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Phospholipase cleavage of glycosylphosphatidylinositol reconstituted in liposomal membranes

Ana Victoria Villar[a], Félix M. Goñi[a,*], Alicia Alonso[a], David R. Jones[b], Yolanda León[b], Isabel Varela-Nieto[b]

[a]*Grupo Biomembranas (Unidad Asociada al C.S.I.C.), Departamento de Bioquímica, Universidad del País Vasco, Aptdo. 644, 48080 Bilbao, Spain*
[b]*Department of Cell Signalling, Instituto de Investigaciones Biomédicas, C.S.I.C., Arturo Duperier 4, 28029 Madrid, Spain*

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Abstract Glycosylphosphatidylinositol (GPI) purified from rat liver lipids was incorporated into lipid bilayers of defined compositions, in the form of large unilamellar vesicles. The GPI concentration in the bilayers was kept constant at 25 mole%, whereas the remaining lipids being phosphatidylcholine, phosphastidylethanolamine, sphingomyelin and/or cholesterol were varied. The resulting liposomes consisted of spherical vesicles, approximately 100 nm in diameter, that could keep their aqueous contents separated from the extravesicular medium. When these liposomes were treated with either *Bacillus cereus* phosphatidylinositol-phospholipase C, *Trypanosoma brucei* GPI-phospholipase C, or bovine serum GPI-phospholipase D, GPI was hydrolyzed at different rates, depending on the enzyme and the bilayer lipid composition. These observations open the way to biophysical and biochemical studies of enzymic GPI cleavage under defined conditions. Extensive GPI hydrolysis was observed in certain cases that could allow the use of these systems for the preparation of inositol phosphoglycans, proposed second messengers of a wide variety of hormones, cytokines and growth factors.

Key words: Glycosylphosphatidylinositol; Phospholipase; Liposomal membrane

1. Introduction

Glycosylphosphatidylinositols (GPI) have become the object of extensive studies in the recent years because of their dual role: as anchors for proteins attached to the outer leaflet of the plasma membrane and as precursors of the putative second messengers termed inositol phosphoglycans (IPG) [1–3]. These water-soluble IPG represent a family of at least two members whose structures are based on a phosphorylated oligosaccharide containing as core a phosphoinositol glycosidically linked to a non-*N*-acetylated hexosamine. The latter are produced in vivo through the action of GPI-specific phospholipases whose activities are positively modulated by a variety of extracellular agonists (reviewed in [3,4]). Thus phospholipase-mediated cleavage of GPI deserves attention as it is the starting step in this novel signal transduction pathway.

Several phospholipases are known to be active on GPI [3–5]. Among these are phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus cereus* [6,7], GPI-specific phospholipase C (GPI-PLC) from *Trypanosoma brucei* [7], and mammalian bovine GPI-specific phospholipase D (GPI-PLD) [8–10]. The substrate specificities of the enzymes have been investigated. The former enzyme, PI-PLC, shows no hydrolytic activity towards phosphorylated forms of PI nor against phosphatidylcholine (PC) or phosphatidylethanolamine (PE) [4,11]. GPI-PLC shows almost exclusive specificity for GPI [7,12]. Only if PI is presented to the enzyme in detergent-based micelles will the enzyme hydrolyze PI [13]. Finally GPI-PLD does not exhibit activity against PI or its phosphorylated forms. Rather it is specific for free GPI lipids [10] and both inositol-acylated and non-inositol-acylated GPI-anchored proteins [14].

All these have been studied on complex cell-derived systems, but a detailed knowledge of their properties and their mechanism of action requires a simpler system in which a defined substrate, GPI, is hydrolyzed by a purified enzyme under controlled conditions. A great deal has been learned about phospholipases A_2 and C from *Bacillus cereus* using artificial phospholipid vesicles (liposomes) as substrates ([15,20]. GPI is composed of a non-*N*-acetylated hexosamine glycosidically linked to the *myo*-inositol ring within PI. This PI contains predominantly saturated fatty acids as opposed to other phosphoinositides whose fatty acid composition is marked by the presence of arachidonic acid and other polyunsaturated fatty acids. The glycan portion of GPI further consists of sugar residues, attached to the non-*N*-acetylated hexosamine, consisting of several galactose units which are phosphorylated. The entire structure gives rise to a highly polar phospholipid whose molecular weight could be calculated to exceed 1500 Da [3,4]. The structure of GPI precludes the formation of bilayers composed exclusively of this lipid. However, it would be feasible to reconstitute purified GPI into bilayers consisting of structural lipids that could not be cleaved by the phospholipase active against GPI. Several phospholipids, and phospholipid-cholesterol mixtures, have been used as bilayer-forming, non-substrate lipids. In this way GPI could be presented as a substrate for the specific phospholipases under carefully regulated conditions.

In this paper we report the preparation of large unilamellar vesicles (LUV) consisting of egg-yolk PC and GPI purified from rat liver. LUV are particularly useful as models for cell membranes. GPI reconstituted in LUV was treated with

*Corresponding author. Fax: +34 (94) 4648500.
E-mail: gbpgourf@lg.ehu.es

Abbreviations: GPI, glycosylphosphatidylinositol; IPG, inositol phosphoglycans; PI-PLC, phosphatidylinositol-specific phospholipase D; GPI-PLD, glycosylphosphatidylinositol-specific phospholipase D; GPI-PLC, glycosylphosphatidylinositol-specific phospholipase C; PC-PLC, phosphatidylcholine-preferring phospholipase C; LUV, large unilamellar vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; PI, phosphatidylinositol; SM, sphingomyelin; Ch, cholesterol; ANTS/DPX, 8-aminonaphthalene-1,3,6-trisulphonic acid/*p*-xylylene bis (pyridinium bromide)

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Bacillus PI-PLC, *Trypanosoma brucei* GPI-PLC and bovine serum GPI-PLD. All three enzymes were found to hydrolyze GPI under conditions that could be conveniently monitored by biochemical and biophysical studies. Unlike other phospholipases C and D types, the enzymes under study did not lead, under our experimental conditions, to vesicle aggregation nor to other major changes in membrane architecture as a result of their hydrolytic activities.

2. Materials and methods

2.1. Materials

GPI-PLC (EC 3.1.4.10) from *Trypanosoma brucei* was supplied by Oxford Glycosystems (Oxford, UK). PI-PLC (EC 3.1.4.10) from *Bacillus cereus* was purchased from Molecular Probes (Eugene, OR, USA). GPI-PLD (EC 3.1.4.50) from bovine serum was from Boehringer Mannheim (Germany). Egg PC and egg PE were purchased from Lipid Products (South Nutfield, UK). Bovine liver PC and PE were from Sigma (Madrid, Spain). Egg sphingomyelin (SM) was from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol (Ch), egg phosphatidic acid (PA), 1,6-diphenylhexatriene and the orcinol and molybdenum blue reagents were from Sigma (Poole, UK). Silica gel G (60 Å) thin layer chromatography (t.l.c. plates) were supplied by Merck (Darmstadt, Germany). All other materials (salts and organic solvents) were of analytical grade or better.

2.2. Purification of rat liver GPI

GPI was purified as detailed in [16]. Briefly, total lipids isolated by chloroform/methanol/hydrochloric acid extraction of 30 rat livers were applied to the origin of heat-activated t.l.c. plates. The plates were then developed twice in the acidic solvent system consisting of chloroform/acetone/methanol/acetic acid/water (10/4/2/2/1, by volume). The material remaining at the origin (from −0.5 cm below to +1 cm above) was eluted from the silica by washing three times with methanol. The eluted material was dried under a gentle stream of nitrogen gas before it was applied to a further set of t.l.c. plates for development in the basic solvent system consisting of chloroform/methanol/ammonia/water (45/45/3.5/10, by volume). GPI was located between the authentic standards PA and PC which had been spotted onto the plates. Confirmation that GPI was indeed the lipid migrating at the stated position was achieved by noting its reaction with iodine vapor, 1,6-diphenylhexatriene (both general lipid stains), the orcinol reagent (specific for glycolipids and sugar residues), ninhydrine (free amino groups) and the molybdenum blue reagent (for phospholipid phosphate). GPI that had not been exposed to the detecting reagents was removed from the silica using methanol as described above. The yield of GPI was calculated by measuring its free amino group content and its total number of phosphate groups by previously published methods [10]. It has been proposed that the stoichiometry of the number of phosphate groups to free amino groups is 3:1 [10]. Possible contamination of GPI with PI and its phosphorylated derivatives was checked by analysing the fatty acid composition of the GPI sample using gas chromatography and mass spectrometry (GCMS) [17]. Briefly, 1% of the purified GPI was transmethylated using 3 M hydrochloric acid in dry methanol. After drying using nitrogen gas, the fatty acid methyl esters (FAMEs) were dissolved in hexane and analyzed by GCMS (5890GC/5972MSD, Hewlett Packard, Stockport, UK) on a polar DB-23 capillary column (0.25 μ film, 0.25 mm × 30 m, J. and W. Scientific, Folsom, CA, USA) using splitless injection at 220°C (12 p.s.i. head pressure, purge after 1 min), a temperature programme of 55°C for 2 min then to 140°C at 70°C/min and finally to 210°C at 1°C/min with interface heating at 270°C. Identification of the FAMEs found in the GPI sample was achieved using authentic FAME standards and by monitoring the presence of their characteristic fragmentation ions (74, 87, 55, 69, 81 and 79 amu).

2.3. Liposome preparation and characterization

Large unilamellar vesicles (LUV) were prepared by the extrusion method [18], using 0.1 mm pore diameter Nuclepore filters (Merck, Darmstadt, Germany) at room temperature, as detailed previously [19,20]. In some LUV preparations their lipid composition was checked and compared with that of the starting lipid mixture. This was achieved by extracting the LUV suspension with chloroform/methanol/hydrochloric acid. The resulting organic phase was evaporated to dryness, then resuspended in a small volume of chloroform/methanol (2/1, v/v) before being transferred to the origin of a t.l.c. plate. The t.l.c. plate was developed in the mobile phase consisting of chloroform/methanol/acetone/acetic acid/water (53/11/21/11/5, by volume). The localization of lipids on the dried t.l.c. plate was achieved by their exposure to iodine vapour. The stained plate was immediately scanned in a CS-930 Shimadzu densitometer to quantitate the spot intensities. Samples for electron microscopy were examined by the negative staining technique, using phosphotungstate as the stain. The average size of LUV was measured by quasi-elastic light scattering using a Malvern Zeta-sizer instrument. LUV loaded with fluorescent probes were freed from non-entrapped fluorophores by diluting the vesicle preparation in an isotonic fluorophore-free buffer prepared in D_2O. The resulting suspension was centrifuged at $436\,000 \times g$ (25°C, 1 h) in a Beckman TLA-100 rotor. Under these conditions LUV float on top of the D_2O buffer and they can easily be recovered to be used in content leakage [21].

2.4. Enzyme assays and turbidity measurements

For optimal catalytic activity all experiments were performed at 39°C, in 10 mM HEPES, 50 mM NaCl pH 7.5, in the presence of 0.1% bovine serum albumin, with continuous stirring. LUV were prepared in 10 mM HEPES, 50 mM NaCl pH 7.5. The lipid concentration was 0.3 mM and all enzymes were used at a final concentration of 0.16 units/ml. Enzyme activity was assayed as follows. Aliquots were removed from the reaction mixture at regular intervals and extracted with chloroform/methanol/hydrochloric acid (200/100/1, by volume). Water-soluble phosphorous was assayed in the aqueous phase according to [22]. A figure of three phosphorous atoms per GPI molecule [10] was assumed for the calculations. The specificity of the enzymes was checked in preliminary experiments by separation of the hydrolytic products, diacylglycerol and PA, by t.l.c. Liposome aggregation was estimated by the increase in turbidity of the buffer, measured as absorbance at 450 nm, in a Cary Varian UV-visible spectrophotometer.

3. Results and discussion

3.1. Liposome preparations

LUV containing GPI and other lipids in varying proportions have been prepared with the compositions detailed in Table 1. The five liposome compositions under study were chosen because similar mixtures, only containing PI instead of GPI, had been found to be good substrates for PI-PLC (A.V. Villar, I. Varela-Nieto, A. Alonso and F.M. Goñi, unpublished work). The GPI-containing lipid mixtures shown in Table 1 gave rise, upon hydration and extrusion, to bilayer structures surrounding vesicles of homogeneous size, as seen by negative staining electron microscopy (results not shown). These vesicles were of the order of 100 nm in diameter, judging from the electron micrographs. Vesicle size was also measured in a more reliable way by using quasi-elastic light scattering spectroscopy. The sizes are also included in Table 1.

Chemical analysis of the LUV revealed that their composition was essentially identical (±10%) the one of the lipid mixture used in their preparation, indicating that the various lipids became evenly incorporated into the bilayers. No specific studies of the orientation of GPI in the vesicle membranes were performed, although being a cone-shaped molecule in the classification of Israelachvili et al. [23], i.e. a lipid with a relatively large polar headgroup, a preference for the outer monolayer was expected. For reasons that may be related to the bulkiness and electrically charged polar group of GPI, we were unable to encapsulate fluorescent solutes (e.g. ANTS/DPX [24]) in GPI-containing liposomes using the standard procedures. These included removing the non-entrapped fluorophores by passing the suspension through Se-

Table 1
Hydrolysis of GPI phospholipids reconstituted in liposomal membranes by PI- and GPI-specific phospholipases[a]

Liposomal composition	Enzyme									
		PI-PLC			GPI-PLC			GPI-PLD		
	Size (nm)	Lag time (min)	Rate (nmol GPI/min)	Hydrolysis (% GPI)	Lag time (min)	Rate (nmol GPI/min)	Hydrolysis (% GPI)	Lag time (min)	Rate (nmol GPI/min)	Hydrolysis[b] (% GPI)
GPI/PC/PE (1:1:2)	111.0 ± 2.5	0	1.1 ± 0.9	23 ± 18	9	1.9 ± 0.7	38 ± 10	0	4.1 ± 0.1	81 ± 1
GPI/PC/PE (1:1:2)[c]	95.1 ± 1.5		1.2 ± 0.5	24 ± 9		n.d.	n.d.		2.9 ± 0.1	57 ± 1
GPI/PC/Ch (1:2:1)	119.6 ± 1.3	0	0.5 ± 0.1	11 ± 2	5	0.3 ± 0.1	5 ± 3	0	2.5 ± 0.5	51 ± 10
GPI/PC/SM (1:2:1)	103.7 ± 2.0		n.d.		7	1.5 ± 0.7	31 ± 15	0	1.1 ± 0.7	21 ± 14
GPI/PE/SM (1:2:1)	121.2 ± 2.8		n.d.		10	0.1 ± 0.1	2 ± 2	2	0.7 ± 0.1	14 ± 2
GPI/SM/Ch (1:2:1)	99.8 ± 3.0		n.d.		0	2.1 ± 0.2	41 ± 4	0	1.6 ± 0.5	32 ± 10

[a]Average values ± S.E.M. ($n = 3$).
[b]% GPI hydrolyzed after 15 min. All preparations contained 3.75 μg GPI at the start.
[c]PC and PE from bovine liver.

phadex columns [19]. Instead we developed a method, as detailed in the corresponding section of the paper, based on vesicle flotation by centrifugation in a D_2O buffer. Under these conditions vesicles containing ANTS/DPX could be isolated in a fluorophore-free medium. Addition of Triton X-100 to these suspensions produced the immediate release of aqueous contents, detected as an increase in fluorescence. Thus GPI-containing bilayers are impermeable to water-soluble molecules, just as biological membranes.

In summary, all the available data indicate that GPI may be incorporated at a 25% molar ratio in a variety of lipidic mixtures resulting in the formation of stable bilayers that surround closed vesicles after hydration.

3.2. Enzyme assays

Purified rat liver GPI was found to contain primarily (97.5%) saturated fatty acids (myristic acid, pentadecanoic acid, palmitic acid and steric acid) with a small proportion (2.5%) of an unsaturated fatty acid (oleic acid) (D.R. Jones and I. Varela-Nieto, unpublished observations). This finding confirmed that the GPI was free from PI and other phosphoinositide contamination due to the failure to detect polyunsaturated fatty acids. The GPI was incorporated into liposomes and they were treated with PI-PLC, GPI-PLC or GPI-PLD as detailed in Table 1. Two representative experiments are shown in Fig. 1. A number of conclusions can be raised on the basis of these experimental results, the first one being that GPI can be used as a substrate by all three enzymes under our experimental conditions. It is equally clear that both the rate and extent of GPI cleavage differ widely depending on the enzyme under question. When both PI-PLC and GPI-PLC were tested they were seen to function at similar rates and hydrolyze GPI at roughly similar extents. GPI-PLD was found to be more active under the same conditions. This agrees with our previous results which showed that the extent of hydrolysis of GPI (purified from human liver) by GPI-PLD was greater as compared to that observed with PI-PLC [10].

Enzyme rates, as recorded in Table 1, are maximum rates which differed from initial rates. This is so because, at least in some cases, the enzymes under study have latency periods of very low activity, termed the lag time, after which a 'burst' of activity followed. Fig. 1 shows two representative examples of the presence (GPI-PLC, Fig. 1A) and absence (GPI-PLD, Fig. 1B) of a lag time. When lag times were observed they are indicated in Table 1. GPI-PLC was the enzyme which particularly displayed this phenomenon. In a previous study from this laboratory the origin of the lag time in another phospholipase C, the phosphatidylcholine-preferring enzyme (PC-PLC) from *Bacillus cereus* was examined in detail [20]. The explanation of the lag time may be similar in both cases, i.e. enzyme activity at low rate until a given fraction of substrate has been cleaved. This is followed by a 'burst' of activity. Interestingly, latency periods are relatively common in lipases of various kinds [25–27]. The extent of hydrolysis recorded in Table 1 corresponds to the value 15 min after enzyme addition. This was virtually the maximum amount of hydrolysis observed as the values at 60 min were similar or only slightly higher than those at 15 min (Fig. 1).

An important conclusion to be drawn from the data in Table 1 is that non-substrate lipids considerably influence the enzyme activities. In all the experiments summarized in Table 1, the GPI concentration was constant, as well as all the other conditions (except the composition of the bilayers in which GPI was reconstituted). The data clearly indicate that lipid composition could influence every enzyme parameter under study: lag time, maximal rate and the extent of GPI hydrolysis. It was found that the lipid composition which allowed the highest enzyme activities was GPI/PC/PE (1:1:2, molar ratio). It should be noted that one out of two molecules in this mixture is PE, a non-bilayer-forming lipid [28]. Non-bilayer, or inverted conical [29] lipids, have been shown to enhance PC-PLC activity (M.B. Ruiz-Argüello and A. Alonso, unpublished work). The influence of non-substrate lipids on GPI-specific phospholipases also illustrates the point that it is the overall properties of the bilayer what modulates the activity of enzymes acting at the bilayer-water interface [30]. This could be an important feature for the regulation of GPI hydrolysis in vivo. Recent advances in lipid cell signalling not only take into account the need for diversity among membrane lipids but also renew the interest in the physical organization of lipids within biomembranes [32]. Thus, these model membrane systems will hopefully provide useful insights into

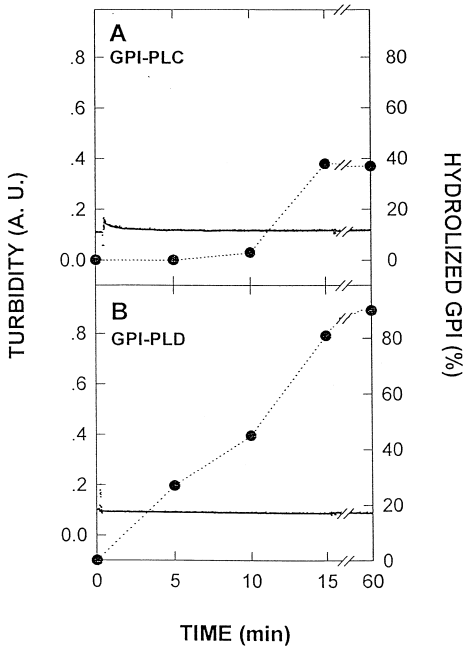

Fig. 1. Hydrolysis of GPI incorporated into large unilamellar vesicles. Bilayer composition was GPI/PC/PE (1:1:2, molar ratio). Preparations contained 3.75 μg GPI at time zero and the final enzyme concentration was 0.16 U/ml. A: Enzyme: *Trypanosoma brucei* GPI-PLC. B: Enzyme: Bovine serum GPI-PLD. Enzyme activity was measured as a function of time by assaying water-soluble phosphorous in successive aliquots from the reaction mixture (broken line). The turbidity of the suspension was recorded simultaneously as the change in absorbance at 450 nm (solid line).

the situation likely to exist in the cell membrane. As is perhaps the case in caveolae [5], the interaction of these particular lipids could promote membrane domain formation.

Fig. 1 shows, in addition to the phosphohydrolase activities of the enzymes in question, the absence of concomitant changes in the suspension turbidity. This is in contrast with other phospholipases, particularly PC-PLC, that induce an increase in turbidity resulting from vesicle aggregation, as soon as the latency period is ended and the burst of activity takes place [20]. The lack of aggregation of GPI-containing vesicles may be due to the presence of the sugar moieties on the bilayer surface. Aggregation requires at least a partial dehydration of the vesicle surfaces in contact. This is not likely to be the case as the carbohydrate residues found within GPI would be expected to co-ordinate to a large amount of water molecules in their vicinity. We have observed in a previous study that even small proportions (< 5 mole%) of PE containing covalently bond poly(ethylene glycol) were enough to reduce the rate and extent of PC-PLC-induced vesicle aggregation [31].

The present report constitutes significant progress in what is known about membrane-binding GPI anchors and of the generation of both hydrophobic and hydrophilic signals from GPI lipids. The possibility of further studying these processes in well-defined model systems will improve our understanding of these important steps which lead to complex metabolic regulation. In addition, extensive hydrolysis of GPI and GPI derivatives under controlled conditions could be used on a preparative scale for the in vivo synthesis of pure biologically active IPG from different sources with excellent yields (Y. León and I. Varela-Nieto, unpublished work). Finally, by studying the action of GPI-specific phospholipases in controlled environments, a new chapter of interfacial biochemistry will be opened to consider the important challenges presented by lipids containing large polar groups at the membrane-water interface.

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