Differential Expression of Glycosylphosphatidylinositol-anchored Proteins in a Murine T Cell Hybridoma Mutant Producing Limiting Amounts of the Glycolipid Core
Implications for Paroxysmal Nocturnal Hemoglobinuria

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Abstract

A T cell hybridoma mutant, which expressed a markedly reduced level of glycosylphosphatidylinositol (GPI)-anchored proteins on the cell surface, was characterized. The surface expression level of Thy-1 was ~ 17% of the wild-type level, whereas the surface expression of Ly-6A was ~ 2.4% of the wild-type level. We show here that these cells synthesized limiting amounts of the GPI core and that the underlying defect in these cells was an inability to synthesize dolichyl phosphate mannose (Dol-P-Man) at the normal level. The defect in Ly-6A expression could be partially corrected by tunicamycin, which blocked the biosynthesis of N-linked oligosaccharide precursors and shunted Dol-P-Man to the GPI pathway. Full restoration of Thy-1 and Ly-6A expression, however, required the stable transfection of a yeast Dol-P-Man synthase gene into the mutants. These results revealed that when the GPI core is limiting, there is a differential transfer of the available GPI core to proteins that contain GPI-anchor attachment sequences. Our findings also have implications for the elucidation of the defects in paroxysmal nocturnal hemoglobinuria. (*J. Clin. Invest.* 1992. 89:1172–1177.) Key words: glycosylphosphatidylinositol anchor • paroxysmal nocturnal hemoglobinuria

Introduction

As an alternative to the use of a transmembrane region, proteins may be attached to membranes via a glycosylphosphatidylinositol (GPI)[1] anchor. A biosynthetic pathway for the GPI anchor has been characterized in mammalian cells, and it shares some similarity with the trypanosomal pathway (1–8). The first two anchor precursors found are N-acetylglucosaminyl-phosphatidylinositol (GlcNAc-PI) and glucosaminyl-phosphatidylinositol (GlcN-PI) which are synthesized by the transfer of GlcNAc from UDP-GlcNAc to phosphatidylinositol (PI) and subsequent N-deacetylation (2–4). Subsequently, GlcN-PI is fatty-acylated in the inositol ring and mannose residues are added from dolichyl phosphate mannose (Dol-P-Man) (1, 2). Finally, one or more residues of ethanolamine phosphate are added to the mannosylated GPI precursors (1, 2). The transfer of the preformed GPI core to the carboxy termini of proteins has not been directly demonstrated in the mammalian system. It has been postulated that the transfer requires a novel transamidase, which cleaves the carboxy termini of proteins that have appropriate cleavage and attachment signals and transfers the preformed cores to them (9–15). Although a consensus sequence has not been defined, a carboxy-terminal hydrophobic domain and small amino acids at the cleavage sites are the general requirements (14, 15).

We have defined defects in GPI anchor biosynthesis in a large panel of T cell mutants (1, 2). These mutants cannot express GPI-anchored proteins on the cell surface because their incompletely formed GPI-anchor precursors cannot be transferred to proteins (16–19). In this paper, we characterize a T cell mutant that expressed reduced levels of GPI-anchored proteins on the cell surface. Interestingly, two proteins studied, Thy-1 and Ly-6A, are not reduced to the same extent. The surface expression level of Thy-1 was 17% of the wild-type level, whereas the surface expression of Ly-6A was 2.4% of the wild-type level. The biosynthetic defect in this mutant was shown to be a deficient production of Dol-P-Man, which is the donor of mannose residues in the biosynthesis of the GPI anchor (1, 20). Predictably, the synthesis of the complete GPI core in this mutant was seen to be severely deficient, which presumably led to the reduced surface expression of GPI-anchored proteins. We were able to rescue the biosynthetic defect with a yeast Dol-P-Man synthase gene (1). With an increased level of Dol-P-Man biosynthesis, the surface expression of Ly-6A and Thy-1 was returned to the wild-type level. This observation implies that when the GPI core was limiting, as in the case of the mutant described here, there was a preferential transfer of the available GPI core to certain proteins that contain GPI-anchor attachment sequences.

Defects in either GPI anchor biosynthesis or attachment have been implicated in the pathogenesis of paroxysmal nocturnal hemoglobinuria (PNH), an acquired hematopoietic stem cell disorder (21–23). A large number of GPI-anchored proteins have been found to be either absent or deficient on the surface of hematopoietic cells (24–28). This can be accounted

1. *Abbreviations used in this paper:* Dol-P-Man, dolichyl phosphate mannose; FITC-GAMG, fluorescein isothiocyanate-goat anti-mouse IgG; GlcN-PI, glucosaminylphosphatidylinositol; GlcNAc-PI, N-acetylglucosaminylphosphatidylinositol; GPI, glycosylphosphatidylinositol; PI, phosphatidylinositol; PNH, paroxysmal nocturnal hemoglobinuria.

for by defects, either partial or complete, in the biosynthesis of the GPI anchor. Recently, it has been shown in several PNH patients that there is a discordance in the expression of GPI-anchored proteins in different cell types (29, 30). The biosynthetic defects in these PNH patients have not been defined, however. Our results, therefore, provide a theoretical explanation for the differential expression of GPI-anchored proteins in granulocytes and erythrocytes derived from PNH patients.

Methods

Reagents and antibodies. The anti-Ly-6A antibody (3E7.1) has been previously described (31). The following mAbs were kindly provided by the laboratories that developed them: anti-Thy-1 (M5/49) (32), and anti-CD3 (145-2C11) (33). Antibodies were used as tissue culture supernatants, containing 10% FCS. The plasmid containing the gene encoding Dol-P-Man synthase was the gift of Drs. P. J. Beck and J. Sambrook (University of Texas Southwestern Medical Center, Dallas, Texas) (34).

Cell culture. T cell hybridomas YH16.33 and M38/20, have been described previously (1, 35). They were maintained in RPMI 1640 medium, which had been supplemented with 10% FCS and penicillin, streptomycin, and fungizone.

Glycolipid labeling and TLC separation. GPI anchor precursors were identified as described previously (1, 2). 2×10^7 cells were labeled for 45 min with [^{3}H]mannose (20 μCi/ml) in low glucose (0.1 g/liter) RPMI in the presence of 10 μg/ml of tunicamycin. The lipids were extracted with $CHCl_3/CH_3OH/H_2O$ (10:10:3). The lipid extract was dried, dissolved in water-saturated butanol and partitioned with H_2O. The butanol phase was dried and blotted on a silica gel 60 plate (EM Science, Gibbstown, NJ). TLC plates were developed in $CHCl_3/CH_3OH/H_2O$ (10:10:3), scanned, and autoradiographed.

Transfection. 2×10^7 mutant cells (M43/8) were washed with PBS twice and resuspended in 1.6 ml of ice-cold PBS. 80 μg/400 μl of undigested plasmid which contained the Dol-P-Man synthase gene was added to the cells. The plasmid was stably transfected into the cells by electroporation (175 V, 1000 μF), using a Cell-Zap IIb electroporator (Owl Scientific, Cambridge, MA) and a Cell-Porator cuvette holder (Gibco Bethesda Research Laboratories, Gaithersburg, MD). After 48 hours, the cells were resuspended in RPMI 1640 medium containing 1.5 mg/ml G418 (G418 sulfate, Geneticin; GIBCO BRL) and aliquoted into microtiter plates. Clones that survived the antibiotic selection were then switched over to regular culture media without G418 and characterized.

Immunofluoresence staining. Indirect immunofluorescent staining was performed as described (35) using an mAb-containing tissue culture supernatant followed by fluorescein isothiocyanate goat anti-mouse IgG (FITC-GAMG) (Caltag Laboratories, San Francisco, CA), under nonlimiting conditions. Fluorescence was quantitated on a FACScan fluorescence-activated cytometer (Becton Dickinson Co., Mountain View, CA). As a negative control, cells were incubated with fresh tissue culture media containing 10% FCS, instead of the specific first antibody, before incubation with FITC-GAMG. For PI-PLC treatment, cells (5×10^6) were incubated for 60 min at 37°C in RPMI 1640 with BSA (2 mg/ml) and 0.1% NaN_3. PI-PLC (0.4 U/ml) was used for the treatment group. Staining and analysis were performed as described above. For tunicamycin treatment, cells were incubated in culture media supplemented with 0.5 mg/ml tunicamycin for 18 h, washed, and stained as described. For the calculation of the percent reduction of surface expression, the following formula was used: [(fluoresence of M43/8 after specific antibody staining) − (fluoresence of M43/8 after nonspecific antibody staining)]/[(fluoresence of YH16.33 after specific antibody staining) − (fluoresence of YH16.33 after nonspecific antibody staining)].

Dol-P-Man synthase assay. Cells were lysed in 0.1 mM N-tosyl-L-lysine chloromethyl ketone (TLCK), 1 mg/ml leupeptin in water, diluted to 10^9 cells/ml with an equal amount of 0.1 M Hepes (pH 7.4) with 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM TLCK, 1 μg/ml leupeptin,

and 20% glycerol, and frozen at −70°C. Defrosted lysates were washed twice with 10 ml of 50 mM Hepes (pH 7.4), 25 mM KCl, 5 mM $MnCl_2$, 0.1 mM TLCK, and 1 mg/ml leupeptin (spun at 6,000 g for 10 min). The pellet was resuspended in the above buffer. The samples (containing membranes from 6×10^6 cells) were assayed in a final volume of 200 μl of 50 mM Hepes (pH 7.4) with 25 mM KCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 0.1 mM TLCK, 1 mg/ml leupeptin, 0.1 mM DTT, 0.0025% NP-40, 100 μg/ml phosphatidylethanolamine, 2.6 μg/ml dolichyl phosphate, and 200,000 cpm of [^{3}H]GDP-mannose. After incubation at 37°C for the appropriate length of time, the reaction was stopped by the addition of 1 ml of 2:1 $CHCl_3/CH_3OH$. After centrifuging, the organic phase was retained and washed three times with $CHCl_3/CH_3OH/H_2O$ (3:48:47), and counted. The samples were also separated by TLC in $CHCl_3/CH_3OH/H_2O$, 60:35:8. Scanning and autoradiography revealed that ~95% of the counts was consistently contained in Dol-P-Man. The area of the TLC corresponding to Dol-P-Man, determined by overlaying autoradiographs, was scraped off the support and the incorporated radioactivity was determined by liquid scintillation counting. The protein content of the cell lysates was assayed with Coomassie Plus Protein Assay (Pierce Chemical Co., Rockford, IL).
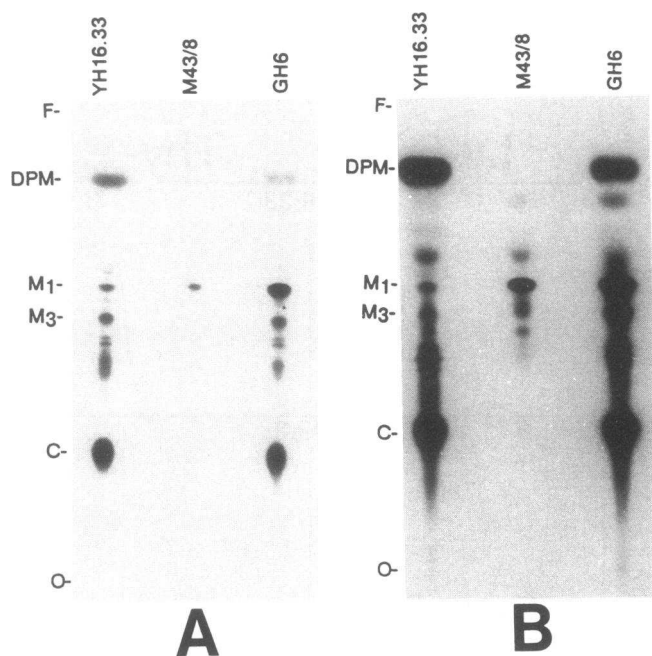
Results

A T cell hybridoma, specific for chicken ovalbumin, YH16.33, was mutagenized and mutant clones were selected for resistance to killing by anti-Ly-6A antibody and complement (35). Clones were examined by FACS analysis for the surface expression of Ly-6A and Thy-1. One clone, M43/8, expressed two GPI-anchored proteins at levels significantly below those of wild-type cells (Fig. 1, *A–D*). Levels of Ly-6A were, on the
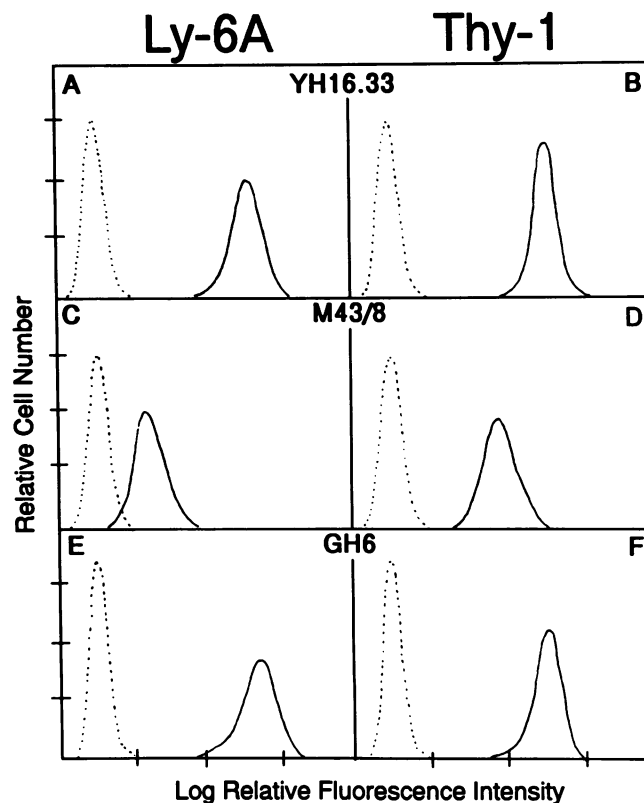
Figure 1. FACS staining of wild type, YH16.33, (*A* and *B*), mutant, M43/8, (*C* and *D*), and transfectant, GH6, (*E* and *F*) cells for Ly-6A (*A, C,* and *E*) and Thy-1 (*B, D,* and *F*). Indicated are the negative control (··········), and specific first-step antibody followed by FITC-GAMG (—).
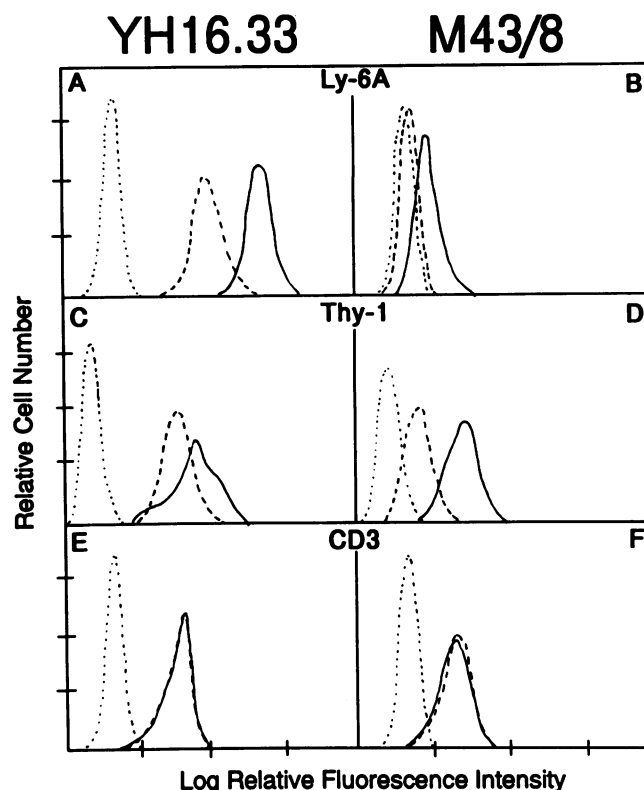
average, ~ 2.4% of normal, while levels of Thy-1 were, on the average, ~ 17% of normal. Similar staining patterns were also observed using different monoclonal antibodies against Ly-6A or Thy-1 (data not shown). The lower level of expression of two GPI-anchored proteins could have been due to a decrease in the production or in the transfer of the preformed GPI core to proteins, or due to a nonspecific defect in protein synthesis. These possibilities could be distinguished by metabolic labeling of the preformed GPI precursors. Thus, the same number of wild-type and mutant cells were labeled with [³H]mannose in the presence of tunicamycin. Labeled glycolipids were extracted, chromatographed, and autoradiographed as described previously (1, 2). Fig. 2 A shows a representative TLC autoradiograph of [³H]mannose-labeled GPI intermediates in the wild type and mutant cells. As shown, the wild type cells form all of the GPI precursors previously defined (2). The mutant cell, however, did not synthesize detectable amounts of mannose-containing GPI precursors (other than M_1) or the complete GPI core (C). In another experiment with longer exposure one could detect a faint spot in the core area (Fig. 2 B). To quantitate the difference, the TLC plates were scanned with a linear radioisotope scanner and the area corresponding to the core in both the wild type and the mutant cells determined. The mutant contained < 1% radioactivity in the core area as

compared to the wild type. This result is reproducible in five separate experiments. Thus, the reduced surface expression of GPI-anchored proteins in the mutant was due to deficient anchor biosynthesis. Since the GPI core was formed at such a low level in the mutant, it was important to determine whether the surface-expressed Thy-1 and Ly-6A were indeed anchored by the GPI tail, and not by alternative means. Fig. 3 shows that both Ly-6A and Thy-1 were sensitive to PI-PLC treatment whereas the non-GPI-anchored CD3ε was not. These results thus established the direct correlation between low GPI core formation and reduced surface expression of GPI-anchored proteins.

Fig. 2 A also reveals the cause of the deficient GPI anchor formation. As shown, Dol-P-Man, the mannose donor for the GPI anchor, was not detectable in the mutant. This was independently confirmed by assaying for Dol-P-Man synthase activity. In Fig. 4 it is shown that the Dol-P-Man synthase activity was at a baseline level, similar to another mutant (M38/20) which had been shown to produce no Dol-P-Man at all. Our inability to detect a low level of Dol-P-Man synthase activity in M43/8 does not mean that M43/8 is completely deficient in this enzyme since (a) a trace amount of GPI core was still synthesized (Fig. 2 B), and (b) the Dol-P-Man assay assesses the steady state level of Dol-P-Man, since enzymes that use Dol-P-Man are present and presumably active. This point was clearly demonstrated by incubating the mutant with tunicamycin, which blocked the transfer of mannose residues from Dol-P-Man to N-linked oligosaccharide precursors and shunted Dol-

Figure 2. Mannose-containing glycolipids in YH16.33, M43/8, and GH6 cells. Plates from two separate experiments were sprayed with fluor and autoradiographed for 3 d (A) or 1 mo (B). It should be noted that 5×10^5 cell equivalents of glycolipid extracts were loaded to each lane. There are at least two glycolipids migrating between M_3 and C. Recent results show that these glycolipids can be labeled with [³H]ethanolamine. We are in the process of further characterizing these glycolipids, which may contain a variable number of ethnolamine phosphate residues and/or other modifications. The pertinent finding for this report is that they are all markedly reduced in the mutant and restored in the transfectant. DPM, dolichyl mannosyl phosphate; C, GPI precursor that contains ethanolamine phosphate; F, front; $M_{1,3}$, GPI precursors containing one or three mannose residues; O, origin. These precursors have been described in detail previously (1, 2).

Figure 3. PI-PLC sensitivity of membrane proteins. YH16.33 (A, C, and E) and M43/8 (B, D, and F) cells were treated with PI-PLC and stained with antibodies to Ly-6A (A and B), Thy-1 (C and D), or CD3 (E and F). Indicated are the negative control (· · · · · · · · ·), mock digestion (—), and PI-PLC digestion (-------).
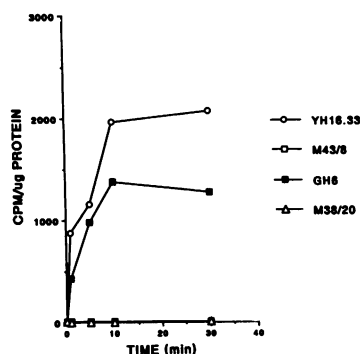
Figure 4. Production of Dol-P-Man in wild-type (*YH16.33*), mutants (*M43/8, M38/20*), and transfectant (*GH6*) cells. Dol-P-Man synthase activity of cell lysates were assayed and organic-soluble compounds were separated by TLC. The areas of the TLC corresponding to Dol-P-Man were scraped off the support and incorporated radioactivity was determined. Incorporated counts were corrected for the protein content of the assayed lysate. Since enzymes that use Dol-P-Man were present and active, this assay represents a steady-state concentration of Dol-P-Man.

P-Man to the GPI pathway (36) (Fig. 5). As shown, in M43/8 cells cultured with tunicamycin for 18 h, the surface expression of Ly-6A had returned to almost wild-type level. However, the surface expression of Thy-1 remained unchanged. This was most likely due to the deleterious effect of tunicamycin on *N*-linked glycosylation, because Thy-1 contained three *N*-linked glycosylation sites whereas Ly-6A had none (37–39). This conclusion was supported by the observation that in wild type cells incubated with tunicamycin for 18 h, the surface
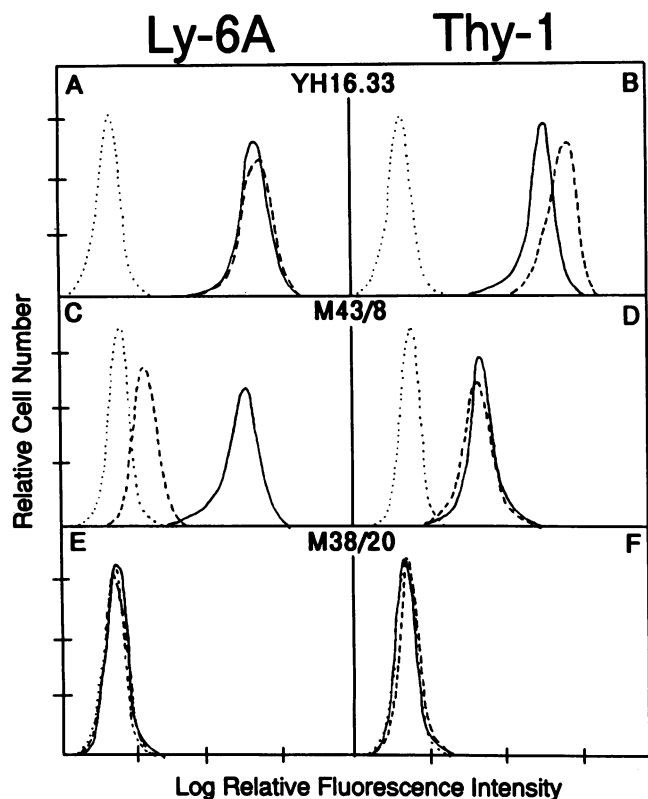

Figure 5. The effect of tunicamycin on Ly-6A or Thy-1 expression in the wild type, YH16.33, (*A* and *B*) or mutants, M43/8 (*C* and *D*) or M38/20 (*E* and *F*). Indicated are the negative control (· · · · ·), sample treated with tunicamycin (———), and without tunicamycin (-------).

expression of Thy-1, but not Ly-6A, was decreased (Fig. 5, *A* and *B*). Restoration of Ly-6A expression in M43/8 demonstrated the presence of endogenous Dol-P-Man because M38/20, which is completely deficient in Dol-P-Man production, could not be corrected by tunicamycin treatment (Fig. 5, *E* and *F*).

Since, tunicamycin did not restore both Thy-1 and Ly-6A expression in the mutant to the wild type level, due to the inherent toxicity of tunicamycin on *N*-glycosylation, which probably interfered with normal folding and/or transport of Thy-1, we attempted to correct the defect by alternative means. We have shown previously that the yeast Dol-P-Man synthase gene can restore GPI anchor synthesis in mutants which cannot synthesize any Dol-P-Man (1). This approach was used to correct the defect in M43/8. Thus, M43/8 was transfected with an expression vector that contained the Dol-P-Man synthase and G418 resistance gene, as previously described (1). 38 subclones survived the G418 selection. 37 of them had surface expression of Thy-1 and Ly-6A corrected to the wild-type levels. It should be noted that GH6 was maintained in culture media in the absence of G418 for several months. Thus, the changes described below cannot be due to metabolic correction with G418. Dol-P-Man mutants transfected with the plasmid containing the Dol-P-Man synthase gene in a reverse orientation did not correct the deficient phenotype (Thomas, L., and E. Yeh, unpublished data). A representative clone, GH6, is shown in Fig. 1. As shown, GH6 expressed Thy-1 and Ly-6A at levels slightly higher than that of the wild type, indicating that the ability to produce GPI anchors had been restored. TLC analysis of GPI biosynthetic intermediates showed that the complete GPI core, as well as Dol-P-Man was produced in GH6 (Fig. 2). The ability to produce Dol-P-Man was also assayed (Fig. 4). In this assay, the maximal level of Dol-P-Man in GH6 was ~ 60% of the wild type's.

Discussion

In this paper we have described a T cell hybridoma mutant which can only synthesize below-normal levels of Dol-P-Man. As a consequence, the mutant synthesized a greatly reduced amount of the complete GPI core, which led to a reduced expression of GPI-anchored proteins on the cell surface. Interestingly, Ly-6A expression was more significantly affected than Thy-1. We demonstrated that the differential expression of GPI-anchored proteins was due to limited availability of preformed GPI core because this defect could be rescued metabolically or genetically.

The rules that govern the attachment of the GPI anchor to proteins are not entirely known. The enzyme that cleaves the carboxy terminus presumably has to recognize a cleavage signal in proteins destined to be GPI anchored, and transfer a preformed GPI core to the attachment site. If the GPI core is produced in excess, the surface expression of GPI-anchored proteins would probably be dependent on the amounts of the proteins synthesized. However, when the core is limiting, there may be a competition for the available core. The factors that determine which proteins get preferential transfer are probably contained within the GPI cleavage and attachment sequence. In M43/8 the differential expression of Ly-6A versus Thy-1 is most likely due to differences in these factors. It should be noted that the basic biosynthetic defect in M43/8 is deficient

Dol-P-Man synthesis, which, predictably, affects *N*-linked glycosylation (Thomas, L., and R. DeGasperi, data not shown). Since Thy-1 is *N*-glycosylated, but Ly-6A is not (37–39), one would expect Thy-1 expression in M43/8 to be more adversely affected than Ly-6A. The observation to the contrary, i.e., that the surface expression of Ly-6A was more diminished than that of Thy-1, suggested that deficient GPI core formation is the primary cause of the differential expression. In the future, when more GPI-anchored protein sequences have been sequenced, an optimal sequence may be deduced. This would be beneficial in the construction of GPI-anchored recombinant proteins, particularly when over-expression is desired.

Significant insight into the biosynthesis of the GPI anchor has been gained by studying a panel of well-defined mutants (1, 2). This is the first time that a partial defect of Dol-P-Man production has been defined and genetically corrected. The methods that we have described in this and previous reports should provide the necessary tools to define the defects in GPI anchor formation in PNH patients (1, 2). Two main lessons are learned from our analysis. The first is that it is often difficult to state unequivocally that a defect in GPI anchor biosynthesis is absolute. In our initial analysis of M43/8, we thought that the complete GPI anchor precursor was not formed at all. With repeated experiments, we then established that the core precursor is formed at ~ 1% of the wild-type level. Thus, when metabolic labeling is used in the analysis of PNH defects, care must be exercised to ascertain whether or not an apparently complete defect could be due to the sensitivity of the detection system. The second lesson is that if the GPI core is limiting, then differential expression of GPI-anchored proteins might result. This may well explain the diminished expression of CD16 in PNH granulocytes that are CD55 and CD59 negative, and diminished expression of LFA-3 and acetylcholinesterase in PNH erythrocytes which are DAF-negative (29, 30).

Acknowledgments

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