Saponin-induced release of cell-surface-anchored Thy-1 by serum glycosylphosphatidylinositol-specific phospholipase D

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A glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) was purified from human serum and used for studies on the release of GPI-anchored Thy-1 glycoprotein from mouse T lymphoma cells Y191. Previous studies have shown that whereas GPI-PLD is highly active against detergent-solubilized GPI-anchored proteins, it is normally unable to release GPI-containing proteins anchored in a lipid bilayer. Confirming these findings, the addition of GPI-PLD to intact Y191 cells did not result in cleavage of Thy-1. However, pretreatment of cells with saponin, a cholesterol-sequestering agent, rendered Thy-1 susceptible to hydrolysis. Very little solubilization of GPI-containing Thy-1 occurred under these conditions. From experiments with reconstituted liposomes it was inferred that the effect of saponin on cells was to aid in the presentation of Thy-1 to GPI-PLD. Furthermore, it was concluded that cholesterol–saponin complexes formed in the membrane were not alone responsible for the effect. Rather, additional molecules in the plasma membrane are possibly involved in the presentation of Thy-1 on saponin-treated cells. This finding may have implications for a physiological role of circulating GPI-PLD in the regulation of GPI-anchored proteins on cells.

INTRODUCTION

A diverse group of plasma membrane glycoproteins are attached to the cell surface by a glycosylphosphatidylinositol (GPI) tail, including enzymes, cell-adhesion molecules, and signal-transducing receptors (for recent reviews see refs. [1–6]). The role of this type of anchoring is not known. Several suggestions have been put forward for the function of GPI on glycoproteins, such as enhancement of lateral mobility of the glycoprotein in the lipid bilayer, sorting signalling for the transport to the apical plasma membrane in polarized cells, and involvement in signal transduction in the cell. An additional possible role is to permit selective release of glycoproteins from the cell surface by the action of GPI-specific phospholipases, thereby regulating the expression of certain glycoproteins on the surface. For such a role to be operative, the susceptibility for cleavage has to be restricted to glycoproteins on certain cells at a given time.

A GPI-specific phospholipase D (GPI-PLD) has been identified in sera from various species [7,8], and subsequently isolated and characterized [9–13]. When assayed *in vitro*, this phospholipase is only active against glycosylated phosphatidylinositol, and is so only in the presence of detergents. High levels of GPI-PLD are normally present in circulation, yet most GPI-anchored glycoproteins on blood cells and on the endothelium are resistant to cleavage. Furthermore, in experiments with cultured cells and liposomes, GPI-anchored glycoproteins are not released after the addition of serum or purified GPI-PLD to the medium [14]. This suggests either that the phospholipase has to be activated (mediated by detergent in the assays), or that the substrate (GPI-containing protein) has to be correctly presented to the phospholipase, in order for cleavage to occur. In either case, such a mechanism would allow for a specific regulation of the release of GPI-anchored proteins to meet the needs of the cells.

In the present study, we have investigated the effects on GPI-PLD-mediated release of the GPI-anchored glycoprotein Thy-1 [15] from a mouse T lymphoma cell line, by changing the membrane structure with saponin. Saponins are amphiphilic molecules, isolated mainly from plants, which were classically identified by their haemolytic activity (reviewed in [16]). Saponins are known to be incorporated into lipid bilayers without solubilizing lipids or membrane proteins, and are therefore not classified as detergents. The haemolytic effect is caused by the generation of pores in the membrane, which is the result of complex formation with cholesterol [17–19]. The results in the present paper show that, upon treatment of cells with saponin, Thy-1 in the membrane becomes susceptible to cleavage by GPI-PLD. Experiments using liposomes indicate that the positive effect of saponin is due to alteration of the membrane structure, and not to a stimulation of the enzyme. Furthermore, conditions favourable for saponin-mediated GPI-PLD cleavage could not be mimicked by cholesterol/phosphatidylcholine-containing liposomes. This finding raises the possibility that endogenous substances may exist in cells that regulate the presentation of GPI-anchored proteins for circulating GPI-PLD.

MATERIALS AND METHODS

Assay of GPI-PLD

The substrate for GPI-PLD was prepared by iodination of Thy-1 purified from human brain [20] or mouse thymocytes [21]. The two preparations were indistinguishable in terms of susceptibility to GPI-PLD cleavage. Approx. 3 μg of Thy-1 was iodinated with ^{125}I (17 Ci/mg, Du Pont) by the chloramine T procedure [22] to a specific radioactivity of approx. 100 μCi/μg of protein. Before use, the substrate was purified further by preparative SDS/PAGE on 10% (w/v) polyacrylamide rod gels [23], and ^{125}I-Thy-1 was eluted from gel pieces in 0.5% Nonidet P-40. This stock solution of substrate was stored in the refrigerator for up to 2 weeks, and was used in the assay after a 25-fold dilution in D-buffer (50 mM Tris/HCl, 10 mM NaCl, 5 mM $CaCl_2$, pH 7.4), giving a final

Abbreviations used: GPI, glycosylphosphatidylinositol; PLC, phospholipase C; PLD, phospholipase D; GPI-PLD, GPI-specific PLD.
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concentration in the assay of 0.01 % Nonidet P-40 and traces of SDS (from the polyacrylamide gel).

Samples to be measured were diluted to 50 μl with D-buffer and mixed with 50 μl of substrate (10–20 nCi) in Eppendorf tubes. After incubation for 30 min at 37 °C, the reaction was stopped by the addition of 800 μl of 10 mM EDTA in PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). A 1:1 suspension (100 μl) of phenyl-Sepharose (Pharmacia-LKB) in PBS was added and the tubes were incubated by end-to-end rotation for 30 min at room temperature. After a brief centrifugation (10000 *g* for 30 s), 800 μl of the supernatants was counted in the γ-counter. Blank samples contained 50 μl of D-buffer and 50 μl of substrate, and were treated identically to GPI-PLD-containing samples.

The percentage hydrolysis was calculated by dividing the radioactivity of the sample supernatant by (0.8 × the radioactivity added to each tube). The radioactivity of the blank supernatant (containing about 10% of the added radioactivity) was subtracted from the samples to give percentage specific hydrolysis. A milliunit of GPI-PLD activity was defined as the activity giving 20 % specific hydrolysis in the assay. This assay is referred to as the 'GPI-PLD assay' in this paper.

Purification of GPI-PLD and production of antibodies

GPI-PLD was purified to homogeneity from fresh human plasma, from which plasminogen had been removed for other purposes. The details of the purification procedure will be described elsewhere. Briefly, after removal of fibrinogen by clotting, the serum was purified by chromatography on DEAE-Sephacel at pH 6.0, phenyl-Sepharose, DEAE-Sephacel at pH 8.0, and gel filtration on a Sephacryl S-300 column (all matrices were obtained from Pharmacia-LKB). The purification was followed by the GPI-PLD assay described above and by SDS/PAGE [23]. The GPI-PLD activity was enriched approx. 6000-fold. N-terminal-sequence analysis revealed a single amino acid in each cycle and showed identity to a previously reported N-terminal sequence of human GPI-PLD [13]. The purified enzyme was stored at −20 °C in 50 % (v/v) glycerol/10 mM Tris/HCl/75 mM NaCl, pH 7.4.

Antiserum was produced by immunizing a rabbit with purified GPI-PLD, using a procedure described before [20]. IgG was purified on a protein A–Sepharose column (Pharmacia-LKB). GPI-PLD-free serum was prepared by incubating fresh human serum with rabbit anti-(human GPI-PLD) IgG coupled to Sepharose. The absence of GPI-PLD activity was confirmed by the GPI-PLD assay.

Metabolic labelling of cells with [^{3}H]ethanolamine and analysis of Thy-1 released after phospholipase treatment

The T lymphoma cell line Y191 [24] was grown in RPMI 1640 medium supplemented with 10% (v/v) fetal-calf serum and antibiotics. Cells (1–5 × 10^6 per ml) were labelled in fresh medium containing 5 μCi/ml [^{3}H]ethanolamine (28 Ci/mmol, Amersham) for 16 h, after which time the cells were washed twice with H-buffer (20 mM Hepes, 150 mM NaCl, 1 mM $CaCl_2$, 0.1 % BSA, pH 7.2). In order to obtain the required amount of cells, in some experiments washed unlabelled cells were added. The cells were suspended in H-buffer containing 2 μM leupeptin and 2 μM pepstatin, and adjusted to 1.2 × 10^8 cells/ml in H-buffer. In some experiments saponin (isolated from *Gypsophila*, Sigma Chemical Co.), dissolved in H-buffer, was added and the cells were incubated on ice for 30 min. To 50 μl of cells (approx. 10 × 10^7 c.p.m.) was added serum, GPI-PLD, or phosphatidylinositol phospholipase C (*Bacillus cereus*, Boehringer–Mannheim) (PLC), all diluted in H-buffer, and the volume was adjusted to 100 μl with H-buffer. The concentration of saponin described in the experiments refers to the final concentration obtained after adjustment to 100 μl. PLC activity was used as defined by the supplier. The cells were incubated for 1 h at 37 °C with gentle agitation and centrifuged at 4 °C for 5 min at 350 *g*, and the supernatant was collected. The supernatant was re-centrifuged twice for 10 min at 10000 *g*.

Thy-1 was immunoprecipitated from the supernatants using a previously described procedure [25], with the exception that protein A–Sepharose was used instead of Pansorbin cells to collect immune complexes. Rabbit anti-(mouse Thy-1) serum (15 μl) [24] was added to each supernatant and the mixture was incubated overnight. Immunoprecipitated material was analysed by SDS/PAGE (12.5 % acrylamide), and the gels were treated for fluorography with Enlightning (Du Pont) and exposed to Hyperfilm MP (Amersham). Radioactive Thy-1 was quantified by scintillation counting after treatment of gel pieces with Solvable (Du Pont), as described elsewhere [25].

The pool of Thy-1 on the surface of Y191 cells that was susceptible to cleavage with PLC was found to be approx. 70 %, which is consistent with results from other cells [26,27].

Triton X-114 partitioning

Phase separation of amphiphilic and hydrophilic molecules was performed with pre-condensed Triton X-114 as described by Bordier [28]. After three cycles of partitioning, Thy-1 was immunoprecipitated from the detergent phase and the water phase as described above.

To ensure that no cleavage of Thy-1 occurred during the phase separation, a control experiment was performed in which fresh serum (final concentration 2 GPI-PLD units/ml) was added to ^{125}I-labelled amphiphilic Thy-1 in 2 % (w/v) Triton X-114. No hydrophilic Thy-1 was generated during the subsequent partitioning. Experiments with the GPI-PLD assay showed that the enzyme is completely inhibited in high concentrations of Triton X-114.

Liposome experiments

Liposomes were made by the method of Brunner et al. [29]. Phosphatidylcholine (30 mg) (egg yolk, type V-E, Sigma Chemical Co.) was dissolved in ethanol/chloroform (1:1, v/v) and dried as a film together with 37.5 mg of sodium cholate (Larodan Fine Chemicals). In some experiments 40 mg of cholesterol (Larodan Fine Chemicals) was included. After two cycles of dissolving in ethanol and drying, the mixture was dissolved in 1 ml of L-buffer (10 mM Tris/HCl, 100 mM NaCl, pH 7.3). After brief centrifugation (1500 *g* for 5 min), 50–100 ng of ^{125}I-Thy-1 (approx. 5 × 10^6 c.p.m.) was added to the supernatant. The solution was applied to a column (20 × 0.8 cm) of Sephadex G-50 medium (Pharmacia-LKB), equilibrated with L-buffer, and was eluted with a flow rate of 2 ml/h. Formed liposomes with incorporated ^{125}I-Thy-1 were obtained in the void volume. In order to isolate small liposomes, the void volume eluate from the Sephadex G-50 column was gel-filtered on a column (25 cm × 1 cm) of Sepharose 4B (Pharmacia-LKB), equilibrated with L-buffer, at a flow rate of 6 ml/h. A homogeneous peak of radioactivity, which was eluted after a minor void volume peak, was collected and used in further experiments. Liposomes were stored at 4 °C and used within 3 weeks.

Phosphatidylcholine content was determined by the method of

Steward as described in ref. [30]. Cholesterol was determined using a test kit manufactured by Boehringer–Mannheim. In liposomes containing both phosphatidylcholine and cholesterol, the molar ratio obtained was 2:1.

Liposomes (450 μg of total lipid) were treated with saponin in L-buffer containing 5 mM $CaCl_2$ for 30 min on ice in a total volume of 355 μl. After addition of PLC, GPI-PLD, or serum, and adjustment of the volume to 374 μl, the mixture was incubated for 1 h at 37 °C. EDTA (40 μl; 0.1 M) was added and the samples were immediately analysed by gel filtration on columns (25 cm × 1 cm) of Sephacryl S-300 (Pharmacia-LKB), equilibrated with L-buffer. The flow rate was 12 ml/h, and fractions (1 ml) were collected and counted for radioactivity. The columns were regenerated by washing with 1% Nonidet P-40 and re-equilibrated with L-buffer.

Control experiments showed that hydrophilic ^{125}I-Thy-1 (released by PLC) was fully recovered (yield > 95%) during gel filtration, whereas the yield of liposome-bound ^{125}I-Thy-1 was 60–80%. Therefore, for the calculation of percentage hydrolysed ^{125}I-Thy-1, the material in fractions corresponding to hydrophilic ^{125}I-Thy-1 (fractions 13–19, see Figure 5b) was compared with the radioactivity applied to the column.

To determine the fraction of ^{125}I-Thy-1 that was located on the outside of the liposomes, excess Pronase digestions (10 μg/ml Pronase in L-buffer containing 5 mM $CaCl_2$, for 1 h at 37 °C) were performed and the material was analysed by gel filtration as above. Pronase-digested radioactive material was eluted in fractions 20–30.

RESULTS

Measurement of enzyme activity and analysis of purified GPI-PLD and antibodies

For the determination of GPI-PLD activity in serum and during purification of the enzyme, an assay was developed based on the conversion of purified amphiphilic Thy-1 into a form lacking the GPI structure. Samples to be measured were incubated together with ^{125}I-labelled Thy-1 in detergent, and the generated hydrophilic ^{125}I-Thy-1 was determined after adsorbing any uncleaved substrate to phenyl-Sepharose. The response was linear up to the point where approx. 30% of the substrate was hydrolysed (Figure 1a). The activity resulting in 20% specific hydrolysis in 30 min at 37 °C was defined as 1 m-unit. Fresh human serum samples showed activities of 30–70 units/ml. Analysis of generated hydrophilic ^{125}I-Thy-1 by SDS/PAGE and autoradiography showed a mobility indistinguishable from that of uncleaved Thy-1 (results not shown). This is consistent with previous reports showing that removal of the GPI anchor from Thy-1 does not influence its migration in SDS/PAGE [27]. The result also shows that the generation of radioactive hydrophilic product was not due to proteolytic fragmentation.

GPI-PLD was purified from human plasma by a series of chromatography steps, yielding a uniform band of apparent molecular mass 120 kDa when analysed by SDS/PAGE (Figure 1b). N-terminal sequence analysis gave a single amino acid derivative in each cycle, indicating that a homogeneous protein was obtained. Of ten identified amino acids, nine were identical to the N-terminal sequence of bovine serum GPI-PLD, derived from the cDNA sequence [12], confirming the relationship between the two enzymes. The sequence also showed identity to a recently published N-terminal sequence of human GPI-PLD [13].

Antibodies were produced by immunizing a rabbit with purified GPI-PLD. The antibodies were found to react predominantly with conformation-dependent epitopes, as treatment of the enzyme with SDS irreversibly destroyed the reactivity (results not shown). When tested on native GPI-PLD in serum, the antibodies effectively inhibited the enzyme activity (Figure 1c). This result shows that the only activity in serum capable of generating hydrophilic Thy-1, under the conditions used in the assay, is GPI-PLD. Complete inhibition was obtained at 20–100-fold excess (mol/mol) of IgG over GPI-PLD.

GPI-PLD-mediated release of Thy-1 after treatment of cells with saponin

The cell line used in the present study was a mouse T-cell lymphoma, designated Y191, which in a previous study was shown to express large amounts of Thy-1 on the cell surface [24]. [^{3}H]Ethanolamine, which is biosynthetically incorporated into the GPI moiety, was used to label Thy-1. By this means, a label was achieved that is retained in Thy-1 after cleavage of the hydrophobic tail, and at the same time a low background in subsequent immunoprecipitations was obtained.

When labelled cells were incubated together with purified GPI-

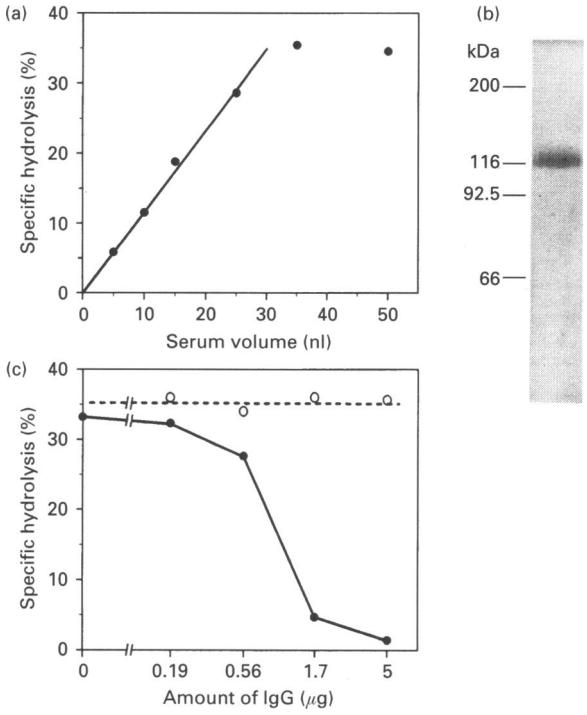

Figure 1 Analysis of GPI-PLD

(**a**) GPI-PLD activity was assayed by hydrolysis of the hydrophobic anchor of ^{125}I-Thy-1 in a Nonidet P-40-containing buffer. The amount of generated hydrophilic ^{125}I-Thy-1 was measured by γ-counting after removing uncleaved substrate by incubation with phenyl-Sepharose. Specific hydrolysis was calculated as unadsorbed radioactivity (c.p.m.) relative to total radioactivity (c.p.m.) added to each tube, minus background levels. Fresh human serum was diluted in buffer and adjusted to 50 μl, to which was added 50 μl of ^{125}I-Thy-1 (approx. 20000 c.p.m.). The response was linear up to approx. 30% specific hydrolysis. (**b**) Purified GPI-PLD was analysed by SDS/PAGE [7% (w/v) polyacrylamide] and visualized by silver staining. Migration of molecular mass standards is shown to the left. (**c**) Inhibition of ^{125}I-Thy-1-hydrolysing activity in serum by rabbit antibodies raised to purified GPI-PLD. Samples (5 μl) of human serum were incubated for 1 h at room temperature with 5 μl of $\log_3$ dilutions of protein A-purified IgG, or without IgG. Remaining GPI-PLD activity was measured by the assay described in (**a**), after a 500-fold dilution. ●, Anti-(GPI-PLD) IgG; ○, pre-immune IgG. The highest amount of IgG (5 μg) corresponds approx. to a 100-fold excess over GPI-PLD (mol/mol).

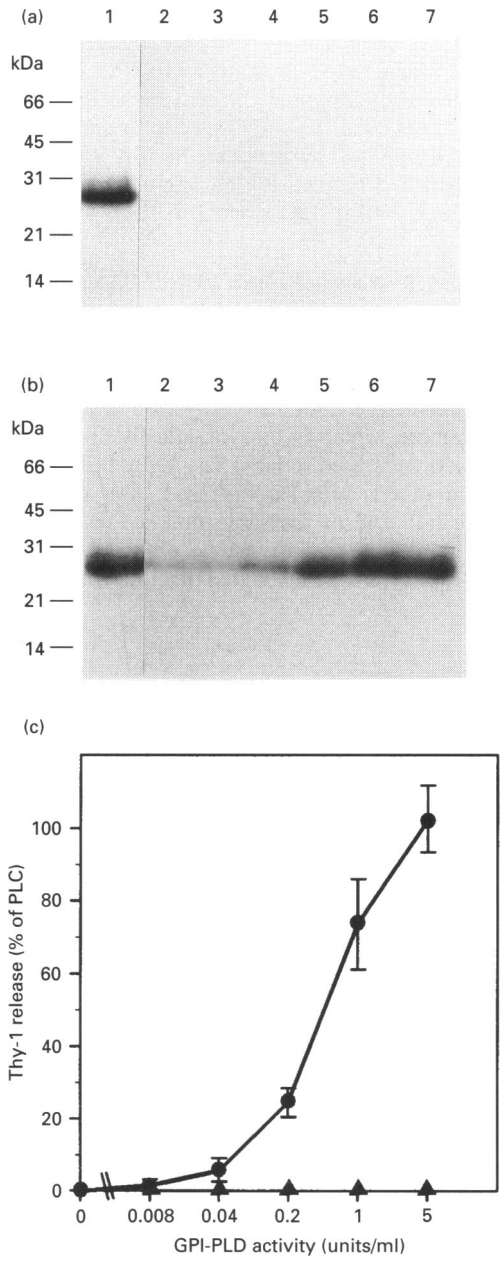

Figure 2 GPI-PLD-mediated release of Thy-1 from cells after addition of saponin

Y191 cells were labelled overnight with [^{3}H]ethanolamine and pre-incubated for 30 min on ice without (**a**) or with (**b**) saponin (final concentration 0.2 mg/ml). The cells were then incubated for 1 h at 37 °C with 0.05 units/ml PLC (lane 1), no enzyme (lane 2), and with 0.04 units/ml (lane 3), 0.2 units/ml (lane 4), 1.0 units/ml (lane 5), 5.0 units/ml (lane 6), and 25 units/ml (lane 7) of purified GPI-PLD. Cells were pelleted and Thy-1 was immunoprecipitated from the supernatants and analysed by SDS/PAGE, followed by fluorography. The graph in (**c**) shows quantification of immunoisolated [^{3}H]Thy-1 by scintillation counting of gel pieces. Key to symbols: ●, with saponin; ▲, without saponin. The release of Thy-1 from cells by GPI-PLD is expressed as a percentage of the release with an excess of PLC, after subtraction of background release (without enzyme). The background level was 5–7% with saponin and negligible without saponin. The data shown are the results (means ± S.D.) of three separate experiments.

PLD, no release of Thy-1 to the medium could be detected (Figure 2a, lanes 3–7, and Figure 2c, triangles). This is consistent with previous reports showing that GPI-anchored proteins bound

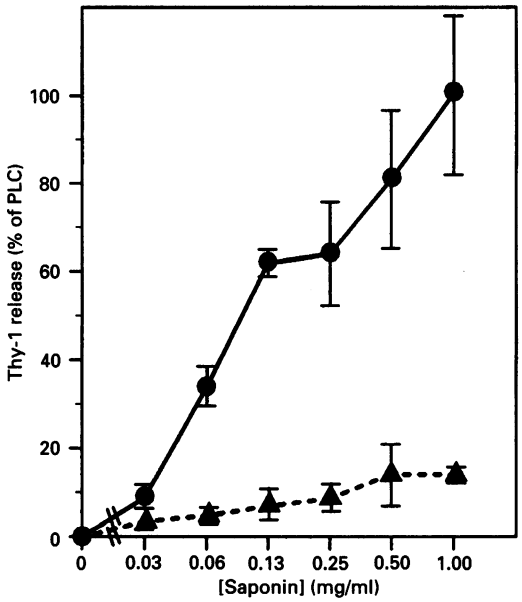

Figure 3 Effect of saponin concentration on the release of Thy-1

[^{3}H]Ethanolamine-labelled Y191 cells were incubated with a series of $\log_2$ dilutions of saponin, or without saponin. After 30 min on ice, GPI-PLD was added to a concentration of 1 unit/ml (●), or no enzyme was added (▲), and the samples were incubated for 1 h at 37 °C. Cells were pelleted, and Thy-1 was immunoprecipitated from the supernatants and analysed by SDS/PAGE and quantified. The release of Thy-1 was expressed as a percentage of the release with PLC, measured in a separate incubation of cells without saponin treatment. The data shown are the results (means ± S.D.) of three separate experiments.

to the cell surface are practically resistant to GPI-PLD [9,14]. In contrast, bacterial phosphatidylinositol-specific PLC, which is commonly used to hydrolyse GPI-anchored proteins on plasma membranes (see for example [31]), readily released Thy-1 (lane 1 in Figures 2a and 2b).

When cells were treated with saponin (0.2 mg/ml), most Thy-1 was retained in the membrane and could be pelleted by centrifugation, leaving only small amounts in the supernatant (Figure 2b, lane 2). However, when GPI-PLD was added to saponin-treated cells, efficient hydrolysis of Thy-1 was achieved (Figure 2b, lanes 3–7, and Figure 2c, circles). Control experiments with Triton X-114 phase partitioning [28] showed that the effect was due to a true cleavage reaction, and not the result of solubilization. Conversely, the small amount of Thy-1 appearing in the supernatant without enzyme present (Figure 2b, lane 2) behaved as native (GPI-containing) Thy-1 in phase partitioning (results not shown). The GPI-PLD activity required to release most of the PLC-releasable Thy-1 was significantly higher than that required for GPI hydrolysis in the GPI-PLD assay where detergent is present. Nevertheless, hydrolysis of Thy-1 on saponin-treated cells occurred with GPI-PLD activities far less than that present in serum (approx. 50 units/ml). Maximal hydrolysis was obtained at approx. 5 units/ml, and addition of higher enzyme activities did not result in further release (see lanes 6 and 7, Figure 2b).

To test for the dependence of saponin concentration on the GPI-PLD-mediated hydrolysis, cells were incubated with increasing concentrations of saponin to which was added GPI-PLD at a fixed concentration (1 unit/ml) (Figure 3, closed circles). The efficiency of hydrolysis was augmented with increasing saponin concentration. Background release of Thy-1 in the presence of saponin gradually increased up to approx. 15%

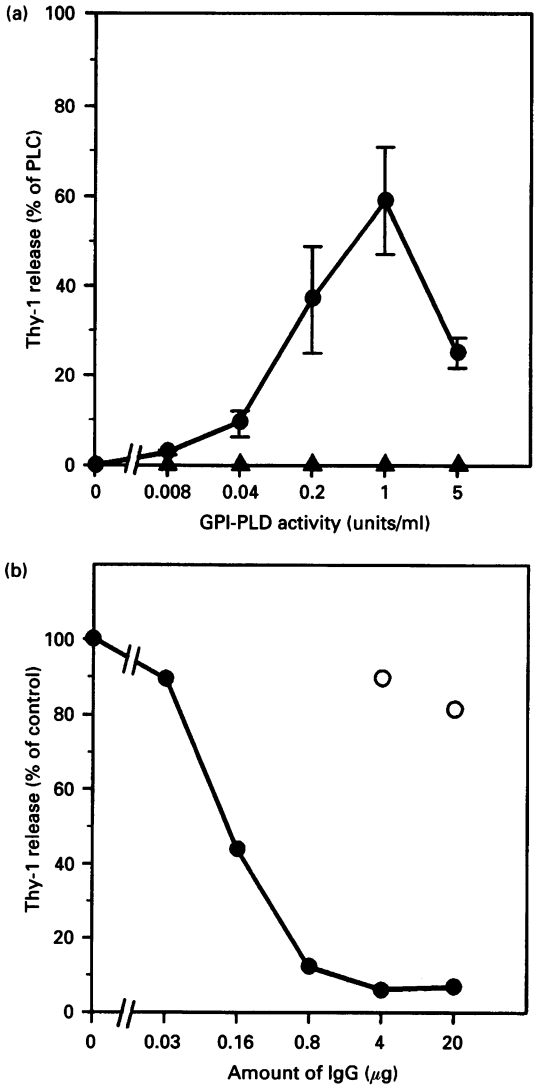

Figure 4 Release of Thy-1 from saponin-treated cells by GPI-PLD in serum

Y191 cells were labelled with [^{3}H]ethanolamine and treated with or without saponin (final concentration 0.2 mg/ml) as described in the legend to Figure 2. (**a**) Cells were incubated for 1 h at 37 °C in the presence of different dilutions of human serum, yielding the indicated GPI-PLD activities as measured by the GPI-PLD assay (see the Materials and methods section). One unit/ml corresponds to a 1:50 dilution of serum. Released Thy-1 was measured as in Figure 2. ●, With saponin; ▲, without saponin. The data shown are the results (means ± S.D.) of three separate experiments. (**b**) Human serum was diluted to give a final activity in cell incubations of 0.2 unit/ml. Diluted serum was pre-incubated for 1 h on ice with indicated amounts of protein A-purified IgG. The mixture were added to saponin-treated cells and the samples were incubated for 1 h at 37 °C. Thy-1 was immunoprecipitated from supernatants and analysed as in (**a**). The release of Thy-1 was expressed as a percentage of control levels (without IgG). ●, Anti-(GPI-PLD) IgG; ○, pre-immune IgG.

at the highest concentration tested (1 mg/ml) (closed triangles in Figure 3). In the same experiments, the leakage of intracellular [^{3}H]ethanolamine-labelled molecules was monitored by scintillation counting of the medium before immunoprecipitation. This leakage gives an indication of the extent of pore formation in the plasma membrane caused by saponin [19]. It was found that pores were generated at lower saponin concentrations (maximal leakage reached at 0.06 mg/ml) than that which stimulated GPI-PLD hydrolysis of Thy-1 (results not shown). This finding indicates that the two processes are not directly correlated.

To confirm that the effect of saponin was specific, and not due to a general 'detergent effect' on the membrane, the same experiment as shown in Figure 3 was performed with Nonidet P-40 instead of saponin. At concentrations where low levels of solubilization was obtained (< 0.5 mg/ml), no stimulatory effect on GPI-PLD-mediated hydrolysis was detected with Nonidet P-40 (results not shown).

Effect of addition of serum to saponin-treated cells

Experiments were performed to see if the results with purified GPI-PLD were also reproducible with crude serum as the source of enzyme (Figure 4a). The addition of serum to untreated cells gave no release of Thy-1 (closed triangles in Figure 4a). With saponin-treated cells (0.2 mg/ml saponin) (closed circles in Figure 4a), release was achieved with an optimum at a serum dilution of 1:50 (1 unit/ml GPI-PLD activity as measured in the GPI-PLD assay). At higher concentrations of serum the response was decreased. The reason for this serum-dependent decrease of Thy-1 release at high serum concentrations is not known, but may be due to the possibility that the effective concentration of saponin is lowered as the result of binding to components in serum, such as lipoproteins.

The possibility that activities in serum other than GPI-PLD are able to release Thy-1 from saponin-treated cells was ruled out by the experiment shown in Figure 4(b). When serum was pre-treated with antibodies against purified GPI-PLD, the release was totally abolished. This experiment shows that GPI-PLD is the only enzyme in serum responsible for the release of Thy-1 from saponin-treated cells.

Liposome experiments

The results presented so far show that saponin has a stimulatory effect on the hydrolysis of GPI-anchored Thy-1 on the plasma membrane. This outcome could be mediated by different possible mechanisms. The effect could be at the enzyme level, where saponin either directly stimulates the activity of GPI-PLD, or removes an inhibiting substance. In the GPI-PLD assay, Nonidet P-40 would perform the same role. Alternatively, the effects of saponin could be at the substrate level. In this case, saponin could either assist in the presentation of the substrate for the enzyme, or relieve the inhibition caused by a blocking component in the membrane. Some of these possibilities can be tested by saponin- and GPI-PLD-treatment of reconstituted artificial liposomes with defined lipid composition.

Liposomes with incorporated ^{125}I-Thy-1 were treated in various ways and the release of Thy-1 was determined by gel filtration, which separates liposome-bound radioactivity from hydrophilic released radioactivity (Figure 5). Untreated phosphatidylcholine liposomes eluted as a homogeneous peak in or near the void volume of the column (fractions 8–10, Figure 5a). Cleavage with PLC (Figure 5b) resulted in an additional peak (fractions 14–16) representing released ^{125}I-Thy-1. Taking into account that not all of the ^{125}I-Thy-1 was located on the outside of the liposomes (see legend to Table 1), it could be calculated that approx. 70% of exposed Thy-1 was cleaved by PLC. This is in agreement with previous results on the susceptibility of Thy-1 to PLC hydrolysis [27]. GPI-PLD treatment of liposomes resulted in minute amounts of hydrophilic Thy-1 being generated (Figure 5c). The extent of hydrolysis by GPI-PLD was not increased by pretreatment of liposomes with saponin (Figure 5d). Taken together, these results clearly indicate

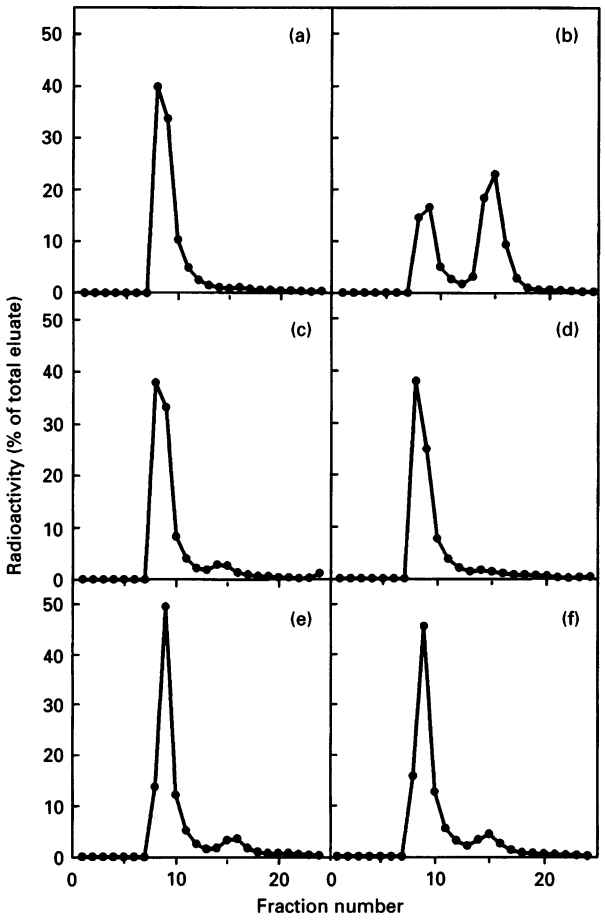

Figure 5 Susceptibility of liposome-bound ^{125}I-Thy-1 to phospholipases

Liposomes containing ^{125}I-Thy-1 were made from phosphatidylcholine (**a–d**) or from a mixture of cholesterol and phosphatidylcholine (final molar ratio 1:2) (**e**, **f**). Liposomes were incubated with phospholipase in the absence (**a–c**, **e**) or in the presence of 0.2 mg/ml saponin (**d**, **f**). (**a**), No enzyme; (**b**), PLC (0.05 units/ml); (**c**), GPI-PLD (5 units/ml); (**d–f**), GPI-PLD (1 unit/ml). Samples were analysed by gel-filtration chromatography on columns of Sephacryl S-300 and eluted fractions were counted for radioactivity.

Table 1 Phospholipase cleavage of liposome-bound ^{125}I-Thy-1

Liposomes containing ^{125}I-Thy-1 were made from phosphatidylcholine (PC), or from a mixture of PC and cholesterol (PC/CH). Liposomes were incubated with or without saponin, serum and purified phospholipase, as described in the Materials and methods section. The samples were analysed by gel filtration chromatography on Sephacryl S-300 as shown in Figure 5. Hydrolysis was calculated as the amount of radioactivity which was eluted in fractions 13–19, relative to total Pronase-accessible radioactivity applied to the column. The Pronase-accessible radioactivity was 64% and 73% for PC liposomes and PC/CH liposomes respectively.

Liposomes	Saponin concn. (mg/ml)	Enzyme concn. (units/ml)		Hydrolysis (%)
PC	–	–		6
PC	–	PLC	(0.05)	73
PC	–	GPI-PLD	(0.2)	7
PC	–	GPI-PLD	(1.0)	8
PC	–	GPI-PLD	(5.0)	11
PC	–	Serum	(0.2)	10
PC	–	Serum	(1.0)	16
PC	–	Serum	(5.0)	22
PC	0.2	–		7
PC	0.2	PLC	(0.05)	60
PC	0.2	GPI-PLD	(1.0)	9
PC	0.2	Serum	(1.0)	14
PC	0.8	–		8
PC	0.8	PLC	(0.05)	62
PC	0.8	GPI-PLD	(1.0)	23
PC	0.8	Serum	(1.0)	30
PC	0.8	GPI-PLD-free serum*		16
PC/CH	–	–		4
PC/CH	–	GPI-PLD	(1.0)	12
PC/CH	–	Serum	(1.0)	17
PC/CH	0.2	GPI-PLD	(1.0)	12
PC/CH	0.2	Serum	(1.0)	18
PC/CH	0.8	–		13
PC/CH	0.8	PLC	(0.05)	63
PC/CH	0.8	GPI-PLD	(1.0)	21
PC/CH	0.8	Serum	(1.0)	27
PC/CH	0.8	GPI-PLD-free serum*		10

* GPI-PLD-free serum was added to a protein concentration corresponding to active serum at 1.0 units/ml.

that saponin does not act at the level of enzyme stimulation or removal of inhibition, as GPI-PLD is fully active under the same conditions as shown in Figure 5(d) when added to cells (see Figure 2c).

The probable mechanism for the effects of saponin is that it interferes with the structure of the plasma membrane in cells, resulting in a presentation of Thy-1 which is favourable for GPI-PLD hydrolysis. If this is true, one has to postulate that other components in the membrane are also participating in this process, as saponin treatment of phosphatidylcholine liposomes gave no effect. An obvious candidate for this is cholesterol, which is known to bind saponin in cell membranes [18]. Liposomes were made containing phospholipid and cholesterol in a proportion comparable with that of plasma membranes [32], into which was incorporated ^{125}I-Thy-1. However, GPI-PLD was not able to hydrolyse Thy-1 to any significant extent, neither on intact liposomes (Figure 5e) nor on saponin-treated liposomes (Figure 5f). Therefore, other components in cell membranes may be important for saponin-mediated GPI-PLD hydrolysis of Thy-1.

In Table 1 the results from a variety of experiments with liposomes are shown. In these results the extent of hydrolysis is expressed as the percentage of radioactivity eluting in fractions corresponding to the PLC-cleaved product (see Figure 5b). The background level in these fractions, obtained from experiments with no enzyme added, was 4–13%. Under no condition tested was the release with GPI-PLD comparable with that of PLC. Serum gave consistently higher release than the same activity of purified GPI-PLD. This can partly be explained by the fact that serum (GPI-PLD-free) gives a higher background level in the gel-filtration assay. Nevertheless, serum was ineffective in hydrolysing Thy-1 on liposomes, with or without saponin treatment.

DISCUSSION

This report describes for the first time conditions needed to alter the structure of the plasma membrane of a cell to allow cleavage of a GPI-anchored protein by GPI-PLD. This and other studies [9,14] have shown that in intact membranes, both on cells and in artificial liposomes, the GPI moiety is protected from cleavage by GPI-PLD. Low and Huang [14] tested several cell lines, and a variety of liposomes with different lipid compositions, to

investigate the requirements for GPI-PLD cleavage of alkaline phosphatase and trypanosome variant surface glycoprotein. In no instance was a significant release detected. These results cast doubts on the role of circulating GPI-PLD for the release of GPI-containing proteins *in vivo*. However, several proteins which are normally anchored to the cell surface by a GPI moiety, are known to exist also as soluble forms in various body fluids (see ref. [3]). We previously characterized a soluble form of Thy-1 present in human cerebrospinal fluid, and found that it contained the glycan moiety of GPI, but lacked the phosphatidic acid part. This is consistent with release by an endogenous GPI-PLD [27]. Although GPI-anchored proteins are resistant to cleavage under 'normal' conditions, it is possible that, under certain circumstances, alterations of the membrane structure render GPI-containing proteins susceptible to phospholipase cleavage.

Although saponins are not found in higher organisms, and can therefore not be physiological regulators of GPI-anchored protein presentation, the elucidation of the mechanism by which saponin exerts its effect *in vitro* may give some hints to a possible regulatory mechanism that is normally operating. Saponins are known to preferentially bind and sequester cholesterol in membranes, but binding to cholesterol-free membranes has also been detected [33]. However, from the results with liposomes in the present study, the conclusion can be drawn that saponin–cholesterol complexes are not alone responsible for the susceptibility of Thy-1 to GPI-PLD. Other components in plasma membranes are likely to be important for correct presentation of the substrate. The nature of these components is completely unknown.

The resistance of GPI-anchored proteins in membranes to GPI-PLD hydrolysis is reminiscent of the situation for other phospholipases acting on membranes. The best-studied example is that of phospholipase A_2, where crystal-structure determinations have pointed to a possible mechanism of action on the phospholipid membrane [34,35]. In this case the substrate (phospholipid) seems to be drawn partly out of the membrane surface into the active site of the enzyme. It may be envisioned that GPI-PLD is using a similar mechanism to 'reach' its substrate, and that components in the membrane can stimulate or hamper this process.

Evidence from several recent studies has suggested that GPI-anchored proteins are clustered in microdomains rich in glycosphingolipids and cholesterol (reviewed in [36]). These microdomains are often associated with uncoated invaginations, called caveolae, which are involved in the uptake of small substances into cells [6]. The maintenance of both GPI-containing protein clusters and caveolae depends on the presence of cholesterol in the membrane. Depletion of cholesterol, either by the addition of cholesterol-binding substances (saponins and polyene macrolide antibiotics) or by growth of cells in the presence of an inhibitor of sterol synthesis, caused a dispersal of GPI-anchored proteins on the membrane [37,38]. This finding implies that the clustering of GPI-anchored proteins is controlled by lipid–lipid interactions between the fatty alkyl/acyl chains of the GPI anchor and the surrounding lipids in the bilayer. This phenomenon can be directly related to the susceptibility of GPI-anchored proteins to GPI-PLD.

We have also tested a cholesterol-binding polyene macrolide, nystatin, for effects on GPI-PLD release of Thy-1. This substance, tested at concentrations up to 0.4 mg/ml, had no influence on the susceptibility of the GPI-anchored protein to the enzyme (results not shown). Even though both saponins and macrolides disperse GPI-anchored proteins in the membrane [37], the mechanism of action may be different (e.g. affecting other molecules than cholesterol differently) due to their dissimilar structures. Further studies are required to investigate the role of cholesterol and other molecules in microdomains in more detail.

In order for GPI-PLD-mediated cleavage of a GPI-anchored protein to occur, it is possible that the GPI-containing protein has to be dispersed on the membrane and allowed to interact with (a) lipid or protein component(s) that facilitate the presentation to GPI-PLD. This presentation, which could be mediated by glycosphingolipids, may promote the accessibility of the substrate, perhaps in a process similar to that described for phospholipase A_2. When normal concentrations of cholesterol are present, tight clustering occurs, which hampers the presentation. Removal of cholesterol by saponin (or by some unknown endogenous molecule *in vivo*) would disrupt the cluster in such a way that the GPI-anchored protein becomes susceptible to cleavage. This model would explain why artificial liposomes are poor at presenting GPI-anchored proteins, both in the absence and presence of cholesterol and saponin. It will be interesting to see if certain components in membranes can be identified that, when incorporated into liposomes, can promote the cleavage of GPI-anchored proteins by GPI-PLD.

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