Subcellular distribution of glycosylphosphatidylinositol-specific phospholipase D in rat liver

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Glycosylphosphatidylinositol (GPI)-hydrolysing enzymes have been described in many mammalian tissues and body fluids; however, their site(s) of action and *in vivo* functions have remained unclear. In order to identify a possible intracellular site of GPI hydrolysis, we studied the subcellular distribution of GPI-hydrolysing activity in rat liver. We found that purified fractions from rat liver hydrolysed the GPI moieties of two GPI-

anchored proteins with the specificity of a phospholipase D. This GPI-specific phospholipase D (GPI-PLD) activity was found to be highly enriched in a lysosomal fraction and showed a similar intracellular distribution to that of typical lysosomal enzymes. Our results indicate that lysosomes may represent a possible intracellular site of GPI-PLD action.

INTRODUCTION

Glycosylphosphatidylinositol (GPI)-anchored cell surface proteins are widely distributed in mammalian tissues. Most of these proteins have been identified by their susceptibility to GPI-hydrolysing phospholipases C and D. GPI-specific phospholipases D (GPI-PLD) have been isolated as 100–115 kDa glycoproteins from mammalian serum [1,2]. In addition, GPI-PLD activities have also been described in brain [3], milk and cerebrospinal fluid [4]. GPI-PLD mRNA has been detected in a number of bovine tissues [5]. Despite the widespread distribution of GPI-PLD, its enzymic action on membrane-bound GPI-anchored proteins has not yet been demonstrated. Although GPI-PLD readily hydrolyses detergent-solubilized GPI-anchored proteins, it is not active towards these substrates when located in membranes [6].

GPI-PLD has also been shown to be secreted from several cell lines in culture [7,8] and was reported to release GPI-anchored proteins from HeLa and bone marrow stromal cells [7,9]. In addition, in studies in which GPI-PLD and GPI-anchored proteins were co-transfected in COS-1 cells, protein GPI anchors were hydrolysed by GPI-PLD [10,11]. Since the membranes encountered by (transfected) GPI-PLD and its potential substrates early in the secretory pathway are believed to be different from those on the cell surface, the above studies may indicate that intracellular compartments represent a better environment for GPI-PLD action. Furthermore it has been shown recently that purified serum GPI-PLD is remarkably resistant towards the action of proteases [12–14] allowing the speculation that GPI-PLD may be active intracellularly, i.e. after uptake from serum, in the degradation of GPI-anchored substrates.

In order to localize a possible intracellular site of GPI-PLD action, we studied the subcellular distribution of GPI-PLD activity in rat liver.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade and from Fluka (Buchs, Switzerland), Sigma (St. Louis, MO, U.S.A.) or Merck (Darmstadt, Germany). Monoclonal antibody 117-1 (purified

IgG₁) against purified GPI-PLD from bovine serum was provided by Dr. B. Nørgaard-Pederson (Copenhagen, Denmark) and Dr. J. Liao. Horseradish peroxidase-conjugated second antibodies were from Dako (Glostrup, Denmark). Acetylcholinesterase (AChE) from bovine erythrocytes [15] and GPI-PLD from bovine serum [4] were purified as described previously. A partially purified GPI-PLD from rat serum was obtained after phase partitioning of rat serum in Triton X-114 followed by DEAE-cellulose and octyl-Sepharose chromatography as described elsewhere [4]. [³H]Myristate-labelled variant surface glycoprotein (VSG) from *Trypanosoma brucei* was prepared by the method of Hereld et al. [16] as described previously [17].

Isolation of rat liver subcellular fractions

Male Wistar rats weighing 300–500 g (Winkelmann, Borken, Germany) were anaesthetized with Nembutal (intraperitoneal administration). For removal of blood, the liver was perfused *in situ* with 0.15 M NaCl (37 °C) via the portal vein, and subsequently excised and homogenized in ice-cold 5 mM Hepes/NaOH buffer, pH 7.4, containing 250 mM sucrose. Subcellular fractions of rat liver were obtained by differential centrifugation as described by de Duve et al. [18]. Fractions enriched in lysosomes were prepared by isopycnic density-gradient centrifugation as described by Trouet [19] and Wattiaux et al. [20]. Fractions enriched in Golgi vesicles were obtained by isopycnic density-gradient centrifugation by the method of Fleischer [21]. All subcellular fractions were resuspended in 5 mM Hepes, pH 7.4, containing 250 mM sucrose, and immediately analysed for marker enzyme activities. Aliquots were shock-frozen in liquid nitrogen and stored at –70 °C until analysed for GPI-PLD activity.

The purities of the preparations were analysed biochemically by measuring marker enzyme activities as described previously [22–24]. The distribution of the constituents and the relative specific activities (RSAs) of the enzymes in the fractions were calculated as described by de Duve et al. [18].

Assay for GPI anchor-hydrolysing activity

All rat liver subcellular fractions were diluted with 50 mM Mes

Abbreviations used: AChE, acetylcholinesterase; GPI, glycosylphosphatidylinositol; GPI-PLD, glycosylphosphatidylinositol-specific phospholipase D; PI-PLC, phosphatidylinositol-specific phospholipase C; RSA, relative specific activity; VSG, variant surface glycoprotein.

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(pH 6.5)/0.1 mM CaCl₂ (assay buffer) to a final protein concentration of approx. 360 µg/ml. GPI hydrolysis was measured using the two GPI-anchored proteins, AChE from bovine erythrocytes and [³H]myristate-labelled VSG from *T. brucei*, as substrates. When AChE was used as substrate, aliquots (50 µl) of diluted subcellular fractions were added to 50 µl of assay buffer containing 4.8 pmol of membrane-form AChE and 0.01–0.07 % Triton X-100 (see below) [4]. After incubation at 37 °C, GPI hydrolysis was stopped by the addition of 800 µl of cold 4 % (w/v) Triton X-114, and the product of the reaction, i.e. soluble AChE, was separated from non-hydrolysed substrate by phase partitioning at 37 °C. AChE activity in the aqueous and detergent-enriched phases was measured by the method of Ellman et al. [25]. GPI-PLD activity was expressed as either percentage substrate conversion or units, with one unit equalling 1 pmol of soluble AChE produced/min (1 pmol of AChE corresponds to 0.43 IU AChE activity [26]).

With VSG as substrate, aliquots (50 µl) of diluted subcellular fractions were added to 50 µl of assay buffer containing Triton X-100 and 4000–12000 c.p.m. of [³H]myristate-labelled VSG [27]. After incubation at 37 °C, released hydrophobic products were extracted sequentially with toluene and water-saturated butan-1-ol, and the distribution of radioactivity between organic and aqueous phases was determined by scintillation counting. Alternatively, the organic extracts were dried under a stream of nitrogen, resuspended in a small volume of water-saturated butan-1-ol, and applied to TLC plates for product identification.

Analysis of [³H]myristate-labelled reaction products by TLC

TLC was performed using silica-gel 60 plates (Merck) developed in solvent system A [chloroform/methanol/acetic acid/0.9 % NaCl in water (100:50:16:5; by vol.)] or B [chloroform/methanol (9:1, v/v)]. After chromatography, the plates were dried and sprayed with En³Hance (DuPont, Boston, MA, U.S.A.) and exposed to a Kodak X-Omat dental film (France) at –70 °C. Alternatively, radiolabelled lipids were quantified by scraping off the relevant regions of silica and counting the radioactivity in a scintillation counter [27].

Generation of radiolabelled TLC standards

[³H]Dimyristoylphosphatidic acid was released from [³H]myristate-labelled VSG by incubation with purified GPI-PLD in assay buffer containing 0.02 % Triton X-100. [³H]Dimyristoylglycerol was obtained by treatment of [³H]myristate-labelled VSG with phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus cereus* (Boehringer, Mannheim, Germany) in 10 mM triethanolamine, pH 7.5, containing 10 mM EDTA and 0.02 % Triton X-100. The radiolabelled reaction products were extracted with water-saturated butan-1-ol and stored at –20 °C.

Protein determination

Protein was determined using the BCA reagent kit (Pierce Chemicals, Rockford, IL, U.S.A.) adapted for microtitre plates or by the method of Lowry et al. [28] with BSA as standard.

RESULTS AND DISCUSSION

GPI-hydrolysing activity in subcellular fractions of rat liver

It has been previously found that a crude preparation of rat liver contains GPI-hydrolysing activity [29]. In order to study the subcellular location of this activity, we fractionated thoroughly

washed rat livers and determined possible GPI-hydrolysing activity in subcellular fractions. Rat liver was fractionated into cytoplasmic extract (fraction E) and nuclear fraction (N). Subsequently, fraction E was separated into heavy mitochondrial fraction (M), light mitochondrial or lysosomal fraction (L), microsomal fraction (P) and final supernatant (S). The purity of the individual fractions was assayed using conventional marker enzymes, and the respective RSAs are given in Table 1. GPI-hydrolysing activity was assayed using the two GPI-anchored proteins, AChE and VSG, as substrates [4,30]. We found that, in the presence of 0.03 % Triton X-100, both AChE and VSG GPI anchors were hydrolysed by fraction E and that the extent of GPI hydrolysis increased in a time-dependent way (Figure 1). Unless otherwise noted, all subsequent experiments to determine GPI-hydrolysing activity were carried out using AChE as substrate.

It has been shown that, in the presence of Triton X-100, maximal activity of purified GPI-PLD is observed at the critical micellar detergent concentration, whereas at higher concentrations of Triton X-100 GPI-PLD activity is strongly inhibited [4]. Similar effects of detergents on enzyme activity have been reported for C-type GPI-hydrolysing phospholipases [27,31]. In addition, we have recently shown that GPI hydrolysis by phospholipases C and D is also inhibited by the presence of purified phospholipids in the assay [31]. Similarly, we here find that the activity of purified bovine serum GPI-PLD is also strongly inhibited by the presence of rat liver subcellular membranes. Furthermore the addition of membrane material to the assay mixture resulted in a shift of the activity maximum of GPI-PLD towards higher Triton X-100 concentrations (results not shown).

Intracellular membranes are known to have variable protein/lipid ratios [32]. Since all subcellular fractions were assayed for GPI-hydrolysing activity using similar amounts of protein, the absolute amounts of lipid in the assay varied slightly. On the basis of the above observation that the activity of purified GPI-PLD was strongly affected by the presence of cell lipids in the assay, it was necessary to determine for each fraction the Triton X-100 concentration at which maximal GPI hydrolysis occurred. In order to minimize the effects of lipids on endogenous GPI-PLD activity, we limited the amounts of membrane material in the assay mixture to approx. 180 µg/ml protein (final concentration). Nevertheless, we found that the activity maxima of GPI hydrolysis varied slightly between the individual subcellular fractions. Maximal GPI-hydrolysing activity of fraction S was observed at 0.020 % Triton X-100, whereas fractions E, N and P had their activity maximum at 0.030 % Triton X-100, and fractions M and L showed maximal GPI hydrolysis at 0.035 % and 0.045 % Triton X-100 respectively. The incubation times varied between 30 and 210 min. In Figure 2, Triton X-100-dependent GPI hydrolysis by fractions E (Figure 2a) and L (Figure 2b) are shown as two representative examples.

Cleavage specificity of GPI hydrolysis

In order to determine the cleavage specificity of the GPI-hydrolysing activity in rat liver, aliquots of a fraction containing high specific GPI-hydrolysing activity (fraction L; see below) were incubated with [³H]myristate-labelled VSG, and the products released were characterized by sequential extraction with toluene followed by water-saturated butan-1-ol, and by TLC. The products of VSG hydrolysis by a phospholipase C are expected to partition into the toluene phase whereas those released by a phospholipase D will partition into the butanol-enriched phase [33]. We found that after treatment of [³H]myristate-labelled VSG with fraction L, approx. 20–35 % of

Table 1 Subcellular distribution of enzymes in rat liver

Rat liver was fractionated by differential centrifugation, and enzyme activities in subcellular fractions were analysed as described in the Materials and methods section. All values are means of triplicate determinations. RSA is % enzyme activity/% protein per fraction; RSA of N + E is taken as 1.0. The protein distribution pattern (160 mg of protein/g wet weight of rat liver) was 8.7% in N, 25.2% in M, 1.8% in L, 19.6% in P and 44.4% in S, with a recovery of 99.7%.

Enzymes	Absolute values (m-units/mg of protein) (N + E)	RSA					Recovery (%)
		N	M	L	P	S	
Lactate dehydrogenase	1794	0.30	0.04	0.11	1.38	1.64	100.0
5'-Nucleotidase	61.1	2.76	0.70	0.72	2.72	0.08	100.6
Glucose-6-phosphatase	47.6	0.87	0.26	2.14	3.47	0.07	97.9
Acid β -N-acetyl-D-hexosaminidase	32.7	0.45	2.26	9.31	0.90	0.12	105.0
Acid β -D-glucuronidase	6.94	0.67	1.12	6.46	2.59	0.08	97.5
Acid phosphatase	25.1	0.27	1.67	9.98	1.54	0.14	98.5
Catalase	524	0.27	0.61	7.82	0.14	1.53	100.3
Monoamino oxidase	0.24	1.06	2.48	1.91	1.26	0.00	101.2
Galactosyltransferase	0.176	0.17	0.07	0.68	3.57	0.13	94.9
GPI-PLD	0.215	0.36	1.16	6.50	1.76	0.49	81.0
GPI-PLD supplemented by serum	0.703	0.22	0.60	0.92	0.66	2.11	86.8

Figure 1 Time-dependent hydrolysis of GPI-anchored proteins by rat liver fraction E

Fraction E (cytoplasmic extract) was incubated at 37 °C in assay buffer containing 0.03% Triton X-100, and the extent of GPI hydrolysis was measured using AChE (●) and VSG (○) as substrates.

the radiolabelled material released was extracted by toluene and 65–80% was recovered in the butanol-enriched phase (range of five experiments). In a separate set of experiments, total radiolabelled reaction products were extracted with water-saturated butan-1-ol and analysed by TLC in solvent system A. As shown in Figure 3, lane b, the major radiolabelled product released from [3 H]myristate-labelled VSG by fraction L co-migrated with authentic dimyristoylphosphatidic acid standard generated by treatment of [3 H]myristate-labelled VSG with purified GPI-PLD (Figure 3, lane c). The remaining radiolabelled products migrated with, or close to, the solvent front. The faint upper band co-migrated with authentic dimyristoylglycerol standard generated by treatment of [3 H]myristate-labelled VSG with PI-PLC (Figure 3, lane a), whereas the lower band of the doublet co-migrated with a fatty acid standard. The identity of the more hydrophobic reaction products as diacylglycerol and fatty acid was confirmed in a second TLC system using solvent B (for the migration of standards in solvent system B see ref. [17]). Together, the identification of [3 H]dimyristoylphosphatidic acid as the

Figure 2 Triton X-100-dependent GPI hydrolysis of rat liver fractions E and L

Fractions E (a) and L (b) were diluted to a protein concentration of 0.18 mg/ml (final concentration) in assay buffer, and aliquots were assayed for AChE GPI-hydrolysing activity in the presence of increasing concentrations of Triton X-100 at 37 °C. The incubation time was 150 min for fraction E and 30 min for fraction L. The values are means $\pm$ S.D. for four to five determinations from two separate experiments. The values for maximal GPI hydrolysis were identical with VSG as substrate (i.e. within the indicated standard deviations).

major radiolabelled product released from [3 H]myristate-labelled VSG after treatment with fraction L clearly demonstrates that rat liver subcellular fraction L contains a D-type GPI-hydrolysing phospholipase. The small amounts of radiolabelled diacylglycerol released can be explained by the combined action of a GPI-PLD and a phosphatidic acid phosphohydrolase activity [34], whereas the presence of radiolabelled fatty acids may be the result of

Figure 3 Identification of radiolabelled products after treatment of [^3H]myristate-labelled VSG with fraction L

Lane a, [^3H]dimyristoylglycerol obtained by incubation of [^3H]myristate-labelled VSG with PI-PLC. Lane b, aliquots (12 000 c.p.m.) of [^3H]myristate-labelled VSG were incubated with fraction L for 30 min at 37 °C, and the products of the reaction(s) were extracted with water-saturated butan-1-ol and analysed by one-dimensional TLC. Lane c, [^3H]dimyristoylphosphatidic acid obtained by incubation of [^3H]myristate-labelled VSG with purified GPI-PLD. The migration of a fatty acid standard is indicated in the right margin. The plate was run in solvent system A. O, origin; FA, fatty acid; F, front.

phospholipase A [35,36] or diacylglycerol lipase [36,37] activity. All these enzyme activities have been reported in rat liver lysosomal fractions.

In order to study the relationship between GPI-PLD activity in subcellular fractions and serum, we compared the pH optima and 1,10-phenanthroline- and EDTA/EGTA-dependence of the activities in fractions E and L with a partially purified GPI-PLD from rat serum. Using VSG as substrate we found that the pH optima for the activities in fractions E and L and for the serum enzyme were all in the range 5.5–6.0 (results not shown). Similar pH optima have been reported for purified GPI-PLD from human [38] and bovine [3] serum. Furthermore GPI-PLD activity in fractions E and L, and the partially purified enzyme from serum, was almost completely (88–98%; range of three determinations for each sample) inhibited by the presence of 100 μM 1,10-phenanthroline in the assay. At 10 μM 1,10-phenanthroline, GPI-PLD activity in fractions E and L was inhibited by $20.6 \pm 7.9\%$ and $30.7 \pm 4.2\%$ respectively, whereas the enzyme from rat serum was inhibited by $46.6 \pm 3.6\%$ (all values are means $\pm$ S.D. for three determinations). In addition, GPI-PLD activities of all three samples were almost completely (> 92%) inhibited by the presence of 0.5 mM EDTA, or 2.0 mM EGTA in the assay. Similar 1,10-phenanthroline- and EGTA-dependencies have been reported for GPI-PLD from human [2,38], rabbit and rat [2] serum and bovine brain [3]. Together these results strongly indicate that the GPI-PLD activities in rat liver subcellular fractions E and L and rat serum are due to similar enzymes.

Intracellular localization of GPI-PLD in rat liver

The subcellular distribution of the enzyme from a given cell can be concluded from its RSA value. The RSA values for GPI-PLD were calculated on the basis of the maximal GPI-PLD activities in the individual fractions (see Figure 2). As shown in Table 1, the highest RSA value for GPI-PLD was found in fraction L (light mitochondrial or lysosomal fraction), suggesting a lysosomal or peroxisomal localization. The subcellular distribution pattern of GPI-PLD, however, rather resembles that of the lysosomal marker enzymes, acid β -N-acetyl-D-hexosaminidase, acid phosphatase and acid β -glucuronidase (Table 1).

A peroxisomal localization of GPI-PLD can probably be excluded since (i) the RSA values of GPI-PLD in fractions M, P and S are different from those of the peroxisomal marker enzyme, catalase, and (ii) the pH optima of purified GPI-PLDs are in the range 5–6 [3,26,38,39], suggesting that the enzyme may be catalytically active in a more acidic environment. The relatively low recovery of GPI-PLD activity after subcellular fractionation as compared with the typical marker enzymes (81% compared with 98–100%; Table 1) may be due to the sensitivity of the enzyme towards the presence of detergents and lipids in the assay or to its instability during preparation of the fractions or storage at $-20\text{ }^{\circ}\text{C}$.

In order to confirm further the lysosomal localization of GPI-PLD, we also prepared rat liver lysosomes by density-gradient centrifugation [19,20]. This procedure involves the use of Triton-WR 1339 which is preferentially incorporated into lysosomes [19] and results in purification factors for lysosomes between 30 and 100 as compared with approx. 9 using differential centrifugation [18]. Unfortunately, we noticed that purified GPI-PLD was strongly inhibited by the presence of Triton-WR 1339 in the assay (P. Bütikofer and M. Boschung, unpublished work) and thus the method had to be abandoned.

As shown in Table 1, the RSA value of GPI-PLD in fraction P (microsomal fraction) was slightly higher than those of the true lysosomal markers, acid phosphatase and acid β -acetyl-D-hexosaminidase. This can be interpreted as GPI-PLD having a heterogeneous intracellular distribution. A similar bimodal distribution has been reported for acid β -glucuronidase which is a constituent of both lysosomes and endoplasmic reticulum [40,41] (see also Table 1). However, it is known that fraction P also contains secretory vesicles containing proteins destined for the secretory pathway. Since the cDNA of GPI-PLD contains a classical signal sequence resulting in secretion of the mature enzyme from cells [10], it is possible that the GPI-PLD activity recovered in fraction P results from the presence of secretory vesicle-associated GPI-PLD. Catalytically active GPI-PLD can be excluded as a true intrinsic constituent of the Golgi system, which also accumulates in the P fraction, on the basis of our findings that the RSA value for GPI-PLD in a highly purified Golgi fraction (136-fold purified on the basis of galactosyltransferase; prepared in a separate experiment by isopycnic density-gradient centrifugation) was 0.50, whereas the RSA values of the lysosomal marker enzymes were higher than 1.0 (e.g. 2.7 for acid phosphatase and 3.4 for acid β -N-acetyl-D-hexosaminidase).

It is well established that mammalian sera contain high amounts of GPI-PLD activity [1,2,4,30]. Thus any contamination of rat liver by serum during isolation and preparation of subcellular fractions could potentially affect GPI-PLD measurements. In order to study the possible contamination of our preparation by serum-derived GPI-PLD, we supplemented washed rat liver before fractionation with small amounts (0.5 ml) of rat serum and studied the distribution of GPI-hydrolysing activity in subcellular fractions. Our results show that, although the RSA values of the marker enzymes were almost identical with those of a serum-free preparation (results not shown), the distribution pattern of GPI-PLD activity was significantly different (Table 1). The highest RSA value for GPI-PLD in the serum-containing preparation was found in fraction S. These findings exclude the possibility that the relative enrichment of GPI-PLD in the lysosomal fraction was due to contamination of the preparation with serum GPI-PLD.

The presence of GPI-PLD in subcellular fractions of rat liver could not be analysed by immunochemical techniques because our monoclonal antibody raised against bovine serum GPI-PLD

did not react with partially purified GPI-PLD from rat serum (results not shown). In general, immunochemical detection of intracellular (i.e. proteolytically degraded) forms of GPI-PLD may be difficult, since several antibodies that were raised against purified intact GPI-PLD from serum have been found not to react with (enzymically active) proteolysed forms of GPI-PLD [13,14].

In summary, our results demonstrate that rat liver contains an enzymic activity that cleaves the GPI anchors of purified AChE and VSG with the specificity of a phospholipase D. Analysis of subcellular fractions reveals that the GPI-PLD activity is mainly associated with a lysosomal fraction and that its relative specific enzyme activity has a similar intracellular distribution to that of a typical lysosomal enzyme. The cDNA sequence of GPI-PLD from bovine liver contains a signal sequence that triggers secretion of the enzyme from cells [10]. The mature enzyme has been found in relatively large amounts in serum, milk and cerebrospinal fluid [1,2,4]; however, it is not known if GPI-PLD has any function in these body fluids. On the basis of our results, we speculate that GPI-PLD is taken up by cells into lysosomes where it may become involved in the cleavage of GPI-anchored substrates. The low pH optimum and relative stability towards proteases [12–14] argue in favour of the proposed lysosomal action of GPI-PLD.

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