Characterization of Putative Glycoinositol Phospholipid Anchor Precursors in Mammalian Cells

LOCALIZATION OF PHOSPHOETHANOLAMINE*

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A number of mammalian cell surface proteins are anchored by glycoinositol phospholipid (GPI) structures that are preassembled and transferred to them in the endoplasmic reticulum. The GPIs in these proteins contain linear ethanolamine (EthN)-phosphate (P)-

6-Man3GlcN core glycan sequences bearing an additional EthN-P attached to the Man residue (Man 1 proximal to GlcN). The biochemical precursors of mammalian GPI anchor structures are incompletely characterized. In this study, putative [3H]Man-labeled GPI precursors were obtained by in vitro GDP-[3H]Man labeling of HeLa cell microsomes and by in vivo [3H]Man labeling of class B and F Thy-1 negative murine lymphoma mutants known to accumulate incomplete GPIs. The high performance liquid chromatography-purified in vitro and accumulated in vivo GPI products were structurally analyzed by nitrous acid deamination, hydrofluoric acid, trifluoroacetic acid hydrolysis, biosynthetic labeling, and exoglycosidase treatment. The data were consistent with a biosynthetic scheme in which Man and EthN-P are added stepwise to the developing glycan. Several additional points were demonstrated: 1) putative mammalian GPI precursors contain incomplete core glycans corresponding to those in previously characterized trypanosome GPI precursors. 2) The proximal EthN-P found in mature mammalian GPI anchor structures is added to Man 1 prior to incorporation of Man 2 and Man 3. 3) Glycans in the incomplete GPIs that accumulate in classes B and F lymphoma mutants consist of Man3GlcN in which EthN-P is linked to Man 1. 4) Distal EthN-P linked to the 6-position of Man, characteristic of the complete GPI core, is found both in a subsequent GPI species with the glyccan sequence EthN-P-6ManMan(EthN-P-)ManGlcN and in a more polar GPI product.

Surface proteins that are membrane-anchored by C-terminal glycoinositol phospholipid (GPI) structures are widely distributed on mammalian as well as on many other eukaryotic cell types (reviewed in Refs. 1 and 2). Experimental data have established that the GPI anchoring units incorporated in these proteins are pre-assembled in the endoplasmic reticulum (ER) and are added en bloc to their primary translation products concurrent with or immediately following the translocation of these peptides across the ER membrane. Biochemical analyses of the anchor moieties on GPI-anchored proteins of diverse origin (3-7) have indicated that they share a common core structure consisting of ethanolamine (EthN)-phosphate (P)-6Mana1-2Mana1-6Mana1-4GlcN linked α1-6 to an inositol phospholipid, but that they exhibit variations in glycan substituents and in inositol phospholipid compositions. One consistently reported feature of the glycans in the anchors of mammalian proteins (4, 5, 7, 8) is that they contain an additional EthN-P (see Fig. 1). In the anchors of rat brain Thy-1, human erythrocyte acetylcholinesterase, and scrapie prion protein this EthN-P has been

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‡The abbreviations used are: GPI, glycoinositol phospholipid; EthN, ethanolamine; ER, endoplasmic reticulum; AHM, anhydromannitol; GPI-PLD, GPI-specific phospholipase D; PI-PLC, phosphatidylinositol-specific phospholipase C; PE, phosphatidylethanolamine; TLC, thin layer chromatography; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Fig. 1. Fragmentation strategy used for glycan analyses. At the top is shown the structure of a mammalian GPI containing an inositol phospholipid with an additional fatty acyl group (−) attached to an inositol hydroxyl group and a complete Man3GlcN core glycan bearing EthN-P groups on the 2-position of Man 1 and 6 position of Man 3. G, GlcN; M, Man; P, phosphate; E, EthN; AHM, 2,5-anhydromannitol. The right side of the figure shows that HNO3 digestion and NaBH4 reduction cleaves GlcN from inositol and reduces the resulting anhydromannose to AHM. Subsequent HF treatment dephosphorylates the product to yield Man3AHM. In 4 M trifluoroacetic acid hydrolysates the Man-6-P linkage remains stable but other phosphate and glycosidic linkages are cleaved. Trifluoroacetic acid hydrolysis of the non HF-treated [3H]Man-labeled fragment thus yields [3H]Man and [3H]Man-6-P in a 2:1 ratio. The left side of the figure shows that treatment of the parental GPI with alkaline phosphatidylinositol-specific phospholipase C (PI-PLC) cleaves EthN-P-ManMan(EthN-P-)ManGlcN-inositol-monophosphate from glycerol. Subsequent treatment of this product with HF yields Man,Glcn-inoisol. Treatment of the parental structure with GPI-specific phospholipase D (GPI-PLD) and alkaline cleaves EthN-P-ManMan(EthN-P-)ManGlcN-acyl inositol from phosphatic acid and desacylates the inositol on the released glycan.
localized to the GlcN-proximal Man (Man 1),2 and in the Thy-1 anchor it has been assigned to the 2-position of this hexose rather than the 6-position as is the terminal EthN-P on Man 3. This contrasts with the glycans in the anchors of Trypanosoma brucei membrane-form variant surface glycoproteins (3), the best characterized GPI-anchored proteins, which contain no additional EthN-P but have a Gal side chain of variable complexity (1) linked α1-3 to Man 1. The GPI-anchoring units of Thy-1 (4) and prion protein (7) also have been found to contain GalNAc and sialyl-GalNAc, respectively, linked β1-4 to Man 1 and another Man linked α1-2 to Man 3.

Recently, it was shown that GPl biosynthesis in trypanosomes could be accomplished in vitro with membranes from hypotonic lysates of cells and sugar nucleotides (9-11). The in vitro biosynthesis yielded a series of GPI species of increasing polarity which were shown to represent anchor precursors consisting of sequentially glycosylated phosphatidylinositol (PI). Among the prominent species were ManGlcN-P1, ManGlcN-P1, and EthN-P-ManGlcN-P1, designated H6, H5, and A' in Ref. (11), respectively. Structures containing identical glycans but PI in which an inositol hydroxyl group is fatty acid acylated were also detected. The spectrum of glycans identified in the in vitro precursors was consistent with prior findings (12) that the addition of Gal residues to Man 1 in trypanosome GPI anchors occurs after EthN-P-ManGlcN core completion and anchor transfer to protein.

In previous studies (13) employing similar methodology in conjunction with membranes from human HeLa cells, mammalian [3H]Man-labeled GPI species (designated H2-H8 in order of increasing polarity) resembling the trypanosome GPI anchor intermediates were identified. The putative mammalian anchor precursors were shown to differ from their trypanosome counterparts in that they exclusively contain acylated (acyl) inositol phospholipids. Two of the species, H6 and H7, were found to contain dephosphorylated core glycans (prepared as described in Fig. 1) corresponding in size to the dephosphorylated ManGlcN core in the fully assembled trypanosome GPI precursor A'. However, these two mammalian GPI species were observed to differ in their susceptibilities to jack bean a-mannosidase consistent with differential EthN-P substitution of distal Man.

In the present study the in vitro GPI products H2-H8 were correlated with GPI products deriving from in vivo labeling of intact HeLa cells and with immature GPI species that accumulate in murine lymphoma mutants that fail to express GPI-anchored Thy-1 (14, 15). The in vitro and in vivo synthesized GPIs were compared structurally to previously characterized trypanosome precursors to define further the mammalian GPI anchor assembly pathway. It was found that mammalian GPI anchor synthesis exhibits many features of trypanosome GPI synthesis but differs in that EthN-P addition to Man 1 occurs as an early event in glycan assembly. To aid in exposition of the results, the respective designations of the mammalian and trypanosome GPI species are listed together with their proposed and known structures in Table I.

### MATERIAL AND METHODS

Reagents and Cells—GDP-[3H]Man (24.3 Ci/mmol) was obtained from Du Pont-New England Nuclear, [β-3H]Man (15 Ci/mmol) and d-([1-14C])Glc (15 Ci/mmol) from American Radiolabeled Chemicals, Inc. (St. Louis, MO), and [1-3H]Ethanol hydrochloride from Amersham Corp. [3H]Glc oligomer standards were provided by Dr. J. Baenziger (Washington University, St. Louis, MO). [1-3H]Man, [3H]EthN hydrochloride from Du Pont-New England Nuclear, [1-2-%]Man (15 Ci/mmol) from Pierce Chemical Co. Glc oligomer standards (1-11-mers) and mannose oligomers from Oxford GlycoSystems (Rosendale, NY). GPI-specific phospholipase D (GPL-PLD) was provided by Dr. K-S. Huang (Hoffman LaRoche).

Classes B (SIA.TB.4.8/2/010X3) and F (ELA.G4.IN/Thy-1.2-2X1.0UAR.1) lymphoma mutants (obtained from R. Hyman, Salk Institute, La Jolla, CA) were maintained in RPMI 1640 supplemented with 10% heat-inactivated horse serum. HeLa cells were cultivated at 37 °C without CO2 under spinner conditions in Eagle’s minimum essential medium for suspension cultures (Whittaker Bioproducts, Walkersville, MD) containing 20 mM HEPES (pH 7.4) and 10% heat-inactivated new born calf serum. Trypanosomes were isolated by DEAE-cellulose chromatography of infected rat blood (16).

### Table I

| Mammalian designation | Structure | Trypanosome designation |
|-----------------------|-----------|------------------------|
| H2                    | ManGlcN   | γ                      |
| H4                    | ManManGlcN| γ                      |
| H5                    | EthN-P-ManManGlcN | δ                 |
| B                     | Man(EthN-P→)→ManGlcN | A'               |
| H6/F6                 | ManMan(EthN-P→)→ManGlcN | A'               |
| H7                    | EthN-P-ManMan(EthN-P→)→ManGlcN | A'               |

Note: Incomplete GPI products that accumulate in classes B and F Thy-1 lymphoma mutants.

### Amersham Corp. [3H]Glc oligomer standards were provided by Dr. J. Baenziger (Washington University, St. Louis, MO).

### References

1. Putative Mammalian GPI Anchor Precursors
2. Table I Nomenclature and proposed glycan structures
acid/NaOH, the resulting glycans were reduced with NaBH₄ as described (11).

Reduced HNO₂ fragments were desphosphorylated by treatment with 49% hydrofluoric acid at -20 °C for 72 h. After neutralization with saturated LiOH, residual salts and nonphosphorylated glycans were removed from samples by passage through a tandem column consisting of Chelex 100, AG3X4, AG50W-X12, and QAE Sephadex A-50 resins. Trifluoroacetic acid hydrolyses were prepared by incubating scraped silica beads bearing [³H]Man-labeled products with 4 M trifluoroacetic acid at 100 °C under argon for 4 h. Hydrolysis mixtures were evaporated and dissolved.

**Analytical Methods**—P-4 chromatography was performed as described (13) employing [³H]Glc standards. Dionex anion-exchange chromatography was performed with a CarboPac PA-01 column and the following gradients using eluants A (0.15 M NaOH), B (0.15 M NaOH, 1.0 M NaAc), and C (H₂O) as follows.

| Gradient | 0 min: A 99%, B 1%; 50 min: A 95%, B 5%; 65-75 min: A 75%, B 25%; flow rate 0.6 ml/min. |
|----------|-----------------------------------------------------------------------------------------------|
| Gradient 2 | 0-15 min: A 10%, C 90%; 17 min: A 61%, B 5%, C 34%; 25 min: A 56%, B 10%, C 34%; 35 min: A 41%, B 25%, C 34%; 45 min: A 36%, B 30%, C 34%; 50 min: B 66%, C 34%; flow rate 1 ml/min. [³H]Man-labeled glycans were detected by scintillation counting of their radioactivity. Added Glic oligomers or other nonphosphorylated and phosphorylated standards were detected via pulsed amperometric detection.

**RESULTS**

**Correspondence of in Vitro and in Vivo Generated GPI Products**—To determine how previously defined GPI species H2-H8 synthesized in vitro (13) relate to GPI species detectable following in vivo labeling of intact cells (14, 15), GPI products derived from GDP-[³H]Man labeling of HeLa cell lysates and from [³H]Man labeling of cultured HeLa cells were compared by TLC. As shown in Fig. 2, panel B, major in vivo bands were observed that corresponded to the three most polar in vitro generated GPI species H6, H7, and H8 (panel A), whereas no prominent bands corresponding to in vitro generated H2-H5 were discernable. To establish if immature Gmennanlipid intermediates that accumulate in GPI anchor defective class F and B Thy-1 negative lymphoma mutants (15) correlate with any of the less polar in vitro GPI species, comparative studies were repeated with these mutant cells. The product that accumulated in [³H]Man-labeled class B cells (panel D) comigrated with a minor in vitro species migrating between H5 and H6 (panel C) while the product that accumulated in class F cells (panel D) comigrated with H6.

**Properties of Core Glycan Structures of Mammalian GPIs—**To investigate the structural relationships of the various GPI species to each other, their glycans were analyzed. For this purpose, the [³H]Man-labeled in vitro GPI products and accumulated GPI species in class B and F mutants were prepared in large quantities, and the in vitro products were separated by Iatrohead HPLC. After verification of homogeneity by TLC, glycan fragments of each species were prepared by HNO₂ deamination and NaBH₄ reduction. This procedure cleaves the GlcN glycosidic linkage to inositol and converts the resulting anhydromannose moiety into 2,5-AHM (see Fig. 1 for predicted products). Portions of the reduced HNO₂ fragments were dephosphorylated with HF (Fig. 1). The sizes of the untreated and HF-treated fragments were then assessed by P-4 chromatography and the values compared to those measured for corresponding fragments from trypanosome GPIs. As summarized in Table II, without HF treatment the fragment from H2 eluted at a position coinciding with that of the untreated fragment (ManAHM) from trypanosyme-γ (ManGlcN-PI), while the fragment of H4 eluted at a position coinciding with that of the HF-treated fragment (Man₄AHM) from trypanosome A' (EthN-P-Man₄GlcN-PI). After HF treatment the fragment from H5 coeluted with trypanosomal Man₅AHM; the fragment from the accumulated species in class B cells eluted at 2.2 Glc units appropriate for Man₅AHM; and the fragments from H6, the accumulated species in class F cells, and H7 co-eluted with trypanosomal Man₆AHM. In contrast to these findings, without HF treatment the fragments from H5, H6, and H7 as well as EthN-P-Man₅AHM from trypanosome A' all eluted at much larger apparent sizes indicative of HF-sensitive substituents in their structures. Attachment of charged groups such as phosphate to glycans is known to shift their elution on P-4 columns to larger apparent Stokes radii (18). The indication of a phosphate group on the H5 fragment is noteworthy in view of its coelution following HF treatment with Man₅AHM, indicating that it contains only one Man adjacent to GlcN.

The core glycan fragments of the mammalian GPIs next were compared to those of the corresponding trypanosome GPIs by a Dionex anion-exchange HPLC procedure previously shown to be sensitive to the glycan structures of trypanosome GPIs. In agreement with the P-4 sizing data, the untreated fragments from H2 and H4 eluted at 1.1 and 2.5

![Fig. 2. Comparison of GPI species synthesized in vitro and in vivo](image-url)

**TABLE II**

| Species | +HF | -HF |
|---------|-----|-----|
| γ⁶ | 1.8 | 1.8  |
| A'⁶ | 3.8 | 8.5 |
| H2 | 1.8 | 1.8 |
| H4 | 3.8 | 3.8 |
| H5 | 1.8 | 6.6 |
| B | 2.2 | 2.2 |
| H6 | 3.8 | 7.0 |
| F | 4.0 | 4.0 |
| H7 | 3.8 | 12.0 |

⁶γ and A' correspond to ManGlcN-PI and EthN-P-Man₄GlcN-PI obtained from trypanosomes. The untreated (-HF) fragment of γ corresponds to Man₅AHM and the HF-treated fragment of A' to Man₆AHM, respectively.

⁷Apparent size relative to Glc standards (see Footnote 3).
that H5, the accumulated class B intermediate, and H6 (which corresponds to the accumulated class F intermediate) contain phosphate groups possibly linked to Man 1, as in mature mammalian protein-attached GPI anchor structures (4, 5, 7). To investigate this possibility, the nondephosphorylated HNO3 fragment from each species was reexamined by Dionex anion-exchange HPLC using a higher molarity NaAc gradient designed to resolve phosphorylated as well as nonphosphorylated glycans. The fragments from H5 (Fig. 3, panel E), the accumulated class B intermediate (not shown), H6 (panel G), and the corresponding accumulated class F intermediate (not shown) all eluted at >8 Dionex units but earlier than doubly charged Man-1-P and Man-6-P. A similar elution position was observed for EthN-P-Man-AHM derived from trypanosome A’ (panel F) and is consistent with the singly charged species expected for this phosphodiester. In contrast, the reduced HNO3 fragment from H7 (panel H) eluted later than Man-1-P and Man-6-P but much earlier than 1,6-fructose diphosphate (not shown) suggestive of a doubly charged glycan bearing two phosphodiester substituents. The indication of a more highly charged structure for this fragment was in accordance with its larger apparent size on P-4 chromatography (Table II). The inference of phosphate groups linked to H5, the accumulated product in the B mutant, and H6 is significant, since H5 and the accumulated species in the B mutant do not include the Man 3 residue that is the only site of phosphate linkage in trypanosome GPIs. Furthermore, although H6 includes Man 3, its sensitivity to jack bean mannosidase (13) indicates that it is unsubstituted on this hexose.

To ascertain whether the phosphodiester-linked substituents inferred from the above analyses involve EthN, HeLa cells were labeled in vivo with [3H]EthN, and the products (Fig. 4) were compared with those derived from labeling with [3H]Man. [3H]EthN-labeled species comigrating with the H5-H6 cluster, with H7, with H8, and with a product of intermediate mobility between H7 and H8 (designated H7’7) were observed. Treatment of the [3H]EthN-labeled sample with HNO3 or GPI-PLD (as diagrammed in Fig. 1) abolished the latter three species, consistent with their identities as GPIs, but overlap with larger phosphatidylethanolamine (PE) and phosphatidylcholine (PC) species.

The relative elution positions of GPI fragments subjected to P-4 chromatography and Dionex anion-exchange HPLC were calibrated by reference to the elution pattern of a standard mixture of Glc oligomers on each system. One Glc unit corresponds to the elution volume of monomeric Glc; two Glc units to the volume of glucosyl-Glc, etc. Since the chemical basis of Glc oligomer chromatography is quite different in the two systems, the Glc unit assignments from one system bear no quantitative relationship to the unit assignments in the other system.

The elution positions of Glc standards and of Man-1-P and Man-6-P were analyzed using gradient 1 and nondephosphorylated fragments (panels E-H) analyzed using gradient 2. The elution positions of Glc standards and of Man-1-P and Man-6-P are shown.

Dionex units (not shown) characteristic of trypanosomal Man-1 and Man-AHM glycan fragments (19). In support of this assignment for the H2 fragment, the putative ManGlcN-inositol derivative of H2 generated by GPI-PLD digestion and alkali treatment (which cleaves inositol from phosphatic acid and deacylates the acyl inositol product (see Fig. 1)) coeluted at 3.1 Dionex units (not shown) with the corresponding ManGlcN-inositol fragment derived from trypanosome-γ (ManGlcN-Pi). The structural relationships of the more polar mammalian GPIs to trypanosome GPIs that were suggested by P-4 chromatography similarly were supported by Dionex analyses of HF-dephosphorylated fragments. As observed for the untreated fragment of H2, the HF-treated fragment from H5 (Fig. 3, panel A) eluted at 1.1 Dionex units identically to trypanosomal Man;AHM, the fragment from the accumulated GPI species in class B cells eluted at 2.2 Dionex units (not shown) as previously reported for trypanosomal Man;AHM (19), and, as observed for the nondephosphorylated fragment of H4, the fragments from H6 (panel C), the accumulated GPI species in class F mutant cells (not shown), and H7 (panel D) all eluted at 2.5 Dionex units identically to trypanosomal Man;AHM derived from A’ (panel B). In accordance with our previous sizing data (13), the findings concerning H4, H6, and H7 establish that these GPIs share a common Man;GlcN core structure with trypanosome A’. They further indicate that the truncated GPI product accumulating in the class F mutant has the same dephosphorylated core structure.

Identification and Localization of EthN-P in More Polar Mammalian GPIs—The P-4 chromatographic size analyses before and after HF dephosphorylation in Table II suggested
lyso-PE peaks obscured the effect of these treatments on the H5-H6 cluster. Parallel in vivo [3H]EthN labeling studies with class B and F cells (not shown), however, yielded [3H] EthN-labeled species that corresponded to the [3H]Man-labeled species accumulating in the two mutant cell types. These observations are consistent with the proposal that the phosphate linkages deduced from the glycan sizing analyses above in fact involve EthN.

Next, the linkage of phosphate to the 6-position of Man in H6, H7, and H8 was investigated in the [3H]Man-labeled GPIs. Trifluoroacetic acid hydrolysates (for predicted products see Fig. 1), in which the Man-6-P linkage remains stable (4) but other Man-phosphate and glycosidic linkages are cleaved (4), were analyzed by Dionex HPLC under conditions that resolve phosphorylated from nonphosphorylated monosaccharides. As seen in Fig. 5, panel B, hydrolysates of H6 showed only labeled Man. This observation indicates that phosphate associated with H6 does not involve a linkage to the 6-position of Man as determined for the terminal EthN-P on Man of trypanosome GPIs, but reflects a trifluoroacetic acid-labile linkage that could involve the 2-position of Man as found in the proximal EthN-P substituent in Thy-1 (4). In contrast, hydrolysates of H7 (panels C and D) showed labeled Man-6-P and Man in a 1:2 ratio (similar to that in hydrolysates of trypanosome A’ (EthN-P-Man3GlcN-PI) (panel A)) as expected (see Fig. 1) from the known Man-6-P at Man of the A’ fragment (EthN-P-Man3AHM), the A’ product (panel B) eluted identically to the untreated A’ fragment (compare Fig. 3, panel F).

Finally, the location of the Man bearing the 6 phosphate linkage in H7 was investigated. For this purpose [3H]NaBH₄-labeled trypanosomal Man₃AHM and the nondephosphorylated HNO₃ fragments of [3H]Man-labeled trypanosome A’, H6, and H7 were incubated with jack bean α-mannosidase. This exoglycosidase should cleave only unsubstituted Man from the nonreducing terminus of glycan fragments. Products were then compared to the untreated fragments by Dionex HPLC. In accordance with the presence of 3 unsubstituted Man residues in trypanosomal Man₃[3H]AHM, the labeled product from Man₃[3H]AHM (Fig. 6, panel A) eluted exclusively at 1.0 Dionex units consistent with free [3H]AHM. Conversely, as expected for the attachment of EthN-P on Man 3 of the A’ fragment (EthN-P-Man₃AHM), the A’ product (panel B) eluted identically to the untreated A’ fragment (compare Fig. 3, panel F). As seen in panel C, the
fragment from H6 was partially digested. As expected for conversion of ManMan(EthN-P→)ManAHM to EthN-P-Man, AHM, two-thirds of the counts were released as free [3H]Man, and the residual product eluted ahead (25 versus 27 min) of the untreated H6 fragment as observed for the H5 fragment (compare Fig. 3, panels E and G). In contrast, the H7 fragment was unaffected by the α-mannosidase treatment as assessed by Dionex HPLC. As shown in panel D, no released [3H]Man was detected and the product eluted identically to the untreated H7 fragment (compare Fig. 3, panel H). Moreover, after further treatment with HF, the α-mannosidase-treated H7 product (panel E) coeluted at 2.5 Dionex units with the corresponding dephosphorylated product (ManαAHM) from A' (panel F). This resistance of H7 to α-mannosidase is predicted if EthN-P is present on Man 3.

**DISCUSSION**

Several contributions are made in the present study. 1) A coherent set of putative GPI anchor intermediates in mammalian cells is identified through the combined use of in vitro and in vivo labeling techniques and of normal and mutant cells. 2) The core glycans of the putative mammalian GPI intermediates are shown to correspond to those of trypanosome GPI precursors by gel exclusion and anion-exchange chromatographic analyses. 3) EthN-P associated with Man 1 as observed in mature mammalian GPI anchors is shown to be added to ManαGlcN-acyl PI prior to further elongation of the core. This modification thus is detected in the major products which accumulate in class B and F Thy-1 negative lymphoma mutant cells. 4) Distal EthN-P attached to the 6-position of Man 3 which characterizes the complete trypanosome GPI anchor core is identified in a subsequent mammalian GPI intermediate H7 as well as in a more polar species H8. Taken together, these findings further define the steps in mammalian GPI anchor assembly and highlight similarities to and differences from the previously characterized synthesis of trypanosome GPI anchors. A pathway for mammalian GPI-anchor assembly based on the findings in this and previous studies (see legend) is depicted in Fig. 7.

Our comparisons of the GPI products deriving from in vitro and in vivo labeling of HeLa cells showed that while the more mature species H6, H7, and H8 are better represented when cells are labeled in vivo, their less completely assembled precursors H2-H5 are proportionately higher in preparations labeled in vitro. The most polar in vitro species H8 was synthesized less efficiently when purified microsomes were used. In contrast, the a-mannosidase-treated H7 product (H5) coeluted at 2.5 Dionex units with the corresponding dephosphorylated product (ManαAHM) from A' (panel F). This resistance of H7 to α-mannosidase is predicted if EthN-P is present on Man 3.

**FIG. 7. Proposed biochemical pathway for mammalian GPI anchor assembly.** Symbols are as indicated in the legend. Initially GlcNAc is transferred from UDP to a PI acceptor (15, 17, 23). The product, GlcNAc-PI, is then deacylated to yield GlcN-PI (15, 17, 23). Following acylation of inositol and presumed ER luminal translocation, the order of which is not known, Man 1 is donated to the resulting GlcN-acyl PI (24). The Man 1 residue in the GlcN-acyl PI product (H2) is then substituted with EthN-P (presumably at the 2-position as observed in the mature anchor of Thy-1) to give EthN-P-ManαGlcN-acyl PI (H5). Man 2 and Man 3 are then sequentially added to H5 to give ManMan(EthN-P→)ManGlcN-acyl PI (H6). Additional EthN-P is incorporated at the 6-position of Man 3 to yield EthN-P-ManMan(EthN-P→)ManGlcN-acyl PI (H7). EthN-P may be incorporated in the 6-position of both Man 2 and Man 3 in H8 and/or other substituents, e.g. additional Man, may be present. Alternative pathways exist for conversion of ManGlcN-acyl PI (H2) to Manα- and ManαGlcN-acyl PI (H4) independent of EthN-P incorporation into Man 1. Many features parallel those of the trypanosome GPI assembly pathway (9–11). The sites of the metabolic blocks in Thy-1 lymphoma mutants B and F are indicated by the corresponding blocked letters.

In the present study we found that the dephosphorylated reduced HNO2 fragment of H5 corresponded to Man, AHM thereby establishing that H5 parallels trypanosome-γ in containing Man, GlcN. Several lines of evidence in the present study indicate that the additional polar substituent in H5 is EthN-P, and that Man 1 in H5 thus is substituted independent of further mannosylation: 1) the apparent size of its reduced HNO2 fragment estimated from P-4 chromatography decreased from 6.6 to 1.8 Glc units following dephosphorylation. 2) Its nondephosphorylated HNO2 fragment ran on Dionex anion-exchange HPLC at a position of >8 Dionex units, approaching the positions of Man-1-P and Man-6-P standards, in accordance with a glycan having a single negative charge characteristic of EthN-P-Man, AHM.
3) Finally, H5, the Man,GlcN-containing product that accumulates in class B Thy-1 negative lymphoma mutants, and H6 as well as the apparently identical Man,GlcN-containing product lacking terminal EthN-P that accumulates in class F mutants all labeled biosynthetically with [3H]EthN as well as with [3H]Man. The association of EthN with phosphates was supported by the finding that the HF-treated fragments of the respective GPI species eluted on both P4 and Dionex anion-exchange chromatography identically to trypanosomal Man₃-, Man₄-, and Man₅AHM. Our findings of EthN-P in the accumulated products in class B and F Thy-1 negative lymphoma cells differ from a report by Sugiyama et al. (15) that the glycans of these products consist of unsubstituted Man₁- and Man₂GlcN. Their inability to identify this modification could be due to loss of the EthN-P-containing species as a result of passage of the nondephosphorylated [3H]Man-labeled glycans through anion-exchange resins prior to characterization in their studies. Results similar to ours concerning the accumulated species in the B mutant were recently reported by Puoti et al. (21).

We found that the later mammalian GPI intermediate H7 contains EthN-P linked to the 6-position of Man 3. This was suggested by previous observations (13) that the dephosphorylated glycan fragments of H7 and H6 obtained by HNO₂ deamination and reduction or by alkali and PI-PLC digestion (prepared as in Fig. 1) are identical and correspond in size, respectively, to Man₃AHM and Man₅GlcN-inositol from trypanosome A', but that H7 and H6 differ in their susceptibilities to jack bean α-mannosidase. Three findings in the present study provide evidence supporting the presence of this second, terminal EthN-P substituent in H7. First, the nondephosphorylated reduced HNO₂ fragment of H7 eluted from P-4 with a larger apparent size (12 Glc units) and from Dionex anion-exchange HPLC at a position of higher net negative charge (>Man-1-P and Man-6-P standards) than did that of H6. Second, its trifluoroacetic acid hydrolysate but not that of H6 contained Man-6-P. Finally, its nondephosphorylated reduced HNO₂ glycan was unaltered by jack bean α-mannosidase treatment in contrast to that of H6. Our additional finding, however, that the more polar product H8 also yielded trifluoroacetic acid hydrolysates containing Man-6-P indicates that two mammalian GPIs with EthN linked to the 6-position of Man exist in mammalian cells. The structure of H8 (and H⁷) remains to be clarified. Preliminary observations that H8 contains a higher proportion of Man-6-P and additional EthN relative to H7 raise the possibility that the EthN-P in H8 is substituted on the 6-position of both Man 2 and Man 3 (as suggested in Fig. 7). A proportion (~15%) of the mature protein-associated GPI anchor of acetycholinesterase recently has been found to contain EthN-P linked to the 6-position of both Man 2 and Man 3 (22). Alternatively, H8 and the intervening species H⁷ could represent GPIs that include a fourth Man residue or GalNAc incorporated into the GPI core in view of the detection of these components in mature anchor structures of Thy-1 and prion protein (4, 7).

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