Tolerance of glycosylphosphatidylinositol (GPI)-specific phospholipase D overexpression by Chinese hamster ovary cell mutants with aberrant GPI biosynthesis

Xiaohan DU*[1], Jiewei CAI*, Jian-zhong ZHOU*, Victoria L. STEVENS† and Martin G. LOW*[2]

*Department of Physiology and Cellular Biophysics, College of Physicians and Surgeons, Columbia University, New York, NY 10032, U.S.A., and †Division of Cancer Biology, Department of Radiation Oncology, Emory University School of Medicine, Atlanta, GA 30335, U.S.A.

Mammalian glycosylphosphatidylinositol (GPI)-specific phospholipase D (GPI-PLD) is capable of releasing GPI-anchored proteins by cleavage of the GPI moiety. A previous study indicated that overexpression of GPI-PLD in mouse RAW 264.7 monocytes/macrophages could be cytotoxic, since survivors of stable transfections had enzymic activity no higher than untransfected cells [Du and Low (2001) Infect. Immun. **69**, 3214–3223]. We investigated this phenomenon by transfecting bovine GPI-PLD cDNA stably into Chinese hamster ovary (CHO) cells using a bi-cistronic expression system. The surviving transfectants showed an unchanged cellular level of GPI-PLD, supporting the cytotoxicity hypothesis. However, when using a CHO mutant defective in the second step of GPI biosynthesis as host, the expression level of GPI-PLD in stable transfectants was increased by 2.5-fold compared with untransfected or empty-vector-transfected cells. To identify the mechanism, we studied another CHO cell mutant (G9PLAP.D5), which seems to be defective at a later stage in GPI biosynthesis. In sharp contrast with wild-type cells, GPI-PLD activity in G9PLAP.D5 transfected with bovine GPI-PLD cDNA was 100-fold higher than untransfected or empty-vector-transfected cells. This was accompanied by a significant release of alkaline phosphatase into the medium and a decrease in membrane-associated alkaline phosphatase. Taken together, our results indicate that overexpression of GPI-PLD is lethal to wild-type cells, possibly by catalysing the overproduction of GPI-derived toxic substances. We propose that cells with abnormal GPI biosynthesis/processing can escape the toxic effect of these substances.

Key words: alkaline phosphatase, CHO cells, cytotoxicity.

INTRODUCTION

Numerous eukaryotic cell-surface proteins, with a wide variety of functions, are anchored to the cell membrane via a C-terminal linkage to glycosylphosphatidylinositol (GPI) [1,2]. Soluble forms of GPI-anchored proteins have also been found in biological fluids, and serum levels of some of these proteins are increased under certain pathological conditions [3–6]. Although GPI-specific phospholipase D (GPI-PLD) isolated from serum is not capable of removing GPI-anchored proteins from cell surfaces, cell-associated GPI-PLD is thought to be responsible for releasing GPI-anchored proteins at an intracellular location ([7–10]; for a review, see [11]). Thus the cellular expression level of GPI-PLD could determine the ultimate destination of newly synthesized GPI-anchored proteins: retention on the cell surface or release into the extracellular fluid.

GPI-PLD is abundant in serum, and the gene expression of GPI-PLD varies dramatically among different tissues [9,12]. Clinical studies have shown that serum GPI-PLD levels in patients with liver diseases, renal diseases and systemic inflammatory response syndrome were significantly different from those in healthy individuals [13–15], suggesting an active regulation of GPI-PLD expression under these pathological conditions. In addition, compared with normal cells, the expression level of GPI-PLD in carcinoma cells was markedly altered [16]. Expression of GPI-PLD was also shown to be regulated by serum stimulation in fibroblasts, by lipopolysaccharide and oxidative stress in macrophages, and by tumour necrosis factor-α ('TNF-α') in human alveolar carcinoma cells [17,18].

Our previous attempts to transfect GPI-PLD cDNA stably in murine RAW 264.7 macrophages suggested that the level of cellular GPI-PLD expression is tightly regulated, and overexpression of GPI-PLD is cytotoxic to cells [17]. In the present study, we show that Chinese hamster ovary (CHO) cells cannot tolerate constitutive overexpression of GPI-PLD either. However, when using CHO cell mutants defective at two distinct steps in the GPI-biosynthesis pathway, we found that they could tolerate GPI-PLD overexpression (2.5-fold and 100-fold for each step respectively). We propose that GPI-derived toxic products are responsible for the inability of CHO cells to withstand overexpression of GPI-PLD.

MATERIALS AND METHODS

Cell lines

CHO cell lines expressing human placental alkaline phosphatase (PLAP), G9PLAP, G9PLAP.85 [19] and G9PLAP.D5 (see the following paragraph) were grown as monolayer cultures in Ham's F-12 medium supplemented with 10% (v/v) fetal bovine serum,

Abbreviations used: GPI, glycosylphosphatidylinositol; GPI-PLD, GPI-specific phospholipase D; PLAP, placental alkaline phosphatase; CHO, Chinese hamster ovary; PI-PLC, phosphatidylinositol-specific phospholipase C; PRIM, proximity imaging; GFP, green fluorescent protein; GFP-GPI, GPI-anchored form of GFP; P-Etn, phosphoethanolamine.

[1] Present address: Maxygen, Inc. 515 Galveston Drive, Redwood City, CA 94063, U.S.A.

[2] To whom correspondence should be addressed (e-mail mgL2@columbia.edu).

300 μg/ml G418, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified incubator at 37 °C in the presence of 5 % CO_2.

The preparation of *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (PI-PLC) was as described previously [20,21]. Parental G9PLAP cells were treated with bacterial PI-PLC, and the PI-PLC-sensitive population was enriched by flow cytometry using a rabbit polyclonal anti-(human PLAP) monoclonal antibody and a magnetic cell separation column (Miltenyi Biotec, Auburn, CA, U.S.A.). The cells were then mutagenized with ethyl methanesulphonate [19]. Surviving cells were allowed to recover for four days, treated with bacterial PI-PLC, and then sorted again by flow cytometry. This time the subpopulation of mutant cells that retained relatively high levels of PLAP after PI-PLC treatment were collected and seeded at limiting dilution in 96-well plates. Colonies that developed from individual cells were screened for the resistance of their GPI-anchored PLAP to PI-PLC digestion using two criteria: (1) percentage of PLAP released by PI-PLC (described below); (2) PI-PLC-induced changes in electrophoretic mobility of butanol-extracted PLAP detected by non-denaturing PAGE in the presence of Triton X-100 [22]. G9PLAP.D5, one of several PI-PLC resistant CHO cell mutants, was used for the experiments reported in the present study. G9PLAP and G9PLAP.D5 express similar amounts of alkaline phosphatase on their cell surface as determined by immunoblotting and enzyme assay (see the Results section).

Alkaline phosphatase assays

To determine the percentage of PLAP released from cells by PI-PLC, cells were seeded on a 96-well plate at a density of 2×10^4 cells/well and grown for 20 h. For each well, cells were washed once with 100 μl of serum-free Ham's F-12 medium, and then covered with 100 μl of the same medium at 37 °C for 1 h. Medium was then removed, and cells in each well were replaced with 40 μl of serum-free Ham's F-12 medium containing PI-PLC (0.05 unit/ml) and incubated at 37 °C for 2 h. The 96-well plate was centrifuged at 1200 ***g*** for 3 min at room temperature, and 20 μl of the supernatant were transferred to a new well containing 20 μl of serum-free Ham's F-12 medium. The rest of the culture supernatant was discarded. Cells were washed with 100 μl of serum-free Ham's F-12 medium and covered with 40 μl of the same medium. Alkaline phosphatase activity in wells containing cells or supernatant was determined by measuring the hydrolysis of *p*-nitrophenyl phosphate, as described previously [17].

To determine the relative level of soluble PLAP secreted into serum medium by CHO cells, cells were seeded at 25 % and covered with serum medium to reach confluence. Cell extracts of each culture were prepared, and the protein concentration was determined as described below. Medium was centrifuged at 16000 ***g*** for 10 min at room temperature. Supernatant corresponding to 500 ng of total cellular proteins was heated at 60 °C for 30 min to inactivate contaminating serum-derived alkaline phosphatase, and the relative activity of PLAP was analysed using a chemiluminescence detection kit from Clontech, Inc. (Palo Alto, CA, U.S.A.).

Proximity imaging (PRIM)

The methodology of using PRIM to quantitatively image homo-oligomerization or clustering processes of green fluorescent protein (GFP)-tagged protein *in vivo* was described by De Angelis et al. [23]. Plasmid pCI-GFP-GPI containing the GPI-anchored form of GFP (GFP-GPI) was generously given by Dino A. De Angelis (Memorial Sloan-Kettering Cancer Center, New York, NY, U.S.A.). CHO cells were seeded at a density of 3.2×10^5 cells/well in a 12-well plate for 20 h, before transient transfection with pCI-GFP-GPI using LIPOFECTAMINE™ PLUS reagent (Life Technologies, Rockville, MD, U.S.A.). Following transfection (5 h), cells were replaced with fresh serum medium, then, 20 h after transfection, cells were dislodged and reseeded in a 60 mm glass bottom microwell (MatTek Corporation, Ashland, MA, U.S.A.) with 2 ml of fresh serum medium. Cells were incubated for a further 24 h at 37 °C before PRIM using a Zeiss Axiovert S100 microscope. Images were acquired, and relative intensities of green fluorescence emitted upon excitation at 410 nm and 470 nm were recorded for the membrane region of each cell using ESee Graphical Interface (Inovision, Raleigh, NC, U.S.A.). For each cell line, the $R_{410/470}$ is depicted as means $\pm$ S.D. for a total of 18 cells randomly chosen from five independent images.

Stable transfection of GPI-PLD

The cDNA of bovine GPI-PLD [8] was PCR-amplified with the primers 5′-dCTTCCCGGGATGTCTGCTTTCAGATTC-3′ and 5′-dCCCCGAATTCTTTAATCTTGGCCGAGC-3′, digested with *Sma*I and *Eco*RI, and then cloned into pIRESpuro2 (Clontech, Inc.) via *Eco*RV and *Eco*RI sites. The resultant plasmid, pPuro/PLD-B, was amplified, purified with EndoFree Maxi Kit (Qiagen, Valencia, CA, U.S.A.), and transfected into CHO cells using LIPOFECTAMINE™ PLUS reagent (Life Technologies). After transfection (5 h), cells were replaced with fresh medium, then, 20 h after transfection, cells were harvested and reseeded at a density of 15 % with selective medium containing 10 μg/ml puromycin (Sigma, St. Louis, MO, U.S.A.). Fresh selective medium was added to cells every 3–4 days until puromycin-resistant colonies appeared. Two independent stable transfections were performed for each cell line, and colonies were then pooled.

Cellular extract and GPI-PLD assay

Preparation of detergent cellular extract, determination of protein concentration, analysis of GPI-PLD-specific activity, and the definition of GPI-PLD activity units were as described previously [17].

To determine the amount of secreted GPI-PLD activity, cells were seeded at 50 % on a 12-well plate and grown for 24 h to reach confluence. Cells were then washed twice with 1 ml of Ham's F-12 serum-free medium, and incubated in 0.8 ml of Ham's F-12 serum-free medium for a further 4 days. The culture supernatant was centrifuged at 16000 ***g*** for 10 min at room temperature, and 10 μl of the supernatant was used for determination of GPI-PLD activity.

RESULTS

G9PLAP.85, but not G9PLAP, can tolerate GPI-PLD overexpression

G9PLAP is a CHO-K1 cell line transfected stably with PLAP cDNA [19]. G9PLAP.85 is a CHO-K1 mutant cell line derived from G9PLAP, defective in the *PIG-L* gene, which is responsible for the second step of GPI biosynthesis [19,24]. Stable transfection of GPI-PLD cDNA into G9PLAP showed no significant increase in GPI-PLD activity level (Figure 1). However, when using G9PLAP.85 as a host, stable tranfection of GPI-PLD cDNA resulted in a 2.5-fold increase in cellular GPI-PLD activity compared with untransfected or empty-vector-transfected cells (Figure 1). The survival rate following transfection with GPI-PLD cDNA was at least 10-fold lower compared with empty

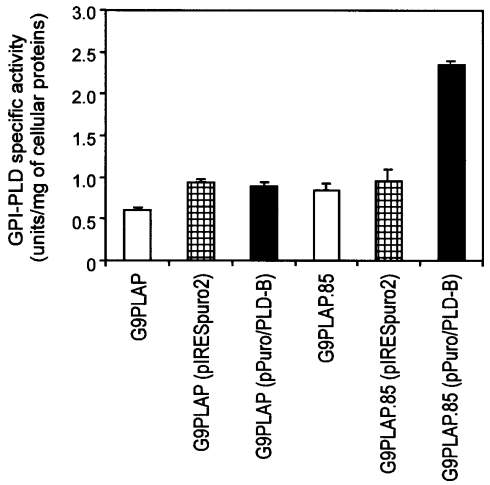

Figure 1 G9PLAP.85, a GPI biosynthesis mutant, can tolerate low levels of GPI-PLD overexpression

G9PLAP and G9PLAP.85 cells were transfected stably with the empty vector (pIRESpuro2) or the GPI-PLD cDNA construct (pPuro/PLD-B). Two independent transfections were performed for each construct and for each cell line, and surviving colonies were pooled as described in the Materials and methods section. The resulting stably transfected lines were designated by their parental cell name, followed by parentheses containing the name of the plasmid construct used in transfection. For determining their cellular GPI-PLD activity, cells were seeded in 60 mm plates. Protein extracts of confluent cells were prepared and analysed for their cellular GPI-PLD specific activities separately. Results are depicted as means ± S.E.M. for three independent experiments.

vector controls for both the G9-PLAP and G9PLAP.85 cell lines (results not shown).

Characterization of G9PLAP.D5, a PI-PLC-resistant CHO cell mutant

To study the mechanism of this difference, we used another CHO cell mutant, G9PLAP.D5, which was selected on the basis of its resistance to PI-PLC. Although the amount of PLAP expressed on G9PLAP.D5 is similar to that on G9PLAP (Figure 2A), the percentage of PLAP that could be released from G9PLAP.D5 by PI-PLC was markedly decreased to 10%, compared with over 70% for G9PLAP (Figure 2B). Furthermore, the acquisition of PI-PLC resistance by G9PLAP.D5 did not have a major effect on the molecular mass or level of PLAP expression, as determined by immunoblotting (results not shown). We propose that PI-PLC resistance of G9PLAP.D5 is due to the absence of the enzyme that catalyses the 'final' step of GPI biosynthesis: inositol deacylation of the GPI anchor. However, the enzyme responsible for inositol deacylation has not been characterized in mammalian cells, and the precise biochemical defect in G9PLAP.D5 remains uncertain.

To determine whether the acquisition of PI-PLC resistance affected the cell-surface distribution of GPI-anchored proteins, PRIM was employed with GFP-GPI as the probe. The results suggested that GFP-GPI on the G9PLAP.D5 cell membrane became more clustered than that on the membrane of G9PLAP cells. The $R_{410/470}$ value of GFP-GPI on G9PLAP cells was determined as 1.58 ± 0.14, similar to the value observed by De Angelis et al. [23] for GFP-GPI on HeLa cells (1.68 ± 0.09). The $R_{410/470}$ value of GFP-GPI on G9PLAP.D5 cells, however, was significantly lower (1.12 ± 0.10), which was close to that observed with the permanent clustering induced by adding a bivalent antibody against GFP on the HeLa cell surface (1.00 ± 0.15; [23]). The clustering of GPI-anchored proteins observed on G9PLAP.D5 could be responsible for resistance of PLAP to release by PI-PLC. However, this seems unlikely because, in a previous study, PI-PLC resistance of PLAP was also observed when butanol-extracted PLAP solubilized in detergent was analysed by non-denaturing PAGE [22].

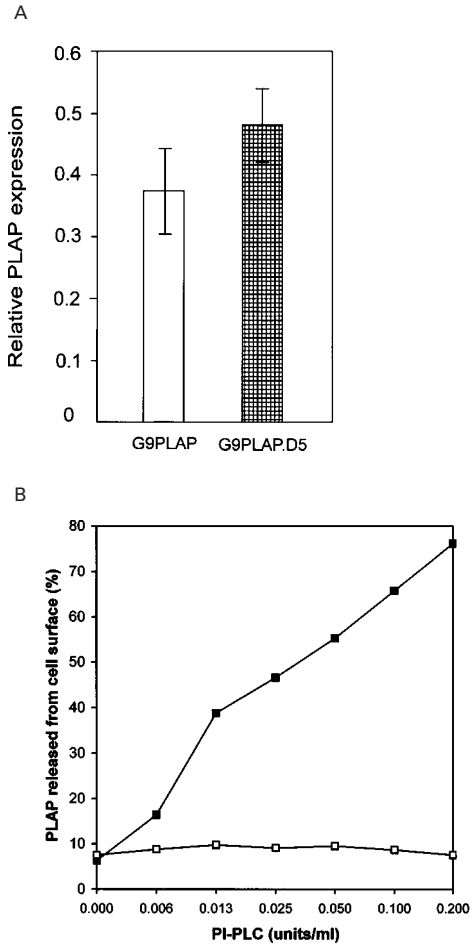

Figure 2 G9PLAP.D5, a phospholipase C-resistant mutant, expresses normal amounts of PLAP

(**A**) The relative amounts of GPI-anchored PLAP expressed on G9PLAP.D5 and G9PLAP cells were similar. (**B**) GPI-anchored PLAP expressed on G9PLAP.D5 cells (open squares) is more resistant to increasing concentrations of bacterial PI-PLC than that expressed on G9PLAP cells (solid squares). The experimental procedure to determine relative PLAP activity (given in arbitrary units) is in the Materials and methods section. Results are shown as means ± S.E.M. for 21 independent experiments in (**A**), and for three independent experiments for each of the conditions in (**B**).

GPI-PLD overexpression in G9PLAP.D5

When G9PLAP.D5 was transfected stably with GPI-PLD cDNA, the cellular enzyme activity of GPI-PLD was increased by 85–100-fold (Figure 3A). As a control, G9PLAP cells transfected stably with GPI-PLD cDNA showed no significant change in cellular GPI-PLD activity, compared with untransfected or empty-vector-transfected G9PLAP (Figure 3A). In contrast with G9PLAP.85 (see above), the survival rate of G9PLAP.D5 cells following transfection with GPI-PLD cDNA was not significantly lower than for the empty vector control. The overexpression of GPI-PLD in G9PLAP.D5 cells resulted in a significant increase in the amount of PLAP released into the medium

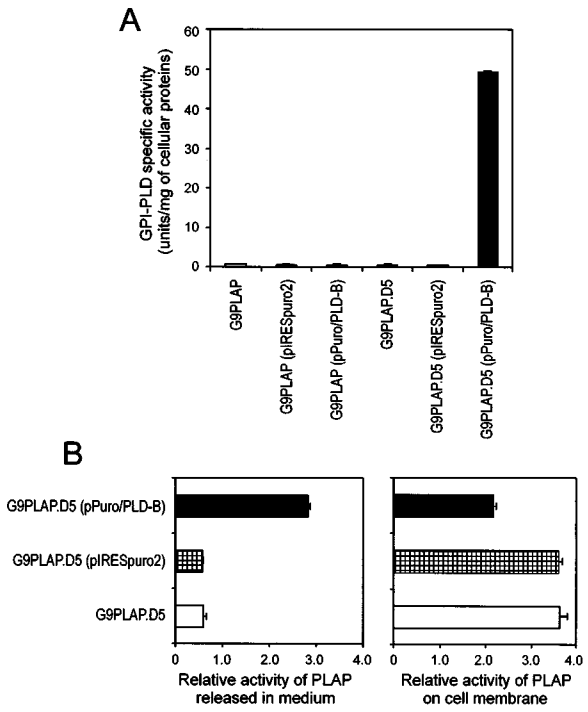

Figure 3 G9PLAP.D5, a phospholipase C-resistant mutant, can tolerate high levels of GPI-PLD overexpression

(**A**) Overexpression of GPI-PLD in G9PLAP.D5. G9PLAP and G9PLAP.D5 cells were transfected stably with empty vector, pIRESpuro2, or GPI-PLD cDNA construct, pPuro/PLD-B. Two independent transfections were performed for each construct and for each cell line. The surviving colonies were pooled as described in the Materials and methods section (refer to the legend for Figure 1 for the designation of stably transfected cell lines). For determination of their cellular GPI-PLD activity, cells were seeded in 60 mm plates. Protein extracts of confluent cells were prepared and analysed for their cellular GPI-PLD specific activities separately. Results are depicted as means ± S.E.M. for three independent experiments. (**B**) Overexpression of GPI-PLD in G9PLAP.D5 cells resulted in increased levels of soluble PLAP and decreased levels of membrane PLAP. The two sets of data are aligned for comparison purposes. Left panel: increased soluble form of PLAP produced by GPI-PLD overexpression. Cells were seeded at 25% confluence, and covered with serum medium until cells became confluent. Relative levels of soluble PLAP in the medium were detected as described in the Materials and methods section. Results are depicted as means ± S.E.M. for three independent experiments. Right panel: decreased cell-surface expression of PLAP by GPI-PLD overexpression. Cells were seeded in 96-well plates, and relative PLAP activity on cells was determined as described in the Materials and methods section. Results are depicted as means ± S.E.M. for six independent experiments.

(Figure 3B; left panel). As a result, the overexpression of GPI-PLD in G9PLAP.D5 cells reduced the surface expression level of GPI-anchored PLAP by approx. 40% (Figure 3B; right panel).

Since the expression level of GPI-PLD in G9PLAP.D5 was so high, the feasibility of using these cells to produce large amounts of mammalian GPI-PLD was evaluated. The secreted GPI-PLD activity in G9PLAP.D5 cells transfected stably with GPI-PLD cDNA [G9PLAP.D5 (pPuro/PLD-B)] was over 100-fold higher than untransfected or empty-vector-transfected G9PLAP.D5 cells. After 4 days, the GPI-PLD level in serum-free culture supernatant reached 2.3–2.8 mg/l.

DISCUSSION

Although GPI-PLD cDNA can be transiently transfected into mammalian cells [8,25,26], our previous attempts to transfect GPI-PLD cDNA stably into the murine monocyte/macrophage cell line, RAW 264.7, did not result in GPI-PLD expression at levels higher than untransfected or empty-vector-transfected cells [17]. In the present work, we also observed that after stable transfection with GPI-PLD cDNA, G9PLAP (a wild-type CHO cell previously transfected with PLAP) did not express a significantly higher level of GPI-PLD activity. This result cannot be attributed to a failure in the transfection protocol for two reasons. First, there is no evidence that G9PLAP is resistant to transfection [19]. Secondly, the cells acquired puromycin resistance. It is also relevant to note that, in the pIRESpuro2 expression system used in these studies, the GPI-PLD and puromycin-resistance genes are controlled by the same promoter. As a consequence, essentially all of the puromycin-resistant colonies would initially have been capable of expressing GPI-PLD. We therefore propose that persistent overexpression of GPI-PLD is cytotoxic, and eventually lethal. Only transfectants with lower levels of GPI-PLD can survive the selection process.

It is not clear why overexpression of GPI-PLD in CHO cells should be cytotoxic. Although its function is unknown, GPI-PLD seems to be responsible for release of GPI-anchored proteins under a variety of physiological or pathological conditions [11]. Overexpression of GPI-PLD could certainly increase the release of GPI-anchored proteins, and thus interfere with cell functions performed by these proteins. In addition, overexpressed GPI-PLD could attack free GPI molecules or their precursors, and decrease the rate of GPI-anchor biosynthesis. However, neither of these possibilities seems a probable cause for cytotoxicity, since numerous mammalian cell lines defective in GPI biosynthesis are known to grow well in culture, in spite of their inability to synthesize GPIs or express GPI-anchored proteins [1,19,24]. Furthermore, in the acquired human blood disease, paroxysmal nocturnal haemoglobinuria, there is a clonal expansion of GPI-deficient cells, indicating that GPIs are not essential for survival *in vivo* either [27]. We believe a more likely explanation for our results is that, rather than depleting the cells of GPI molecules, overexpression of GPI-PLD leads to the accumulation of excessive amounts of novel, 'toxic' glycosylated inositols, which the cell cannot tolerate.

This hypothesis is supported by our stable transfection experiments carried out in CHO cell mutants with aberrant GPI biosynthesis. In contrast with the CHO wild-type G9PLAP, both of the CHO cell mutants, G9PLAP.85 and G9PLAP.D5, showed a marked increase in GPI-PLD activity after being transfected stably with GPI-PLD cDNA. The fact that overexpression of GPI-PLD is tolerated by cells with abnormal GPI biosynthesis suggests that the effects of the hypothetical toxic product are 'neutralized' by abnormal GPI biosynthesis, and are thus likely to be GPI-related. Two classes of compound are expected to exist in wild-type cells, but not in the mutants: (i) GlcNα1-6PtdIns in G9PLAP.85 cells, and (ii) relatively 'mature' GPI molecular species that are PI-PLC-sensitive, i.e. [phosphoethanolamine (P-Etn)]$_x$(Man)$_y$GlcNα1-6PtdIns in G9PLAP.D5 cells (where the 'x' and 'y' subscripts denote the variable stoichiometry of the glycan chain). GPI-PLD could convert these compounds into GlcNα1-6Ins and (P-Etn)$_x$(Man)$_y$GlcNα1-6Ins respectively. Therefore we propose that at least one of them is involved in the cytotoxic effect induced by GPI-PLD overexpression. Alternatively, the phosphatidic acid, also produced by GPI-PLD-mediated breakdown of these lipids, could contribute to the toxicity by virtue of its role in intracellular signalling [10]. However, the physiological effect of GPI-derived phosphatidic acids would necessarily be a minor one, because it would be submerged in the high metabolic turnover of phosphatidic acids resulting from normal lipid metabolism in both wild-type and mutant cells.

The large differences in cytotoxicity resulting from GPI-PLD overexpression that are exhibited by the two mutants could

readily be explained either by differences in the relative amounts of precursors that accumulate (i.e. GlcNAcPtdIns or PI-PLC-resistant GPIs), by their intrinsic toxicity, or by their sensitivity to hydrolysis by GPI-PLD. Even though early steps of GPI synthesis occur on the cytoplasmic side of the endoplasmic reticulum and the precursors could be transferred 'directly' to the plasma membrane, the majority of free GPIs (and all the GPI-anchored proteins) reach the cell surface via vesicular transport through the endoplasmic reticulum/Golgi [28]. Consequently, they will be continually exposed to attack by GPI-PLD. It is relevant to note that a wide variety of GPIs and their degradation products have previously been shown to exert profound effects on cell physiology [29–31]. We speculate that similar effects may mediate the cytotoxicity observed following overexpression of GPI-PLD in CHO cells.

Although purified mammalian GPI-PLD and bacterial PI-PLC are both capable of hydrolysing many different GPI structures, GPI-PLD is also able to hydrolyse PI-PLC-resistant GPIs [32–35]. However, since purified GPI-PLD is unable to release GPI-anchored proteins from cell surfaces, it has not been possible to determine whether GPI-PLD is capable of hydrolysing PI-PLC-resistant GPIs in or on cells [7]. The data presented here therefore provide the first 'direct' evidence that GPI-PLD can release GPI-anchored proteins under physiological conditions from PI-PLC-resistant GPI anchors. Whether PI-PLC resistant GPIs are a 'preferred' substrate for GPI-PLD is an interesting question that merits further investigation.

Although the acquisition of PI-PLC resistance occurs early in GPI biosynthesis, its physiological purpose is currently unknown. A GPI inositol deacylase gene has recently been cloned from *Trypanosoma brucei*, which has sequence similarity with a mammalian enzyme acyloxyacyl hydrolase. However, the observation that inositol-acylated GPI biosynthesis in *T. brucei* continues when this gene is knocked out raises some interesting questions regarding the mechanism, function and control of inositol acylation in both trypanosomes and mammals [36].

Purification of mammalian GPI-PLD is routinely performed using serum as the starting material [37,38]. The complex nature of serum not only requires multiple purification steps, but also results in a low and variable yield, and this has been a major limitation on structure–function studies of GPI-PLD. In an attempt to overcome these disadvantages, we have evaluated different expression systems, including *Escherichia coli*, yeast and baculovirus-infected insect cells. Only the baculovirus expression system produced active GPI-PLD molecules with a yield of 0.7–1.5 mg/l (X. Du and M. G. Low, unpublished work). However, GPI-PLD produced by this method was extremely unstable, and required a completely different purification protocol, possibly due to the lack of normal post-translational modification in baculovirus-infected insect cells (X. Du and M. G. Low, unpublished work). The studies on G9PLAP.D5 (pPuro/PLD-B) reported here indicate that these cells could be the basis of a superior expression system for GPI-PLD. The GPI-PLD produced by these cells should retain the typical mammalian post-translational modifications and an almost doubled yield without the complications of the insect expression system. Most importantly, the amount of GPI-PLD secreted by G9PLAP.D5 (pPuro/PLD-B) reached a level similar to that present in serum. With fewer proteins in the cell-culture medium, G9PLAP.D5 (pPuro/PLD-B) might provide a simpler method for production of large quantities of purified GPI-PLD, and thereby facilitate structure–function studies.

The observation that overexpression of GPI-PLD is cytotoxic in two different mammalian cell lines (CHO cells and the monocyte/macrophage cells line RAW 264.7) could also explain our failure to obtain GPI-PLD expression in yeast cells (J.-Y. Li and M. G. Low, unpublished work), where GPIs are essential for biosynthesis of the cell wall [39,40]. In contrast, as noted above, we have been able to express GPI-PLD in insect cells (X. Du and M. G. Low, unpublished work). This result could be explained by a recent observation made by Azzouz et al. [41]. They demonstrated that, although uninfected insect H5 cells are able to synthesize GPI and GPI-anchored proteins, an early step of GPI biosynthesis is abolished in cells following baculovirus infection [41]. Thus insect cells could, upon infection with recombinant baculovirus containing GPI-PLD cDNA, become defective in GPI biosynthesis and therefore provide an intracellular environment resistant to the toxic effects of GPI-PLD overexpression, just like the CHO cell mutants.

We thank Dino A. De Angelis for generously providing the plasmid pCI-GFP-GPI. We are also grateful to Theresa Swayne and the Optical Microscopy Facility at Columbia University (supported by National Institutes of Health grants S10 RR10506, S10 RR13701, P30 CA13696 and by the Lieber Foundation) for assistance with the PRIM measurements. This work was supported by National Institutes of Health grants GM-40083 and GM-35873 (to M.G.L.) and GM-54191 (to V.L.S.).

REFERENCES

1 Stevens, V. L. (1995) Biosynthesis of glycosylphosphatidylinositol membrane anchors. Biochem. J. **310**, 361–370
2 McConville, M. J. and Menon, A. K. (2000) Recent developments in the cell biology and biochemistry of glycosylphosphatidylinositol lipids. Mol. Membr. Biol. **17**, 1–16
3 Metz, C. N., Brunner, G., Choi-Muira, N. H., Nguyen, H., Gabrilove, J., Caras, I. W., Altszuler, N., Rifkin, D. B., Wilson, E. L. and Davitz, M. A. (1994) Release of GPI-anchored membrane proteins by a cell-associated GPI-specific phospholipase D. EMBO J. **13**, 1741–1751
4 Smith, G. M., Biggs, J., Norris, B., Anderson-Stewart, P. and Ward, R. (1997) Detection of a soluble form of the leukocyte surface antigen CD48 in plasma and its elevation in patients with lymphoid leukemias and arthritis. J. Clin. Immunol. **17**, 502–509
5 Middelhoven, P. J., van Buul, J. D., Kleijer, M., Roos, D. and Hordijk, P. L. (1999) Actin polymerization induces shedding of FcγRIIIb (CD16) from human neutrophils. Biochem. Biophys. Res. Commun. **255**, 568–574
6 Wilhelm, O. G., Wilhelm, S., Escott, G. M., Lutz, V., Magdolen, V., Schmitt, M., Rifkin, D. B., Wilson, E. L., Graeff, H. and Brunner, G. (1999) Cellular glycosylphosphatidylinositol-specific phospholipase D regulates urokinase receptor shedding and cell surface expression. J. Cell. Physiol. **180**, 225–235
7 Low, M. G. and Huang, K.-S. (1991) Factors affecting the ability of glycosylphosphatidylinositol-specific phospholipase D to degrade the membrane anchors of cell surface proteins. Biochem. J. **279**, 483–493
8 Scallon, B. J., Fung, W.-J., Tsang, C., Li, S., Kado-Fong, H., Huang, K.-S. and Kochan, J. P. (1991) Primary structure and functional activity of a phosphatidylinositol-glycan specific phospholipase D. Science **252**, 446–448
9 Tsujioka, H., Misumi, Y., Takami, N. and Ikehara, Y. (1998) Posttranslational modifications of glycosylphosphatidylinositol (GPI)-specific phospholipase D and its activity in cleavage of GPI anchors. Biochem. Biophys. Res. Commun. **251**, 737–743
10 Tsujioka, H., Takami, N., Misumi, Y. and Ikehara, Y. (1999) Intracellular cleavage of glycosylphosphatidylinositol by phospholipase D induces activation of protein kinase Cα. Biochem. J. **342**, 449–455
11 Low, M. G. (2000) Structure and function of GPI-specific phospholipases. In Paroxysmal Nocturnal Hemoglobinuria and the Glycosylphosphatidylinositol-Linked Proteins (Young, N. S. and Moss, J., eds.), pp. 239–268, Academic Press, San Diego, CA
12 LeBoeuf, L. C., Caldwell, M., Guo, Y., Metz, C., Davitz, M. A., Olson, L. K. and Deeg, M. A. (1998) Mouse glycosylphosphatidylinositol-specific phospholipase D (*Gpld1*) characterization. Mamm. Genome **9**, 710–714
13 Rhode, H., Lopatta, E., Schulze, M., Schubert, K., Schubert, H., Reinhart, K. and Horn, A. (1999) Glycosylphosphatidylinositol-specific phospholipase D in blood serum: is the liver the only source of the enzyme? Clin. Chim. Acta **281**, 127–145
14 Maguire, G. A. and Gossner, A. (1995) Glycosyl phosphatidylinositol phospholipase D activity in human serum. Ann. Clin. Biochem. **32**, 74–78
15 Raymond, F. D., Fortunato, G., Moss, D. W., Castaldo, G., Salvatore, F. and Impallomeni, M. (1994) Inositol-specific phospholipase D activity in health and disease. Clin. Sci. **86**, 447–451

16 Ross, D. T., Scherf, U., Eisen, M. B., Perou, C. M., Rees, C., Spellman, P., Iyer, V., Jeffrey, S. S., Van de Rijn, M., Waltham, M. et al. (2000) Systematic variation in gene expression patterns in human cancer cell lines. Nat. Genet. **24**, 227–235
17 Du, X. and Low, M. G. (2001) Down-regulation of glycosylphosphatidylinositol-specific phospholipase D induced by lipopolysaccharide and oxidative stress in the murine monocyte-macrophage cell line RAW 264.7. Infect. Immun. **69**, 3214–3223
18 Iyer, V. R., Eisen, M. B., Ross, D. T., Schuler, G., Moore, T., Lee, J. C. F., Trent, J. M., Staudt, L. M., Hudson, Jr J., Boguski, M. S. et al. (1999) The transcriptional program in the response of human fibroblasts to serum. Science **283**, 83–87
19 Stevens, V. L., Zhang, H. and Harreman, M. (1996) Isolation and characterization of a Chinese hamster ovary (CHO) mutant defective in the second step of glycosylphosphatidylinositol biosynthesis. Biochem. J. **313**, 253–258
20 Low, M. G., Stiernberg, J., Waneck, G. L., Flavell, R. A. and Kincade, P. W. (1988) Cell-specific heterogeneity in sensitivity of phosphatidylinositol-anchored membrane antigens to release by phospholipase C. J. Immunol. Methods **113**, 101–111
21 Low, M. G. (1992) Phospholipases that degrade the glycosyl-phosphatidylinositol anchor of membrane proteins. In Lipid Modification of Proteins: a Practical Approach (Hooper, N. M. and Turner, A. J., eds.), pp. 117–154, Oxford University Press, Oxford
22 Wong, Y. W. and Low, M. G. (1992) Phospholipase resistance of the glycosyl-phosphatidylinositol membrane anchor on human alkaline phosphatase. Clin. Chem. **38**, 2517–2525
23 De Angelis, D. A., Miesenböck, G., Zemelman, B. V. and Rothman, J. E. (1998) PRIM: Proximity imaging of green fluorescent protein-tagged polypeptides. Proc. Natl. Acad. Sci. U.S.A. **95**, 12312–12316
24 Nakamura, N., Inoue, N., Watanabe, R., Takahaski, M., Takeda, J., Stevens, V. L. and Kinoshita, T. (1997) Expression cloning of PIG-L, a candidate *N*-acetylglucosaminylphosphatidylinositol deacetylase. J. Biol. Chem. **272**, 15834–15840
25 Li, J.-Y. and Low, M. G. (1999) Studies of the role of integrin EF-hand, Ca^{2+}-binding sites in glycosylphosphatidylinositol-specific phospholipase D: reduced expression following mutagenesis of residues predicted to bind Ca^{2+}. Arch. Biochem. Biophys. **361**, 142–148
26 Küng, M., Bütikofer, P., Brodbeck, U. and Stadelmann, B. (1997) Expression of intracellular and GPI-anchored forms of GPI-specific phospholipase D in COS-1 cells. Biochim. Biophys. Acta **1357**, 329–338
27 Takeda, J., Miyata, T., Kawagoe, K., Iida, Y., Endo, Y., Fujita, T., Takahashi, M., Kitani, T. and Kinoshita, T. (1993) Deficiency of the GPI anchor caused by a somatic mutation of the *PIG-A* gene in paroxysmal nocturnal hemoglobinuria. Cell **73**, 703–711
28 Baumann, N. A., Vidugiriene, J., Machamer, C. E. and Menon, A. K. (2000) Cell surface display and intracellular trafficking of free glycosylphosphatidylinositols in mammalian cells. J. Biol. Chem. **275**, 7378–7389
29 Tachado, S. D., Mazhari-Tabrizi, R. and Schofield, L. (1999) Specificity in signal transduction among glycosylphosphatidylinositols of *Plasmodium falciparum, Trypanosoma brucei, Trypanosoma cruzi* and *Leishmania* spp. Parasite Immunol. **21**, 609–617
30 Almeida, I. C., Camargo, M. M., Procópio, D. O., Silva, L. S., Mehlert, A., Travassos, L. R., Gazzinelli, R. T. and Ferguson, M. A. J. (2000) Highly purified glycosylphosphatidylinositols from *Trypanosoma cruzi* are potent proinflammatory agents. EMBO J. **19**, 1476–1485
31 Jones, D. R., Avila, M. A., Sanz, C. and Varela-Nieto, I. (1997) Glycosyl-phosphatidylinositol-phospholipase type D: A possible candidate for the generation of second messengers. Biochem. Biophys. Res. Commun. **233**, 432–437
32 Roberts, W. L., Myher, J. J., Kuksis, A., Low, M. G. and Rosenberry, T. L. (1988) Lipid analysis of the glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase. Palmitoylation of inositol results in resistance to phosphatidylinositol-specific phospholipase C. J. Biol. Chem. **263**, 18766–18775
33 Ferguson, M. A. J. (1992) Site of palmitoylation of a phospholipase C-resistant glycosyl-phosphatidylinositol membrane anchor. Biochem. J. **284**, 297–300
34 Field, M. C. and Menon, A. K. (1992) Biosynthesis of glycosyl-phosphatidylinositol membrane protein anchors. In Lipid Modification of Proteins: a Practical Approach (Hooper, N. M. and Turner, A. J., eds.), pp. 155–190, Oxford University Press, Oxford
35 Priest, J. W., Xie, L. T., Arrowood, M. J. and Lammie, P. J. (2001) The immunodominant 17-kDa antigen from *Cryptosporidium parvum* is glycosylphosphatidylinositol-anchored. Mol. Biochem. Parasitol. **113**, 117–126
36 Güther, M. L. S., Leal, S., Morrice, N. A., Cross, G. A. M. and Ferguson, M. A. J. (2001) Purification, cloning and characterization of a GPI inositol deacylase from *Trypanosoma brucei*. EMBO J. **20**, 4923–4934
37 Davitz, M. A., Hom, J. and Schenkman, S. (1989) Purification of a glycosyl-phosphatidylinositol specific phospholipase D from human plasma. J. Biol. Chem. **264**, 13760–13764
38 Huang, K.-S., Li, S., Fung, W.-J. C., Hulmes, J. D., Reik, L., Pan, Y.-C. E. and Low, M. G. (1990) Purification and characterization of glycosyl-phosphatidylinositol-specific phospholipase D. J. Biol. Chem. **265**, 17738–17745
39 Lu, C.-F., Montijn, R. C., Brown, J. L., Klis, F., Kurjan, J., Bussey, H. and Lipke, P. N. (1995) Glycosyl phosphatidylinositol-dependent cross-linking of α-agglutinin and β1,6-glucan in the *Saccharomyces cerevisiae* cell wall. J. Cell Biol. **128**, 333–340
40 Leidich, S. D., Drapp, D. A. and Orlean, P. (1994) A conditionally lethal yeast mutant blocked at the first step in glycosyl phosphatidylinositol anchor synthesis. J. Biol. Chem. **269**, 10193–10196
41 Azzouz, N., Kedees, M. H., Gerold, P., Becker, S., Dubremetz, J. F., Klenk, H. D., Eckert, V. and Schwarz, R. T. (2000) An early step of glycosylphosphatidyl-inositol anchor biosynthesis is abolished in lepidopteran insect cells following baculovirus infection. Glycobiology **10**, 177–183

Received 11 May 2001/26 September 2001; accepted 23 October 2001