Towards the in vitro reconstitution of caveolae. Asymmetric incorporation of glycosylphosphatidylinositol (GPI) and gangliosides into liposomal membranes

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Abstract Large unilamellar vesicles consisting of phospholipids with or without cholesterol have been prepared containing GPI and/or gangliosides asymmetrically located in the outer leaflet of the bilayer. Such asymmetric distribution of GPI and gangliosides is found in 'rafts' and caveolae. Using these vesicles, GPI can be readily hydrolysed by phospholipases. Both cholesterol and ganglioside are seen to inhibit, in an additive way, the hydrolytic activity of GPI-specific phospholipase D.

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Key words: Raft; Caveola; Asymmetry; Glycosylphosphatidylinositol; Ganglioside; Phospholipase

1. Introduction

Glycolipid membrane domains are currently attracting a considerable degree of attention. Assemblies ('rafts') of glycosphingolipids and cholesterol are believed to form microdomains with specific functions in membrane traffic and signal transduction. Flask-shaped 60 nm invaginations of the cell plasma membrane, termed caveolae, may be the sites at which these domains cluster and self-stabilise [1–4]. Rafts and caveolae recruit specific membrane proteins which are implicated in cell signalling [5,6].

These domains have a particular asymmetric disposition as glycosphingolipids and glycosylphosphatidylinositol (GPI)-linked proteins locate preferentially in the outer leaflet [1–3,7,8]. While artificial lipidic vesicles (liposomes) have been extremely useful in the understanding of multiple aspects of membrane structure and function, no simple technology is available for the reliable preparation of liposomes with asymmetrically distributed lipids. In view of the structural and functional importance of rafts and caveolae, the obtaining of synthetic vesicles with an asymmetric distribution of sphingolipids and GPI lipids is highly attractive.

In a previous study [9], we described the preparation of large unilamellar vesicles (~100 nm in diameter) incorporating GPI in such a way that the glycosidic fraction could be cleaved by specific phospholipases. In a further step towards the modelling of rafts and caveolae, we have attempted the preparation of liposomes with GPI and glycosphingolipids located preferentially in the outer monolayer. The present paper summarises our progress in this direction.

2. Materials and methods

2.1. Materials

α-Galactosidase (EC 3.2.1.22) from green coffee beans, neuraminidase (EC 3.2.1.18) from *Clostridium perfringens*, bovine serum albumin (BSA), resorcinol (1,3-benzenediol), Triton X-100 (*t*-octylphenoxypolyethoxyethanol), FITC-dextran (fluorescein isothiocyanate-dextran, average molecular weight 4400 Da), Sephacryl HR-300-S, 1,6-diphenylhexatriene, orcinol and molybdenum blue reagent were from Sigma (St. Louis, MO, USA). Sephadex G-75 was purchased from Pharmacia Biotech (Uppsala, Sweden). GPI-phospholipase D (EC 3.1.4.50) was purified from bovine serum [10]. Monosialoganglioside (GM_3) was from Matreya Inc. (Gap, PA, USA). Egg phosphatidylcholine (PC) and egg phosphatidylethanolamine (PE) were purchased from Lipid Products (South Nutfield, UK). Cholesterol (Ch), egg phosphatidic acid (PA), 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), *p*-xylenebis(pyridinium bromide) (DPX) were supplied by Molecular Probes Inc. (Eugene, OR, USA). Silica gel G (60 Å) thin layer chromatography (TLC) plates were supplied by Merck (Darmstadt, Germany).

2.2. Purification of rat liver GPI

GPI was purified as detailed in [11]. Briefly, total lipids isolated by chloroform/methanol/hydrochloric acid extraction of 30 rat livers were applied to the origin of heat-activated TLC 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 TLC 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 vapour, 1,6-diphenylhexatriene (both general lipid stains), the orcinol reagent (specific for glycolipids and sugar residues), ninhydrin (amino group detector) and the molybdenum blue reagent (reacting with 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 from its free amino group content and its total number of phosphate groups measured by previously published methods [12]. It has been proposed that the stoichiometry of the number of phosphate groups to free amino groups is 3:1 [12].

2.3. Liposome preparation

PE/PC/Ch (2:1:1 molar ratio) and PE/PC (2:1 molar ratio) large unilamellar vesicles (LUV) were prepared by the extrusion method [13], using 0.1 μm pore diameter Nuclepore filters (Merck, Darmstadt, Germany) at room temperature, as detailed previously [14]. The average size of LUV was measured by quasi-elastic light scattering using a

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Malvern Zeta-sizer instrument. Liposomal lipid concentrations (0.3 mM in all experiments) were determined by phosphate analysis [15].

2.4. Asymmetric incorporation of glycolipids

To incorporate GPI or GM_3 to liposomes prepared as described above, the glycolipids in organic solvent were dried and resuspended in methanol (5% of the vesicle suspension volume). Vesicles were added to this methanolic glycolipid solution. After vortex mixing they were left to incubate for 15 min at room temperature.

2.5. Enzyme assays

For galactosidase assays, the enzyme was used at 0.5 U/ml in the presence of 0.1% BSA at 39°C with continuous stirring. For optimal catalytic activity, these experiments were performed in 10 mM sodium acetate, 50 mM NaCl, pH 6.1. LUV were prepared in the same buffer. Enzyme activity was monitored following two different methods. (a) Phosphate mass assay. Aliquots were removed from the reaction mixture at regular intervals and extracted with chloroform/methanol/hydrochloric acid (200/100/1, by volume). Water-soluble phosphorus was assayed in the aqueous phase according to [15]. A number of three phosphorus atoms per GPI molecule [12] was assumed for the calculations. (b) Alternatively, enzyme activity was followed by assaying water-soluble sugars. Aliquots were removed as described above and sugar determinations were made by the phenol-sulphuric acid method described by [16].

In order to determine sialic acid formed after treatment of liposomes containing GM_3 with neuraminidase, a modification of the resorcinol-sialic acid assay [17] was used. The conditions for optimal enzyme activity were established as: 10 mM HEPES, pH 5.6, 39°C with continuous stirring. The enzyme concentration used was 0.16 U/ml. Aliquots were removed from the reaction mixture as described above. GPI-PLD hydrolysis was assayed by the determination of the water-soluble phosphate content of aliquots removed from the reaction mixture at defined intervals (using the method described above).

2.6. Fluorimetric assays

Vesicle contents leakage was detected using the ANTS/DPX fluorescent probe system described by [18]. Labelled liposomes were separated from unbound probes in a Sephadex G-75 chromatography column. Osmolalities of extra- and intravesicular solutions were checked using an Osmomat 030 cryoscopic osmometer (Gonotec, Berlin, Germany). The 100% leakage fluorescence signal was achieved by the addition of Triton X-100 (up to 0.5%, w/v). A Shimadzu RF-540 spectrofluorometer was used at room temperature, with continuously stirred cuvettes. Excitation and emission wavelengths were 355 and 530 nm respectively. A cut-off filter (470 nm) was used to prevent contamination from scattered light.

3. Results and discussion

When LUV composed of PE, PC and Ch (2:1:1 molar ratio) at 0.3 mM lipid concentration were treated with a small volume of methanolic solution of GPI (final GPI concentration 0.03 mM) as indicated in Section 2, GPI becomes incorporated into the lipidic phase. This was demonstrated by partition experiments. When GPI in methanolic solution was incorporated into a chloroform:methanol:HCl:water (2:1:0.03:0.6, v/v/v/v) mixture, GPI fully partitioned into the upper, methanol:water, phase as indicated by TLC of the aqueous and organic phases. However, when the methanolic GPI was added to the LUV suspension, it fully partitioned into the chloroform phase, indicating its association with the lipid bilayer. Furthermore, when multilamellar vesicles (PE:PC:Ch, 2:1:1 molar ratio, total lipid concentration 2 mM) were treated with methanolic GPI (0.03 mM) and then centrifuged (Beckman TLA 100 rotor, $436\,000 \times g$, 23°C, 1 h) no GPI could be recovered in the supernatants, indicating its association to the vesicles. Bilayer-associated GPI remained asymmetrically located on the outer lipid monolayer.

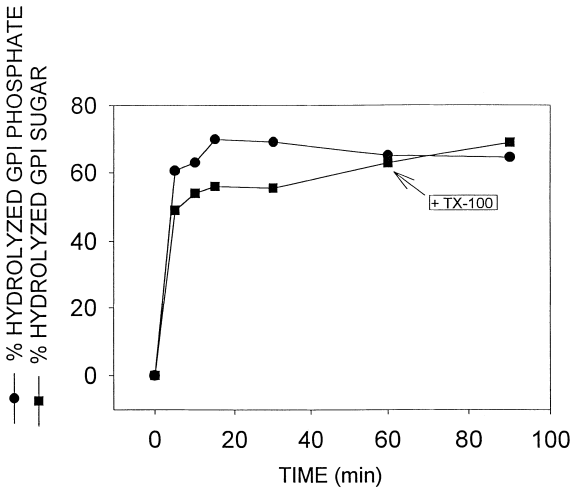


Fig. 1. Time course of GPI hydrolysis by α-galactosidase. GPI was incorporated into preformed LUV (PE:PC:Ch, 2:1:1 molar ratio). The total lipid concentration was 0.3 mM. The GPI concentration was 0.03 mM. The enzyme concentration was 0.5 U/ml. The arrow indicates when Triton X-100 (0.025% w/v) was added to the reaction medium.

This is shown by enzymic hydrolysis experiments (Fig. 1) in which the GPI-containing vesicles were treated with 0.5 U/ml α-galactosidase, an enzyme known to degrade the glycosidic portion of GPI, releasing phosphorylated sugars [19]. LUV lipids apart from GPI were not hydrolysed by galactosidase. Furthermore, galactosidase cannot cross the membrane and reach the inner liposomal compartment. The enzyme activity is shown as a function of time in Fig. 1. Both water-soluble carbohydrate and water-soluble phosphate assays revealed very similar results. After 60 min, when approximately 65% of the GPI appeared to have been cleaved, Triton X-100 (0.025% w/v final concentration) was added in order to terminate the membrane barrier effect. (Triton X-100 under these conditions permeabilises the LUV membranes to large molecules, see below.) No further hydrolysis occurred during the following 30 min. This was interpreted as an indication that most, if not all, of the GPI molecules were originally located in the outer monolayer and were accessible to galactosidase. The lack of further hydrolysis after membrane permeabilisation by Triton X-100 was not due to a detergent-induced enzyme inactivation, because virtually identical hydrolysis occurred when the vesicles were treated with Triton X-100 prior to enzyme addition (data not shown).

The process of GPI incorporation and galactose digestion was examined in further detail using vesicles prepared as above but containing the water-soluble low molecular weight fluorescent probes ANTS and DPX [18]. DPX is an efficient quencher of ANTS fluorescence, acting through collisional quenching, provided they are both at high concentrations. Initially both ANTS and DPX were trapped at suitably high concentrations inside the vesicles, so that the fluorescence is low. However, any leakage of vesicular aqueous contents would lead to dilution of the probes, and to an increase in fluorescence. Fig. 2 shows that when methanol (final concentration 5% v/v, i.e. the same concentration attained when GPI was added in methanolic solution) was added to PE:PC:Ch (2:1:1 molar fraction) LUV, the fluorescence did not change, indicating that under these conditions methanol by itself did not disrupt the membrane permeability barrier to small sol-

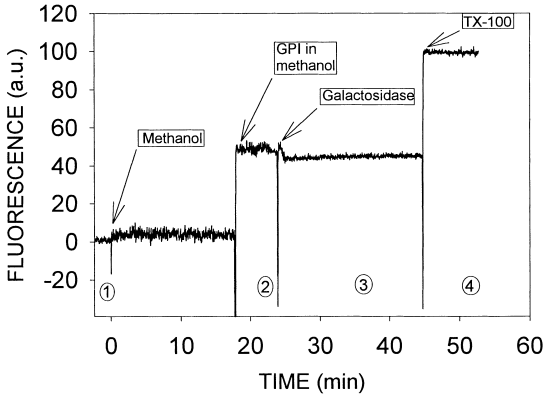


Fig. 2. Effect of GPI insertion on the permeability barrier properties of LUV bilayers (PE:PC:Ch, 2:1:1 molar ratio). The vesicles contained the low molecular weight fluorescent probes ANTS/DPX. The various reagents were added as indicated by the arrow (see text for details). The encircled figures at the bottom correspond to the times at which average diameters were measured by QELS. Average diameters were: 1, 139 ± 1.5 nm; 2, 130.8 ± 8.9 nm; 3, 136.7 ± 6.4 nm; at stage 4, no reliable particle size could be measured, instead the polydispersity was 1.0 (maximal), indicating complete heterogeneity of particle sizes.

utes such as ANTS or DPX. A second similar dose of methanol had no effect either (data not shown). However, a methanolic solution of GPI (0.03 mM final GPI concentration) did elicit a sizable release of aqueous liposomal contents (approximately 50%). This leakage is probably due to molecular rearrangements following insertion of the GPI non-polar moiety. Galactosidase did not by itself induce any leakage (Fig. 2). In fact, a small decrease in fluorescence was detected, presumably due to the non-specific binding of ANTS and/or DPX to the protein molecule. Finally, addition of 0.025% (w/v) Triton X-100 released, as expected, the whole vesicular contents.

These observations confirm that neither methanol nor galactosidase by themselves substantially modify the vesicle architecture. According to the results in Fig. 1, the partial leakage induced by GPI insertion does not reflect a transbilayer movement of this lipid. In fact, the extremely bulky polar headgroup of GPI would most likely prevent any flip-flop movement. It should also be noted that, according to quasi-elastic light scattering measurements, the size of the vesicles did not change with either the methanol, GPI or galactosidase treatments. As expected, the size of the vesicles was significantly altered by Triton X-100 (see legend to Fig. 2).

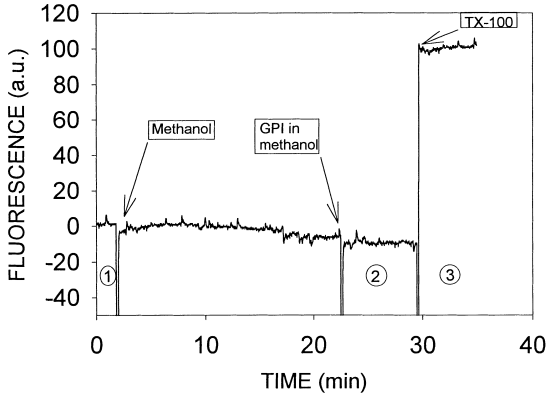


Fig. 3. Effect of GPI insertion on the permeability barrier properties of LUV bilayers. All conditions as in Fig. 2, except that the vesicles contain FITC-dextran (average molecular weight 4400 Da). Average particle diameters were: 1, 131.9 ± 1.1 nm; 2, 129.7 ± 2.3 nm; 3, complete heterogeneity (polydispersity 1.0).

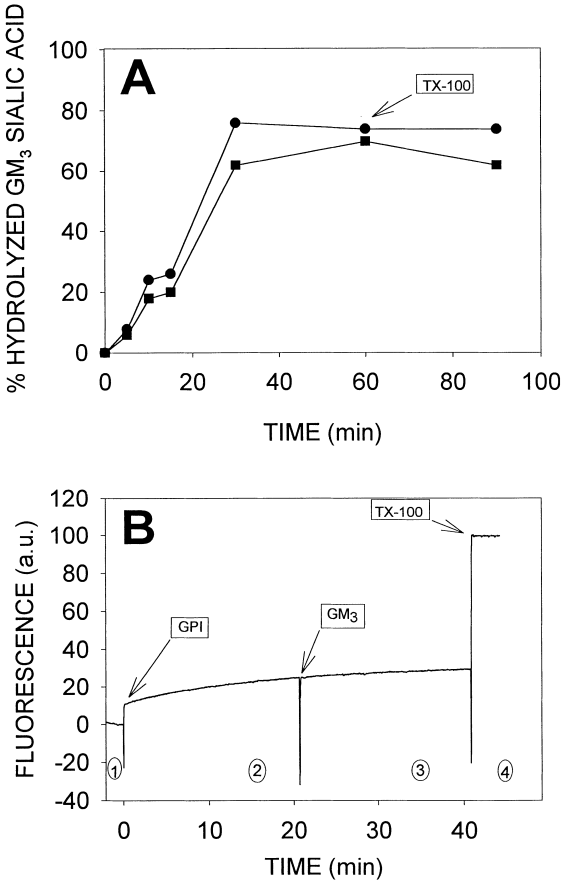


Fig. 4. A: Time course of GM_3 hydrolysis by neuraminidase. GM_3 was incorporated into preformed LUV (PE:PC:Ch, 2:1:1 molar ratio). The total lipid concentration was 0.3 mM. The GM_3 concentration was 0.015 mM. The enzyme concentration was 0.16 U/ml. The arrow indicates when Triton X-100 (0.025%, w/v) was added to the reaction medium. B: The effect of GM_3 insertion on the permeability barrier properties of LUV bilayers. All conditions as in Fig. 2 except that the additions marked by the arrows correspond to 0.015 mM GPI in methanol, 0.015 mM GM_3 in methanol and 0.025% (w/v) Triton X-100, respectively. The average particle diameters were: 1, 148.1 ± 2.2 nm; 2, 201.2 ± 8.6 nm; 3, 215.3 ± 3.3 nm; 4, complete heterogeneity (polydispersity 1.0).

Exactly the same results were obtained when LUV composed of PE:PC (2:1 molar ratio), i.e. cholesterol-free, were used. In this case the vesicle size before Triton addition was approximately 155 nm. The mild character of the bilayer perturbation caused by GPI was demonstrated by an additional experiment (Fig. 3), in which larger fluorescent molecules (FITC-dextran, molecular weight 4400 Da) were entrapped in the vesicles. These molecules are self-quenching and their fluorescence increases upon dilution [20]. As shown in Fig. 3, neither methanol nor GPI in methanolic solution was able to release FITC-dextran molecules from the LUV. As expected, Triton X-100 did destroy the membrane barrier. This experiment reinforces the idea that, according to Fig. 1, galactosidase only acts on the outside of the vesicles in the absence of detergent, and that it may access the vesicle interior only after addition of Triton X-100.

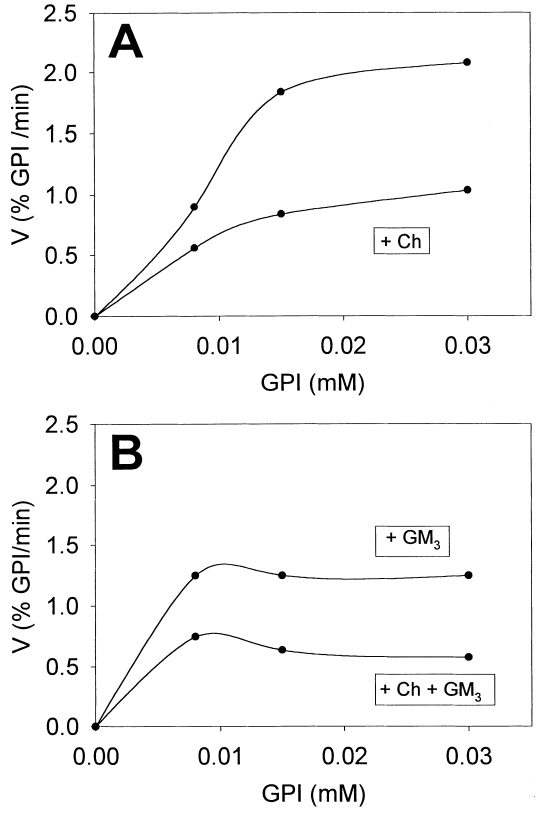


Fig. 5. Hydrolysis of GPI by GPI-specific phospholipase D. 'Velocity versus substrate concentration' curves. GPI and GM_3 were externally added to preformed LUV (see text for details). LUV composition was PE:PC (2:1 molar ratio) or PE:PC:Ch (2:1:1 molar ratio) as required. The concentration of GPI was always 10 mol% of total lipid whereas the concentration of GM_3 was always 5 mol% of total lipid. The average values of three independent experiments are indicated.

Similarly, addition of GM_3 ganglioside in methanolic solution to preformed LUV of either PE:PC:Ch (2:1:1) or PE:PC (2:1 molar ratio) led to the asymmetric insertion of GM_3 into the outer monolayer. This was demonstrated by the GM_3 cleavage experiment using externally added neuraminidase described in Fig. 4A. As expected, the results in Fig. 4B, obtained with vesicles loaded with ANTS/DPX, indicate the partial leakage upon GPI insertion (0.015 mM final GPI concentration). In the same figure, addition of GM_3 (0.015 mM final GM_3 concentration) to vesicles already containing GPI was seen to induce no further leakage. Changing the order of addition did not modify the result in that GM_3 added first did not induce leakage, whereas further addition of GPI did. While the origin of this different behaviour could be traced to the unequal lipid moieties of these molecules, glycerol-based in GPI, sphingosine-based in GM_3, further tests would be necessary in order to clarify this phenomenon.

The results in Figs. 1–4 suggest that both GM_3 and GPI can be asymmetrically inserted into the outer monolayer of preformed LUV containing PE and PC, with or without cholesterol. This is important because the vesicles herein obtained reproduce, for the first time, part of the asymmetry of rafts and caveolae found in animal cell membranes. In addition, the method allows a more effective use of costly GPI, and provides an ideal tool to assay GPI-specific phospholipase activity in a variety of lipid environments. An example of this is shown in Fig. 5. The 'velocity versus substrate concentration' plots for GPI hydrolysis by GPI-specific phospholipase D are indicated. This assay was already described in our previous work [9]. The enzyme appears to follow non-Michaelian kinetics. Interestingly, enzyme activity appears to be highly dependent on lipid composition, PE:PC bilayers led to highest activities, whereas cholesterol and GM_3 (the latter even at low concentrations) inhibited the enzyme in an additive manner.

In conclusion, an extremely easy method has been developed that allows the preparation of LUV containing GPI and/or gangliosides asymmetrically inserted into the outer monolayer, the same orientation found in rafts and caveolae. These vesicles could be useful in presenting GPI or GPI derivatives to specific enzymes, antibodies or cells.

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