A Candidate Mammalian Glycoinositol Phospholipid Precursor Containing Three Phosphoethanolamines*

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The glycoinositol phospholipid (GPI) anchors of mammalian proteins contain linear ethanolamine (EthN)-P-6ManManManGlcN glycan sequences that bear additional EthN-P substituents and in some cases include a fourth Man and a GalNAc or sialic acid-GalManolamine. Precursors of these anchoring structures are preassembled in the endoplasmic reticulum by sequential glycosylation of inositol phospholipid. In previous studies (Hirose, S., Prince, G. M., Sevlever, D., Ravi, L., Rosenberry, T. L., Ueda, E., and Medoff, M. E. (1992) J. Biol. Chem. 267, 16968-16974), a series of putative intermediates of this assembly process were isolated from human HeLa cells and murine lymphomas, and several of the more polar products were found to contain a second EthN-P attached to the Man residue (Man 1) proximal to GlcN. In this study, the most polar HeLa cell GPI species was purified by normal phase Iatrobead high performance liquid chromatography, and its glycan was characterized. Dionex anion exchange chromatographic analyses of fragments produced by nitrous acid deamination, hydrofluoric acid ricinate in the endoplasmic reticulum and transferred en bloc are discussed.

A candidate GPI species was purified by normal phase Iatrobead high performance liquid chromatography, and its glycan was characterized. Dionex anion exchange chromatographic analysis of fragments produced by nitrous acid deamination, hydrofluoric acid dephosphorylation, and trifluoroacetic acid hydrolysis in conjunction with biosynthetic labeling studies indicated a structure containing a third EthN-P substituent linked to the 6-position of Man 2. The polar GPI product exhibited a ManManManManGlcN core lacking additional Man or GalNAc. The implications of the identification of this triply phosphoethanolamine-substituted species to mammalian GPI anchor biosynthesis are discussed.

A number of cell surface proteins are anchored by posttranslationally added glycoinositol phospholipid (GPI)1 structures (reviewed in Refs. 1 and 2). These structures are prefabricated in the endoplasmic reticulum and transferred en bloc to the endoplasmic reticulum and transferred en bloc to the nascently synthesized protein. Their glycans contain linear ethanolamine (EthN)-P-ManManManGlcN core sequences that are variably decorated in different organisms. In GPI-anchored proteins that have been examined from mammalian cells (3-5), a second EthN-P substituent is characteristically present on the Man residue (Man 1) proximal to GlcN, and a third EthN-P may be present on the adjacent Man 2 residue (6).

The biochemical pathway providing for assembly of GPI-anchoring units was analyzed initially in Trypanosoma brucei. Studies in these cells (7, 8) showed that the first two intermediates of the pathway, GlcNAc- and GlcN-PI, are formed by transfer of GlcNAc from UDP to an inositol phospholipid and deacetylation of the product. EthN-P-ManGlcN-PI then sequentially is assembled as a result of stepwise addition of Man 1, Man 2, Man 3, and terminal EthN-P to the nascently formed GlcN-PI. In previous studies (9-15) employing cultured human HeLa and murine lymphoma cell lines, putative mammalian GPI intermediates corresponding to the trypanosomal precursors were identified. The mammalian GPI mannolipid species (designated H2-H8 in Refs. 9 and 11) were found to differ from their trypanosomal counterparts, however, in that 1) an inositol hydroxyl group is uniformly acetylated (9) as a result of acylation of GlcN-PI (15, 16) and 2) upon incorporation of Man 1, a proximal EthN-P is added yielding EthN-P-ManGlcN-acyl-PI (H5) (11). Subsequent mammalian GPI products (11, 12) corresponding to Man(EthN-P-+)-ManGlcN-acyl-PI, ManMan(EthN-P-+)-ManGlcN-acyl-PI (H6), and EthN-P-ManMan(EthN-P-+)-ManGlcN-acyl-PI (H7) were identified, but the most polar GPI product (H8) remained incompletely characterized.

In the present study, [3H]Man-labeled GPs were prepared by GDP-[3H]Man labeling of HeLa cell microsomes and [14C]H Man labeling of intact HeLa cells, and H8 was purified by Iatrobead HPLC of the in vitro and butanol:diisopropylether (DIPole) saline partitioned in vivo products. The extent of phosphorylation and nature of the core glycans of purified H8 were examined by Dionex HPLC analyses of its nitrous acid deamination (HNO3) fragment before and after hydrofluoric acid (HF) dephosphorylation. The EthN content of H8 was assessed by [3H]EthN labeling, and the linkages of EthN-P substituents in H8 were evaluated by analyses of Man and Man-6-P in trifluoroacetic acid hydrolysates of the [3H]Man-labeled product.

MATERIALS AND METHODS

Reagents—GDP-[3H]Man (24.3 Ci/mmole) and UDP-[3H]GlcNac (26.8 Ci/mmole) were purchased from Du Pont-New England Nuclear, 2-[3H]Man (15 Ci/mmole) from American Radiolabeled Chemicals, Inc., and [3H]EthN (3-30 Ci/mmole) from Amersham Corp. [3H]-NaBH4-labeled trypanosomal Man-2,5-anhydromannitol (AHM) was provided by Dr. M. Ferguson (Univ. of Dundee). Glucose (Glc) oligomer standards (1-11-mers) were bought from Oxford Glyco-Systems (Rosedale, NY). Tunicamycin and dithiothreitol were ob-
tained from Sigma. TLC Silica 60 plates were purchased from Merck (Darmstadt, Germany).

**Bioanalytical Labeling.**—$^{3}H$-Labeled GPs were prepared as previously described (9, 11). Briefly, for *in vitro* labeling, 1.5 mg of unwashed HeLa cell microsomes (density = 1.55-1.80 as prepared in Ref. 9) in 2 ml of 2 mM unlabeled UDP-GlcNAc, 5 mM MnCl$_2$, 1 mM ATP, 0.5 mM dithiothreitol, and 0.2 mM $\mu$g/ml tunicamycin were added to 100 $\mu$M of dried GDP-[H]Man, and the mixture was incubated at 37°C for 90 min. For *in vivo* labeling, HeLa cells (2 x 10$^6$) in 100 ml of glucose-free RPMI/10% dialyzed fetal bovine serum (RPMI/FBS) were incubated with 10 pg/ml tunicamycin and then labeled at 37°C for 2 h with 1 $\mu$Ci of $^{3}H$Man. In some experiments, 2 x 10$^6$ cells in 10 ml of RPMI/FBS were labeled with 500 $\mu$Ci of $^{3}H$Man. Following incubations, reaction mixtures were extracted with equal volumes of chloroform and methanol calculated to give chloroform:methanol:water (10:10:3) (see Fig. 1), the dried extracts were partitioned in butanol:water, and butanol-associated lipids were dried.

**Mannolipid Purifications and Glycan Preparations.**—For HPLC separations of *in vitro*-labeled products, preliminary purification of extracts using butanol:DIPESaline to remove extraneous neutral lipids was performed. Butanol-associated lipids of *in vitro* [H]Man-labeled products were dried and partitioned twice with two parts of butanol:DIPE (3:7) and 1 part of 50 mM saline (17) (see Fig. 1). More polar glycolipid precursors partitioned in the saline phase, whereas less polar precursors and neutral lipids were retained in the organic phase, as confirmed by TLC (not shown). The pooled aqueous phases were further partitioned three times in butanol:water, and the butanol phases were dried. In *in vitro* and butanol:DIPESaline-partitioned in *in vivo* [H]Man-labeled lipid products were dissolved in 100 $\mu$l of chloroform:methanol (80:20) and separated on a 6RSP-8100 (4.6 x 250 mm) Iatrobhead HPLC column (Iatron Laboratories) using the following gradient: 0-20 min: 80% chloroform, 20% methanol to 55% chloroform, 45% methanol; 20-70 min: 55% chloroform, 45% methanol to 50% chloroform, 41% methanol, 3% water; 70-85 min: 40% chloroform, 41% methanol, 9% water to 44% chloroform, 44% methanol, 15% water; flow rate 1 ml/min.

**Trifluoroacetic Acid Hydrolysates.**—[H]Man-labeled H$_8$ and H$_7$ were isolated by scraping beads containing the separated GPs from TLC plates developed with chloroform:methanol:water (10:10:3) (9, 11). The scraped silica beads bearing the purified products were incubated with 4 $\mu$l trifluoroacetic acid at 100°C under argon for 4 h. The beads were pelleted, [H]Man products in supernatants were collected, and the dried residue was suspended in water. Unlabeled Man and Man-6-P were added as internal standards.

**Analytical Methods.**—Dionex anion exchange chromatography was performed using a CarboPac PA-01 column (11). The following linear gradients with eluants A (0.15 M NaOH), B (0.15 M NaOH, 1.0 M NaAc), and C (H$_2$O) were used. Gradient 1: 0-5 min (99% A, 1% B to 96% A, 4% B); 5-60 min (75% A, 25% B); flow rate 0.6 ml/min. Gradient 2: 0-45 min (95% A, 5% B); 45-65 min (95% A, 5% B); 65-75 min (100% A); flow rate 0.6 ml/min. Gradient 3: 0-15 min (10% A, 90% C); 15-25 min (10% A, 90% C to 61% A, 5% B, 34% C); 25-35 min (61% A, 5% B, 34% C to 36% A, 30% B, 34% C); 35-50 min (36% A, 30% B, 34% C to 66% B, 34% C); flow rate 1 ml/min.

**RESULTS**

**Purification of H$_8$**—In previous studies (9, 11), Iatrobhead HPLC was exploited to purify *in vitro* prepared mammalian GPI intermediates. Elution with a gradient extending from chloroform:methanol (80:20) to chloroform:methanol (100:5) followed by addition of water to chloroform:methanol:water (41:50:9) yielded dolichol-phosphoryl-Man (Dol-P-Man) and six discrete peaks corresponding to ManGlcN-acyl-PI (H$_2$) through EthN-P-EManMan(EthN-P-)-ManGlcN-acyl-PI (H$_7$). The gradient, however, released only minimal amounts of the most polar product H$_8$ (9, 11). To improve the recovery of H$_8$, the polarity of the latter part of the gradient was increased by adding water to chloroform:methanol (95:5) in increments of 5% water. Although this resulted in a large late peak of radioactivity eluted at fraction 90, ~5 min following completion of the last gradient step. After collection, >90% of the radiolabel recovered in this peak migrated on TLC as a single peak at a position corresponding to that of H$_8$. To verify that the *in vitro* product corresponded to the endogenous *in vivo* synthesized product, polar GPs products recovered in the aqueous phase after [H]Man labeling of intact HeLa cells and butanol:DIPESaline partitioning of products to deplete phospholipids were separated by the identical Iatrobhead HPLC procedure. As seen in Fig. 2B, the most polar *in vivo* product co-eluted with the *in vitro* prepared H$_8$ product. After isolation, it exhibited identical migration on TLC (not shown).

**Comparative Properties of the Glycans of H$_8$, H$_7$, and Trypanosomal A**—in previous studies (11), Dionex anion exchange chromatography was shown to provide good resolution of the glycans from mammalian and trypanosomal GPI intermediates. Using this structure-sensitive analysis, ~85-75 min HNO$_3$ fragments of the various GPI species were examined without or with HF dephosphorylation. The phosphorylated fragments were separated utilizing an extended 20-500 $\mu$l NaAc gradient that permitted analysis of EthN-P-containing
structures. It was found that due to its additional EthN-P substituent on Man 1 (11), the phosphorylated A fragment (EthN-P-6ManMan(EthN-P→)ManAHM) of H7 eluted later than the (EthN-P-MamAHM) of the fully assembled trypanosomal precursor A'. To determine the relationship of the glycan of H8 to the previously characterized glycans of H7 and trypanosomal A', the behaviors of the phosphorylated reduced HNO2 fragments of the three species on this chromatographic system were compared. As shown in Fig. 3, A-C, the H8 fragment eluted even later than the H7 fragment, at a position between that of Man-6-P and 1,6-Fru-di-P which bear two and four negative charges, respectively. The order and general elution positions of the H8, H7, and A' fragments would be appropriate if an additional EthN-P substituent were present in the H8 structure.

To establish that the additional charged substituent in the H8 fragment is phosphodiester-linked and to determine the relationship of the core glycans of H8, H7, and trypanosomal A', the HF-dephosphorylated reduced HNO2 fragments of the three species were re-examined by Dionex anion exchange chromatography using a shallow 10-50 mM NaAc gradient previously employed to distinguish trypanosomal Man-6-P AHM neutral glycan fragments (15). As seen in Fig. 3, D-F, the HF-treated reduced HNO2 fragment of H8 like that of H7 (11) co-eluted at 2.5 Dionex units with that of trypanosomal A' and with authentic (Na[3H]BH4-labeled) Man-3[3H]AHM from trypanosomal A', indicative of a core glycan structure consisting of ManManManGlcN.

To confirm that the phosphate linkages inferred in H8's phosphorylated glycan involve EthN, HeLa cells were labeled alternatively with [3H]Man or [3H]EthN, and [3H]Man- and [3H]EthN-labeled GPI products on TLC plates were correlated. Integration of H counts under the alternatively labeled Hs and H7 peaks and comparison of the results showed that the ratio of [3H]EthN to [3H]Man counts in H8 was 1.97, while that in H7 was 0.92, a difference indicative of more EthN in H8 than in H7.

Localization of EthN-P in H8—In 4 mM trifluoroacetic acid hydrolysates, the Man-6-P linkage in GPI structures remains stable (3), whereas all other Man- and Man glycosidic linkages are cleaved. In previous studies (11), analyses of 4 mM trifluoroacetic acid hydrolysates for [3H]Man and [3H]Man-6-P were used to assign the linkages of EthN-P substituents in H7 and earlier mammalian intermediates. These analyses showed that the proximal EthN-P linked to Man 1 in H7 and its precursors is attached to a position other than the 6-position, consistent with the 2 linkage that has been reported for the proximal EthN-P in the protein-associated GPI anchor of Thy-1 (3). In contrast, the second EthN-P identified in H7 is linked to the 6-position of Man 3 identically with the terminal Man 3-linked EthN-P in trypanosomal A'. To ascertain the position of the third HF-sensitive substituent in H8 and verify its phosphodiester linkage, the TLC separated species was subjected to trifluoroacetic acid hydrolysis, and the content of Man and Man-6-P was compared to that in trifluoroacetic acid hydrolysates of H7. As shown in Fig. 4, A and B, in contrast to hydrolysates of H7 which showed [3H]Man and [3H]Man-6-P in a 2:1 ratio, hydrolysates of H8 showed [3H]Man and [3H]Man-6-P in a 1:2 ratio. Since Man 2 and Man 1 in GPI core structures are in 1-6 linkage and the 6-position of Man 1 is thus unavailable, the indication of 2 Man-6-P residues and 1 Man residue in H8 indicates that the third EthN-P in H8 is linked to the 6-position of Man 2.

**DISCUSSION**

Following [3H]Man labeling of mammalian cells, a series of [3H]Man-containing GPI products with properties of GPI anchor pathway intermediates are detectable. In previous studies (9,11), several but not the most polar of these putative anchor precursors were characterized and shown to include a two EthN-P-containing structure; EthN-P-6ManMan(EthN- P→)ManGlcN-acyl-PI (H7). In the present study, the most polar species was isolated by Iatrobead HPLC and analyzed. Dionex anion exchange chromatographic analyses of its HNO2 fragment before and after HF dephosphorylation in conjunction with comparative [3H]Man and [3H]EthN biosynthetic labeling and compositional studies of trifluoroacetic acid hydrolysates for Man and Man-6-P showed that it corresponds to a structure containing three EthN-Ps; EthN-P-6Man(EthN-P-6)Man(EthN-P→)ManGlcN-acyl-PI. This three-EthN-P GPI structure posed greater difficulties in purification than encountered with other GPI species. Elution from TLC plates with methanol, chloroform:methanol, chloroform:methanol:water, and a number of other solvents failed to yield amounts of sample adequate for analysis. Attempts at direct nitrous acid deamination of the GPI on silica beads likewise were not successful. Although the Iatrobead purification procedure developed in this study permitted greatly improved recovery of H8 and of its reduced HNO2 fragment (~60% yield approaching that of trypanosomal A'), the recovery of the H8 fragment from Dionex anion exchange chromatography was lower than that of the A' fragment. The low recovery could reflect poor elution due to the high net negative charge of the H8 glycan or conceivably its breakdown during chromatography. While the presence of...
FIG. 3. Comparative Dionex HPLC anion exchange of the reduced HNO₃ fragments of H8, H7, and trypanosomal A'. A-C, nondephosphorylated fragments were analyzed using gradient 2. D-F, HF-dephosphorylated fragments were analyzed using gradient 1. In both cases, 0.6-ml fractions were collected. The elution positions of Glc oligomer standards indicated by the number of Glc residues in the oligomer and of Man-1-P, Man-6-P, and 1,6-Fru-diP are shown.

FIG. 4. Comparative Dionex HPLC analyses of Man and Man-6-P content in H7 and H8. Trifluoroacetic acid hydrolysates of in vivo prepared [³H]labeled H7 (A) and H8 (B) were analyzed using gradient 3 with 1-ml fractions. The elution positions of internal unlabeled Man and Man-6-P standards are shown.

Another fragment cannot be excluded, no other peaks were detectable even in 1 M NaAc washes of Dionex columns.

Our Dionex analyses performed following removal of EthN-P substituents by HF showed that the core glycan of H8 consists of an undecorated three-Man (ManManManGlcN) structure. This structure corresponds to that found in the mature protein-associated GPI anchor of human erythrocyte (E¹⁺) acetylcholinesterase (4). In other studies, comparative TLC analyses have indicated that the major [³H]Man-labeled GPI products synthesized by K562 cells, human polymorphonuclear cells (9), and human lymphocytes (19) correspond to those in HeLa cells. These observations suggest that if additional substituents are present on the GPI core in these cell types, such as the additional Man residue on Man 3 and the GalNac or sialic acid-Gal-GalNac substituent on Man 1 that have been detected in the protein-associated GPI-anchors of rat brain Thy-1 (3) and prion protein (5), they must be incorporated after anchor transfer to protein.

The GPI species that serve(s) as anchor donor(s) in mammalian cells has (have) not yet been identified. In trypanosomes, it has been shown that in vitro preparations, EthN-P-Man₃GlcN-PI, -acyl-PI, and -lyso-PI can all function in this capacity (20). Recent analyses of E¹⁺ acetylcholinesterase (6) have indicated that ~15% of the polypeptides bear three-EthN-P-containing GPI anchor structures, and ~80% bear two-EthN-P-containing structures. Analyses of [³H]Man-labeled GPI products following [³H]Man labeling of mammalian cell lines frequently have shown a preponderance of H8, but the proportions of H8 and H7 in different cell types have been noted to vary (9-11). Although protein transfer of GPIs with variations in their glycans has not been documented, the identification of free mammalian GPI species with both three- and two-EthN-P substituents raises the possibility that both can serve as donors. Alternatively, in view of the observations in trypanosomes (21) that galactosylation of Man 1 can occur either before or after transfer of the free GPI to protein (22), it is possible that the number of EthN-P substituents also may be altered after as well as before GPI transfer to protein.

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Note Added in Proof—Following submission of this paper, evidence for a GPI species containing EthN-P was reported by Kamitani et al. (Kamitani, T., Menon, A. K., Hallaq, Y., Warren, C. D., and Yeh, E. T. (1992) J. Biol. Chem. 267, 24611–24619).

REFERENCES