiency (data not shown).

**DISCUSSION**

The main findings of the present study are (i) the identification of intracellular GPI in human cells that uniformly exhibit properties of acylated inositol phospholipids, (ii) the demonstration that two of the most polar of these species, H6 and H7, contain core glycans corresponding in size to that of Tryp A’ (P2) and exhibiting $\alpha$-mannosidase sensitivities character-

*Figure 4.* Iatrobead-purified H7 (Lower) and Tryp A’ (Upper) were treated with alkali followed by PI-PLC. After dephosphorylation of the released glycans with HF, the products were mixed with $[^14C]$Glucose monosaccharide and compared on AX-5 (Left) and P-4 (Right). $V_0$, void volume; numbers correspond to positions of mono- and oligoglucose standards prepared from dextran/borohydrate.

**Fig. 5.** HeLa cell microsomes (360 $\mu$g) were labeled for 90 min with GDP-$[^3H]$Man (A), PMNs from normal control S.H. (B), or PNH patients J.V. and L.D. (C and D) were labeled with $[^3H]$Man, and products were compared by TLC. (Insets) Flow cytograms of anti-CD59-stained cells from each donor. Positions of in vitro HeLa cell standards run internally are indicated. D, Dol-P-Man; 2–8, H2–H8.

Table 1. Properties of $[^3H]$Man-labeled GPI glycans

<table>
<thead>
<tr>
<th>Species</th>
<th>$\alpha$-Mannosidase*</th>
<th>NH$_4$/PI-PLC</th>
<th>H$_2$O/L</th>
<th>H$_2$O/Ax-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>H6</td>
<td>34.0</td>
<td>6.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>H7</td>
<td>8.0</td>
<td>6.2</td>
<td>3.5</td>
<td>4.0</td>
</tr>
<tr>
<td>A'</td>
<td>1.5</td>
<td>6.2</td>
<td>3.7</td>
<td>4.0</td>
</tr>
<tr>
<td>$\zeta$</td>
<td>3.9</td>
<td></td>
<td>37.0</td>
<td></td>
</tr>
<tr>
<td>$\delta$</td>
<td>37.0</td>
<td></td>
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*Percentage release into aqueous phase of butanol/water partitions.

The conversion by GPI-PLD of H2–H7 into products characteristic of acyl inositol glycans and the aqueous-phase release of $[^3H]$Man label in these species by PI-PLC only in conjunction with alkali pretreatment suggests that inositol in these intermediates is acylated. This interpretation is supported by the findings that butanol phase-retained products
(i) following GPI-PLD treatment were converted to aqueous-phase products by alkali and (ii) following alkali pretreatment exhibited TLC mobilities characteristic of partially deacylated derivatives and were cleaved by PI-PLC. The human GPIs thus correspond to the acylated Tryp intermediates β and ζ (P3) (1). Our finding that human GPI mannosipids, that uniformly contain acylated inositol, are present in cells bearing surface proteins with GPI-anchor structures that contain predominantly unsubstituted inositol (as described in HeLa cell DAF molecules (13)), implies the existence of a terminal deacylase.

The cleavage and partitioning of H2-H7 into the aqueous phase following alkali treatment alone suggests that they contain diacylglycerol as do Tryp intermediates (1). However, further studies will be required to allow distinction between exclusively diacyl and mixed diacyl and alkylacyl phospholipids and clarification of the relationship between inositol phospholipids in these manno lipid GPI precursors and those in mature human GPI-anchor structures (13). Comparative analyses of the HNO2-generated glycan fragments of the aqueous phase-released and butanol phase-re tained products indicated that the incomplete alkali release did not reflect heterogeneity of glycans.

The observation of only 30–40% release of [3H]Man label from H3, H4, and H6 following jack bean α-mannosidase digestion is most likely due to reduced efficiency of enzymatic cleavage of glycolipid as compared to glycan since similar release was measured with Tryp δ. Another factor, however, may be that during GPI-anchor core glycan assembly, Man 1 in human GPI precursors is substituted with P-Etn, in accordance with the presence of this substituent in mature human GPI-anchor structures (14, 15). Such substitution would account for similarities in TLC mobilities between the human and Tryp species despite the presence of the third inositol-associated acyl substituent in the former. Observations of shifts in mobilities of the HF and non-HF reduced HNO2 fragments of H5 and H6 on Dionex chromatography (S.H., G.M.P., L.R., and M.E.M., unpublished observations) are consistent with this interpretation.

The greater sensitivity of H6 than the more polar H7 to jack bean α-mannosidase suggested that a distal Man residue in H7 was substituted. The findings that the (dephosphorylated) glycan derivatives of HNO2 deamination as well as alkali pretreatment and PI-PLC digestion of both H6 and H7 exhibited virtually identical elution behavior on both P-4 and AX-5 columns, which coincided in all cases with the corresponding fragments (Man-anhydromannitol and Man-GlcN-inositol) from Tryp A′ (P2), argue that human and Tryp intracellular GPIS share common core glycans.

Our previous findings that affected PMNs of two PNH patients assemble GlcNAc-PI and GlcN-PI with efficiency equal to normal cells predicted that the defect in these patients must reside in subsequent steps in GPI-anchor assembly or incorporation into protein. Our observation in the present study, that cells from three other patients similarly synthesize GPI-A and -B but that cells from all of four patients studied lack H6–H8 and at least two show complete absence of earlier intermediates localizes the biochemical block to (i) GlcN-PI acylation or luminal translocation, (ii) P-Etn or Man incorporation, or, conceivably, (iii) altered regulation. Further analyses are necessary to permit delineation of the precise alteration(s) and confirmation of whether the disease is heterogeneous as previously postulated (2, 5).

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