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Factors affecting the ability of glycosylphosphatidylinositol-specific phospholipase D to degrade the membrane anchors of cell surface proteins

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Mammalian serum and plasma contain high levels of glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD). Previous studies with crude serum or partially purified GPI-PLD have shown that this enzyme is capable of degrading the GPI anchor of several purified detergent-solubilized cell surface proteins yet is unable to act on GPI-anchored proteins located in intact cells. Treatment of intact ROS17/2.8, WISH or HeLa cells (or membrane fractions prepared from them) with GPI-PLD purified from bovine serum by immunoaffinity chromatography gave no detectable release of alkaline phosphatase into the medium. However, when membranes were treated with GPI-PLD in the presence of 0.1 % Nonidet P-40 substantial GPI anchor degradation (as measured by Triton X-114 phase separation) was observed. The mechanism of this stimulatory effect of detergent was further investigated using [^{3}H]myristate-labelled variant surface glycoprotein and human placental alkaline phosphatase reconstituted into phospholipid vesicles. As with the cell membranes the reconstituted substrates exhibited marked resistance to the action of purified GPI-PLD which could be overcome by the inclusion of Nonidet P-40. Similar results were obtained when crude bovine serum was used as the source of GPI-PLD. These data indicate that the resistance of cell membranes to the action of GPI-PLD is not entirely due to the action of serum or membrane-associated inhibitory factors. A more likely explanation is that, in common with many other eukaryotic phospholipases, the action of GPI-PLD is restricted by the physical state of the phospholipid bilayer in which the substrates are embedded. These data may account for the ability of endothelial and blood cells to retain GPI-anchored proteins on their surfaces in spite of the high levels of GPI-PLD present in plasma.

INTRODUCTION

A large number of cell surface proteins are attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor (reviewed by Low, 1989; Cross, 1990). Although the precise physiological role played by the GPI anchor remains to be defined, it has been suggested that release of proteins by specific anchor-degrading enzymes may provide the cell with a novel mechanism for modulating the expression of proteins at the cell surface. Indeed, proteins which are anchored by this method are sensitive to release from the cell surface by the action of bacterial phosphatidylinositol-specific phospholipase C (PI-PLC). Using a variety of purified detergent-solubilized GPI-anchored proteins as substrates it has proved possible to identify several phospholipases from eukaryotic organisms which are capable of degrading GPI anchors. All of these phospholipases are GPI-specific and may therefore participate in release of GPI-anchored proteins from cellular surfaces. The parasite *Trypanosoma brucei* contains a membrane-associated GPI-specific PLC (GPI-PLC) with a molecular mass of 37–39 kDa (Hereld *et al.*, 1986; Fox *et al.*, 1986; Bulow & Overath, 1986). An enzyme with similar properties, but with a molecular mass of 58 kDa, has been isolated from rat liver plasma membranes (Fox *et al.*, 1987). The cDNA-derived sequence of the trypanosomal GPI-PLC does not predict an *N*-terminal leader peptide and suggests that the polypeptide is translated on cytoplasmic ribosomes (Hereld *et al.*, 1988; Carrington *et al.*, 1989). This observation is supported by immunogold labelling studies wherein the GPI-PLC appears to be located at the cytoplasmic surface of intracellular vesicles (Bulow *et al.*, 1989). However, the mechanism of the interaction between such a cytoplasmic-oriented enzyme and a cell surface GPI-anchored substrate (i.e. variant surface glycoprotein; VSG) is difficult to envisage at present.

Mammals (and possibly other higher eukaryotes) also produce a GPI-specific phospholipase D (GPI-PLD). This enzyme is larger than the GPI-PLCs (i.e. molecular mass 100–110 kDa) and, unlike GPI-PLC, requires divalent cations for activity (Davitz *et al.*, 1989; Huang *et al.*, 1990). While the GPI-PLD was identified originally in tissue homogenates and membrane fractions (Malik & Low, 1986) its abundance in both plasma and serum (Davitz *et al.*, 1987; Cardoso de Almeida *et al.*, 1988; Low & Prasad, 1988) makes it likely that much of the activity observed in unperfused tissues is due to blood contamination. Information concerning the tissue/cell distribution of the GPI-PLD is sparse due to this complicating factor. In spite of the uncertainty regarding the source of the enzyme, the high level of GPI-PLD activity that is present in plasma raises a number of interesting questions regarding its regulation. Since endothelial and blood cells express GPI-anchored proteins on their cell surface it might be expected that such proteins would be released continually from the surface of these cells due to their constant exposure to high levels of GPI-PLD. However, preliminary studies (Davitz *et al.*, 1989; M. G. Low, unpublished work) indicate that treatment of cultured cells with serum or partially purified preparations of GPI-PLD does not produce significant release of GPI-anchored proteins. The purpose of this study was

Abbreviations used: GPI, glycosylphosphatidylinositol; PI-PLC, PI-specific phospholipase C; GPI-PLD, GPI-specific phospholipase D; HBSS, Hanks balanced salt solution; NP-40, Nonidet P-40; VSG, variant surface glycoprotein.

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to investigate in detail the apparent resistance of GPI-anchored proteins on the surface of cultured cells to the action of GPI-PLD in order to gain insights into the molecular mechanism of GPI-PLD action.

EXPERIMENTAL

Materials

Lipids were obtained from Serdary Research Laboratories (London, Ontario, Canada) or Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{3}H]Myristate-labelled VSG was prepared as described previously (Hereld *et al.*, 1986; Huang *et al.*, 1991). Amphipathic alkaline phosphatase was prepared from human placenta by butanol extraction and purified by phase separation in Triton X-114 and gel filtration (Huang *et al.*, 1991). PI-PLC was purified from culture supernatants of *Bacillus subtilis* BG2320 containing a high copy number plasmid with the gene encoding *Bacillus thuringiensis* PI-PLC (Henner *et al.*, 1988; Low, 1991). The purification procedure was essentially as described previously for *Bacillus thuringiensis* (Low *et al.*, 1988), except that chloramphenicol (15 μg/ml) was included in the medium and the volume of culture medium was reduced to 500 ml per 2 litre flask (Low, 1991). GPI-PLD was purified from bovine serum by immunoaffinity chromatography on an anti-GPI-PLD monoclonal antibody column followed by chromatography on wheat germ lectin and Mono Q columns (Huang *et al.*, 1990). The purified enzyme was dialysed overnight at 4 °C with 250 vol. of buffer A (150 mM-NaCl/10 mM-Hepes/NaOH, pH 7.0) to remove the CHAPS used during purification. The dialysed GPI-PLD was stored in aliquots at −20 °C for several months without appreciable loss of activity. Some of the earlier experiments in this study utilized partially purified GPI-PLD from rat or rabbit plasma prepared by gel filtration on Sephacryl S-300 (Low & Prasad, 1988). Other early experiments used enzyme purified from bovine serum with the first four steps of the eight-step procedure reported by Huang *et al.* (1990) and dialysed with buffer A before use. The preparations were approx. 15–20- and 75-fold-purified respectively. No differences between these preparations were noted; in this study immunoaffinity-purified bovine serum GPI-PLD was used unless specifically mentioned.

Membrane fractions were prepared from confluent cultures of cells (see below) grown in two 150 cm^2 flasks. Culture media were removed and the cells were incubated with 20 ml of Hanks balanced salt solution (HBSS; Gibco BRL, Gaithersburg, MD, U.S.A.)/flask at 37 °C for 20 min in order to reduce contamination with GPI-PLD derived from the culture medium. The cells in each flask were scraped with 3 × 2 ml aliquots of ice-cold 0.25 M-sucrose/10 mM-Hepes/NaOH, pH 7.3. The scrapings were combined and homogenized on ice using a Polytron for 2 min. The homogenate was centrifuged at 2000 *g* for 15 min and the resulting supernatant was centrifuged at 200000 *g* for 60 min. The 2000 *g* and 200000 *g* pellets were resuspended in 5 ml and 1 ml of homogenization buffer respectively and aliquots were stored at −20 °C until required for experimentation. The majority of the alkaline phosphatase activity sedimented at 2000 *g* and this fraction was used for all experiments except where indicated. Attempts were made to prepare a 'microsomal' membrane fraction by including an intermediate centrifugation at 10000 *g* but this yielded insufficient material for experimentation.

Release of alkaline phosphatase from cells and membranes with GPI-PLD

HeLa, ROS 17/2.8 and WISH cells were cultured in 12-well cluster dishes (4 cm^2/well) until confluent. The cells were washed with 2 × 0.5 ml of HBSS and then incubated in quadruplicate with 0.5 ml of HBSS alone or GPI-PLD or PI-PLC diluted in HBSS/well for 1 h at 37 °C. The medium was removed and the cells washed with 2 × 0.5 ml of HBSS. The incubation medium and the washings were combined and centrifuged at 13600 *g* in a microcentrifuge for 1 min. The supernatant than was ultracentrifuged at 100000 *g* (36000 rev./min in a Beckman 50.4 rotor) for 20 min and the final supernatant was sampled for determination of alkaline phosphatase activity. The cells remaining on the plate were scraped in three successive 0.5 ml aliquots of 1% Triton in HBSS and combined with the pellets from the first centrifugation of the incubation medium. This procedure ensured that detached cells were included in the analysis. Duplicate aliquots of the supernatants and cells were analysed for alkaline phosphatase as described below. Results are expressed as the ratio of the activity in the 100000 *g* supernatant to the total activity (supernatant plus cell-associated activity).

Membrane fractions (0.02 ml; 4–20 μg of protein depending on cell type and membrane fraction) prepared as described above were incubated in a final volume of 0.05 ml with 0.01 ml of buffer A, GPI-PLD or PI-PLC (diluted in buffer A) for 60 min at 37 °C. The incubation mixtures were diluted with 0.95 ml of ice-cold buffer A and a 0.2 ml sample was removed for determination of total alkaline phosphatase activity. The remainder was ultracentrifuged at 100000 *g* (36000 rev./min in a Beckman 50.4 rotor) for 20 min and 0.4 ml of the supernatant was removed. Duplicate samples of the diluted incubation mixture and the supernatant were assayed for alkaline phosphatase activity. Released alkaline phosphatase activity is expressed as a percentage of the total activity in each incubation. Incubations were done in quadruplicate for each condition. In experiments with ROS cells or membranes there was often a significant effect of PI-PLC on the total alkaline phosphatase activity, although the magnitude of this change varied substantially between experiments. The explanation of this effect is not known, but presumably is due to the different solubility of the released versus the membrane-bound enzyme. Consistent with this explanation, PI-PLC generally had no effect on total alkaline phosphatase activity in experiments with HeLa or WISH cells. GPI-PLD had no effect on total alkaline phosphatase activity with any of the cell types used here.

Determination of alkaline phosphatase anchor degradation by Triton X-114 phase separation

In order to test the effect of detergents on the action of GPI-PLD on membrane-bound alkaline phosphatase it was necessary to modify the procedure described above. Pilot experiments showed that inclusion of 0.1% (w/v) Nonidet P-40 (NP-40) in the incubations resulted in substantial solubilization of GPI-anchored alkaline phosphatase which was not reversed by 20-fold dilution in buffer prior to ultracentrifugation. To avoid this problem, the extent of anchor degradation was determined by the Triton X-114 phase separation technique described previously (Bordier, 1981). Membranes were incubated with phospholipases in microcentrifuged tubes in a final volume of 0.05 ml as described above [in the presence or absence of 0.1% (w/v) NP-40] and then diluted on ice with 0.2 ml 0.15 M-NaCl/0.1 mM-$MgCl_2$/0.01 mM-zinc acetate/10 mM-Hepes/NaOH, pH 7.0, and 0.25 ml of 2% (w/v) Triton X-114. A 0.1 ml sample was removed for determination of total alkaline phosphatase activity. The tubes were then incubated at 37 °C for 10 min to promote phase separation, centrifuged for 5 min at 13600 *g* and a 0.1 ml sample of the upper phase was removed. Anchor degradation was determined from the percentage of alkaline phosphatase activity that was in the upper phase. In unincubated controls this value was generally about 20% of the total. In some cases incubation

of membranes at 37 °C without added phospholipases led to significant increases in the upper phase activity, suggesting that the membranes contained endogenous anchor-degrading activity, possibly GPI-PLD. Attempts to reduce this by thorough washing of the cells prior to membrane preparation had only partial success. For simplicity, data quoted have been corrected for the control (i.e. no phospholipase) values.

Degradation of GPI-anchored proteins reconstituted into phospholipid vesicles

Phospholipids dissolved in chloroform (see below) were dried under N_2 and dissolved in 1.2 ml of 10% (w/v) sodium cholate, CHAPS or n-octyl glucoside. [^{3}H]Myristate-labelled VSG (60 μg) or human placental alkaline phosphatase was added and the mixture was incubated for 1 h at 0 °C. The mixture was then diluted with 1 ml of buffer A and dialysed with 3 × 500 ml of buffer A for 5, 18 and 24 h at 4 °C. The dialysate was removed and the volume adjusted to 3 ml with buffer A. The phosphatidylcholine/VSG vesicles (0.1 ml) were incubated with buffer A or phospholipases diluted in buffer A in a final volume of 0.2 ml for 1 h at 37 °C. Some incubations also contained NP-40 at a final concentration of 0.25%. Incubations were stopped by the addition of 0.5 ml of butan-1-ol saturated with 1 M-NH_4OH. The upper butanol phase containing the products of the reaction, [^{3}H]1,2-dimyristoylglycerol or [^{3}H]dimyristoylphosphatidic acid, was removed and the radioactivity determined. In some experiments the dialysed vesicles were ultracentrifuged (see below) prior to phospholipase treatment. For this purpose the material floating in the top one-sixth of the gradient was used as substrate.

The composition of the phospholipid vesicles was varied as follows: (i) phosphatidylcholine (10 mg) from either egg yolk or soybean was used alone, or (ii) soybean phosphatidylinositol (1 mg), egg phosphatidylethanolamine (1 mg), dimyristoylphosphatidic acid (1 mg) or cholesterol (1 mg) was mixed with 10 mg of phosphatidylcholine (egg yolk or soybean) prior to evaporation of the solvent. The lipid compositions and detergents used in particular experiments are specified in Figures or Tables.

In some experiments human placental alkaline phosphatase was used as the substrate instead of VSG. Egg phosphatidylcholine and sodium cholate were used for preparation of the vesicles. In these experiments anchor degradation was determined from the distribution of alkaline phosphatase activity after Triton X-114 phase separation as described above.

Determination of extent of reconstitution of GPI-anchored proteins into phospholipid vesicles

[^{3}H]Myristate-labelled VSG or alkaline phosphatase was mixed with detergents and phospholipids as described above. Control mixtures contained detergent but no phospholipids. After removal of the detergent by dialysis the dialysate was adjusted to 40% (w/v) sucrose by addition of 70% (w/v) sucrose in buffer A. Portions of 1 ml were placed in the bottom of three 12 ml centrifuge tubes and the tubes were then filled with 30% (w/v) sucrose in buffer A. The tubes were centrifuged at 200000 *g* in a Beckman SW41 rotor for 18 h at 4 °C and 1 ml fractions were removed. Proteins not incorporated into phospholipid vesicles pelleted on the bottom of the tube and were solubilized by washing the tube with 1 ml of 1% (w/v) SDS. The ^{3}H radioactivity or alkaline phosphatase activity in each fraction was determined and expressed as a percentage of total recovered from the gradient.

Biochemical assays

Alkaline phosphatase was assayed using *p*-nitrophenyl phosphate as substrate in 1 M-diethanolamine/HCl buffer at pH 10 (Malik & Low, 1986); 1 unit is defined as 1 μmol of substrate hydrolysed/min. Triton X-100 (0.2%) was included during the incubation. In order to eliminate turbidity resulting from Triton X-114 separations, 0.05 ml of 1% (w/v) sodium deoxycholate was added after these incubations. The protein content of membrane fractions was determined using the BCA Protein Assay (Pierce, Rockford, IL, U.S.A.). PI-PLC was assayed as described previously (Low *et al.*, 1988) using [^{3}H]-phosphatidylinositol in 0.1% (w/v) sodium deoxycholate as substrate; 1 unit of PI-PLC activity is defined as 1 μmol of substrate hydrolysed/min. GPI-PLD activity was assayed as described previously using [^{3}H]myristate-labelled VSG in 0.1% (w/v) NP-40 as substrate (Huang *et al.*, 1990, 1991). In this study, 1 unit of GPI-PLD activity is defined arbitrarily as 1% of substrate hydrolysed/min.

RESULTS

GPI-PLD is unable to release alkaline phosphatase from intact cells and cell membranes

Previous studies of GPI-PLD have used purified detergent-solubilized proteins such as VSG and placental alkaline phosphatase as substrates. In order to measure the activity of GPI-PLD under more physiological conditions, the effect of GPI-PLD on cells which express high levels of alkaline phosphatase was investigated. Incubation of intact HeLa, ROS or WISH cells with an excess of immunoaffinity-purified bovine GPI-PLD (100 units/ml) for 60 min did not lead to significant release of alkaline

Table 1. Effect of phospholipases on release of alkaline phosphatase from intact cells

Cells were incubated in the presence of GPI-PLD (100 units/ml), PI-PLC (1.5 units/ml) or medium alone (control) for 60 min at 37 °C as described in the Experimental section. The amount of alkaline phosphatase released into the supernatant is expressed as a percentage of the total. Values are means of quadruplicate incubations (±S.D.); each line refers to an independent experiment. All values for PI-PLC incubations were significantly different from control or GPI-PLD treatments ($P < 0.01$). The values for GPI-PLD incubations were not significantly different from control incubations except where indicated (* significantly different from control; $P < 0.05$). ND, no detectable alkaline phosphatase in supernatant (i.e. < 0.02%).

	Alkaline phosphatase release (%)		
Cell line	Control	+GPI-PLD	+PI-PLC
ROS cells	0.24±0.47	0.20±0.38	93.23±0.87
	1.07±0.28	0.51±0.18*	94.56±1.39
	0.29±0.09	0.75±0.15*	96.50±0.74
	0.61±0.24	0.35±0.10	98.11±1.07
	1.62±2.33	0.28±0.08	95.37±0.61
	1.24±0.56	0.67±0.19	89.5±0.94
HeLa cells	0.63±0.53	0.94±0.45	12.3±0.88
	1.05±0.55	0.87±0.33	18.4±2.00
	0.64±0.44	ND	13.41±0.79
	0.13±0.31	0.04±0.65	14.03±2.44
	0.68±0.87	1.07±0.73	4.77±1.03
	0.03±0.14	0.21±0.23	9.79±0.82
	0.06±0.17	0.28±0.16	5.89±0.43
	0.20±0.15	ND	6.18±0.88
	0.97±0.72	0.41±0.25	8.61±1.40
WISH cells	0.15±0.08	0.27±0.10	10.33±0.41
	0.06±0.02	0.04±0.01	4.93±0.79
	0.04±0.02	0.12±0.13	4.24±0.59

Table 2. Effect of phospholipases on release of alkaline phosphatase from membranes

Membrane fractions prepared from cells were incubated in the presence of GPI-PLD (100 units/ml), PI-PLC (1.5 units/ml) or medium alone (control) for 60 min at 37 °C as described in the Experimental section. The amount of alkaline phosphatase released into the supernatant is expressed as a percentage of the total. Values are means of quadruplicate incubation (±S.D.); each line refers to an independent experiment. The values for GPI-PLD incubations were not significantly different from control incubations except where indicated (* significantly different from control; $P < 0.05$).

	Alkaline phosphatase release (%)		
	Control	GPI-PLD	PI-PLC
ROS cell membranes	3.92±1.18	3.89±1.00	80.29±2.18
	4.76±0.72	7.00±1.46	73.80±1.60
	11.77±1.61	9.98±1.72	72.75±2.15
	0.64±0.59	0.89±0.25	58.66±1.58
	0.94±0.32	0.73±0.31	64.31±1.21
	0.59±0.40†	1.92±0.38*	75.79±7.64
HeLa cell membranes	1.18±0.22	0.63±0.22*	2.85±0.34
	1.96±0.68	1.53±0.48	5.20±0.66
	1.74±0.48†	2.52±0.82	10.40±0.40
WISH cell membranes	0.58±0.03	0.54±0.09	1.53±0.13
	5.05±1.83	4.40±0.70	6.60±0.67
	3.89±1.17†	6.85±1.84*	12.28±1.31

† 100000 *g* fraction used for these experiments.

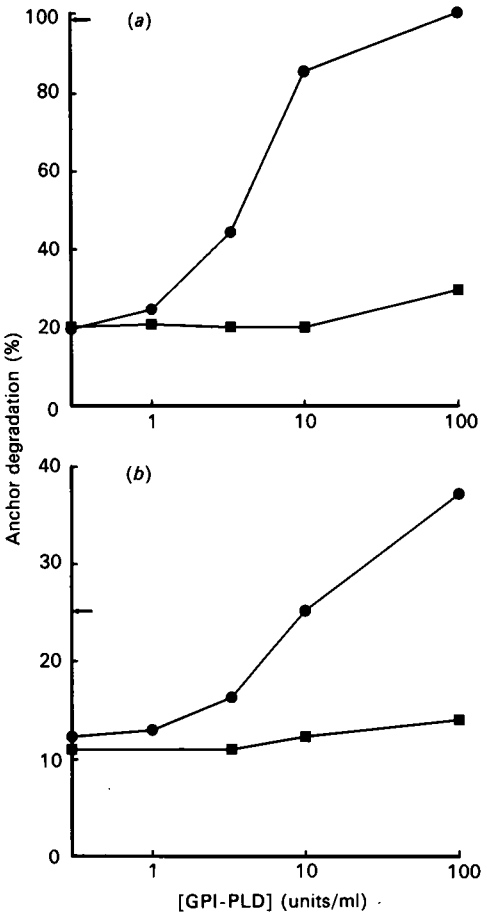

Fig. 1. Effect of detergent on degradation of membrane alkaline phosphatase by GPI-PLD

Membrane fractions from (*a*) ROS and (*b*) HeLa cells (200000 *g* pellet) were incubated with GPI-PLD in the presence or absence of NP-40 for 60 min at 37 °C. Anchor degradation was then determined by Triton X-114 phase separation as described in the Experimental section. Values are means of duplicate incubations from a single representative experiment, one of three giving similar results. ■, No NP-40; ●, 0.1% (w/v) NP-40. Arrow indicates the amount of anchor degradation occuring after incubation with PI-PLC (0.3 units/ml) in the absence of NP-40.

phosphatase activity into the medium (Table 1). Comparable results (not shown) were obtained using fetal bovine or rabbit serum, or GPI-PLD partially purified from plasma or serum (rat, rabbit and bovine; see the Experimental section).

To exclude the possibility that attachment of the cells to the culture dishes restricted access of the GPI-PLD, the cells were scraped from the flasks and homogenized, and crude particulate/membrane fractions were prepared as described in the Experimental section. Treatment of these fractions with immunoaffinity-purified bovine GPI-PLD (100 units/ml for 60 min at 37 °C) also did not release detectable alkaline phosphatase into the supernatant (Table 2). It should be emphasized that the amount of GPI-PLD activity used in these experiments would be sufficient to give complete degradation of VSG or placental alkaline phosphatase when used as substrate under standard assay conditions (Huang *et al.*, 1990, 1991).

Is latency responsible for the resistance of alkaline phosphatase to GPI-PLD?

Latency of the alkaline phosphatase due to location inside the cells or in inside-out plasma membrane vesicles might account for the failure of the enzyme to be degraded by GPI-PLD. However, substantial release of alkaline phosphatase occurred when ROS cells or membranes were incubated with PI-PLC from *B. thuringiensis* (Tables 1 and 2), indicating that the alkaline phosphatase in these cells was in a location accessible to macromolecules. By contrast, relatively little alkaline phosphatase was released from HeLa or WISH cells by the PI-PLC (Table 1), raising the possibility that most of the alkaline phosphatase in these cells was not accessible to GPI-PLD. However, other interpretations of this result are possible and these are explored in more detail later. The amount of PI-PLC-induced release of alkaline phosphatase from membranes was generally less than that obtained with the intact cells. This may be due to aggregation of membrane vesicles, but interpretation of the data is complicated by the high control release seen in several of the experiments with membranes (Table 2).

GPI-PLD is able to release alkaline phosphatase from ROS cell membranes in the presence of detergent

Since GPI-PLD activity is readily detectable with detergent-solubilized GPI-anchored proteins as substrates, the effect of detergent on the release of alkaline phosphatase from cell membranes by GPI-PLD was determined. The standard assay for GPI-PLD uses 0.1% NP-40 for solubilizing the VSG substrate, and this detergent was therefore used in experiments described here. This concentration of NP-40 is sufficient to solubilize substantial alkaline phosphatase from cell membranes of ROS and HeLa cells (results not shown). Thus it was not possible to use a simple ultracentrifugation step for measuring degradation of the GPI anchor upon GPI-PLD action. Instead, phase separation by Triton X-114 was used for this purpose. When ROS cell membranes were incubated in buffer, the majority of the alkaline phosphatase partitioned into the detergent-rich

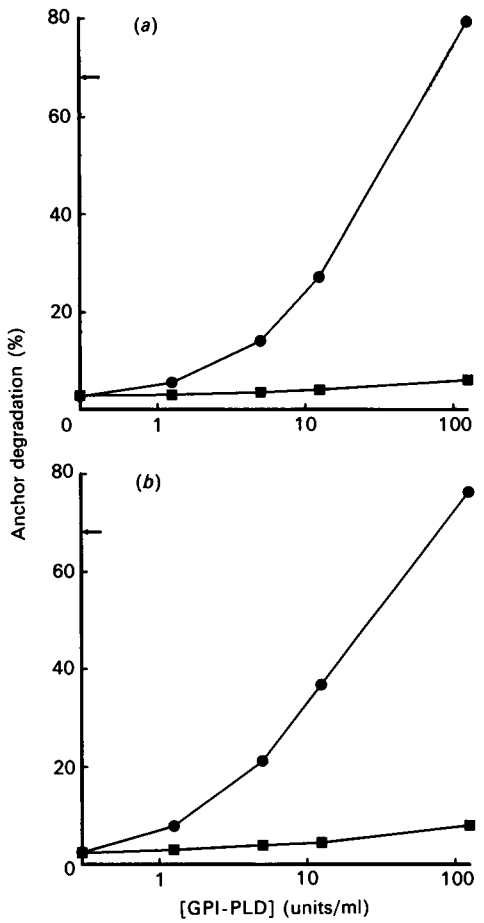

Fig. 2. Effect of detergent on GPI-PLD-mediated degradation of VSG incorporated into phospholipid vesicles

Phospholipid vesicles prepared as described in the Experimental section from (*a*) VSG/egg phosphatidylcholine/sodium cholate and (*b*) VSG/egg phosphatidylcholine/phosphatidylinositol/n-octyl glucoside were incubated with GPI-PLD in the presence or absence of NP-40 for 60 min at 37 °C. Anchor degradation was then determined by butanol extraction. Values are means of duplicate incubations from a single experiment; data from these and similar experiments using different vesicle compositions are summarized in Table 3. ■, No NP-40; ●, 0.25% (w/v) NP-40. Arrow indicates the amount of anchor degradation occurring after incubation with PI-PLC (0.3 unit/ml) in the absence of NP-40.

phase (indicating the presence of an intact GPI anchor). This result was not substantially changed when the membranes were incubated with GPI-PLD up to 10 units/ml (Fig. 1*a*). Even at the highest concentration used (100 units/ml) the increase in anchor degradation only amounted to approx. 10%. However, when the detergent NP-40 was included there was a dramatic increase in the sensitivity to GPI-PLD. Comparison of the amount of GPI-PLD required to produce similar amounts of anchor degradation indicated that there was an approx. 100-fold increase in sensitivity in the presence of NP-40 (Fig. 1*a*). A similar increase in sensitivity was noted when ROS cell membranes were incubated with crude bovine serum instead of the immunoaffinity-purified GPI-PLD, suggesting that serum factors had little influence on the activity of GPI-PLD on membranes (results not shown).

As noted above, a small amount of degradation was observed routinely in the absence of NP-40 at the highest GPI-PLD concentration (Fig. 1*a*). This result was unexpected because incubations done under identical conditions did not release alkaline phosphatase from the membrane into the supernatant (Table 2). This difference might result from the two different techniques used to monitor anchor degradation in these two types of experiment, i.e. ultracentrifugation and Triton X-114 phase separation. The reason for this discrepancy is not known at present, but the most likely explanation is that it is an artefact due to activation of GPI-PLD by Triton X-114 during phase separation. When high concentrations of GPI-PLD (i.e. 100 units/ml) were added to controls, which were then immediately subjected to the Triton X-114 phase separation, some anchor degradation was usually observed. However, although this amount of anchor degradation was generally lower than that observed in samples which were incubated at 37 °C for 60 min prior to Triton X-114 phase separation, the magnitude of the increase varied substantially between experiments. Consequently, no definite conclusion could be reached concerning the exact contribution of post-incubation degradation to the results obtained with the highest concentration of GPI-PLD.

Treatment of ROS cell membranes with PI-PLC from *B. thuringiensis* resulted in complete anchor degradation even in the absence of NP-40 in the incubation medium (Fig. 1*a*). This result confirms that obtained previously by ultracentrifugation (see above and Table 2) and indicates that most of the alkaline phosphatase in these membrane preparations is accessible to macromolecules and is not located on the luminal surface of closed membrane vesicles.

Alkaline phosphatase in HeLa and WISH cell membranes is relatively resistant to degradation by phospholipases

The same type of experiment was attempted using membranes from HeLa cells (Fig. 1*b*) and WISH cells (results not shown), and the results were qualitatively similar to those obtained with ROS cell membranes. Incubation with GPI-PLD (100 units/ml for 60 min at 37 °C) had no reproducible effect on the distribution of alkaline phosphatase in the Triton X-114 phase separation. Inclusion of NP-40 during the incubation gave an increase in the amount of alkaline phosphatase in the upper phase, but this was a small proportion of the total (approx. 10–20% and 20–40% for WISH and HeLa cells respectively; see Fig. 1*b*). The relatively small increases in degradation precluded an accurate determination of the effect of NP-40 on sensitivity to GPI-PLD.

Treatment of HeLa (Fig. 1*b*) and WISH (results not shown) cell membranes with PI-PLC gave some anchor degradation when assayed by Triton X-114 phase separation. However, this was generally less than that obtained with GPI-PLD (see Fig. 1*b*) and was not increased by the inclusion of NP-40 (results not shown). The reason for the resistance of most of the alkaline phosphatase in these two cell types to anchor degradation by phospholipases, shown above (Tables 1 and 2), is unknown, but apparently is not a result of inaccessibility of alkaline phosphatase in closed membrane vesicles.

Detergent stimulates GPI-PLD-mediated degradation of GPI-anchored proteins reconstituted in phospholipid vesicles

From the above evidence it seemed likely that the stimulatory effect of detergent on GPI-PLD-mediated degradation of alkaline phosphatase was due to a physical effect on the membrane rather than disruption of a specific interaction between the enzyme and a GPI-PLD 'inhibitor' at the cell surface. Many phospholipases are stimulated by detergents, and this is usually attributed to an effect on substrate presentation and organization rather than on the phospholipase itself. To provide additional experimental support for this idea, [^{3}H]myristate-labelled VSG was reconstituted into phosphatidylcholine vesicles by the detergent dialysis technique. The phosphatidylcholine/VSG vesicles were then incubated with different concentrations of GPI-PLD and

Table 3. Effect of detergent on GPI-PLD-mediated degradation of VSG incorporated into phospholipid vesicles

VSG anchor degradation by GPI-PLD was determined as described in the legend to Fig. 2. Data from those and other experiments using different vesicle compositions are summarized here. An estimate of substrate accessibility in each vesicle preparation was made by determining (in parallel incubations) the amount of VSG degraded by PI-PLC (0.3 unit/ml) in the absence of NP-40. Values are the means of duplicate incubations; each line refers to an independent experiment done with a different preparation of phospholipid vesicles. Experiments in Groups I and II were done using different preparations of VSG. † indicates that alkaline phosphatase was used as substrate instead of VSG; * indicates that bovine serum containing comparable levels of GPI-PLD activity was used instead of the immunoaffinity-purified enzyme. PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; dmPA, dimyristoylphosphatidic acid.

Vesicle composition/detergent	Anchor degradation (%)			
		GPI-PLD		
Group I	PI-PLC	50 units/ml; no NP-40	5 units/ml; plus NP-40	1.67 units/ml; plus NP-40
Egg PC/sodium cholate	71.0	9.7	30.8	8.9
Egg PC/sodium cholate	71.8	7.6	33.5	9.6
Egg PC/sodium cholate*	73.8	6.7	42.3	19.3
Egg PC/sodium cholate*	80.6	10.9	31.3	12.9
Egg PC/cholesterol/sodium cholate	70.8	14.2	31.3	12.3
Egg PC/cholesterol/sodium cholate	75.0	11.7	33.1	12.8
Egg PC/PI/sodium cholate	73.2	10.5	25.5	11.8
Egg PC/PI/sodium cholate	73.1	15.4	32.7	12.9
Egg PC/PE/sodium cholate	78.3	13.3	29.1	13.3
Egg PC/n-octyl glucoside	79.2	12.6	39.8	16.4
Egg PC/n-octyl glucoside	78.2	9.7	32.7	16.2
Egg PC/n-octyl glucoside	76.0	9.0	29.1	14.0
Egg PC/PE/n-octyl glucoside	74.1	12.2	27.8	11.8
Soybean PC/n-octyl glucoside	75.7	12.6	41.6	16.6
Egg PC/sodium cholate†	74.5	9.8	11.8	5.8
Group II		125 units/ml; no NP-40	5 units/ml; plus NP-40	1.25 units/ml; plus NP-40
Egg PC/sodium cholate	68.6	3.2	11.4	2.9
Soybean PC/sodium cholate	82.0	10.7	15.3	4.01
Egg PC/dmPA/sodium cholate	75.3	11.5	1.6	0.4
Egg PC/dmPA/sodium cholate	68.5	5.3	1.4	0.1
Soybean PC/n-octyl glucoside	67.0	4.3	6.4	2.2
Egg PC/PI/n-octyl glucoside	69.2	5.6	18.7	5.4
Egg PC/sodium cholate†	67.7	5.6	17.3	2.1

the extent of GPI anchor degradation was determined from the amount of ^3H radioactivity released into the butanol phase of an aqueous butanol extraction (Fig. 2). As with the cell membranes, GPI-PLD had relatively little activity in the absence of detergent up to GPI-PLD concentrations of about 5 units/ml. At the highest concentrations used (i.e. 50–125 units/ml) the amount of degradation approached only about 10–15% of the total (Fig. 2 and Table 3). A dramatic increase in the sensitivity of the substrate to GPI-PLD was observed when 0.25% NP-40 was included in the incubation mixture. Comparison of the amounts of GPI-PLD required to produce a similar extent of anchor degradation indicated an increase in sensitivity of at least 25–30-fold in the presence of NP-40.

Similar results were obtained when crude bovine serum (Table 3; see data marked by asterisk) or partially purified bovine serum GPI-PLD (results not shown) containing comparable amounts of GPI-PLD activity were used instead of the immunoaffinity-purified enzyme. This confirms the result obtained with alkaline phosphatase in ROS cell membranes and reinforces the conclusion that other serum factors have little or no influence on GPI-PLD activity towards substrates located in phospholipid bilayers.

Human placental alkaline phosphatase was also reconstituted into phosphatidylcholine vesicles and its sensitivity to purified GPI-PLD was examined using procedures similar to those described above for VSG (Table 3). In this case the extent of anchor degradation was determined by measuring the distribution of alkaline phosphatase in a Triton X-114 phase separation. As in the experiments with VSG described above, alkaline phosphatase in phospholipid vesicles was relatively insensitive to GPI-PLD. Incubation with GPI-PLD at 50 units/ml for 60 min at 37 °C resulted in less than 10% degradation. Addition of NP-40 markedly increased sensitivity to GPI-PLD (Table 3).

It was of interest to determine if NP-40 was able to reverse completely the apparent inhibition of GPI-PLD activity by phosphatidylcholine. The activities of intermediate concentrations of GPI-PLD towards VSG in the presence and absence of phosphatidylcholine were therefore compared (Fig. 3). With 0.05% NP-40, phosphatidylcholine inhibited the amount of degradation by approx. 60%. However, when the detergent concentration was increased above 0.1% the extent of inhibition was substantially decreased. The large stimulatory effect of NP-40 (at 0.05–0.1%) on degradation of the VSG/phosphatidylcholine substrate was not seen with VSG alone and probably reflects conversion of the phospholipid vesicles to mixed micelles, since the activity reaches a maximum when the detergent (approx. 1.5–4 mM) and phosphatidylcholine (approx. 2 mM) are present at approximately equimolar concentrations (Fig. 3). However, the activity of GPI-PLD towards VSG alone was markedly

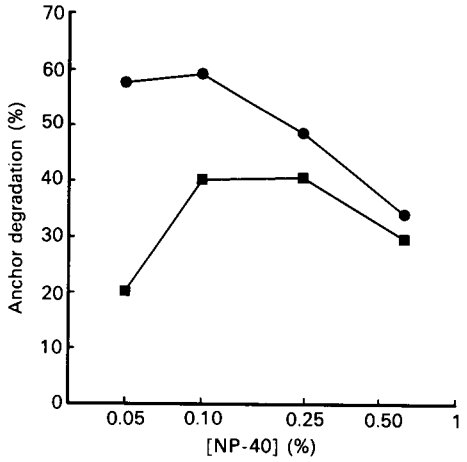

Fig. 3. Effect of phospholipid on detergent-stimulated degradation of VSG by GPI-PLD

VSG, with or without phosphatidylcholine, was incubated with GPI-PLD (12.5 units/ml) and NP-40, at the concentrations indicated, for 60 min at 37 °C. Anchor degradation was then determined by butanol extraction as described in the Experimental section. Values are means of duplicate incubations from a single experiment which was one of three giving similar results. The amount of degradation obtained in parallel experiments with the same concentration of GPI-PLD but no NP-40 was usually 5% or less for either type of substrate. ■, Phospholipid vesicles prepared from VSG/soybean phosphatidylcholine/sodium cholate; ●, VSG without phospholipid.

stimulated by NP-40 at much lower concentrations. Activity was maximal at 0.05% NP-40, and half-maximal activity was achieved at concentrations as low as 0.01% (results not shown).

Resistance of reconstituted VSG to degradation by GPI-PLD is independent of phospholipid vesicle composition

The results above suggested that GPI-PLD, like other phospholipases acting on simpler substrates, expresses relatively poor activity towards a substrate incorporated into a phospholipid bilayer. To ensure that this result was not an artefact of the reconstitution procedure used, the method for preparing the phospholipid vesicles was varied in four ways.

(1) Three different readily dialysable detergents (i.e. sodium cholate, n-octyl glucoside and CHAPS) were used for solubilizing the phospholipid. Similar results were obtained with the first two of these detergents (Table 3). In contrast, two experiments with vesicles prepared from egg yolk phosphatidylcholine dissolved in CHAPS demonstrated substantial degradation of VSG with low levels of GPI-PLD employed in the absence of NP-40 (results not shown). These observations were not reproducible. Two other experiments with CHAPS gave results similar to the other two detergents. Because of the variability in results obtained with CHAPS, this detergent was not used for preparation of phospholipid vesicles in subsequent experiments.

(2) Attempts were made to reconstitute VSG by adding it directly to preformed phospholipid vesicles which were prepared without detergent. However, these experiments were unsuccessful and no data are shown. When VSG was added to phosphatidylcholine vesicles prepared by sonication the measurements of sensitivity to PI-PLC and GPI-PLD and the effects of detergent were not reproducible between experiments. Multilamellar phosphatidylcholine vesicles prepared without sonication were also evaluated, but in preliminary experiments it was found that these vesicles did not incorporate sufficient VSG to render them useful as substrates.

(3) Ultracentrifugation of the VSG/phosphatidylcholine substrate prior to use had no substantial effect on the results (not shown). This suggested that the small amount of degradation occurring at the highest concentration of GPI-PLD was not due to a selective degradation of VSG molecules which were not incorporated into phosphatidylcholine vesicles, since centrifugation would have removed free VSG molecules.

(4) Finally, the lipid composition of the vesicles was varied. These variations included alteration of the degree of fatty acid saturation (Table 3) in the phosphatidylcholine (the predominant phospholipid in all experiments) and the inclusion of cholesterol, phosphatidylinositol or phosphatidylethanolamine. These variations in lipid composition were not designed either to mimic the composition of a plasma membrane or to produce phospholipid vesicles with defined physical features. However, collectively the changes in composition used would be expected to produce vesicles varying substantially in fluidity, surface charge and lipid organization. As shown in Fig. 2 and Table 3, VSG incorporated into all of these different types of vesicles was relatively insensitive to GPI-PLD. Furthermore, sensitivity to GPI-PLD was increased to a similar degree by NP-40 in all cases.

An unexpected finding in this series of studies was made with vesicles prepared from a mixture of egg yolk phosphatidylcholine and dimyristoylphosphatidic acid (10:1, w/w). VSG in these vesicles was resistant to degradation by GPI-PLD even in the presence of detergent (Table 3). The reason for this inhibition is not clear, although preliminary studies have indicated that the inhibitory effect is relatively specific for phosphatidic acids and does not require the presence of phosphatidylcholine and other phospholipids (M. G. Low, unpublished work). It remains to be determined if inhibition of GPI-PLD by phosphatidic acid is due to a purely physical effect on substrate presentation or to a form of 'product inhibition' based on the chemical structure of this molecule.

GPI-anchored proteins are accessible on the surface of phospholipid vesicles

A possible explanation for the low activity of GPI-PLD in the absence of NP-40 was that the VSG or alkaline phosphatase was unable to be incorporated into the phospholipid vesicle. After removal of the detergent by dialysis, the proteins would probably aggregate, and this might mask the GPI-PLD-sensitive site. The extent of incorporation of VSG into phospholipid vesicles was therefore tested by ultracentrifuging the vesicles in a discontinuous sucrose density gradient. Under these conditions, vesicles and associated proteins float to the top, whereas free proteins sediment to the bottom of the tube. As shown in Table 4, the majority of the recovered ^3H radioactivity was located in the top one-third of the gradient, compared with approx. 10–20% in the bottom one-third and the pellet. By contrast, control VSG/detergent mixtures containing no phospholipids which were dialysed and ultracentrifuged under similar conditions revealed about 80% of the radioactivity in the pellet and less than 10% in the upper one-third of the gradient. Similar results were obtained with placental alkaline phosphatase when distribution of the protein was monitored by enzyme activity (Table 4). These results indicated that the majority of the substrate molecules were incorporated into phospholipid vesicles and therefore are unlikely to be inaccessible to GPI-PLD solely because of the formation of large aggregates.

In order to assess the possibility that the VSG or alkaline phosphatase substrates might be resistant to the GPI-PLD because of the orientation of these proteins and their anchors towards the inside of the vesicle, the vesicles were incubated with PI-PLC from *B. thuringiensis*. In the presence of NP-40, PI-PLC

Table 4. Incorporation of [^{3}H]myristate-labelled VSG or alkaline phosphatase into phospholipid vesicles

[^{3}H]Myristate-labelled VSG or alkaline phosphatase was mixed with phospholipids and detergents or detergents alone as indicated. After dialysis to remove the detergent, samples were fractionated in a discontinuous sucrose density gradient as described in the Experimental section. Combined radioactivity or enzyme activity in the upper and lower three fractions of the gradient and in the pellet are expressed as a percentage of the total recovered from the gradient. Values are means (±S.D.) of triplicate fractionations done with the same preparation of phospholipid vesicles; each line refers to an independent experiment. PC, phosphatidylcholine; dmPA, dimyristoylphosphatidic acid.

Vesicle composition/detergent	^{3}H radioactivity or enzyme activity (% of total recovered)		
	Upper fractions	Lower fractions	Pellet
VSG/egg PC/sodium cholate	91.07±3.18	1.62±0.40	–
VSG/egg PC/sodium cholate	64.82±6.25	7.25±2.79	8.42±1.16
VSG/egg PC/n-octyl glucoside	71.97±20.38	8.36±4.52	–
VSG/egg PC/n-octyl glucoside	54.86±15.70	6.51±5.80	17.58±3.95
VSG/egg PC/n-octyl glucoside	61.88±2.45	3.96±1.11	24.32±5.21
VSG/soybean PC/sodium cholate	74.62±5.87	2.95±1.75	7.72±0.51
VSG/egg PC/CHAPS	74.39±1.74	1.74±0.25	8.28±0.24
VSG/egg PC/dmPA/n-octyl glucoside	78.60±4.84	1.65±0.38	9.72±3.25
VSG/cholate	5.32±1.58	5.65±0.21	82.37±1.92
VSG/cholate	1.52±0.04	7.91±2.30	87.86±2.38
VSG/n-octyl glucoside	4.13±0.49	9.42±4.30	79.53±3.04
VSG/n-octyl glucoside	3.95±0.51	5.74±0.43	82.81±0.94
Alkaline phosphatase/egg PC/ sodium cholate	82.75±6.23	3.65±0.47	1.36±0.90
Alkaline phosphatase/sodium cholate	13.28±2.06	30.47±6.05	35.84±7.07

degraded 90–100% of the VSG (results not shown). In the absence of NP-40, the amount of degradation was significantly less, but generally greater than 70% (Fig. 2 and Table 3). This result indicated that the majority of the VSG was accessible at the surface of the vesicle.

DISCUSSION

GPI-PLD activity was originally observed as a result of its ability to degrade the GPI anchor of alkaline phosphatase during extraction of this protein from membrane fractions and tissue homogenates with butanol (Low & Zilversmit, 1980; Malik & Low, 1986). Identification of a similar activity in serum and plasma has permitted purification of GPI-PLD, and its cDNA recently has been isolated and sequenced (Davitz *et al.*, 1989; Huang *et al.*, 1990; Scallon *et al.*, 1991). In spite of these advances in our knowledge of GPI-PLD structure, relatively little is known of its mechanism and sites of action *in vivo*. All of the assay systems currently used for GPI-PLD require substrates which are solubilized in detergent. When the ability of GPI-PLD to degrade GPI anchors was measured under more physiological conditions (e.g. substrates in cells or membrane fractions), no activity was observed (Davitz *et al.*, 1989; M. G. Low, unpublished work). Although the mechanism of resistance to GPI-PLD of GPI-anchored proteins located in membranes has not been investigated previously the question is not without physiological relevance. The concentration of GPI-PLD in plasma or serum is in the approximate range of $(1–5) \times 10^3$ units/ml (1 unit is defined as 1% degradation of GPI substrate/min). Nevertheless, endothelial and blood cells are capable of retaining GPI-anchored proteins on their cell surfaces in spite of continuous exposure to this high concentration of GPI-PLD.

In the present study, therefore, our goals were 2-fold: (1) to determine if the inability of the GPI-PLD to act on membranes is an intrinsic property of the GPI-PLD polypeptide itself or is due to a distinct inhibitory protein found in the serum or on the membrane containing the substrate; and (2) to reconstitute resistance to GPI-PLD in a simplified system consisting of purified lipids and GPI-anchored proteins. Such studies would allow closer examination of the mechanism of resistance, and permit modelling *in vitro* of potential regulatory mechanisms which GPI-PLD might use *in vivo*.

The results of this study suggest that the low activity of serum or plasma GPI-PLD against GPI-anchored proteins located in membranes is not due entirely to the presence of inhibitory factors either in the serum or on the membranes. First, the behaviour of the GPI-PLD in serum and after purification is very similar with respect to sensitivity to stimulation by NP-40, suggesting that an inhibitory substance was not removed during purification. It is conceivable that some inhibitor remains in the GPI-PLD preparation after purification but was not detectable by SDS/PAGE. However, to account for the lack of activity of the purified GPI-PLD, an inhibitor would need to be the same size as the GPI-PLD, effective at relatively low stoichiometry or not detectable by protein-staining techniques (e.g. a lipid). Second, NP-40 markedly stimulates the activity of GPI-PLD towards membranes. NP-40 is a relatively mild non-denaturing detergent which does not usually disrupt specific protein–protein interactions (e.g. antigen–antibody, cytoskeletal proteins etc.). It therefore seems unlikely that it would prevent the GPI-PLD from associating with specific protein inhibitors on the membrane.

The observation that GPI-PLD was unable to act on substrates in cell membranes and that this 'inhibition' could be reversed by the addition of detergent is reminiscent of previous findings with several other phospholipases degrading less complex lipid structures (Waite, 1987). For example, pancreatic phospholipase A_2 and the broad-specificity phospholipase C from *Bacillus cereus*, both of which hydrolyse phosphatidylcholine and other simple glycerophospholipids, have relatively low activity against cell membranes compared with phospholipase A_2 from snake venom and the broad-specificity phospholipase C from *Clostridium perfringens* respectively. The cytosolic inositol-phospholipid-specific phospholipases C from mammalian tissues also are relatively inactive against membrane substrates (Low

et al., 1986). In contrast, the bacterial PI-PLCs readily attack phosphatidylinositol and GPI-anchored proteins in membranes. Generally, the inhibition of some phospholipases that is evident with substrates located in membranes can be overcome by the addition of detergents. Studies on model membrane systems such as monolayers, phospholipid vesicles and detergent/phospholipid mixed micelles have indicated that the organization of the phospholipids in the membrane is responsible for this restriction of phospholipase activity (Waite, 1987).

In view of this information, it was of interest to determine whether GPI-PLD would act on GPI-anchored proteins inserted into model membranes consisting entirely of defined lipid components. Our studies with VSG and alkaline phosphatase reconstituted into phospholipid vesicles of various compositions indicate that GPI-PLD is relatively ineffective against these substrates also. This result suggests that, in common with many other phospholipases, GPI-PLD is influenced by the microenvironment in which the substrate is located.

Although the results obtained with natural membranes and phospholipid vesicles were qualitatively similar, some quantitative differences were noted. At the highest concentration of GPI-PLD used, small, but significant, levels of anchor degradation were observed with phospholipid vesicles but not with intact ROS cells. This might suggest that additional factors unique to the cell membrane were also involved in the resistance to GPI-PLD. However, the results obtained with the ROS cell membrane fractions complicated this interpretation, since different results were obtained using two different methods for detecting anchor degradation (i.e. ultracentrifugation and Triton X-114 phase separation). Thus in experiments using ultracentrifugation to detect release no effect of GPI-PLD was observed. By contrast, membranes incubated with GPI-PLD under identical conditions often gave detectable degradation when Triton X-114 phase separation was used to monitor anchor degradation. A contribution of cell-associated inhibitory factors to the observed resistance to GPI-PLD is therefore not excluded by the present data.

Some evidence suggests that GPI-PLD molecules may associate into higher-molecular-mass complexes. For example, gel filtration under non-denaturing conditions of serum or plasma from several mammalian species indicates a molecular mass of 200–500 kDa for the majority of the GPI-PLD activity (Low & Prasad, 1988). The GPI-PLD purified from bovine serum is mainly in a 200 kDa form (possibly a dimer), but significant amounts of higher-molecular-mass forms also are present (Huang *et al.*, 1990). If the complexes have relatively low activity compared with the monomeric form of GPI-PLD and are also dissociated by NP-40, then our results could be explained by an effect of the detergent on the enzyme itself rather than on the organization of the lipid in the substrate particle. Our data do not exclude this possibility, but it is relevant to note that the aggregated forms of purified bovine GPI-PLD were detected during gel filtration in the presence of CHAPS, indicating that they are not particularly sensitive to disruption by detergent (Huang *et al.*, 1990). In this respect, it is relevant to note results in the present study showing that GPI-PLD activity is relatively independent of detergent concentration when phospholipids are not present. Furthermore, the purified human GPI-PLD reported by Davitz *et al.* (1989) appears to be monomeric, as it has the same size (approx. 110 kDa) when analysed either by gel filtration under non-denaturing conditions or by SDS/PAGE. Still, this enzyme apparently is unable to release decay-accelerating factor from erythrocytes (Davitz *et al.*, 1989). It seems, therefore, that dimerization or aggregation of GPI-PLD *per se* is not responsible for the low activity of this enzyme, seen in the absence of detergent.

The relative insensitivity of alkaline phosphatase in WISH and HeLa cells to degradation by phospholipases was unexpected, and while this result is not directly related to the main purpose of this study, it does merit some comment. The resistance to PI-PLC or GPI-PLD plus NP-40 could result from membrane attachment by a hydrophobic polypeptide anchor or a modified GPI molecule. A hydrophobic polypeptide suitable for membrane anchoring might result from ineffective removal of the *C*-terminal signal peptide during GPI attachment, or from alkaline phosphatase molecules which are polypeptide-anchored due to differential processing of the primary RNA transcript. However, there is no evidence from previous studies with alkaline phosphatase for the presence of polypeptide-anchored forms. By contrast, there is a precedent for the utilization by proteins of a GPI anchor which is modified on the inositol ring by a long-chain acyl group and is known to be PI-PLC-resistant. These acyl GPIs have been described in human erythrocytes, where they anchor proteins such as acetylcholinesterase (Roberts *et al.*, 1987, 1988) and decay-accelerating factor (Walter *et al.*, 1990), and also in *T. brucei* where a protein-anchoring function is suspected but has not yet been demonstrated (Clayton & Mowatt, 1989). These molecules are reported to be GPI-PLD-sensitive, but it should be emphasized that our criteria for alkaline phosphatase anchor degradation (i.e. release from the membrane or redistribution in a Triton X-114 phase separation) are indirect ones which essentially measure the hydrophobic/hydrophilic balance of the protein and its anchor. Therefore it is possible that extensive degradation of HeLa and WISH cell alkaline phosphatase did occur in the presence of GPI-PLD and NP-40 but was not detectable due to the presence of a long-chain acyl group on the inositol ring. This acyl group would not be removed by GPI-PLD, but might influence partitioning in Triton X-114. This idea is supported by earlier work showing that GPI-PLD-treated human erythrocyte acetylcholinesterase remained associated with Triton X-100 micelles during non-denaturing electrophoresis because of the single acyl group on the inositol ring, which was retained after degradation by GPI-PLD (Toutant *et al.*, 1989). Further studies, in which the PI-PLC-resistant alkaline phosphatases in HeLa and WISH cells are analysed directly for the presence of acyl GPI anchors, will be required to resolve this question.

The results of the present studies have broad implications for the regulation of GPI-PLD activity *in vivo*. GPI-PLD in either a crude or a partially purified state has been reported to act on a wide variety of detergent-solubilized GPIs (Davitz *et al.*, 1987, 1989; Low & Prasad, 1988; Roberts *et al.*, 1988; Hooper & Turner, 1989; Masterson *et al.*, 1989; Stochaj *et al.*, 1989; Hoener *et al.*, 1990; Huang *et al.*, 1990; Mayor *et al.*, 1990), and our findings should therefore be applicable to other GPI-anchored proteins. It is conceivable that individual GPI-anchored proteins or the membrane in which they are located will differ in their properties to such an extent that the effects of the phospholipid bilayer on GPI-PLD action are removed or enhanced. However, it is not possible to either predict or model experimentally such properties for all the potential GPI-PLD substrates. For the purposes of the following discussion, therefore, we will make the assumption that the behaviour of alkaline phosphatase and VSG observed here is typical for other substrates.

It seems very unlikely that naturally occurring analogues of the known GPI-PLD activators (i.e. detergents and butanol) could be involved in regulating GPI-PLD activity *in vivo*. One effect of this type of agent is to produce complete disruption of membrane structure and solubilization of many membrane components. However, it is possible that in certain pathological situations (e.g. biliary obstruction) membranes may become more susceptible to the action of GPI-PLD, resulting in the

release of proteins such as alkaline phosphatase into the plasma (McComb *et al.*, 1979). Ikehara and co-workers have observed a hydrophilic species of alkaline phosphatase in the bile-duct-ligated rat which is consistent with this idea (Miki *et al.*, 1985; Kominami *et al.*, 1985). Alternatively, anchor degradation could occur subsequent to release from the membrane, since liver alkaline phosphatase circulating in human serum is known to exist in both aggregated (i.e. anchor-intact) and non-aggregated (i.e. anchor-degraded) forms (Bailyes *et al.*, 1987; Traynor *et al.*, 1986).

The occurrence of GPI-PLD in plasma suggested initially that it might act on GPI-anchored proteins on cell surfaces. However, there is no reason to exclude the possibility that GPI anchor degradation actually takes place in intracellular compartments where the environment, such as low pH, might favour GPI-PLD activity, and that the enzyme activity found in plasma actually represents 'spillage' from such compartments. There is some evidence to suggest that GPI-anchored proteins might exhibit unusual intracellular trafficking (Lisanti & Rodriguez-Boulan, 1990) or locate to specialized compartments (Rothberg *et al.*, 1990*a*,*b*), although neither the mechanism nor the physiological purpose of these phenomena has been established. There is currently no information on the intracellular trafficking and subcellular localization of GPI-PLD. In spite of this, some recent evidence does provide support for an intracellular site of action for GPI-PLD. In COS cells co-transfected with GPI-PLD and placental alkaline phosphatase the majority of the alkaline phosphatase gets secreted into the medium instead of being associated with the membrane (Scallon *et al.*, 1991). By contrast, the GPI-PLD secreted into the media of GPI-PLD transfectants is unable to release alkaline phosphatase from alkaline phosphatase transfectants. This intriguing result suggests that intracellular compartments might provide a more favourable environment for GPI-PLD action (Scallon *et al.*, 1991).

Interpretation of our results in the context of the regulation of GPI-PLD *in vivo* is complicated by the fact that the origin and sites of action of plasma GPI-PLD currently are unknown. Although recent immunolocalization studies on mammalian tissues have suggested its presence only in discrete locations (e.g. sub-populations of mast cells, neurons and keratinocytes), these data have not yet been confirmed by biosynthetic labelling studies (Gleichauf *et al.*, 1990; Sesko & Low, 1991). It is conceivable that GPI-PLD is synthesized/secreted by cells in response to an acute stimulus along with activators or in an active but unstable form. Consequently the enzyme would exert its effects locally on the secreting cell or its neighbours before it became inactivated *en route* to the plasma. In this case, inactivation would be the retention of catalytic activity but elimination of the ability to interact with substrates in the membrane. The majority of the GPI-PLD in plasma would therefore be a physiologically inactive species by virtue of its location. Conversely, the GPI-PLD may be constitutively synthesized/secreted in a stable form which can act systemically over longer periods. In this case, plasma GPI-PLD might be regulated acutely by circulating or cell-associated factors which would allow the enzyme to overcome its inability to act on membranes. Unfortunately, it is not possible to distinguish between these two extreme possibilities at present, since the full extent of the cellular origins and sites of action of GPI-PLD is uncertain. In spite of these unresolved questions, our data do provide a plausible explanation for the ability of blood and endothelial cells to retain GPI-anchored proteins on their cell surfaces in the presence of large amounts of GPI-PLD catalytic activity in the plasma. It seems that GPI-anchored proteins located in membranes are relatively unfavourable substrates for plasma GPI-PLD. Understanding the mechanism and full physiological significance of this observation awaits further investigation.

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