Expression and secretion of glycosylphosphatidylinositol-specific phospholipase D by myeloid cell lines

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Glycosylphosphatidylinositol (GPI)-specific phospholipase D (GPI-PLD) is abundant in mammalian plasma. It could potentially regulate the surface expression of GPI-anchored proteins, but it remains to be established which tissue(s) or cell type(s) are the principal sources of the circulating enzyme. Here we report that all the myeloid cell lines tested, including K562 (multipotential blast), KG-1 (human myeloblast), HL-60, NB4, PLB-985 (human promyelocyte), U937 (human promonocyte), THP-1 (human monocyte) and J774, RAW264.7 (mouse monocyte/macrophage), contained GPI-degrading activity. T.l.c. analysis of reaction products confirmed the activity as a phospholipase D. These cells also exhibited positive immunofluorescent staining with an anti-GPI-PLD monoclonal antibody.

The expression of GPI-PLD activity was not substantially reduced when the cells were cultured in either serum-free medium or GPI-PLD-depleted regular medium. Both granulocytic and monocytic differentiation of myelomonoblastic lines (e.g. HL-60) induced by dimethyl sulphoxide or phorbol diester respectively was accompanied by a 2–3-fold increase in GPI-PLD activity. J774 and HL-60 cells secreted GPI-PLD into the medium constitutively. Taken together, these data suggest that myeloid cells are a potential contributor to the circulating GPI-PLD pool. As leucocytes express many important GPI-anchored surface antigens, these cells may prove to be a valuable model system for studying the physiological functions of GPI-PLD.

INTRODUCTION

An increasing number of cell surface proteins have been found to be anchored to the plasma membrane by a complex glycolipid, glycosylphosphatidylinositol (GPI) (Low, 1989; Cross, 1990; Ferguson, 1992). GPI-anchored proteins are functionally diverse and include complement regulatory factors, cell-adhesion molecules, ectoenzymes, lymphocyte differentiation antigens, tumour markers, cell surface receptors, both the normal and scrapie forms of the prion, as well as the majority of protozoal parasitic cell surface antigens. One proposed role for the GPI anchor is that it facilitates the release of the anchored proteins from the membrane by serving as a target substrate for anchor-specific phospholipases. Several soluble forms of GPI-anchored proteins have been described, e.g. Thy-1 (Almqvist and Carlsson, 1988), N-CAM-120 (He et al., 1987), 5'-nucleotidase (Klemens et al., 1990) and decay-accelerating factor (Metz et al., 1993), which have structural features reminiscent of cleavage by phospholipases.

A GPI-specific phospholipase D (GPI-PLD) has been purified from human and bovine serum (Davitz et al., 1987, 1989; Low and Prasad, 1988; Huang et al., 1990). Two GPI-PLD cDNAs have been cloned and sequenced from liver (Scallan et al., 1991) and pancreatic islet (Tsang et al., 1992) cDNA libraries, suggesting that more than one gene encodes this enzyme. At present GPI-PLD is the only GPI-degrading enzyme in mammals and humans that has been characterized extensively at the molecular level. However, a direct demonstration of cleavage of cell-associated GPI-anchored proteins by either crude or purified plasma GPI-PLD has not been achieved (Davitz et al., 1989; Low, 1990; Low and Huang, 1991). Therefore conclusive evi-

dence for the physiological role of GPI-PLD is still lacking. The major obstacle in this endeavour is the lack of suitable model systems in which both GPI-PLD and its substrates are expressed.

Although plasma contains a large quantity of GPI-PLD (5–10 µg/ml), it remains to be established which tissue(s) or cell type(s) are the principal sources of the circulating enzyme. Davitz and colleagues have found that the pancreatic islets of Langerhans and mast cells express and secrete GPI-PLD (Metz et al., 1991, 1992), and our laboratory has observed that GPI-PLD is also present in keratinocytes and neuronal cells (Sesko and Low, 1991; Xie et al., 1993). However, biochemical assays and immunological studies have shown that many other cell types do not express GPI-PLD (Metz et al., 1991; Xie et al., 1993). Here we demonstrate that myeloid cells also express and secrete GPI-PLD.

MATERIALS AND METHODS

Materials

Monoclonal antibodies (mAbs) against bovine serum GPI-PLD were as described previously (Huang et al., 1990). Fluorescein isothiocyanate-conjugated rat anti-mouse IgG was from Jackson ImmunoResearch (West Grove, PA, U.S.A.). [³H]Myristate-labelled variant surface glycoprotein ([³H]VSG) was prepared as described previously (Huang et al., 1991). GPI-PLD-depleted complete medium was prepared by passing the medium through an mAb 216-conjugated affinity column (Huang et al., 1990). All culture media and sera were purchased from Gibco-BRL (Grand Island, NY, U.S.A.). Nutridoma-SP was from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). Silica-gel plates were

Abbreviations used: GPI, glycosylphosphatidylinositol; GPI-PLD, GPI-specific phospholipase D; PA, phosphatidic acid; DAG, diacylglycerol; NP-40, Nonidet P40; VSG, variant surface glycoprotein; PMA, phorbol 12-myristate 13-acetate; mAb, monoclonal antibody; DMSO, dimethyl sulphoxide; SLO, streptolysin O; Myr, myristic acid.

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from Whatman (Clifton, NJ, U.S.A.). All other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell lines and culture conditions

The following cell lines were from A.T.C.C.: K-562 (CCL 243), KG-1 (CCL 246), HL-60 (CCL 240), U-937 (CRL 1593), THP-1 (TIB 202), J774 (TIB 67), RAW264.7 (TIB 71), MOLT-4 (CRL 1582). NB4 and PLB-984 cells were from Dr. Jianye Teng. J774 and RAW264.7 cells were grown in Dulbecco's modified Eagle's medium (without pyruvate) supplemented with 10% (v/v) donor calf serum. All the other cell types were grown in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum. For serum-free culture of these cell lines, cells were first washed once with unsupplemented medium and then cultured in medium supplemented with 1% Nutridoma-SP. For some experiments, cells were cultured in GPI-PLD-depleted complete medium.

Monocytic or granulocytic differentiation of myeloid precursor cells (e.g. HL-60) was achieved by treatment for 2–3 days with 100 nM phorbol 12-myristate 13-acetate (PMA) or 1.25% dimethyl sulphoxide (DMSO) respectively (Collins, 1987).

Immunostaining of cultured cells

Cells were first washed three times with PBS and then attached to microscope slides using a cytocentrifuge (800 rev./min for 5 min). The attached cells were fixed with 4% *p*-formaldehyde in PBS for 10 min at room temperature followed by three PBS washes. Fixed cells were blocked with 10% (w/v) non-fat dry milk in PBS (with 0.1% Triton X-100) for 1.5 h at room temperature followed by two PBS washes. Blocked cells were incubated with PBS (as a control) or various antibodies (30 µg/ml) in PBS supplemented with 1% non-fat dry milk and 0.1% Triton X-100 for 1 h at room temperature followed by three PBS washes. Finally, cells were incubated with fluorescein isothiocyanate-conjugated rat anti-mouse IgG antibodies (10 µg/ml) in PBS supplemented with 1% non-fat dry milk and 0.1% Triton X-100 for 1 h at room temperature followed by three PBS washes. The slides were mounted with a coverslip in preserving solution [50% (v/v) glycerol, 0.8 mg/ml *p*-phenylenediamine in 0.2 M Na₂CO₃ buffer (pH 9.0)] for fluorescence microscopy and microphotography.

Preparation of detergent cellular extracts

Cells were harvested and washed two to three times with ice-cold PBS. The cell pellets were resuspended in an appropriate volume of 0.5% Nonidet P40 (NP-40) in buffer A (150 mM NaCl, 10 mM Hepes, pH 7.0, 0.1% Na₂S₂O₈). After vigorous vortex mixing and incubation on ice for 10–30 min, the NP-40 extracts were centrifuged at 4 °C for 5 min at 15000 *g*. The NP-40-insoluble pellet was discarded and the supernatant was assayed for GPI-degrading activity.

Localization of GPI-PLD in myeloid cells

Two methods were employed to determine the subcellular localization of GPI-PLD in myeloid cells. The first method employed sonication to break down cells with a Branson cell sonifier (power 4, 30% intermittent for 4 min). Unbroken cells and nuclei were pelleted with a spin at 500 *g* for 10 min and

discarded. The supernatant was further centrifuged at 100000 *g* for 1 h at 4 °C. The relative distribution of GPI-PLD activity in the 100000 *g* supernatant and pellet fractions was then determined. The second method used streptolysin O (SLO) to permeabilize the plasma membrane to release the cytosolic contents. First, 1×10^6 – 2×10^6 cells in 1 ml of PBS were preincubated for 20 min on ice with 10–12 units of activated SLO (Gravotta et al., 1990). Unbound SLO was washed away with ice-cold PBS. Permeabilization was induced by incubating 0.2 ml of final cell suspension at 37 °C for 20 min. Finally the GPI-PLD activity and the cytosolic marker enzyme lactate dehydrogenase activity released into the incubation medium were determined.

GPI-degradation assay

GPI degradation by cells was monitored by using VSG as substrate as described previously (Huang et al., 1991; Low, 1992) with slight modifications. Briefly, 10 µl of NP-40 cellular extracts and 90 µl of buffer A were mixed in microcentrifuge tubes. The reaction was started by adding 0.1 ml of a substrate mixture containing 40 mM Tris/maleate (pH 7.0), 0.1% NP-40, and 14000 c.p.m. [³H]VSG. In some experiments, 0.5–1 mM 1,10-phenanthroline (GPI-PLD inhibitor) or 20 mM NaF (phosphatidate phosphohydrolase inhibitor) were also included in the assay. The reaction mixture was incubated at 37 °C for 60 min or as indicated and then stopped by the addition of 0.5 ml of 1 M NH₄OH-saturated butanol. After 2 min of vortex mixing, phase separation was achieved by 3 min centrifugation in a Microfuge. Then 0.3 ml of the upper organic phase was sampled, mixed with 5 ml of Ecoscint (National Diagnostics, Atlanta, GA, U.S.A.) and analysed by liquid-scintillation counting. One unit of GPI-PLD activity is arbitrarily defined as the amount of enzyme required to hydrolyse 1% of [³H]VSG in 1 min.

T.l.c. analysis of VSG-degradation products

A modification of a previously described procedure (Metz et al., 1991; Xie et al., 1993) was used. In brief, 0.45 ml of the upper phase from the butanol extraction in the standard assay (see above) was sampled and dried using a Speed-Vac concentrator. The dried samples were resuspended in 50 µl of chloroform/methanol (2:1, v/v) and spotted on to silica-gel 60A K6 plates. The plates were developed in chloroform/methanol/0.25% KCl (55:45:10, by vol), air-dried, sprayed with EN³HANCE, and subjected to fluorography for 2 weeks at –80 °C. This t.l.c. system gave a good separation of phosphatidic acid (PA; *R_F* = 0.68) from diacylglycerol (DAG; *R_F* = 1), and fatty acid/monoacylglycerol (*R_F* = 0.85–0.94) as determined with purified lipid standards.

Determination of GPI-PLD secretion

Cells were washed three times with regular medium and used immediately. Regular culture medium plus 1% Nutridoma SP and secretagogues or secretion inhibitors were mixed with cells. Experiments using a different reaction medium (125 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 25 mM Hepes, pH 7.5, 5 mM glucose, 1 mg/ml BSA) gave similar results. The reaction was initiated by incubating the mixture at 37 °C. To examine the effect of reaction temperature on myeloid GPI-PLD release, reaction mixtures were also incubated on ice or at 17 °C. Small samples of the reaction medium were withdrawn at different times after the reaction started. Finally the GPI-PLD activity in the medium samples and cell extracts were determined as described above.

Miscellaneous procedures

The protein content of detergent cellular extracts was determined using the BCA protein assay kit (Pierce). Lactate dehydrogenase activity was assayed using the LDH diagnostic kit (Sigma). Cell viability was determined by Trypan Blue (0.4 %) exclusion.

RESULTS

Myeloid cell lines contain GPI-degrading activity

A culture of HL-60 promyelocytic leukaemia cells newly derived from a frozen A.T.C.C. stock culture was serendipitously found to contain a very high level of GPI-degrading activity (about 50 units/mg of protein). Inexplicably, this HL-60 GPI-degrading activity decreased to approx. 7 units/mg of protein after 1 month of culture and remained stable at this level for the remainder of the study period. This finding prompted us to survey a wider range of myeloid cell lines at different stages of differentiation for their ability to degrade GPI. All lines tested exhibited significant GPI-degrading activity as shown in Table 1. Of the nine lines examined, J774 murine macrophages exhibited the highest level of GPI-degrading activity (about 50 units/mg of protein). This high activity remained unchanged over the entire study period of 10 months. The other myeloid cell lines, ranging from multipotential blasts (K562) to monocytes (THP-1 and RAW 264.7), all contained much lower, but still significant, levels of GPI-degrading activity (4–10 units/mg of protein; Table 1). We also examined a T-lymphoblastic leukaemia cell line MOLT-4 and found that it also exhibited a low, but detectable, level of GPI-degrading activity (Table 1).

Characterization of the myeloid GPI-degrading activity

In order to determine the nature of the myeloid GPI-degrading activity, the reaction products were analysed by t.l.c. As shown in Figure 1 all the myeloid cell lines tested generated [3 H]phosphatidic acid ([3 H]PA) when incubated with [3 H]VSG.

Table 1 GPI-degrading activity in cultured haematolymphoid cell lines

Detergent extracts of cells cultured in the presence or absence of serum (2–3 days) were prepared and assayed for [3 H]Myr-labelled VSG degradation as described in the Materials and methods section. Values represent means $\pm$ S.D. (no. of independent experiments).

Cell line (lineage)	GPI-degrading activity (units/mg of protein)	
	+ Serum	– Serum
Mouse cells		
J774 (monocyte/macrophage)	52.05 $\pm$ 20.11 (5)	36.08 $\pm$ 2.69 (3)
RAW 264.7 (monocyte/macrophage)	4.55 $\pm$ 0.07 (2)	–
Human cells		
HL-60 (promyelocyte)	6.93 $\pm$ 1.33 (10)	4.02 $\pm$ 0.73 (3)
KG-1 (myeloblast)	5.01 $\pm$ 2.02 (4)	–
K562 (multipotential blast)	3.45 $\pm$ 0.14 (3)	4.07 $\pm$ 0.81 (2)
PLB-985 (promyelocyte)	8.94 $\pm$ 1.58 (5)	5.79 $\pm$ 0.76 (4)
NB4 (promyelocyte)	4.25 $\pm$ 0.72 (4)	–
U-937 (promonocyte)	5.63 $\pm$ 0.08 (2)	–
THP-1 (monocyte)	4.12 $\pm$ 0.19 (3)	–
MOLT-4 (T-lymphoblast)	2.57 $\pm$ 0.33 (2)	5.11 $\pm$ 0.77 (2)

This result unequivocally demonstrated that myeloid cells contained GPI-PLD activity. However, in addition to [3 H]PA, variable amounts of [3 H]dimyristoylglycerol ([3 H]DAG) and [3 H]myristic acid ([3 H]Myr) accumulated also, suggesting the additional presence of PA phosphohydrolase and DAG lipase and/or GPI-PLC activities in the cell extracts. With the lymphoid MOLT-4 cell line, only [3 H]DAG and [3 H]Myr accumulated and [3 H]PA was not detectable (Figure 1, lane 20). To evaluate the contribution of GPI-PLC to the overall VSG degradation, 1 mM 1,10-phenanthroline, a known specific inhibitor of GPI-PLD (Low and Prasad, 1988; Low, 1990, 1992), was included in the reaction mixture. Phenanthroline abolished [3 H]PA accumulation and greatly reduced the intensity of the [3 H]DAG band (Figure 1, compare lanes 2 and 1, 7 and 4, 10 and 9). Phenanthroline (0.5–1 mM) generally elicited a 50–90 % reduction in the formation of the total [3 H]VSG-degradation products for all the cell extracts tested (results not shown). In some cases, [3 H]Myr still accumulated even when [3 H]PA and [3 H]DAG production was inhibited (Figure 1, compare lanes 2 and 1, 7 and 4, 10 and 9). This observation suggests that a small proportion of VSG-degrading activity is due to direct cleavage by phospholipase A rather than secondary deacylation of PA or DAG. On this basis, we conclude that the majority, if not all, of the myeloid GPI-degrading activity is a phospholipase D.

Figure 1 [3 H]Phosphatidic acid was produced by the myeloid GPI-degrading activity

[3 H]Myristate-labelled VSG was incubated for 3 h at 37 °C with carrier control (lane 22), 5 μ g of J774 cell extracts or 50 μ g of detergent extracts for all other cells. 1,10-Phenanthroline (Phe; 1 mM) or NaF (20 mM) was also included in the reaction mixture as indicated. HL-60 cells in lanes 5 and 6 had been differentiated for 2 days with 1.25 % DMSO and 100 nM PMA respectively. The reaction products were analysed by t.l.c. and fluorography. The mobilities of pure standard lipids, dimyristoylglycerol (DAG), myristic acid (Myr) and phosphatidic acid (PA) are indicated at the left of the fluorograms.

To characterize the origin of [3 H]DAG and [3 H]Myr further the role of PA phosphohydrolase was examined. When NaF (20 mM), a known inhibitor of PA phosphohydrolase (Jamal et al., 1991; Heller et al., 1992; Xie et al., 1993), was included in the incubation mixture, the intensity of the [3 H]DAG band was reduced with a concomitant increase in [3 H]PA band intensity for J774, HL-60, KG-1, PLB-985 and THP-1 (Figure 1, compare lanes 3 and 1, 8 and 4, 11 and 9, 15 and 14, 19 and 18). This result indicated that myeloid cells express PA phosphohydrolase activity which converts the [3 H]PA produced by GPI-PLD action into [3 H]DAG. Inclusion of NaF in the MOLT-4 reaction mixture also allowed [3 H]PA accumulation with a decrease in [3 H]DAG band intensity (Figure 1, compare lanes 21 and 20), suggesting that the low level of GPI-degrading activity in MOLT-4 cells can also be attributed to GPI-PLD action. We also noted that [3 H]Myr accumulation was not significantly altered by NaF (Figure 1, compare lanes 3 and 1, 8 and 4, 11 and 9, 15 and 14, 19 and 18, 21 and 20), further suggesting the presence of phospholipase A activity. In contrast, NaF had no effect on the product profile obtained with K562 and U-937 cells (Figure 1, compare lanes 13 and 12, 17 and 16), suggesting that these cells express predominantly a NaF-insensitive isoenzyme of PA phosphohydrolase (Jamal et al., 1991; Kanoh et al., 1992). It is interesting to note that when HL-60 cells were differentiated either monocytically (Figure 1, lane 5) or granulocytically (Figure 1, lane 6), the ratio of DAG/Myr to PA greatly increased over that of the control cells (Figure 1, lane 4). This observation suggests that differentiation of myeloid progenitor cells induces increased expression of PA phosphohydrolase.

Origin of myeloid GPI-PLD: endogenous synthesis or uptake from medium?

As abundant GPI-PLD is present in the bovine serum, which was used in culturing the cells, it is possible that these cells acquired GPI-PLD from the culture medium. To examine this possibility, cells were cultured in the absence of serum, and cellular GPI-PLD activity was subsequently determined. After 2–3 days in serum-free culture, the majority of GPI-PLD activity still remained in the cells (Table 1). The GPI-degrading activity in the medium increased significantly (results not shown). This increase is probably due to a combination of secretion (see below) and cell lysis (as indicated by the appearance of Trypan Blue-stained cells after many hours of serum-free culture; results not shown). It was difficult to monitor cells during long-term serum-free culture (i.e. > 3 days) because even in the presence of Nutridoma SP, there was significant cell lysis. In an attempt to circumvent this problem, cells were cultured in GPI-PLD-depleted complete medium. Similar results were obtained with no substantial reduction in cell-associated GPI-PLD activity after 2–3 days (results not shown). Significantly, with J774 cells, the total GPI-PLD activity (cell-associated plus medium) at the end of a 2-day culture in GPI-PLD-depleted medium increased approx. 50% over that of the starting culture. However, it was noted that culture in GPI-PLD-depleted medium for this length of time also resulted in a greater amount of cell death than cultures in serum-free medium. The reason for this is unknown but it is possible that the GPI-PLD depletion procedure (mAb 216 affinity chromatography) also depleted essential serum component(s) and/or contaminated the medium with toxic material.

To determine whether myeloid cells synthesize GPI-PLD, J774 cells were labelled with [35 S]methionine and immunoprecipitated with an antibody known to bind rat serum GPI-PLD (i.e. mAb 191). However, radioactive bands in the expected 100 kDa region were not observed. This negative result might also be due to a

low rate of GPI-PLD turnover. To examine this possibility, the effect of cycloheximide, a protein-synthesis inhibitor, on GPI-PLD expression was tested. Short-term (up to 6 h) treatment of J774 and HL-60 cells with 10–60 μ g/ml cycloheximide did not significantly alter their GPI-PLD content whereas 24 h treatment caused only a 20% reduction.

Immunostaining of cultured myeloid cells

To demonstrate further that myeloid cells express GPI-PLD, cells were stained with several anti-bovine serum GPI-PLD mAbs. J774 cells were strongly stained intracellularly with mAb 191 (Figure 2a). By comparison with the phase-contrast micrograph (Figure 2b), all J774 cells were stained to some extent, although heterogeneity of staining intensity existed among individual cells. This may reflect the different positions of cells fixed on the slides, as there is a strikingly uneven distribution of bright staining within each cell (Figure 2a). Bright staining is mainly clustered in the perinuclear region and beneath the cell surface (Figure 2a). Several mAbs including 190, 205, 196 and 200 which recognize different epitopes of bovine serum GPI-PLD from mAb 191 also showed significant, though less intense, staining of J774 cells, whereas others (e.g. 212, 216) were completely negative (results not shown). The irrelevant mAb T-173 gave faint non-specific staining of nuclei (Figure 2c). HL-60 cells were stained positively by two mAbs, 191 (Figure 2d) and 196 (not shown) whereas staining by other mAbs was negative. U-937 (Figure 2e), THP-1 (Figure 2f) and all other myeloid cell lines were stained by mAb 191. Consistent with its low GPI-PLD content, T-lymphoid MOLT-4 cells were barely stained by mAb 191 (results not shown). With the exception of K562 and MOLT-4 cells, all human cell lines tested showed a similar mAb 191 staining pattern: bright dots in the perinuclear region and around the cell surface, and weak to strong staining of the nuclei, particularly nucleoli (Figures 2d–2f). With K562 cells, the bright dots were absent. It should be pointed out that the punctate staining in the perinuclear region and at the cell surface of most cell types is specific for mAb 191 whereas the nuclear staining is non-specific, as even the irrelevant mAb T-173 gave a similar staining pattern.

Localization of GPI-PLD in myeloid cells

The punctate immunostaining pattern of myeloid GPI-PLD suggests that it is concentrated in limited intracellular locations, presumably secretory vesicles. This suggestion was supported by permeabilization experiments with SLO which induces pore formation specifically on the plasma membrane (Gravotta et al., 1990). When either J774 or HL-60 cells were permeabilized by SLO, most of the cytosolic enzyme, lactate dehydrogenase, was released into the medium whereas GPI-PLD was not released (results not shown). To characterize the intracellular distribution of GPI-PLD further, cellular fractionation of J774 and HL-60 cells was performed. Cells were disrupted by sonication, the soluble and membrane fractions separated by ultracentrifugation, and GPI-PLD activity was determined in the total sonicate, supernatant and membrane fractions. As shown in Table 2, the GPI-PLD activity in the membrane fraction accounted for approx. 60% of the total cellular activity for both J774 and HL-60 cells. The supernatant fraction contained almost all of the remaining activity. Myeloid cells generally possess a large number of granules and vesicles which are quite small (100–500 nm) and can survive sonication (Uvnas, 1974). In contrast, the soluble luminal contents of the endomembrane system, mainly endoplasmic reticulum and Golgi, to a large extent, are released

Figure 2 Immunofluorescence staining of myeloid cell lines

Myeloid cells, maintained in suspension culture, were spun on to microslides with a cytospin, stained with an mAb to GPI-PLD (mAb 191) and then visualized by fluorescence microscopy. (a) J774 murine macrophages stained with mAb 191. All cells became stained to some extent as indicated by comparison with the phase contrast of the same field (b). (c) J774 cells stained with irrelevant control mAb, T-173. The faint non-specific staining appears to be entirely located in the nuclei as judged by comparison with the phase contrast (not shown). (d) HL-60 human promyelocytes, (e) U-937 human promonocytes and (f) THP-1 human monocytes stained with mAb 191. Note the numerous brightly stained dots, presumably granules, in these cells. These three cell types also exhibited the faint non-specific nuclear staining described above for J774 when T-173 was used as a control (results not shown).

Table 2 Distribution of GPI-PLD activity in J774 and HL-60 cells

Cells were sonicated and fractions prepared as described in the Materials and methods section. The GPI-PLD activities in total sonicate, supernatant and membrane fractions were determined. The activity in total sonicate was approximately (with a deviation of $< \pm 5\%$) the sum of the activity in the supernatant fraction and that in the membrane fraction. The percentage of activity in each fraction was calculated as a percentage of recovered activity. Values represent means $\pm$ S.D. (no. of independent experiments).

Fraction	Percentage of total recovered GPI-PLD activity	
	J774	HL-60
Supernatant	40.7 $\pm$ 4.6 (4)	42.2 $\pm$ 1.7 (2)
Membrane	59.3 $\pm$ 4.6 (4)	57.8 $\pm$ 1.7 (2)

immunostaining data are consistent with the suggestion that myeloid GPI-PLD is localized mainly in vesicles with the remainder in endoplasmic reticulum and Golgi.

Effect of differentiation on GPI-PLD activity in myeloid progenitor cells

The promyelocytic leukaemia cell lines HL-60, NB4 and PLB-985 are bipotential: they can be differentiated into either neutrophil-like cells (by such inducers as DMSO, retinoic acid and dibutyl cyclic AMP) or macrophage-like cells (by such inducers as phorbol diesters and vitamin D₃) (Collins, 1987). With the differentiation of these promyelocytes, the expression of many GPI-anchored proteins are up-regulated and some are released into the medium (Maliszewski et al. 1985). To examine the possibility that there is also an accompanying increase in GPI-PLD expression with differentiation, GPI-PLD activity was determined in differentiated promyelocytes. As shown in Table 3, HL-60 cells differentiated with either DMSO or PMA for 2–3

into the supernatant fraction by the sonication procedure (Kreibich and Sabatini, 1974). These results combined with the

Table 3 Differentiation of myeloid precursor cells induced enhanced expression of GPI-PLD activity

HL-60 and KG-1 cells were cultured in the presence of carrier control, 1.25% DMSO, or 100 nM PMA for 2–3 days. The cells were harvested and extracted with 0.5% NP-40, and the GPI-PLD activity was determined. Values represent means $\pm$ S.D. (no. of independent experiments).

Treatment	GPI-PLD specific activity (units/mg of protein)	
	HL-60 cells	KG-1 cells
Control	6.93 $\pm$ 1.33 (10)	5.01 $\pm$ 2.02 (4)
DMSO	14.48 $\pm$ 4.13 (4)	—
PMA	14.04 $\pm$ 3.98 (6)	12.92 $\pm$ 2.63 (2)

Figure 3 Secretion of GPI-PLD by J774 cells

J774 cells, cultured in 100 mm $\times$ 20 mm dishes (5×10^6 cells in each dish), were washed three times with serum-free Dulbecco's modified Eagle's medium by aspiration and then incubated in 4 ml of the same medium supplemented with 1% Nutridoma SP. The secretion reaction was started immediately by adding 2 mM ATP and 500 nM PMA or carrier as indicated followed by incubation at 37 °C. Small portions of reaction medium were sampled at 0, 0.5, 1 and 2 h after the start of the reaction and assayed for the GPI-PLD activity. Total activity in each dish was approx. 100 units. A representative experiment is shown, one of two giving similar results. ○, Control; ●, +PMA; △, +ATP; ▲, +PMA +ATP.

days resulted in a consistent 2- to 3-fold increase in GPI-PLD activity. Essentially the same results were obtained for PBL-985 and NB4 cells (results not shown). KG-1 cells are in an earlier (myeloblast) stage of development and can be induced by phorbol esters to differentiate into macrophage-like cells (Koeffler et al., 1979). As shown in Table 3, PMA-induced differentiation of KG-1 cells also led to a similar 2- to 3-fold increase in GPI-PLD activity.

Secretion of GPI-PLD by myeloid cells

To explore the possibility that myeloid cells may be a significant contributor to the GPI-PLD in the plasma, we determined whether the cultured cell lines could release GPI-PLD activity into culture medium. As shown in Figure 3, J774 cells constitutively released GPI-PLD activity into extracellular medium at a rate of about 1 unit/h per 10^6 cells (equivalent to approx. 5% of

cellular GPI-PLD per h). This time-dependent release did not result from cell lysis as (i) no dead cells were detached from the culture dishes and (ii) there was no increase in the number of Trypan Blue-stained cells during the experimental period. Release continued for at least 4 h (the longest time tested). Secretion experiments with long-term serum-free cultured cells were complicated by cell lysis as described above. HL-60 and PLB-985 promyelocytes also exhibited similar rates of constitutive release of GPI-PLD into extracellular medium (results not shown). To explore the possibility that myeloid cells release GPI-PLD through the classical exocytotic pathway, we repeated these experiments under conditions known to disrupt the secretory process. Temperatures below 18 °C inhibit membrane fusion and thus most of the membrane traffic process (Kuismanen and Saraste, 1989). Incubating HL-60 cells and J774 cells on ice or at 17 °C inhibited GPI-PLD release by 50–70%. Another well-characterized secretion inhibitor is brefeldin A, which blocks transport of proteins into post-Golgi compartments and eventually disassembles Golgi (Lippincott-Schwartz et al., 1990). Surprisingly, treatment of cells with 10–30 μ g/ml brefeldin A for 2–4 h caused only a modest inhibition of 5–20%.

In an attempt to stimulate the basal release of GPI-PLD, the effects of some J774 cell agonists were examined. J774 cells possess two types of ATP receptors: occupation of one class of receptor leads to calcium mobilization whereas occupation of the other type results in permeabilization of plasma membrane (Murgia et al., 1993). However, addition of 2 mM ATP did not increase J774 GPI-PLD release. The protein kinase C activator, PMA (500 nM), stimulated J774 cell secretion of GPI-PLD by 10–20%, and this effect was enhanced by ATP to give an overall increase in secretion rate of approx. 80% in the first hour (Figure 3).

DISCUSSION

In spite of extensive characterization of the biochemical and molecular properties of GPI-PLD over the past several years (Scallion et al., 1991; Hoener and Brodbeck, 1992; Low and Huang, 1991, 1993; Li and Low, 1993), its physiological role remains undefined. It has long been proposed that GPI-PLD modulates the surface expression of GPI-anchored proteins by converting them from the membrane-anchored form into the soluble form (Low, 1989). However, the plasma GPI-PLD (either purified or crude) fails to hydrolyse GPI-anchored proteins in intact cells, isolated membranes or artificial liposomes (Davitz et al., 1989; Low and Huang, 1991). Therefore it is reasonable to assume that only endogenous GPI-PLD is capable of hydrolysing membrane-associated substrates. This view is supported by co-transfection experiments in which COS cells co-transfected with cDNAs for GPI-PLD and GPI-anchored alkaline phosphatase released alkaline phosphatase into the culture medium (Scallion et al., 1991). However, physiological cleavage of endogenous GPI-anchored proteins by an endogenous GPI-PLD has not been unequivocally demonstrated. Thus far, several cell types, including pancreatic α and β cells (Metz et al., 1991), mast cells (Metz et al., 1992), keratinocytes and neuronal cells (Sesko and Low, 1991; Xie et al., 1993), have been shown to contain GPI-PLD as determined by enzyme activity and/or immunoreactivity. Unfortunately none of the above cell types is well suited for studying GPI-PLD function, as GPI-anchored proteins have not been identified/characterized (in α and β cells, keratinocytes) or sufficient cell material is difficult to obtain (for mast cells and neuronal cells). The present study represents our continued effort to identify other GPI-PLD-expressing cells which may be more practical model systems for studying GPI-PLD function.

This study demonstrates that all the myeloid cell lines tested also express and release GPI-PLD. These myeloid cell lines represent different stages of myeloid differentiation ranging from early progenitor cells (K562 and KG-1) to terminal cells (J774 and RAW 264.7) (Table 1). There is no obvious correlation between the stage or pathway of cell differentiation and the level of GPI-PLD content. Most cells contain 4–9 units of GPI-PLD activity/mg of protein except J774 cells which express substantially higher levels of GPI-PLD activity (approx. 50 units/mg of protein). Currently we do not have a plausible explanation for the high expression of GPI-PLD activity by J774 cells or a similarly high, but transient, expression of GPI-PLD by HL-60 cells. A similar mouse monocyte/macrophage line RAW264.7 expresses an at least 10-fold lower level of GPI-PLD activity. We also examined one T-lymphoblastic leukaemia line, MOLT-4, and found that it also contained a modest level of GPI-PLD activity (approx. 2 units/mg of protein). These findings were unexpected because an earlier study (Metz et al., 1991) failed to detect any GPI-PLD activity in cells purified from human blood including erythrocytes, lymphocytes, macrophages and neutrophils. Such a discrepancy could be explained by the loss of GPI-PLD expression after normal cell maturation and the non-physiological acquisition of GPI-PLD by these cancerous cell lines. Another more likely possibility is that different assay conditions used in these two studies were responsible. The assay conditions used in that study, e.g. high concentrations of detergent [now known to be inhibitory to GPI-PLD (Low and Huang, 1991, 1993; Hoener and Brodbeck, 1992)], might have precluded detection of a low level of GPI-PLD activity. Consistent with this explanation, significant GPI-PLD activity (approx. 2 units/mg of protein) was detected in purified human neutrophils using a lower detergent concentration (M. Xie and M. G. Low, unpublished work). This observation indicates that a re-examination of GPI-PLD expression in other primary mature myeloid cells is warranted.

We have shown previously (Low and Prasad, 1988) that plasma is the major location of rat GPI-PLD, although all tissues examined contained significant amounts of GPI-PLD activity (Xie et al., 1993). Because of the extensive vascularization, the GPI-PLD activity in tissues could still be accounted for by the contamination of plasma GPI-PLD even after perfusion. Therefore it is very difficult to determine unequivocally the tissue sources of circulating GPI-PLD. The present study, using cultured cells, suggests that myeloid cells are a likely source of plasma GPI-PLD. Myeloid cells would in fact be ideally suited for such a role. All the myeloid cells except macrophages (present in most tissues) reside in the bone marrow and blood, which constitute one continuously renewing system. Furthermore, several lines of evidence support the suggestion that the GPI-PLD in myeloid cells is unlikely to have resulted from contamination by or uptake from the serum. (i) The majority of myeloid GPI-PLD activity remained associated with the cells after 2–3 day culture in either serum-free medium or GPI-PLD-depleted regular medium. (ii) Preliminary immunocapture assays (Xie et al., 1993) indicate that little of the GPI-PLD extractable from J774 cells is bound to mAb 191 in contrast with GPI-PLD from bovine serum (M. Xie and M. G. Low, unpublished work). (iii) Several myeloid cell lines (e.g. J774, HL-60, U-937) exhibited barely detectable binding or uptake of 125 I-labelled bovine serum GPI-PLD (D. J. Silience and M. G. Low, unpublished work). Taken together, these results suggest that the majority of myeloid GPI-PLD is synthesized *de novo*.

In spite of this evidence for an endogenous origin of GPI-PLD, we have, as yet, been unable to confirm GPI-PLD biosynthesis by myeloid cells. Preliminary experiments using

[35 S]methionine metabolic labelling were unsuccessful. This was not unexpected since, as described above, the majority of GPI-PLD in the J774 cells did not bind to mAb 191. Furthermore, the inability of cycloheximide to reduce enzyme activity suggests that the GPI-PLD turnover rate is relatively low ($t_{1/2} > 24$ h). A combination of low turnover rate and low mass abundance (approx. 0.01 % of total cellular protein) of GPI-PLD may also have contributed to the failure of the biosynthetic labelling experiment.

Further evidence supporting the notion that myeloid cells are potential contributors of plasma GPI-PLD comes from the GPI-PLD localization and secretion experiments. The punctate immunofluorescence staining pattern with mAb 191 suggests that some of the myeloid GPI-PLD might be localized in vesicles. Consistent with this possibility were the permeabilization and cell fractionation data which indicated that the majority of myeloid GPI-PLD activity was either bound to membranes or located inside sonication-resistant intracellular organelles (Uvnas, 1974). These findings are in contrast with our earlier results with keratinocytes where no punctate staining was observed and the majority (70–75 %) of cellular GPI-PLD activity went into the supernatant fraction after sonication (Xie et al., 1993). We also showed that myeloid cells released GPI-PLD activity into the culture medium constitutively. This release was greatly inhibited by low temperature (below 18 °C), suggesting involvement of membrane traffic in the process (Kuismanen and Saraste, 1989). However, the GPI-PLD release was only modestly reduced by brefeldin A treatment. A combination of the low biosynthetic rate of myeloid GPI-PLD and the likely localization of the majority of this enzyme in post-Golgi vesicles may well explain this weak inhibition by brefeldin A. As brefeldin A only blocks transport of proteins across the Golgi (Lippincott-Schwartz et al., 1990), release from post-Golgi compartments should not be affected. Together these data strongly suggest that most, if not all, of the released GPI-PLD is derived from the post-Golgi compartments (possibly vesicles) most likely through the classical exocytotic mechanism.

This study also shows that either monocytic or granulocytic differentiation of myeloid progenitor cells is accompanied by a 2–3-fold increase in GPI-PLD activity. PA phosphohydrolase activity also increases significantly with myeloid differentiation. Previous studies have shown that granulocytic differentiation of HL-60 cells led to a dramatic increase in phosphatidylcholine-specific phospholipase D activity (Xie et al., 1991). A plethora of recent data have strongly implied that phosphatidylcholine-specific PLD and PA phosphohydrolase are important components of cellular signal-transduction pathways (Billah and Anthes, 1990). Therefore the current findings are consistent with the suggestion that GPI-PLD and PA phosphohydrolase in addition to phosphatidylcholine-specific PLD all play important roles in the myriad myeloid functions that are acquired only after full myeloid maturation.

The finding that myeloid cells express GPI-PLD is particularly interesting, as many GPI-anchored proteins are known to be expressed in myeloid cells. Therefore the myeloid cell lines will provide an excellent model system for studying GPI-PLD function. The soluble forms of many of the GPI-anchored proteins present in myeloid cells, e.g. CD14 (Bazil et al., 1989), CD16 (Huizinga et al., 1990), decay-accelerating factor (Medof et al., 1987) and CD59 (Stefanová et al., 1989), have been found in normal plasma as well as in synovial fluid and urine in some patients. The GPI-PLD-mediated anchor cleavage is a potential mechanism for generating these soluble proteins. Consistent with this suggestion is the observation that a substantial proportion of plasma-soluble CD14, decay-accelerating factor and CD59 have

the same molecular mass as the GPI-anchored form (Medof et al., 1987; Bazil et al., 1989; Stefanová et al., 1989). Further corroborating evidence for GPI-PLD action in myeloid cells is provided by our preliminary observation that HL-60 cells labelled with [³H]ethanolamine (a component of the GPI anchor), when cultured in the presence of PMA, released several ³H-labelled proteins into the culture medium (M. Xie and M. G. Low, unpublished work). Future characterization of these [³H]-ethanolamine-labelled proteins using myeloid cell models should provide clues to both the mechanism and physiological significance of GPI-PLD action in general.

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