Defective Glycosyl Phosphatidylinositol Biosynthesis in Extracts of Three Thy-1 Negative Lymphoma Cell Mutants

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The glycosyl phosphatidylinositol (GPI) anchors that attach certain proteins to membranes are preassembled by sequential addition of glycan components to phosphatidylinositol (PI) before being transferred to nascent polypeptide. A cell-free system consisting of trypanosome membranes has been reported to catalyze GPI biosynthesis (Masterson, W. J., Doering, T. L., Hart, G. W., and Englund, P. T. (1989) Cell 56, 793–800; Menon, A. K., Schwarz, R. T., Mayor, S., and Cross, G. A. M. (1990) J. Biol. Chem. 265, 9033–9042). We now describe conditions for studying the initial steps of GPI biosynthesis in extracts of murine lymphoma cells. Two chloroform-soluble products, tentatively identified as [6-3H]GlcNAc-PI and [6-3H]GlcN-PI, were generated during incubations of EL4 cell lysates with UDP-[6-3H]GlcNAc. The involvement of PI in the reaction was established by the sensitivity of the products to hydrolysis by PI-specific phospholipase C and the finding that the addition of exogenous PI to the incubation stimulated the reaction. The minor, more polar product was sensitive to nitrous acid cleavage and was converted to the major product, as judged by TLC, after treatment with acetic anhydride. The glycolipids generated in lymphoma extracts appeared to be the same as the products produced in parallel incubations with trypanosome membranes. Analysis of available lymphoma mutants deficient in Thy-1 surface expression revealed that extracts of the class A, C, and H mutants are completely defective in synthesizing GlcNac-PI and GlcN-PI.

Certain proteins in eukaryotic cells are attached to membranes by glycosyl phosphatidylinositol (GPI)-anchored proteins (1, 2). They include coat proteins (VSG of trypanosomes), enzymes (alkaline phosphatase and acetylcholinesterase of animal cells), and cell adhesion proteins (LFA-3). Despite this diversity, the core structures of the different GPI anchors are relatively conserved (3–6). Minimally, the inositol moiety of the anchor is linked directly to a GlcN unit, followed by three mannose residues and one phosphoethanolamine. The latter is attached via an amide linkage to the C terminus of the protein. The sensitivity of GPI anchors to cleavage by PI-specific phospholipase C or nitrous acid (indicative of a GlcN moiety) has been used to characterize GPI-linked proteins and GPI precursors.

Radiolabeling experiments (7, 8) with VSG strongly suggest that the anchor is preassembled and transferred en bloc to nascent protein. Recent work with trypanosome membranes indicates that the GPI anchor is synthesized by sequential addition of individual sugars to PI (9–11). A cell-free system in which trypanosome membranes, UDP-GlcNAc, and radiolabeled GDP-mannose (9–11) are incubated to generate putative precursors of GPI has been described and partially characterized (12, 13). As yet, however, none of the enzymes involved has been purified, and net product synthesis from exogenous PI has not been demonstrated.

While not as abundant as VSG, the numerous mammalian proteins that are modified by a GPI membrane anchor play important roles in cell physiology, and free GPI has been implicated as a source of second messengers in response to insulin (14–16). We have therefore chosen to investigate GPI biosynthesis in a murine lymphoma cell line in which mutants defective in this pathway are available. These mutants were selected because they do not express the antigen Thy-1 on their surface (17, 18). Of the six complementation groups described, enzymatic defects have been determined for only class E (19) and class F (20). The biochemical lesions in the other complementation groups remain undefined.

We now describe a novel cell-free system for assaying the transfer of GlcNAc from UDP-GlcNAc to PI in animal cell membranes. The two predominant products are tentatively identified as GlcNac-PI and GlcN-PI. They appear to be the first two intermediates in GPI biosynthesis. A survey of the available Thy-1-deficient mutants revealed that extracts of class A, C, and H mutants are unable to generate these products.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum was obtained from HyClone Laboratories (Logan, UT). Dulbecco's modified Eagle's medium was purchased from Gibco. UDP-[6-3H]GlcNAc (18.9 Ci/mmol) was obtained from Du Pont-New England Nuclear. PI-phospholipase C from Boecillius cereus was obtained from Boehringer Mannheim, and PI-phospholipase C from Boecillius thuringennessis was from AMAC, Inc. (Westbrook, ME). PI from soybean and bovine liver, PS from bovine brain, PC (dipalmitoyl), UDP-GlcNaC, GDP-mannose, and all other chemicals were purchased from Sigma. Silica Gel 60 TLC plates (E. Merck) were from VWR Scientific (New York).

Cell Culture—The Thy-1 positive mouse lymphoma cell lines EL4, S49.1, SIA.TB.4.8.2, TIMI.4, BW5147.3, and the Thy-1 deficient mutant S49(Thy-1-a) were purchased from the American Type Culture Collection (Rockville, MD). The other mutant cell lines SIA(Thy-1-b), TIMI(Thy-1-c), BW5147(Thy-1-e), EL4(Thy-1-f), and S49(Thy-1-b) were the generous gift of Dr. R. Hyman (Salk Institute). The cells were routinely grown in suspension culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO2 at 37 °C. The cells were passaged every 3 or 4 days and seeded at a density of 1 x 10^6 cells/ml. Cell numbers...
Enzymatic Synthesis of GPI Precursors

In Vitro Biosynthesis of GPI Precursors—Unless otherwise indicated, all incubations were carried out at 37°C for 1 h in 13 x 100-mm glass tubes equipped with Teflon-lined caps. The volume of the reaction mixture was 300 pl. The UDP-[6-3H]GlcNAc, which was purchased as an ethanol solution, was dried under nitrogen and chloroform/methanol (1:1, v/v) containing 0.1 M HCl. Exogenous lipids were added to some incubations by first drying the indicated amount of the lipid (in chloroform) in the incubation tube under nitrogen. The residue was then dissolved in the reaction mixture in the presence of 0.01% Triton X-100 and was subjected to two different organisms resulted in complete disappearance of both compounds, indicating that they are PI derivatives. Mild alkaline hydrolysis of the initial reaction products, fol-

RESULTS

Fig. 1. Characterization of products radiolabeled by UDP-[6-3H]GlcNAc. EL4 cell lysates were incubated with UDP-[6-3H]GlcNAc. The chloroform-soluble products were isolated, characterized by chemical or enzymatic means, and subjected to TLC and autoradiography, as described under “Experimental Procedures.” The arrows indicate the lipids tentatively identified as GlcNAc-PI and GlcN-PI by comparison with authentic samples. lane 1, no treatment; lane 2, base hydrolysis; lane 3, mock PI-phospholipase C (no enzyme); lane 4, B. cereus PI-phospholipase C; lane 5, B. thuringiensis PI-phospholipase C; lane 6, mock nitrous acid (no sodium nitrite); lane 7, nitrous acid; lane 8, mock acetylation (no acetic anhydride); lane 9, acetylation.
lowed by reextraction to isolate the remaining chloroform-soluble substances, resulted in the loss of 80–85% of the radiolabel. The remaining CHC\textsubscript{3}-soluble material was separated by TLC (Fig. 1, lane 2). Two species were resolved which were more hydrophilic than the original compounds. Because base hydrolysis cleaves ester-linked but not ether-linked chains, these results suggest that 15–20% of the GlcNAC-labeled products contain one alkyl chain.

Nitrous acid treatment of the reaction products resulted in disappearance of the more slowly migrating band (Fig. 1, lanes 6 and 7). Because only unacetylated amines are sensitive to this treatment, the lower product probably contains GlcN. This was confirmed by treatment of the reaction products with acetic anhydride. The lower band disappeared (Fig. 1, lanes 8 and 9), but the amount of radioactivity in the upper band increased correspondingly. The acetylation was done in the presence of methanol to favor N-acetylation.

Effects of Exogenous PI on Product Formation—To further investigate the involvement of PI in the formation of the radiolabeled products, various phospholipids were added to the system. The amount of the two major products formed in each case is shown in Fig. 2. The addition of 50 \mu\text{g} of soybean PI or bovine liver PI resulted in a 4–5-fold increase in the amount of product formed. Higher levels of these lipids (150 \mu\text{g}) also stimulated product formation, but to a lesser extent. Product formation did not increase when PC was added, and it went up only 1.5–2-fold with exogenously supplied PS. The small increase observed with PS may be due to some contamination of this lipid with PI, since it was isolated from a biological source (bovine brain). Alternatively, PS may have some physical effects because of its negative charge. The stimulation by exogenously supplied PI suggests that this phospholipid is involved as the second substrate.

Comparison of Lymphoma and Trypanosome Extracts—Doering et al. (10) have reported that two products are radiolabeled by UDP-[\textsuperscript{6-\text{H}}]GlcNAC when incubated with trypanosome membranes. These have been tentatively identified as GlcNAC-PI and GlcN-PI (10, 11) though not by NMR spectroscopy or mass spectrometry. Interestingly, these compounds appear to be substrates for mannose additions (9, 11), as expected for early intermediates in GPI biosynthesis. The products formed by EL4 lysates were compared with those formed by trypanosome membranes in parallel incubations. As shown in Fig. 3, lanes 1 and 3, the two products generated by EL4 lysates migrated the same during TLC as did the major products formed by trypanosome membranes. The total radioactivity recovered in the chloroform phase was decreased by approximately 50%, and three more slowly migrating lipids were generated by the trypanosome membranes when 10 \mu\text{M} GDP-mannose was included in the incubation (Fig. 3, lane 4). Because the addition of mannose residues makes the products more hydrophilic, the decrease in the amount of radiolabeled lipid recovered in the CHC\textsubscript{3} phase may be due to the fact that some of these products do not partition into CHC\textsubscript{3}.

There was no decrease in the amount of radiolabeled material recovered or any appearance of more slowly migrating products when GDP-mannose was added to EL4 lysates (Fig. 3, lane 2).

Class A, C, and H Thy-1\textsuperscript{−} Mutants Do Not Make GlcNAC-PI and GlcN-PI—Having established conditions to study the reactions that appear to generate the first two intermediates in GPI biosynthesis, we next tested extracts of the available lymphoma cell mutants that do not express Thy-1 on their surface. Lysates from all parental cell lines, as well as those from class B, E, and F mutants, generated the putative GlcNAC-PI and GlcN-PI, when incubated with UDP-[\textsuperscript{6-\text{H}}]GlcNAC (data not shown). However, no chloroform-soluble products were generated by lysates of class A, C, and H mutants. A typical result is shown in Fig. 4 for the wild-type EL4 and S49.1 lysates and for the two mutants derived from the latter, S49(Thy-1\textsuperscript{a}) and S49(Thy-1\textsuperscript{h}). No products were formed when equal amounts (by protein) of the lysates of the three mutants were mixed in pairs (data shown for S49(Thy-1\textsuperscript{a}) and S49(Thy-1\textsuperscript{h}) in Fig. 4), despite their ability to complement each other following cell fusion. Mixing of the

![Fig. 2. Effects of exogenous phospholipids on GlcNAC-PI and GlcN-PI formation. Either 50 \mu\text{g} (solid bars) or 150 \mu\text{g} (hatched bars) of the indicated phospholipids were included during the incubations of EL4 cell lysates with UDP-[\textsuperscript{6-\text{H}}]GlcNAC, as described under “Experimental Procedures.” The amount of product formed was quantitated by scraping the bands on the TLC plates corresponding to GlcNAC-PI and GlcN-PI and counting with a scintillation counter. The numbers shown represent the average of three samples ± standard error.](image)

![Fig. 3. Comparison of EL4 and trypanosome membrane-generated products. Parallel incubations of EL4 lysates (lanes 1 and 2) and trypanosome membranes (lanes 3 and 4) with UDP-[\textsuperscript{6-\text{H}}]GlcNAC were carried out in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 10 \mu\text{M} GDP-mannose. PI migrates just above GlcNAC-PI and lyso-PI migrates just below GlcN-PI in this TLC system (not shown). Conditions for TLC and autoradiography are the same as in Fig. 1.](image)
may reflect lower levels of endogenous dolichol-phosphate than in trypanosome membranes.

Our in vitro system for the generation of GlcNAc-PI and GlcN-PI differs significantly from that described by Saltiel and co-workers (27, 28), who incubated liver microsomes with CDP-diacylglycerol and myo-[3H]inositol to obtain radiolabeled PI as an in situ acceptor of GlcNAc and other sugars. Although it will be important to demonstrate the participation of PI by direct methods, it appears that the product obtained by Saltiel and co-workers (27, 28) is actually lyso-PI (29).

Three of the existing lymphoma cell mutants that are defective in the export of Thy-1 to the cell surface produced no GlcNAc-PI or GlcN-PI under our conditions (Fig. 4). The fact that three complementing mutants are defective in what would appear to be a single reaction suggests either that the GlcNAc-PI synthase consists of several subunits or that there may be a problem in the transport of UDP-GlcNAc to the reaction site, presumed to be the luminal surface of the endoplasmic reticulum. The possibility that any of the mutants might be defective in the generation of PI has been excluded previously (20). Further, class A and C mutants appear to produce normal high mannose glycoproteins, a process that requires UDP-GlcNAc (18). Whatever the final explanation, these mutants offer a unique system for dissecting the enzymology and molecular biology of GPI biosynthesis.

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