Effect of glycosylphosphatidylinositol (GPI)-phospholipase D overexpression on GPI metabolism

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GPI-PLD [glycosylphosphatidylinositol (GPI)-specific phospholipase D (PLD)] is a secreted mammalian enzyme that specifically cleaves GPI-anchored proteins. In addition, the enzyme has been shown to cleave GPI anchor intermediates in cell lysates. The biosynthesis of the GPI anchor is well characterized; however, the mechanisms by which the levels of GPI anchor intermediates are regulated are still unknown. To investigate whether GPI-PLD plays a role in this regulation, we isolated stable HeLa cells overexpressing the enzyme. GPI-PLD-HeLa (GPI-PLD-transfected HeLa) cells showed a 3-fold increase in intracellular GPI-PLD activity and drastically decreased the levels of GPI-anchored proteins when compared with untransfected HeLa controls. Intracellular cleavage of GPI-anchored proteins has been suggested to occur early in the secretory pathway and, in agreement with this proposal, GPI-PLD activity in GPI-PLD-HeLa cells was detected not only in the endoplasmic reticulum and Golgi apparatus, but also in the plasma membrane. The enzyme was also active in lipid rafts, membrane microdomains in which GPI-anchored proteins and GPI anchor intermediates are concentrated, indicating that intracellular GPI-PLD cleavage may also occur in this compartment. Pulse–chase paradigms revealed the turnover rate of the last intermediate of the GPI anchor pathway in GPI-PLD-HeLa cells to be accelerated compared with the controls. Furthermore, 1,10-phenanthroline, a GPI-PLD inhibitor, reversed this effect. Our studies demonstrated that GPI-PLD can cleave not only GPI-anchored proteins, but also GPI anchor intermediates intracellularly. This observation opens the possibility that GPI-PLD can influence the steady-state levels of GPI-anchored proteins by hydrolysing the anchor before and after its attachment to proteins.

Key words: glycosylphosphatidylinositol (GPI), glycosylphosphatidylinositol-anchored protein, glycosylphosphatidylinositol-specific phospholipase D, HeLa cell, lipid raft.

INTRODUCTION

In eukaryotes, a group of cell-surface proteins is attached to the outer leaflet of the plasma membrane by GPI (glycosylphosphatidylinositol) anchors. These GPI structures anchor most cell-surface proteins in protozoan parasites [1] and a significant proportion of plasma-membrane proteins in yeast [2]. In mammals, on the other hand, GPI anchoring is required for attaching a smaller but functionally diverse subset of cell-surface proteins, including enzymes, receptors, cell adhesion molecules and differentiation antigens. GPI-anchored proteins participate in many important cellular functions, including immune recognition, complement regulation and intracellular signalling [3]. In addition to providing the attached proteins with a stable association with the outer leaflet of the plasma membrane, the GPI anchor targets GPI-anchored proteins to cholesterol- and sphingolipid-rich membrane microdomains termed lipid rafts [4].

GPI anchor synthesis and its attachment to proteins, a process that includes the removal of the C-terminal GPI signal sequence from the proteins and its replacement by a pre-assembled anchor, occur in the ER (endoplasmic reticulum) [5]. Synthesis of the anchor in the ER starts with the addition of GlcNAc to PI (phosphatidylinositol), followed by deacetylation of GlcNAc-PI to GlcN-PI. Downstream steps of the mammalian biosynthetic pathway consist of the sequential addition of GPI anchor components to GlcN-PI (i.e. palmitic acid, mannose and phosphoethanolamine groups). The pathway ends with the assembly of two GPI anchor intermediates and putative anchor donors, H7 and H8. GPI anchor intermediates can also disengage from the biosynthetic pathway and migrate from the ER to other intracellular compartments and the plasma membrane.

Cell-surface GPI anchor intermediates, i.e. those without attached proteins, were first reported in *Leishmania* parasites [6,7]. The presence of GPI anchor intermediates in the plasma membrane of mammalian cells was established using three different approaches: (i) subcellular fractionation [8], (ii) covalent labelling with cell-impermeant probes [9] and (iii) virus budding from either the apical or basolateral surface of polarized epithelia [10]. This approach took advantage of the presence of plasma-membrane lipids (including GPI anchor intermediates) in the virus envelope to show that mature GPI anchor intermediates are present in both the apical and basolateral plasma membranes, in contrast with the typical apical localization of GPI-anchored proteins [11,12]. The non-polarized distribution of GPI anchor intermediates suggested that they reside in the cytoplasmic leaflet of the plasma membrane [10], since the inner leaflet in the plasma membrane of polarized epithelia is continuous, allowing for free mixing of apical and basolateral membrane components. This proposal was further supported by the cytoplasmic orientation of early GPI anchor intermediates (i.e. GlcNAc-PI and GlcN-PI). However, Singh et al. [9] and, later, Baumann et al. [13], using membrane-impermeant probes, showed that H8 is present in the outer leaflet of the plasma membrane. Both groups also determined that the transport of H8 to the plasma membrane can be blocked under conditions known to slow or disrupt the vesicular transport between the ER and the Golgi apparatus. Thus it was concluded that this is the main route followed by GPI anchor intermediates to reach the plasma membrane.

Abbreviations used: DAF, decay accelerating factor; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; PI, phosphatidylinositol; PLD, phospholipase D; PNT, 1,10-phenanthroline; Tos-Lys-CH_2Cl, tosyl-lysylchloromethane.

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Most of the genes involved in the GPI anchor biosynthetic pathway have been cloned [14], and activities encoded by some of these genes have been characterized [15]. In contrast with the knowledge on the biosynthesis of GPI anchors, there is a complete lack of information on their degradation. Our previous kinetic analyses of GPI synthesis suggested the existence of catabolic pathways influencing the turnover rates of GPI anchor intermediates [16]. One potential player in this process is GPI-PLD [GPI-specific PLD (phospholipase D)], a secreted enzyme that cleaves the linkage between the phosphate and inositol in GPI structures [17]. GPI-PLD is very abundant in mammalian serum, yet the circulating enzyme does not cleave GPI-anchored proteins on the cell surface. Intracellular GPI-PLD, on the other hand, has been proposed to be responsible for the presence of soluble forms of DAF (decay accelerating factor) as well as other GPI-anchored proteins in the circulation and in extracellular fluids [18,19]. Furthermore, in certain pathologies such as cancer in which the enzyme is upregulated [20], tumour-associated GPI-anchored proteins (i.e. carcinoembryonic antigen and urokinase receptor) are found in the serum of patients [21].

The proposal that endogenous GPI-PLD is involved in the catabolism of GPI anchor intermediates is based on the following evidence: (i) endogenous GPI-PLD has been shown to cleave GPI anchor intermediates in membrane preparations [22] and (ii) GPI-anchored proteins are cleaved by GPI-PLD in the secretory pathway while in transit to the plasma membrane [23,24]. GPI anchor intermediates also reach the cell surface via the same pathway [9,13] and, therefore, they are probably exposed to GPI-PLD [19].

In the present study, we determined that, in addition to GPI-anchored proteins, GPI-PLD cleaves GPI anchor intermediates intracellularly. We also identified intracellular locations of GPI-PLD activity that include the ER and lipid rafts.

MATERIALS AND METHODS

Reagents

DMEM (Dulbecco's modified Eagle's medium), glucose- and inositol-free DMEM, LIPOFECTAMINE™ Plus, Geneticin and Protein A–agarose were obtained from Invitrogen (Carlsbad, CA, U.S.A.). Amplify and ECL® reagents were from Amersham Biosciences (Piscataway, NJ, U.S.A.). Methionine- and cysteine-free DMEM and Tran^{35}S-label (70 % from [^{35}S]methionine and approx. 15 % from 1175 Ci/mmol [^{35}S]cysteine) were obtained from ICN (Irvine, CA, U.S.A.) and silica gel 60 TLC plates from Merck (Gibbstown, NJ, U.S.A.). [2-^{3}H]Mannose (20 Ci/mmol) and [2-^{3}H]inositol (20 Ci/mmol) were obtained from ARC (St. Louis, MO, U.S.A.). Rabbit polyclonal anti-GPI-PLD antibody was prepared as described previously [25]. Anti-DAF 2H6 monoclonal antibody was a gift from Dr Ed Medof (Case Western Reserve University, Cleveland, OH, U.S.A.).

Isolation of HeLa cells stably expressing GPI-PLD

The coding region for mouse pancreatic GPI-PLD was isolated from full-length GPI-PLD cDNA [26] and subcloned into the *Eco*RI–*Xho*I sites of a pcDNA3.1 vector (Invitrogen). The full-length cDNA was modified by removing the 5′- and 3′-untranslated regions and adding the Kozak sequence within the ATG site to enhance translation. The resultant plasmid was amplified and purified using a Quantum Prep kit (Bio-Rad Laboratories), and was used to generate stable GPI-PLD-HeLa (GPI-PLD-transfected HeLa) transfectants as follows. HeLa S3 cells were plated on 6-well plates, and on the following day, 1 μg of plasmid was transfected using LIPOFECTAMINE™ Plus reagent according to the manufacturer's instructions. After 48 h, the cells were transferred to 150 mm dishes, and on the following day, selection of expressing clones was started by supplementing HeLa culture medium [DMEM containing 10 % (v/v) foetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine] with 300 μg/ml Geneticin. Clones were screened by measuring the levels of GPI-PLD secreted into serum-free medium over a 24 h period on Western blots probed with a polyclonal anti-GPI-PLD antibody. GPI-PLD activity in conditioned media was quantified using GPI-anchored placental alkaline phosphatase as the substrate. One positive clone (GPI-PLD-HeLa), having approx. 3-fold higher activity in the medium and in cell lysates (using ^{3}H-mannose-labelled GPI anchor intermediates as substrates) compared with that of untransfected HeLa cells, was used for the studies.

[^{3}H]Inositol labelling of HeLa D cells and lysis at acidic and basic pH

HeLa D cells, isolated in our laboratory [27], were labelled with 10 μCi of [^{3}H]inositol for 3 days in two 100 mm plates in inositol-free DMEM, supplemented similar to HeLa medium but with 10 % (v/v) dialysed newborn calf serum instead of 10 % foetal calf serum. After the incubation period, the cells were washed and one-fifth of the cells were extracted in CMW (chloroform/methanol/water, 10:10:3). The remaining cells were divided into four tubes and resuspended in 50 μl of either 50 mM Tris/acetate buffer (pH 5.5) or 50 mM Tris/HCl (pH 8.0). One tube for each pH was supplemented with PNT (1,10-phenanthroline) to a final concentration of 500 μM. The four tubes were incubated overnight at 37 °C and extracted in CMW by adding 100 μl of water and 850 μl of chloroform/methanol (1:1). CMW extracts were dried down and partitioned between water and butanol as described previously [28]. Butanol phases were subjected to TLC using CMW (10:10:3) as the developing solvent. The chromatograms were analysed in a BioScan scanner and then subjected to fluorography after spraying them with EN^{3}HANCE (PerkinElmer).

[^{3}H]Inositol labelling and analysis of GPI-anchored proteins

Duplicate 150 mm plates of S3 and GPI-PLD-HeLa cells at 30–40 % confluency were incubated with 150 μCi of [^{3}H]inositol in inositol-free DMEM, supplemented as described above. After 3 days, the cells were collected, washed and extracted on ice with 700 μl of extraction buffer consisting of 20 mM sodium phosphate buffer (pH 6.9), containing 1 mM EDTA, 1 mM EGTA, 150 mM NaCl and 2 % (w/w) Triton X-114 and supplemented with 1 μg/ml each of leupeptin and pepstatin. The cell extracts were incubated for 30 min on ice with intermittent vortex-mixing, and the lysates were then centrifuged for 10 min at 14 000 ***g***. The pelleted material was re-extracted with 300 μl of extraction buffer and both extracts were combined. Phase separation was achieved by warming up the samples to 37 °C, followed by a brief centrifugation at low speed. The upper aqueous phase was removed and replaced with a pre-equilibrated aqueous phase for a second-phase partition. Both the aqueous and detergent phases were brought to 1 ml with phosphate buffer, and proteins were precipitated in 10 % (w/v) trichloroacetic acid. Trichloroacetic acid pellets were washed with cold acetone, resuspended in loading buffer, and the proteins were analysed by SDS/PAGE. After the run, the gels were soaked in Amplify and subjected to fluorography.

^{35}S pulse–chase analysis of DAF

HeLa cells in 6-well plates were washed twice with methionine- and cysteine-free DMEM and the cells were then incubated for 20 min at 37 °C with 1 mCi of Tran^{35}S-label (70 % [^{35}S]methionine and approx. 15 % [^{35}S]cysteine) in 0.5 ml of methionine- and cysteine-free DMEM, supplemented as described for the [^{3}H]inositol labellings. After the pulse, the cells were washed twice, and either the cells were extracted as indicated below or the label was chased in 1 ml of HeLa medium for 2 h. Cells from the pulse and the chase were extracted in 1 ml of RIPA buffer [50 mM Tris/HCl (pH 8.0), supplemented with 1 % Nonidet P40, 0.1 % SDS and 0.5 % deoxycholate]. The extracts were incubated overnight in a rocker at 4 °C with 30 μl of Protein A–agarose beads and 1 μg/ml 2H6 anti-DAF antibody. The beads were collected by centrifugation, washed three times and boiled in loading buffer to elute DAF. The eluted proteins were resolved on 10 % SDS/polyacrylamide gel (Bio-Rad Laboratories). After the run, the gels were processed as described for ^{3}H-inositol-labelled proteins.

GPI-PLD activity in membrane fractions

HeLa cells (5×10^7) were collected and incubated for 1 h at 37 °C in 10 ml of glucose-free DMEM containing 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 10 % dialysed newborn calf serum and supplemented with 10 μg/ml tunicamycin and 100 μg/ml glucose (Man-labelling medium). The cells were then labelled for 1 h with 500 μCi of [^{3}H]mannose. The unincorporated [^{3}H]mannose was removed by three washes with PBS and the radiolabelled cells were combined with 5×10^7 unlabelled HeLa cells and resuspended in 5 ml of ice-cold 0.25 M sucrose in 10 mM Hepes buffer (pH 7.5), supplemented with 1 mM dithiothreitol, 1 mM PMSF, 1 μg/ml leupeptin and 0.1 mM tosyl-lysylchloromethane (Tos-Lys-CH_2Cl; 'TLCK'). During all the subsequent steps, the sample was kept at 4 °C. The cells were disrupted using a nitrogen cavitation bomb and subcellular fractions were prepared as described in detail elsewhere [8]. Briefly, after holding the cells at approx. 3100 kPa for 30 min, the cells were further disrupted in a glass homogenizer in the presence of 1.5 units of *Staphylococcus aureus* nuclease. Heavy membranes were then pelleted at 10 000 ***g*** for 15 min. The supernatant was layered on top of a discontinuous sucrose gradient composed of 2 ml each of 38, 30 and 20 % (w/v) sucrose in 10 mM Hepes (pH 7.5) and centrifuged for 2 h at 140 000 ***g*** in a Beckman SW41 rotor. Three opalescent bands at the sucrose interfaces and the pellet at the bottom of the tube were collected. The three membrane fractions were pelleted by dilution of the sucrose with 10 mM Hepes and centrifugation for 2 h at 230 000 ***g*** in the same rotor. The top fraction of the sucrose gradient was enriched in plasma-membrane markers, whereas the middle fraction contained a mixture of Golgi and plasma-membrane markers, and both the lower fraction and the pellet were enriched in ER markers (most probably representing the smooth and rough ERs; results not shown). The lower fraction and the pellet were combined for analysis. The organelle-specific marker enzymes used to determine the purity of the fractions were alkaline phosphodiesterase I for the plasma membrane, α-mannosidase II for Golgi and dolicholphosphomannose synthase for ER, as described elsewhere [8]. Equal amounts (based on protein content) of the three ^{3}H-mannose-labelled subcellular fractions were then incubated overnight at 37 °C in 150 μl of GPI-PLD buffer [50 mM Mes (pH 6.5) containing 0.05 % Triton X-100 and 0.1 mM $CaCl_2$], supplemented with PNT to inhibit GPI-PLD. On the following day, ^{3}H-mannose-labelled GPI anchor intermediates were extracted with CMW and analysed by TLC as described above.

When GPI-PLD activity was assessed in lipid rafts, 5×10^7 cells were labelled with 500 μCi of [^{3}H]mannose in Man-labelling medium as described previously, and rafts were prepared by floatation in a sucrose gradient as described in detail in [8]. Briefly, the cells were lysed with a glass homogenizer for 1 h at 4 °C in 25 mM Hepes (pH 7.4), 1 % Triton X-100, 150 mM NaCl and 5 mM EDTA, supplemented with 1 μg/ml each of leupeptin and pepstatin and 0.1 mM Tos-Lys-CH_2Cl. Unbroken cells were removed by low-speed centrifugation and the supernatant was transferred to an ultracentrifuge tube and adjusted to 40 % sucrose. A discontinuous sucrose gradient was then formed on top of the 40 % layer by overlaying 6 ml of 38 % sucrose and 3 ml of 5 % sucrose. After centrifugation for 19 h in a SW41 rotor at 230 000 ***g***, rafts floated to the interface between 5 and 38 % sucrose and appeared as an opalescent band. Rafts were isolated by pelleting the membranes, after dilution of sucrose with 50 mM Hepes supplemented with proteinase inhibitors, and centrifugation for 2 h at 230 000 ***g*** in the same rotor.

[^{3}H]Mannose pulse–chase

HeLa cells (3×10^6) were incubated for 1 h at 37 °C in 5 ml of Man-labelling medium as described in the previous section. After a 1 h pulse labelling with 250 μCi of [^{3}H]mannose, one-fourth of the cells was extracted with CMW. The rest of the cells were resuspended in 5 ml of PBS containing 5 mM unlabelled mannose (chase medium) and incubated for 4 h at 37 °C. One-third of the cells was extracted with CMW after the initial chase and the remaining two-thirds of the cells were divided into two parts and incubated in fresh chase medium for an additional 3 h in the presence and absence of 500 μM PNT. CMW extracts from the pulse and 4 and 7 h chases were dried and partitioned between butanol and water. The butanol phases were analysed by TLC using CMW as the developing solvent.

RESULTS AND DISCUSSION

Analysis of the rate of synthesis and decay of H6, H7 and H8 (the last three GPI anchor intermediates of the biosynthetic pathway) using [^{3}H]mannose pulse–chase paradigms in wild-type cells (e.g. HeLa and K562) revealed that the label in H6 and H7 disappeared quite rapidly, but was not quantitatively recovered in H8, which decays more slowly, with a half-life of > 1 h [16]. GPI anchor intermediates also decay, albeit at slower rates, in K cells, a K562 mutant that is deficient in the transfer of GPI anchor intermediates to proteins. This indicates that in cells, GPI turnover is not exclusively due to their transfer to proteins. These results prompted us to propose the existence of a catabolic pathway for GPI anchor intermediates.

In preliminary studies to establish whether intracellular trafficking and/or lysosomal functions are required for GPI degradation, we investigated the following reagents: monensin (to block intra-Golgi transport), A23187 (a calcium ionophore), chloroquine and NH_4Cl (to inhibit lysosomal function). One of the reagents that consistently affected GPI metabolism was NH_4Cl. NH_4Cl is believed to act by increasing the pH value of intracellular compartments, including lysosomes. We repeated these preliminary experiments by pulsing mutant K cells with [^{3}H]mannose, followed by a 4.5 h chase in the presence or absence of NH_4Cl. H6 and H7 levels were 2-fold higher in cells incubated with NH_4Cl when compared with the controls. This result suggested that GPI degradation may take place in an acid

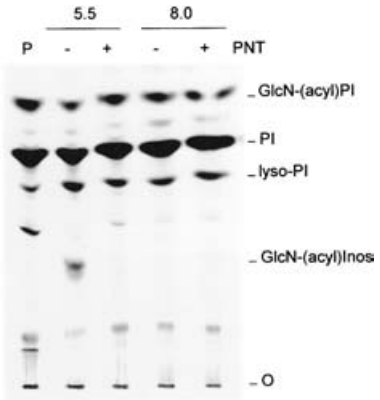

Figure 1 GPI-PLD cleaves membrane-bound GPI anchor intermediates at acidic pH

HeLa D cells were labelled for 3 days with 20 μCi of [^{3}H]inositol. After the incubation, one-fifth of the cells were extracted with chloroform/methanol/water. Half of the remaining cells were lysed in 50 mM Tris/acetate (pH 5.5) and the other half in Tris/HCl (pH 8.0) buffers, and the two groups of cell lysates were incubated overnight at 37 °C in the absence (−) and presence (+) of PNT. Lipids from the cell lysates and the untreated cells (P) were extracted and analysed by TLC, as described in the Materials and methods section. The origin of the run is indicated by the letter O.

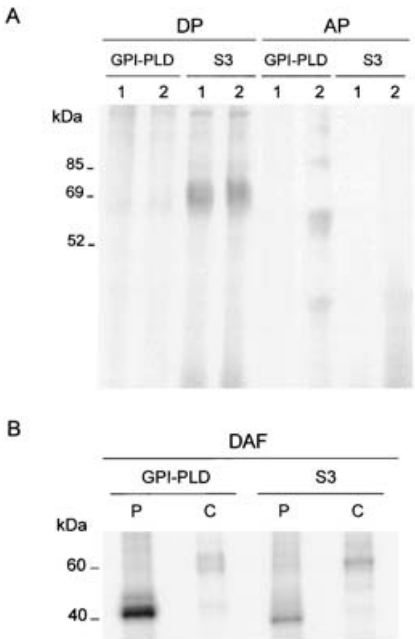

Figure 2 GPI-PLD overexpression decreases the intracellular levels of GPI-anchored proteins

(**A**) S3 and GPI-PLD-HeLa cells were labelled for 3 days with 300 μCi of [^{3}H]inositol. The cells were extracted in a buffer containing 2 % Triton X-114. After phase separation, ^{3}H-inositol-labelled proteins in the detergent and aqueous phases (DP and AP respectively) from Triton X-114 extracts were analysed by SDS/PAGE, followed by fluorography. Independent duplicate experiments for each cell line were performed. (**B**) HeLa cells were labelled with 1 mCi of Tran^{35}S-label for 20 min. After the pulse, the cells were either extracted or chased for 2 h in DMEM. ^{35}S-labelled DAF from pulse (P) and chase (C) cell extracts was immunoprecipitated and analysed by gel electrophoresis as described in the Materials and methods section.

environment and/or that the enzymes involved have an acidic optimum pH. To confirm this, ^{3}H-mannose-labelled HeLa and mutant K cells were lysed in the absence of detergent at either pH 5.5 or 8.0. We consistently observed a decrease in GPI levels and the presence of putative GPI-PLD cleavage products at pH 5.5, but not at pH 8.0 (results not shown). To establish that these species are in fact derived from GPI-PLD hydrolysis, we repeated the above experiment, but with ^{3}H-inositol-labelled HeLa D cells. HeLa D is a subline of HeLa S3 cells that accumulates GlcN-(acyl)PI [27], the third intermediate of the GPI biosynthetic pathway. The advantage of using this approach is that GlcN-(acyl)Inos, the GPI-PLD cleavage product of GlcN-(acyl)PI, can be easily identified, because it moves further away from the origin of the TLC plate [29], in contrast with the GPI-PLD products of mannosylated GPI anchor intermediates that stay at or very close to the origin. As shown in Figure 1, when ^{3}H-inositol-labelled HeLa D cells were lysed in acidic pH, GlcN-(acyl)PI was decreased and a species migrating at the position of GlcN-(acyl)Inos was detected. This fragment has been extensively characterized in a previous study by a variety of methods, including hydrophobic chromatography and MS [29]. Moreover, GlcN-(acyl)Inos was not present when these lysates were incubated in the presence of PNT (an inhibitor of GPI-PLD) or at pH 8.0 (Figure 1). These results, along with the observed protective effect of increasing the intracellular pH value in intact cells on GPI decay, seem to suggest that GPI-PLD is involved in the catabolism of GPI anchor intermediates. Significantly, the optimum pH value of purified GPI-PLD is 6.5 [30].

In our initial approach to investigate the involvement of GPI-PLD in the catabolism of GPI anchor intermediates in intact cells, we inhibited the enzyme with PNT. However, these studies unexpectedly revealed that PNT also blocks the synthesis of phosphoethanolamine-containing GPI anchor intermediates (i.e. H6, H7 and H8) due to the inhibition of phosphoethanolamine transferases [22]. This complicates the use of PNT as a tool to study the role of GPI-PLD in GPI metabolism, because it is difficult to separate the effects of PNT on the activities of GPI-PLD and phosphoethanolamine transferases. Therefore, as an alternative approach, we decided to study the role of GPI-PLD on GPI metabolism by increasing the endogenous GPI-PLD activity by means of recombinant GPI-PLD expression.

GPI-anchored proteins are drastically decreased in GPI-PLD-HeLa cells

It has been reported previously that higher levels of intracellular GPI-PLD activity increase the secretion of GPI-anchored proteins into the medium [20,31,32]. Therefore we initiated the characterization of GPI-PLD-HeLa cells by measuring the cellular levels of endogenous GPI-anchored proteins. Steady-state levels of GPI-anchored proteins in S3 and GPI-PLD-HeLa cells were assessed by metabolic labelling of the cells with [^{3}H]inositol for 3 days. [^{3}H]Inositol uptake was similar (approx. 2 %) in both cell lines. [^{3}H]Inositol is only incorporated into GPI-anchored proteins, and these proteins can be recovered in the detergent phase of Triton X-114 extracts after phase separation. Analysis by SDS/PAGE of the ^{3}H-inositol-labelled proteins in the detergent phase of S3 cells revealed a broad band of proteins with a molecular mass of approx. 70 kDa (Figure 2A). This band probably contains DAF, a 68 kDa protein, which is one of the most abundant GPI-anchored proteins in HeLa cells. The detergent phase of the GPI-PLD transfectant extracts was almost free of radiolabelled proteins; however, weak bands, with lower molecular mass compared with the band in the detergent phase of S3 cells, were visible in the aqueous phase (Figure 2A). The recovery of ^{3}H-inositol-labelled GPI-anchored proteins in the aqueous phase of GPI-PLD-HeLa cell extracts is consistent with the loss of their hydrophobicity owing to the removal of phosphatidic acid from the GPI anchor by GPI-PLD. Bands with

a molecular mass different from that of the main bands observed in HeLa S3 cells are present in the aqueous phases of GPI-PLD-HeLa cells (Figure 2A). These bands probably originated by proteolysis of the GPI-PLD-cleaved GPI-anchored proteins. ^{3}H-inositol-labelled proteins are probably released into the medium as a result of intracellular cleavage by GPI-PLD (see results in the next section). Thus fewer proteins are recovered in the aqueous phase of the GPI-PLD-HeLa cells compared with the detergent phase of S3 cells (Figure 2A).

To analyse the effect of GPI-PLD on the turnover rates of GPI-anchored proteins, the cells were pulse-labelled with [^{35}S]methionine/cysteine, followed by a 2 h chase. Analysis of the ^{35}S-labelled immature DAF (approx. 43 kDa) after the pulse showed a much stronger signal in GPI-PLD-HeLa cells compared with the untransfected controls (Figure 2B), probably because ^{35}S incorporation was higher in GPI-PLD-HeLa cells (19 % versus 7 % respectively). During the chase, the cell-associated ^{35}S counts decreased by 56 % in the GPI-PLD-HeLa cells and by 40 % in S3 cells from their values after the pulse. More importantly, during the 2 h chase, the signal in DAF decreased by 70 % in GPI-PLD-HeLa cells, whereas the counts in DAF in S3 cells remained almost constant (Figure 2B), again indicating increased cleavage and release of GPI-anchored proteins in the GPI-PLD-HeLa cells. The doublet in the mature DAF band (68 kDa) after the chase in GPI-PLD-HeLa cells was consistently observed (Figure 2B). This may be due to the presence of a pool of DAF without the GPI anchor, since GPI-DAF and anchorless DAF have slightly different gel mobility. Analysis by immunoprecipitation of the chase medium showed the presence of DAF in GPI-PLD-HeLa cells, but not in S3 cells (results not shown). In summary, this experiment shows that, in GPI-PLD-HeLa cells, the synthesis of proteins destined to be GPI-anchored is normal, but GPI-anchored proteins are at or below detectable levels due to their intracellular cleavage by GPI-PLD.

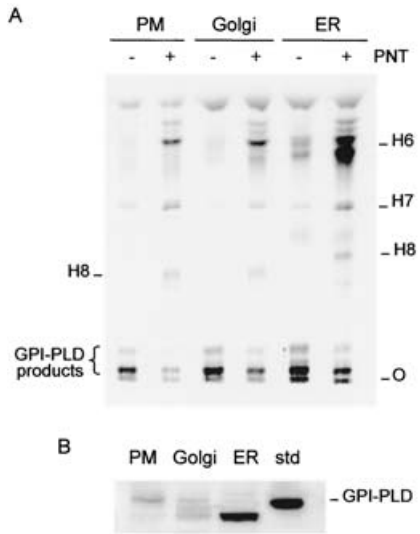

Figure 3 GPI-PLD in plasma membrane and subcellular fractions

(**A**) GPI-PLD-HeLa cells were labelled with 500 μCi of [^{3}H]mannose, and plasma-membrane (PM) and subcellular fractions were prepared as described in the Materials and methods section. Equal amounts (based on protein determination) of membranes were resuspended in GPI-PLD buffer [50 mM Mes (pH 6.5) containing 0.05 % Triton X-100 and 0.1 mM $CaCl_2$] and each fraction was incubated overnight at 37 °C in the presence (+) and absence (−) of 500 μM PNT. At the end of the incubation period, ^{3}H-mannose-labelled GPI anchor intermediates in each sample were extracted. GPI-PLD activity was determined by TLC analysis of GPI anchor intermediates. (**B**) Membrane fractions (10 μg each) were resolved by SDS/PAGE and their GPI-PLD levels were assessed by Western-blot analysis using an anti-GPI-PLD antibody as probe. GPI-PLD purified from human serum was run alongside as a positive control (std). The origin of the run is indicated by the letter O.

Intracellular distribution of GPI-PLD activity

The intracellular compartment in which GPI-PLD cleaves GPI-anchored proteins is still undetermined, with one report claiming that it occurs in a post-Golgi compartment or at the plasma membrane [24], whereas a more recent study proposes that the cleavage occurs in the ER [33].

To determine the intracellular localization of GPI-PLD activity, we isolated subcellular fractions from metabolically ^{3}H-mannose-labelled GPI-PLD-HeLa cells and used the labelled endogenous GPI anchor intermediates as substrates. Equal amounts of three subcellular fractions enriched in plasma membrane, Golgi and ER markers were then incubated overnight at 37 °C in GPI-PLD buffer with and without PNT to inhibit GPI-PLD. GPI anchor intermediates were then extracted and recovered in butanol. To determine the GPI-PLD activity in each subcellular fraction, the extent of GPI decay was assessed by TLC analysis of the butanol phases. This analysis also detects the formation of GPI-PLD cleavage products of GPI anchor intermediates (acylated inositol glycans), since they are partially recovered in the butanol phase. The last three GPI anchor intermediates of the biosynthetic pathway H6, H7 and H8 were present in all three membrane fractions examined, but the levels of H6 were much higher in the ER (Figure 3A). In the absence of PNT, the GPI levels in all the fractions were significantly lower and the amount of GPI-PLD cleavage products of GPI anchor intermediates was higher than that in the samples with PNT, indicating the presence of GPI-PLD activity (Figure 3A). The detection of low levels of GPI cleavage products in the samples incubated with PNT (Figure 3A, lanes marked '+') suggests that some cleavage occurred before and/or during isolation of the subcellular fractions. We observed a similar distribution of GPI-PLD activity when subcellular fractions were prepared from untransfected HeLa S3 cells (results not shown), demonstrating that GPI-PLD is targeted to the same compartments in GPI-PLD-HeLa cells. The fact that immature GPI-PLD (the band with low molecular mass in the ER fraction in Figure 3B) is active in the ER seems to support the proposal that GPI-PLD cleavage of GPI-anchored proteins occurs in the ER. Our comparative analysis of immature DAF by a short ^{35}S pulse does not appear to support this proposal, since, in numerous experiments, we failed to observe a weaker signal of immature DAF in GPI-PLD-HeLa cells (Figure 2A). However, to make a more definitive conclusion, Triton X-114 partition experiments must be performed to establish whether, in GPI-PLD-HeLa cells, DAF in the ER contains a GPI anchor.

To investigate the distribution of GPI-PLD protein in the subcellular fractions, each fraction was subjected to Western-blot analysis (Figure 3B). In contrast with approximately similar levels of GPI-PLD activity among subcellular fractions, the protein was found almost exclusively in the ER (Figure 3B). This could be the result of some misfolded inactive GPI-PLD accumulating in that compartment as a result of the overexpression of the protein, despite efficient secretion of GPI-PLD into the medium (results not shown). The distribution of GPI-PLD species in the subcellular fractions provided a good assessment of the purity of these fractions, since smaller immature species were present in the ER, a mixture of immature and mature forms were present in the Golgi and only mature species were present in the plasma-membrane fraction (Figure 3B).

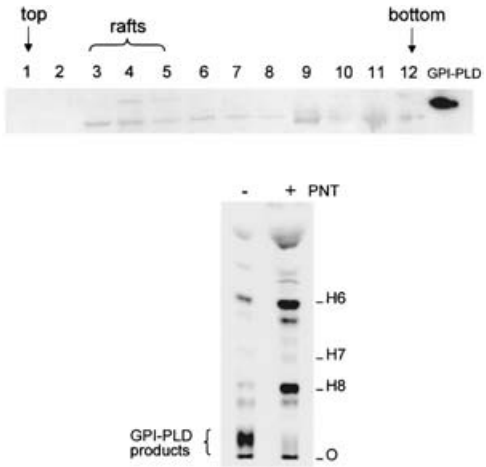

Figure 4 GPI-PLD is present and active in lipid rafts

GPI-PLD-HeLa cells were labelled with 500 μCi of [^{3}H]mannose and a Triton X-100 lysate was subjected to floatation to equilibrium. Top panel: aliquots of the sucrose gradient were subjected to Western-blot analysis using a polyclonal anti-GPI-PLD antibody. The top and the bottom of the gradient are indicated with arrows. Bottom panel: fractions of the sucrose gradient containing lipid rafts (top panel, lanes 3–5) were pooled and incubated overnight in GPI-PLD buffer in the presence and absence of PNT. ^{3}H-mannose-labelled GPI anchor intermediates were then extracted and analysed by TLC.

GPI-PLD is active in lipid rafts

The other intracellular compartment that we examined for the presence of GPI-PLD was lipid rafts. These membrane microdomains were discovered by monitoring the acquisition of detergent insolubility by GPI-anchored placental alkaline phosphatase as the enzyme travelled from the ER to the plasma membrane [34]. Since this initial report, numerous GPI-anchored proteins were found to be present preferentially in lipid rafts, and it has been shown that the anchor alone is sufficient to target GPI-anchored proteins to lipid rafts [35]. Our report, showing that GPI anchor intermediates are also concentrated in lipid rafts [8], further supports the notion that the GPI anchor targets GPI-anchored proteins to lipid rafts.

To assess the distribution of GPI-PLD activity in lipid rafts, GPI-PLD-HeLa cells were metabolically labelled with [^{3}H]mannose and rafts were prepared using a standard method, i.e. lysis of the cells in 1% Triton X-100 at 4 °C, followed by floatation to equilibrium in a discontinuous sucrose gradient. We analysed the distribution of GPI-PLD protein in 12 fractions of the sucrose gradient by Western-blot analysis. Characterization of the sucrose gradient using the prion protein (an endogenous GPI-anchored protein in HeLa cells) as a marker of rafts showed that prion was recovered in fractions 3 and 4 (results not shown). Both mature and immature (probably ER-derived) GPI-PLD species (Figure 4, top panel) were found in rafts. The distribution of GPI-PLD in the gradient indicates that, in GPI-PLD-HeLa cells, the enzyme is present in two pools: one raft-associated and the other located outside these membrane domains. The partition of GPI-PLD, normally a secreted protein, in rafts was not due to the accumulation of the enzyme in the ER, since mature forms were also present in rafts. The presence of secreted proteins without transmembrane domains in rafts, such as GPI-PLD, has been reported previously [36]. GPI-PLD may localize in raft membranes through interaction with raft components such as its substrates, GPI anchor intermediates and GPI-anchored proteins, or lectins.

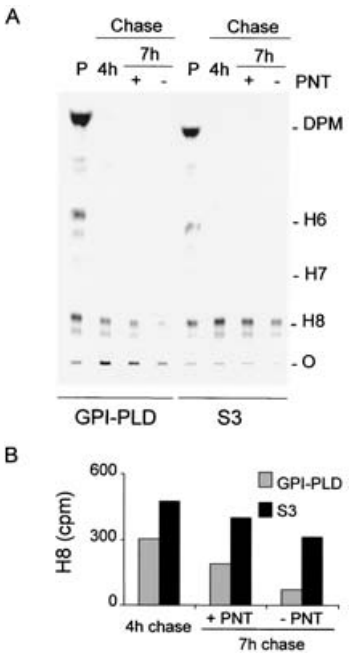

Figure 5 Increased GPI-PLD activity accelerates the metabolism of H8

(**A**) TLC analysis of lipid extracts of S3 and GPI-PLD-HeLa cells after a 1 h pulse (P) with 250 μCi of [^{3}H]mannose and 4 and 7 h chases in the presence and absence of PNT. (**B**) H8 was quantified in the two chase time points using the integration software of a TLC scanner. A representative experiment out of three is shown.

To assess the activity of GPI-PLD in raft fractions, we took advantage of endogenous ^{3}H-mannose-labelled GPI anchor intermediates present in these fractions to use them as substrates. Raft fractions were pooled and pelleted by ultracentrifugation. The membranes were then resuspended in GPI-PLD buffer and incubated overnight in the presence and absence of 500 μM PNT. TLC analysis of the samples showed that, in the absence of PNT, H6, H7 and H8 were degraded, and GPI-PLD cleavage products of GPI anchor intermediates were detected close to the origin of the plate (Figure 4, bottom panel). Quantification of H6 and H8 indicated that they decreased by 64 and 74% respectively from their levels in the presence of PNT. The presence of GPI-PLD activity in lipid rafts suggests that these membrane microdomains may be an additional cellular compartment in which GPI-PLD cleavage occurs.

GPI turnover is affected by GPI-PLD overexpression

Decay of GPI anchor intermediates during the chase procedures is probably due to the combined effects of their conversion into downstream intermediates and their degradation. Therefore, to confine the analysis to degradation, we focused on the last GPI anchor intermediate of the pathway (H8) by chasing the label for 4 h. At that point, we divided the cells into two pools and chased each pool for an additional 3 h in PBS containing 5 mM mannose and supplemented with and without 500 μM PNT. The samples with PNT were included to demonstrate that any observed decay of H8 during the chase was the result of GPI-PLD activity. By focusing on H8, we also avoid any PNT inhibitory effect on the synthesis of upstream GPI anchor intermediates [22]. After the first 4 h of chase, the only species left in both the S3 and GPI-PLD-HeLa cells was H8 (Figure 5A). Furthermore, at that point, the level of H8 in GPI-PLD-HeLa cells was already lower compared with S3 cells, despite its initial higher level in the transfectant after

the pulse. During the subsequent 3 h of chase in the absence of PNT, H8 decayed by an additional $74 \pm 4\%$ in GPI-PLD-HeLa cells, whereas in S3 cells it decreased only by $35 \pm 5\%$ (Figure 5B). PNT significantly decreased the rapid decay of H8 in the GPI-PLD-HeLa transfectant and did so to a lesser extent in S3 cells (Figure 5B), confirming that GPI-PLD is responsible for most, if not all, of the catabolism of H8 in intact cells. This effect is not due to mistargeting of GPI-PLD in GPI-PLD-HeLa cells, since we verified that the enzyme is active in the same subcellular compartments in GPI-PLD-HeLa cells as in untransfected controls (results not shown).

Most of the H8 had probably left the ER after 4 h of chase and it was probably located in either late compartments of the secretory pathway or at the plasma membrane. Therefore the GPI-PLD cleavage of H8 observed after 4 h probably occurs in late Golgi and/or just before arrival to the plasma membrane. Our measurements of intracellular H8 cleavage are probably underestimated, because H8 arriving at the cell surface is no longer susceptible to GPI-PLD due to the topology of H8 at the plasma membrane and the fact that the secreted enzyme does not cleave GPI anchor intermediates present on the cell surface. Additionally, in our experimental paradigm, we cannot detect any possible cleavage occurring in the ER. The susceptibility of H8 to GPI-PLD indicates that this GPI is luminally orientated in transport vesicles and it is delivered to the plasma membrane by a vesicular mechanism, in agreement with previous reports [9,13].

Our isolation of HeLa cells stably expressing GPI-PLD contrasts with a recent report, which concluded that stable overexpression of GPI-PLD cannot be achieved in cell lines expressing GPI-anchored proteins, probably due to cell toxicity of GPI-PLD cleavage products of GPI anchor intermediates and GPI-anchored proteins [37]. However, HeLa cells not only express normal levels of GPI-anchored proteins, but also are one of the few cell lines in which GPI anchor intermediates are easily detectable. Therefore the lack of tolerance to stable expression of GPI-PLD in Chinese-hamster ovary cells and other cell lines tested by Du et al. [37] is probably due to other mechanisms.

In summary, our characterization of GPI-PLD overexpression in HeLa cells showed that (i) the cellular levels of both GPI anchor intermediates and GPI-anchored proteins are significantly decreased when GPI-PLD activity is increased and (ii) GPI-PLD is present and active in isolated raft and ER membranes. The implications of our finding that GPI-PLD cleaves GPI anchor intermediates intracellularly are two-fold. First, it opens the possibility that GPI-PLD regulates GPI-anchored protein levels both by decreasing the amount of anchor available for transfer and by cleaving GPI-anchored proteins in the secretory pathway. Secondly, glycan fragments generated by GPI-PLD cleavage of GPI anchor intermediates as well as phosphatidic acid, produced by the cleavage of GPI anchor intermediates and GPI-anchored proteins, have the potential to be biologically active. Structurally related glycan fragments from parasite GPI anchor intermediates have been shown to elicit immune responses in cells, and glycans from mammalian GPI anchor intermediates have been implicated as an insulin mimetic in a controversial proposal [38]. Furthermore, a recent report suggested that GPI-PLD activates protein kinase Cα through the generation of diacylglycerol from phosphatidic acid, which is produced by the intracellular cleavage of GPI-anchored proteins by GPI-PLD [33]. It is tempting to speculate that GPI-PLD not only regulates intracellular levels of GPI anchor intermediates and GPI-anchored proteins but may also (i) release GPI-anchored proteins in a soluble form, so that they can exert their functions in distant locations and (ii) generate by-products that can act as second messengers.

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