Isolation and characterization of a Chinese hamster ovary (CHO) mutant defective in the second step of glycosylphosphatidylinositol biosynthesis

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Mutant cell lines defective in the biosynthesis of glycosylphosphatidylinositol (GPI) described to date were isolated by selecting cells which no longer expressed one or more endogenous GPI-anchored proteins on their surface. In this study, a new mutant in this pathway was isolated from ethylmethanesulphonate-mutagenized Chinese hamster ovary cells stably transfected with human placental alkaline phosphatase (PLAP) as a marker of GPI-anchored proteins. A three-step protocol was employed. In the first step, cells with decreased surface expression of PLAP were selected by four rounds of complement-mediated lysis with an anti-(alkaline phosphatase) antibody. The surviving cells were cloned by limiting dilution and those with low levels of total alkaline phosphatase activity were selected in the second step. Finally, the ability of each clone to synthesize the first three intermediates in GPI biosynthesis *in vitro* was assessed to determine which cells with low alkaline phosphatase activity harboured a defect in one of these reactions. Of 230 potential mutants, one was defective in the second step of GPI biosynthesis. Microsomes from this mutant, designated G9PLAP.85, were completely unable to deacetylate either endogenous GlcNAc-phosphatidylinositol (PI) synthesized from UDP[6-^{3}H]GlcNAc or exogenous GlcNAc-PI added directly to the membranes. Complementation analysis with the Thy-1-deficient murine lymphoma cells demonstrated that G9PLAP.85 has a molecular defect distinct from these previously described mutants. Therefore, these results suggest that mutants in GPI biosynthesis could be selected from almost any cell line expressing a GPI-anchored marker protein.

INTRODUCTION

Numerous eukaryotic proteins are attached to the cell surface by a glycosylphosphatidylinositol (GPI) membrane anchor. The conserved core of this glycolipid consists of an inositol-containing lipid [usually phosphatidylinositol (PI)], glucosamine, three mannose residues and a phosphoethanolamine (see [1] for review). The GPI core is assembled in the endoplasmic reticulum and then transferred as a whole to a newly synthesized protein [2,3]. The membrane anchor is attached to the protein by a peptide bond between the C-terminus and the amine of the phosphoethanolamine.

The pathway for the biosynthesis of GPI has been elucidated by analysis of the structure of biosynthetic intermediates from trypanosomes [4–7], *in vitro* studies of the synthesis of GPI precursors [8–10], and the characterization of mammalian cells unable to synthesize GPI [11,12]. This pathway as it occurs in mammals and yeast is shown in Figure 1. In the first step, GlcNAc is transferred from UDP-GlcNAc to PI. The discovery that three complementing mutants (classes A, C and H) were unable to catalyse this reaction indicated that three separate gene products are required for this step [13,14]. Two of these gene products (classes A and H) have been expression cloned using these mutants [15,16] and defects in the class A gene have been found to be responsible for the disease paroxysmal nocturnal haemoglobinuria [17,18]. In the second step, GlcNAc-PI is deacetylated to form GlcN-PI. This reaction is stimulated *in vitro* in mammalian cells by GTP [19]. GlcN-PI is acylated on the inositol ring in the third step of the pathway in mammals and yeast but not trypanosomes. The structural elucidation of the glycolipid that accumulated in the class E lymphoma cell and yeast *dpm1* mutants unable to add mannoses to the GPI core [12,20] as GlcNAc-PI(acyl) provided the first evidence that this reaction was obligatory in these species. Subsequent characterization in yeast [21] and mammalian cells [22] indicated that the acyl donor is probably an acyl-CoA in the former and a phospholipid in the latter.

Following inositol acylation, three mannoses are added to the GPI core. The finding that GPI biosynthesis was affected in the class E mutant unable to synthesize dolichol phosphomannose [11] indicated that this glycolipid was the endogenous donor for at least the first mannose residue. Subsequent experiments with trypanosomes demonstrated that all of the core mannoses are derived from dolichol phosphomannose [23]. In the final step in the pathway, phosphoethanolamine is transferred from phosphatidylethanolamine to the three-mannose-containing core [24]. Analysis of the glycolipids that accumulate in the class B and F lymphoma mutants indicated that these cells are defective in the addition of the third mannose and the terminal phosphoethanolamine respectively [14]. Furthermore, additional phosphoethanolamines were found attached to the first and second mannoses, suggesting that additional modifications are made to the core in mammals before GPI biosynthesis is completed [25–29]. cDNAs which correct the class B [30] and F [31] defects have been expression cloned.

Clearly, the study of mutants defective in GPI biosynthesis has provided invaluable information about this pathway which would have been very difficult to obtain by other means. However, there

Abbreviations used: GPI, glycosylphosphatidylinositol; PI, phosphatidylinositol; PLAP, placental alkaline phosphatase; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; PI-PLC, PI-specific phospholipase C; PEG, polyethylene glycol; EMS, ethylmethanesulphonate; DTT, dithiothreitol; SSC, 0.15 M NaCl/15 mM trisodium citrate; TLCK, tosyl-lysylchloromethane.

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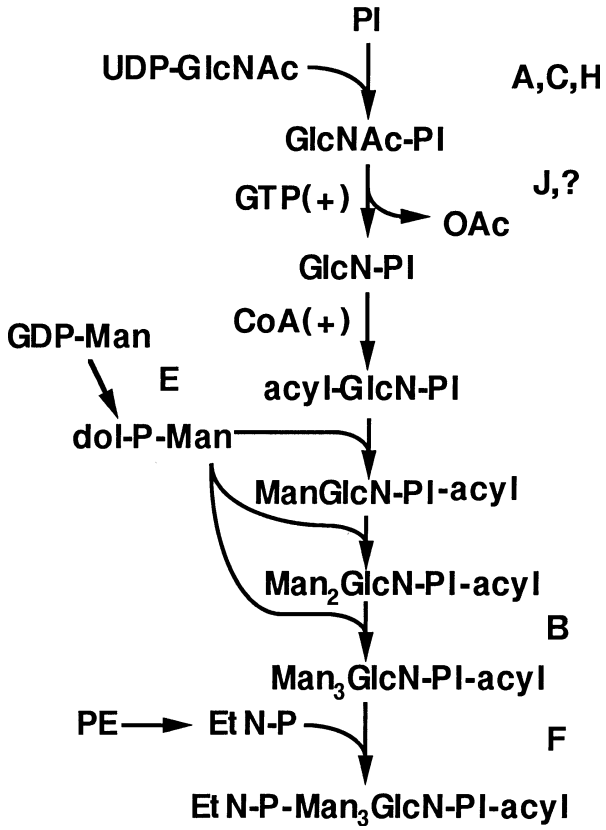

Figure 1 GPI biosynthesis in mammalian cells

The pathway for GPI biosynthesis in yeast and mammals is shown. The effectors of the second and third reactions in mammalian cell-free systems are shown to the left of the appropriate steps and the complementation groups assigned to these reactions are shown on the right.

are several reactions for which no mutant has yet been isolated. Most of the existing mammalian mutants defective in GPI biosynthesis were obtained by selecting cells which do not express a GPI-anchored protein on their surface, either by complement-mediated lysis or flow cytometry. The choice of cell lines available for mutant selection has been limited to those which express known GPI-anchored proteins for which antibodies are available. In this study, a potentially new mutant in GPI biosynthesis was isolated by complement-mediated lysis of Chinese hamster ovary (CHO) cells that expressed human placental alkaline phosphatase (PLAP) which had been introduced by transfection. Analysis of the GPI biosynthetic ability of the mutant revealed that it was defective in the second step of the pathway, the deacetylation of GlcNAc-PI. While surface expression of alkaline phosphatase was lost, mRNA for this protein was still produced, indicating that the DNA coding sequence for this GPI-anchored marker protein was retained through the selection. Complementation analysis with the Thy-1-deficient murine lymphoma mutants demonstrated that this cell represents a distinct complementation group.

MATERIALS AND METHODS

Materials

Fetal bovine serum, horse serum, Ham's F-12 medium, Dulbecco's modified Eagle's medium (DMEM) and G418 were purchased from GIBCO. UDP-[6-^{3}H]GlcNAc (25 Ci/mmol) was obtained from American Radiolabelled Chemicals. [α-^{32}P]dATP (3000 Ci/mmol) was from ICN Radiochemicals. Silica gel 60 TLC plates (E. Merck) were purchased from VWR Scientific. DEAE cellulose, GTP, ATP and polyethylene glycol (PEG) were obtained from Sigma. PI-specific phospholipase C (PI-PLC) was purified from *Bacillus subtilis* BG2320 cells which had been transformed with a high-copy-number plasmid encoding *Bacillus thuringiensis* PI-PLC (a gift from Dr. Martin Low, Columbia University, New York, NY, U.S.A.) as described by Low [32]. Rabbit polyclonal anti-(human PLAP) antibody was purchased from DAKO.

Cell culture

CHO-K1, G9PLAP and G9PLAP.85 cells were routinely grown in monolayer cultures in Ham's F-12 medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin in an atmosphere of 5% CO_2 at 37 °C. G418 (300 μg/ml) was also included in the culture medium of the G9PLAP.85 cells. Cells were routinely passaged by removing the medium, washing with PBS and detaching from the tissue-culture dish with 0.5 mM EDTA in PBS.

The Thy-1-deficient murine lymphoma cell lines S49(Thy-1$^-$a) (from the American Type Culture Collection), SIA(Thy-1$^-$b), TIMI(Thy-1$^-$c), BW5147(Thy-1$^-$e), EL4(Thy-1$^-$f) and S49(Thy-1$^-$h) (all generously provided by Dr. R. Hyman, Salk Institute, San Diego, CA, U.S.A.) were grown in suspension culture in DMEM supplemented with 10% (v/v) horse serum, 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were passaged every 3 or 4 days and seeded at a density of 1×10^5 cells/ml. Cell numbers were determined by counting with a haemacytometer.

Transfection of CHO-K1 cells

The cDNA for human PLAP was obtained from Dr. Sidney Udenfriend (Roche Institute of Molecular Biology, Nutley, NJ, U.S.A.) in the plasmid pGEM-4Z/PLAP#6. The 2200 bp *Eco*R1–*Eco*R1 fragment containing the alkaline phosphatase coding sequence was removed from pGEM-4Z/PLAP#6 by restriction with this enzyme and ligated into the *Eco*R1 cloning site of pSFFV.neo [33] adjacent to the SFFV LTR promoter element. CHO-K1 cells were transfected with pSFFV.neo/PLAP (10 μg) by electroporation at 580 V using an Electro Cell Manipulator 600 (BTX). Cells stably expressing PLAP were isolated by selecting colonies that grew in 500 μg/ml G418 and cloned by limiting dilution. One colony, designated G9PLAP, was found to have high alkaline phosphatase activity (see below) and was selected for use in subsequent studies.

Characterization of alkaline phosphatase activity

Cells were scraped from tissue-culture dishes, pelleted by centrifugation (1000 **g**, 5 min), washed with PBS and resuspended in 100 mM aminomethylpropanol, pH 10.5. Alkaline phosphatase activity was determined by measuring the hydrolysis of *p*-nitrophenyl phosphate as described by Nair et al. [34].

Susceptibility to cleavage by PI-PLC was used to verify that PLAP was expressed as a GPI-anchored protein. Washed G9PLAP cells (prepared as described above) were incubated in 25 mM Tris/HCl, pH 7.5, 0.25 M sucrose and 10 mM glucose with 10 m-units of *B. thuringiensis* PI-PLC for 1 h at 37 °C. Cells were then pelleted by centrifugation (1000 **g** for 15 min) and the amount of PLAP remaining associated with the cells was measured. The percentage of PLAP which was not released by

PI-PLC treatment was determined by dividing the cell-associated alkaline phosphatase activity after PI-PLC treatment by the total alkaline phosphatase activity (determined by assaying cells taken through the incubation and re-isolation procedure but not exposed to PI-PLC).

Complement-mediated lysis of alkaline phosphatase-expressing cells

G9PLAP cells were mutagenized with ethyl methanesulphonate (EMS, 200 μg/ml) for 24 h and allowed to recover for 48 h, before selection of cells which no longer expressed alkaline phosphatase on their surface by complement-mediated lysis. The mutagenized cells ($> 5 \times 10^7$ per selection) were incubated with anti-PLAP antibody (1 mg/ml) in Ham's F-12 medium on a rocker plate for 1 h at room temperature. The medium was then removed and replaced with Ham's F-12 medium supplemented with 20 % (v/v) human serum. The cells were incubated at 37 °C for 1 h during which the complement components in the serum assembled to form the membrane attack complex which killed alkaline phosphatase-expressing cells. The surviving cells were allowed to repopulate the flask and subjected to three more rounds of complement-mediated lysis. The surviving cells were cloned by limiting dilution.

Preparation of cell lysates and microsomes

Cell lysates were prepared by sonic disruption of cells suspended in 10 mM Hepes (pH 7.5), 0.5 mM dithiothreitol (DTT), 0.1 mM tosyl-lysylchloromethane (TLCK) and 1 μg/ml leupeptin as previously described [13]. These lysates were used immediately for analysis of GPI biosynthesis. Microsomes were prepared as described previously [19] and stored for up to 6 months before being used. Protein was quantified using the bicinchoninic assay of Smith et al. [35].

In vitro biosynthesis and analysis of GPI precursors

Biosynthesis of GPI intermediates from UDP-[6-^{3}H]GlcNAc (1 μCi) by cell lysates (approx. 150 μg of protein) or microsomes (approx. 60 μg of protein) was measured in a reaction mixture of 50 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM TLCK, 1 μg/ml leupeptin, 0.2 μg/ml tunicamycin, 1 mM ATP and 1 mM EDTA (total volume of 300 μl). Deacetylation of [^{3}H]GlcNAc-PI (10000 c.p.m.), enzymically prepared as previously described [19], was measured using the same conditions except that ATP and EDTA were omitted from the reaction mixture. GTP (1 mM) was included as indicated. After incubation for the indicated time at 37 °C, the reaction was stopped by the addition of 0.5 ml of water and 3 ml of chloroform/methanol (2:1, v/v) containing 0.1 M HCl. The radiolabelled GPI precursors were then extracted using the method of Bligh and Dyer [36] and analysed by TLC as previously described [22].

Fractionation of GPI precursors

GPI precursors were separated on the basis of charge by chromatography on DEAE-cellulose as previously described [19]. The net-neutral, zwitterionic precursors, GlcN-PI and GlcN-PI(acyl), did not bind to the DEAE-cellulose and were recovered when the column was washed with chloroform/methanol/water (2:3:1, by vol.). GlcNAc-PI, which is negatively charged, was eluted with chloroform/methanol/50 mM ammonium acetate (2:3:1, by vol.).

Complementation analysis

G9PLAP.85 and CHO-K1 cells were fused with each of the Thy-1-deficient murine lymphoma cells essentially as described by Mohney et al. [37]. Briefly, cells were harvested by centrifugation, washed with PBS, and 2.5×10^6 of each type to be fused combined in L-glutamine-free DMEM. The cells were then pelleted by centrifugation and resuspended in 1 ml of 50 % PEG in L-glutamine-free DMEM (prepared by microwaving the solution to boiling several times until the PEG was dissolved) over a 1 min time period with constant mixing. This solution was then diluted by addition of 1 ml of L-glutamine-free DMEM over 1 min followed by 5 ml of DMEM containing 10 % (v/v) horse serum over 3 min. The cell solution was then centrifuged (1000 rev./min, 5 min) and the cells resuspended in 5 ml of DMEM containing 10 % (v/v) horse serum in a 100 cm^2 culture dish and incubated at 37 °C. After at least 24 h, complementation was assessed by determining the percentage of fused cells which expressed alkaline phosphatase. This activity was detected histochemically directly in the culture dish using naphthol AS-MX phosphate as the substrate as described by Yoon et al. [38].

Northern blot analysis

Total RNA was isolated from CHO-K1, G9PLAP and G9PLAP.85 cells using the acid guanidinium–phenol–chloroform method [39]. The RNA (20 μg/lane) was then fractionated by electrophoresis on a 1 % agarose–formaldehyde gel and transferred to a Nytran$^+$-supported nylon membrane. Alkaline phosphatase message was detected by hybridization of a ^{32}P-labelled DNA probe generated from the PLAP coding sequence of pGEM-4Z/PLAP#6 with the Random Primers DNA Labelling System (Gibco/BRL) to the membrane. The filter then was washed twice with 2 × SSC (SSC: 0.15 M NaCl, 15 mM trisodium citrate)/0.1 % SDS at 25 °C for 5 min each, twice with 0.2 × SSC/0.1 % SDS at 25 °C for 5 min each, twice with 0.2 × SSC/0.1 % SDS at 42 °C for 15 min each, and twice with 0.1 × SSC/0.1 % SDS at 68 °C for 15 min each. The PLAP message was then visualized by autoradiography on Kodak XAR-5 film.

To verify that equivalent amounts of RNA had been loaded in each lane of the gel, the membrane was probed for β-actin message with a ^{32}P-labelled probe prepared by random priming using the 2 kb insert of the plasmid pA1, which contains the full-length coding sequence of β-actin, as a template [40].

RESULTS AND DISCUSSION

Selection of cells not expressing alkaline phosphatase on their surface

The choice of cell lines is likely to very important in the isolation of mutants in GPI biosynthesis that are distinct from those previously described. This is because the class A gene, which encodes one of the proteins required for the first step in the pathway [13,14], resides on the X chromosome [17]. Therefore, because only one copy of a gene must be affected to obtain a cell defective in the activity it encodes, the frequency of occurrence of class A mutants is approx. 1 in 10^6. If the other genes required for GPI biosynthesis reside on autosomal chromosomes, then two copies must be mutated for the gene product to be lost and the frequency of occurrence of these mutants is expected to be much less. To avoid isolating predominantly class A mutants, we have chosen to isolate new mutants in GPI biosynthesis in CHO cells. These cells are functionally hemizygous, expressing proteins from only one allele [41]. Therefore, the probability of isolating

Table 1 Alkaline phosphatase activity in parental and mutant cell lines

Alkaline phosphatase activity was measured in wild-type CHO-K1, PLAP-transfected G9PLAP, and three putative mutant cells as described in the Materials and methods section. Abbreviation: n.d. not determined.

Cell	Alkaline phosphatase activity (nmol/min per mg of protein)	
	Total	PI-PLC
CHO-K1	4.07	n.d.
G9PLAP	485	91.1
G9PLAP.85	8.81	n.d.
G9PLAP.67	7.02	n.d.
G9PLAP.72	8.85	n.d.

a CHO mutant defective in any gene should be approximately equal.

Since the surface expression of alkaline phosphatase in G9PLAP cells is dependent on the biosynthesis of GPI, mutants defective in these pathway were sought by selecting cells which did not have this protein on their plasma membrane. This was accomplished with complement-mediated lysis of EMS-mutagenized G9PLAP cells with a polyclonal anti-PLAP antibody and human serum. This treatment killed more than 95% of G9PLAP cells but less than 2% of CHO-K1 cells, which do not express PLAP. After four rounds of complement-mediated lysis, negligible additional killing of cells was observed and the surviving cells were cloned by limiting dilution. A total of 230 individual colonies were harvested and grown up for further characterization.

While proteins which are normally GPI-anchored are still translated in cells unable to synthesize this glycolipid, they are either secreted [42–44] or degraded intracellularly [43,45]. Therefore, as a first measure of whether GPI biosynthesis was affected in the putative mutants, alkaline phosphatase activity was measured. Of the 230 original clones, 153 were found to have significantly lower alkaline phosphatase activity (by at least 50%) than the parental G9PLAP cells.

Characterization of the biosynthesis of the first three GPI intermediates in the putative mutants

The synthesis of GlcNAc-PI, GlcN-PI and GlcN-PI(acyl) from UDP-[6-^{3}H]GlcNAc was measured in lysates prepared from the putative mutants to determine if any of these cells were defective in the initial steps of GPI biosynthesis. Of the 153 clones with low alkaline phosphatase activity, 30 appeared to be affected in one of the first three steps of GPI biosynthesis (results not shown). Three cells, designated G9PLAP.67, G9PLAP.72 and G9PLAP.85, appeared unable to synthesize either GlcN-PI or GlcN-PI(acyl) in the initial screen. The alkaline phosphatase activity of these cells, which is shown in Table 1, was roughly 2% of that in the parental G9PLAP cell line. This level was only slightly above that found in CHO-K1 cells, which do not express this enzyme and so serves as a measure of the level of detection of this assay. A similar reduction in alkaline phosphatase protein was seen in G9PLAP.85 relative to that in G9PLAP when the enzyme was detected by immunoblotting (results not shown).

The lysate assay for biosynthesis of GPI precursors provides a somewhat less sensitive measure of the first three steps in this pathway than does the microsomal system. Although we have not investigated the reason for this, it is probably because of the lower level of GPI biosynthetic enzymes and the presence of other activities which are capable of degrading UDP-[6-^{3}H]GlcNAc in the lysate, which are not in a microsomal

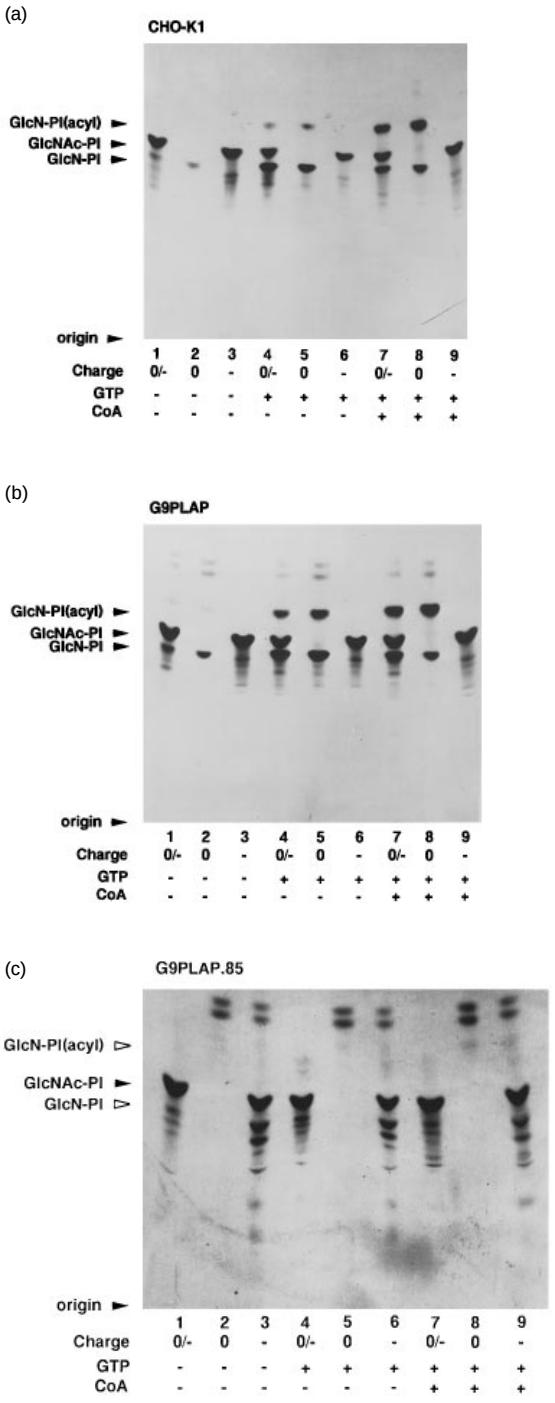

Figure 2 Biosynthesis of GPI intermediates by microsomes prepared from wild-type and mutant cells

Microsomes from untransfected CHO-K1 cells (**a**), parental G9PLAP cells (**b**) and mutant G9PLAP.85 cells (**c**) were incubated with UDP-[6-^{3}H]GlcNAc in the presence of no effectors (lanes 1–3), 1 mM GTP (lanes 4–6), or GTP + 1 μM CoA (lanes 7–9). GPI precursors were extracted and either analysed by TLC prior to (lanes 1, 4 and 7) or after DEAE cellulose chromatography to separate the neutral (lanes 2, 5 and 8) and acidic (lanes 3, 6 and 9) lipids. The arrowheads on the left indicate the mobility of standards for GlcNAc-PI, GlcN-PI and GlcN-PI(acyl).

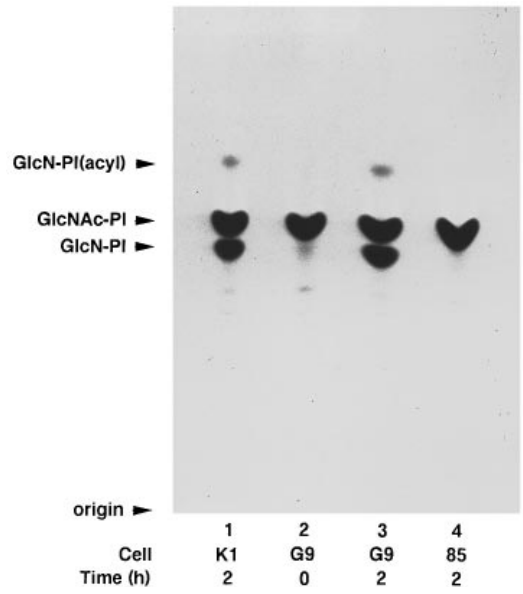

Figure 3 Deacetylation of [^{3}H]GlcNAc-PI

Microsomes from CHO-K1 (K1), G9PLAP (G9), or G9PLAP.85 (85) cells were incubated with 5000 c.p.m. of [^{3}H]GlcNAc-PI in the presence of 1 mM GTP for either 0 or 2 h (as indicated). The radiolabelled lipids were then extracted and resolved by TLC as described in the Materials and methods section.

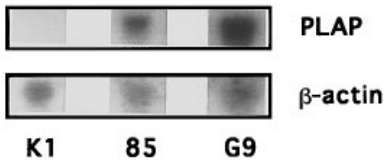

Figure 4 Northern blot analysis for PLAP mRNA

Total RNA was extracted from CHO-K1 (K1), G9PLAP (G9), and G9PLAP.85 (85) cells, resolved by electrophoresis on a formaldehyde–agarose gel, and transferred to a nylon membrane. PLAP and β-actin mRNAs were detected using ^{32}P-labelled DNA probes as described in the Materials and methods section.

preparation. The lysate assay was used for the initial screening of the potential mutants because of the ease of preparation of the cells. However, this assay is expected to falsely identify some normal cells as defective in GPI biosynthesis but not miss any potential mutants. To determine if the three putative mutants identified with the lysate assay were really defective in GlcNAc-PI deacetylation, this reaction was measured in microsomes prepared from these cells. The ability of the parental cell lines CHO-K1 and G9PLAP, and the putative mutant cell line G9PLAP.85, to synthesize the first three intermediates in GPI biosynthesis is shown in Figures 2(a), 2(b) and 2(c) respectively. In each case, the reaction was carried out using conditions to maximize synthesis of GlcNAc-PI (no effectors were added, lanes 1–3), GlcN-PI (GTP was added, lanes 4–6), and GlcN-PI(acyl) (GTP and CoA were added, lanes 7–9). Because tunicamycin does not completely inhibit the biosynthesis of dolichol-linked GlcNAc-containing lipids in these CHO-derived cell lines, it was necessary to chromatograph the radiolabelled products on DEAE cellulose prior to TLC analysis to separate the neutral products [GlcN-PI and GlcN-PI(acyl)] from the acidic glycolipids (GlcNAc-PI and the dolichol-linked lipids). Therefore, in Figures 2(a), 2(b) and 2(c), the unfractionated products are shown in lanes 1, 4 and 7, while the neutral lipids are in lanes 2, 5 and 8 and the acidic lipids are in lanes 3, 6 and 9. Comparison of the results in Figures 2(a) and 2(b) shows that there is little difference in the biosynthesis of the first three intermediates in the pathway by CHO-K1 and G9PLAP cells, indicating that expression of alkaline phosphatase did not affect GPI biosynthesis. G9PLAP.85 cells synthesized GlcNAc-PI normally (see Figure 2c, lanes 1 and 3) but did not make GlcN-PI (lane 2). The addition of GTP to the incubation, either alone (lane 5) or with CoA (lane 8), did not correct the inability of the mutant to make the second GPI intermediate. Therefore, G9PLAP.85 appears to be totally defective in GlcNAc-PI deacetylation. Further analysis of GPI biosynthesis in the other two putative deacetylase mutants revealed that this reaction was only partially affected in G9PLAP.67 and G9PLAP.72 cells (results not shown).

While the analysis of GPI biosynthesis from UDP-[6-^{3}H]GlcNAc indicates that the defect in G9PLAP.85 cells is in the second step of the pathway, the possibility that these cells do not synthesize GlcN-PI because of some problem in the delivery of GlcNAc-PI to the deacetylase cannot be ruled out by these results. Therefore, the GlcNAc-PI deacetylase activity of G9PLAP.85 cells was measured directly with exogenously supplied [^{3}H]GlcNAc-PI. These results are shown in Figure 3. More than 98% of the radiolabel added to the microsomes was [^{3}H]GlcNAc-PI, as is shown in the zero time point for G9PLAP in lane 2. CHO-K1 (lane 1) and G9PLAP (lane 3) microsomes deacetylated approx. 20% of the [^{3}H]GlcNAc-PI in 2 h. There was no detectable deacetylation of [^{3}H]GlcNAc-PI by G9PLAP.85 microsomes in the same period (lane 4). Therefore, the GPI biosynthetic defect in this mutant is in the deacetylation of GlcNAc-PI.

Retention of the PLAP gene by G9PLAP.85 cells

Besides cells which no longer express alkaline phosphatase on their surface because of defects in GPI biosynthesis, the complement-mediated lysis screen will also select cells that have lost or are unable to transcribe the PLAP gene. To verify that the PLAP gene was still present and transcribed in the G9PLAP.85 mutant, the mRNA encoding this protein was measured by Northern blot analysis. The expression of PLAP mRNA by CHO-K1, G9PLAP.85 and G9PLAP cells is shown in Figure 4. As expected, no PLAP mRNA was detected in the untransfected CHO-K1 cells. The message was clearly detected in both the G9PLAP.85 and G9PLAP cells, although the level was somewhat lower in the mutant cell. The mRNA for β-actin is also shown to demonstrate that nearly equal amounts of RNA from each cell was loaded on the gel. These results show that the PLAP gene is present and transcribed in the G9PLAP.85 cells and, therefore, the loss of surface expression of alkaline phosphatase is due to the defect in GPI biosynthesis.

G9PLAP.85 represents a complementation group distinct from the Thy-1-deficient mutants

To determine if the defect in GPI biosynthesis in the G9PLAP.85 cells was distinct from those in the Thy-1-deficient mutants, the CHO-derived mutant was fused with each of murine lymphoma cells and alkaline phosphatase expression assessed. As shown in Table 2, alkaline phosphatase was expressed at levels comparable with the G9PLAP parental cell line in almost all the fusion

Table 2 Alkaline phosphatase expression in fused cells

G9PLAP.85 or CHO-K1 cells were fused with Thy-1-deficient lymphoma cells as described in the Materials and methods section. Alkaline phosphatase expression was assessed in the fusion products, which were identified by their large size. The results shown are the percentage of 100 fused cells which expressed alkaline phosphatase. In each case, the experiment was done at least twice and nearly identical results were found in each instance. The results of a representative experiment are shown here. Abbreviation: n.d., not determined.

	G9PLAP.85	CHO-K1
S49(Thy-1$^-$a)	95	n.d.
SIA(Thy-1$^-$b)	98	n.d.
TIMI(Thy-1$^-$c)	92	n.d.
BW5147(Thy-1$^-$e)	99	0
EL4(Thy-1$^-$f)	95	n.d.
S49(Thy-1$^-$h)	96	n.d.

products. No enzyme activity was detected in the fusion products of CHO-K1 and BW5147(Thy-1$^-$e), as expected because neither of these cells normally express this enzyme. Therefore, as suggested by the finding of a unique biochemical phenotype for G9PLAP.85, these results confirm that this cell line represents a new complementation group of mutants in GPI biosynthesis. Two potential GlcNAc-PI deacetylase mutants have been isolated and described by Medof and colleagues [37,46]. One of these K562 cell lines, designated IV D, has been assigned to complementation group J [37]. Whether IV D and G9PLAP.85 harbour the same genetic defect has not yet been determined. Therefore, G9PLAP.85 will not be assigned to a complementation group until complementation studies between the two mutants have been done.

Like the other mutants in GPI biosynthesis, G9PLAP.85 is expected to be very useful in the study of this pathway. The GlcNAc-PI deacetylase is the only enzyme of the pathway that has been characterized biochemically. Milne et al. [47] partially purified this enzyme from trypanosomes and characterized its substrate specificity. These investigators found it very difficult to purify significant amounts of the GlcNAc-PI deacetylase without loss of enzymic activity. Therefore, it may be easier to study this enzyme by first cloning it and expressing the protein from the cDNA sequence. The G9PLAP.85 mutant provides the necessary tools for expression cloning of this enzyme. Furthermore, the fact that GlcNAc-PI deacetylation is stimulated by GTP in mammalian cells but not yeast or trypanosomes suggests that there may be important differences in this enzyme in these species. Elucidation of the cDNA sequence for the mammalian GlcNAc-PI deacetylase should demonstrate whether this enzyme binds GTP directly and provide insight into this regulation.

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