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Insertion of a Glycosylphosphatidylinositol-Anchored Enzyme into Liposomes

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Abstract. Incorporation of alkaline phosphatase (AP), a glycosylphosphatidylinositol (GPI)-anchored protein, into liposomes containing detergent, followed by detergent removal with hydrophobic resin was performed. Incorporation media were collected during different steps of detergent removal and were analyzed by flotation in sucrose gradient. The presence of protein was checked by measuring enzymatic activity, while the presence of ^{3}H-radio-labelled liposomes was followed by determination of the radioactivity. The incorporation yield of the protein into liposomes increased with incubation time in presence of hydrophobic resin. Protein was also incorporated at different protein/lipid ratios. At the highest protein lipid ratio, our data showed that 260 molecules of GPI-linked AP (AP-GPI) could be associated with one liposome, corresponding to 65% vesicle coverage. Finally, observations by electron cryomicroscopy indicated (i) that the protein seemed exclusively associated with the lipid bilayer via the GPI-anchor, as shown by the distance—about 2.5 nm—between the protein core and the liposome membrane; (ii) that the AP-GPI distribution was heterogeneous on the liposome surface, forming clusters of protein.

Key words: Lipidic anchors — Alkaline phosphatase — Liposomes — Electron cryomicroscopy — Reconstitution — Protein organization into liposomes

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Abbreviations: AP, alkaline phosphatase; AP-GPI, glycosylphosphatidylinositol-linked alkaline phosphatase; EM, electron microscopy; EPA, egg phosphatidic acid; GPI, glycosylphosphatidylinositol; OctGlc, n-octyl β-D-glucoside; PtdCho, egg yolk phosphatidylcholine; PtdIns-PLC, glycosylphosphatidylinositol-specific phospholipase C.
Enzymes: Alkaline phosphatase, orthophosphoric-monoester phosphohydrolase (EC 3.1.3.1); glycosylphosphatidylinositol-specific phospholipase C (EC 3.1.4.10).

Introduction

GPI-anchored proteins are proteins localized on the outer leaflet of the plasma membrane (Nosjean, Briolay & Roux, 1997; Ferguson, 1999). GPI anchor is a mode of membrane attachment for more than 200 eukaryotic cell-surface proteins (Ferguson, 1999). They are functionally diverse, including hydrolytic enzymes, protozoan surface proteins, adhesion proteins, surface antigens, receptors and prion protein (Hooper, 1992). Most of them present important biological functions, especially in signal transduction and in the recognition process (Nosjean et al., 1997). Particularly the GPI moiety was assumed to be a sorting signal for the anchored proteins, like the N-linked sugar chains, which seem also necessary (Benting, Rietveld & Simons, 1999a).

The detailed molecular structure of GPI anchors was determined for several proteins (Ferguson, 1999). They conserve a basic core covalently attached to the C-termini of proteins. It consists of a glycan moiety bonded to phosphatidylinositol with two acyl chains anchored into the plasma membrane. Evidence is now accumulating that the plasma membrane is organized into different lipid and protein subdomains (Jacobson, Sheets & Simson, 1995; Mouritsen & Jørgensen, 1997). Thus, GPI-anchored proteins are clustered into sphingolipid- and cholesterol-rich membrane domains, also called rafts, containing other signalling proteins such as tyrosine-kinases (Cinek & Horejsi, 1992; Harder & Simons, 1997; Hooper, 1999). Rafts are postulated to act as moving relay stations in membrane trafficking and in signal transduction (Harder & Simons, 1997; Rietveld & Simons, 1998; Simons & Ikonen, 1997; Brown & London, 1998). This raft hypothesis seems well confirmed by fluorescence energy transfer microscopy (Varma & Mayor, 1998) or by cross-linking experiments (Friedrichson & Kurzchalia, 1998). Besides, using differential detergent solubilization, Madore

et al., (1999) reported that functionally different GPI-anchored proteins are organized in different subdomains at the neuronal cell surface.

Among the different groups of GPI-linked proteins, mammalian AP (EC 3. 1.3.1), a widely distributed enzyme, was one of the first proteins that was shown to be GPI-anchored in the plasma membrane (Low & Zilversmit, 1980). This enzyme is homodimeric and hydrolyzes non-specifically phosphate monoesters at alkaline pH to produce inorganic phosphate and an alcohol (Coleman & Gettins, 1983). The X-ray–resolved structure of AP from human placenta has been recently published (Le Du et al., 2001). It presents about 90% sequence similarity with intestinal AP, whose molecule dimensions can be assimilated to a 10 nm × 5 nm prolate ellipsoid.

Biomimetic systems are very useful to understand the organization and the interactions taking place between proteins and lipids in cell membranes. Many methods are available to insert membrane proteins into artificial membranes. Most of them usually involve the use of detergent. Data on the anchored protein insertion process into lipid bilayers are limited. Such information is useful not only for the optimization of functional membrane systems but also for the formation of ordered protein assemblies required in structural analysis of the proteins, i.e., 2-D crystals.

Previously, we investigated the incorporation conditions of the GPI-anchored AP in membrane systems such as liposomes (Angrand et al., 1997; Nosjean & Roux, 1999; Morandat, Bortolato & Roux, 2002) or Langmuir films (Ronzon et al., 2002a, 2002b, 2002c). In this report, we demonstrate that the unpurified and partially lipid-extracted AP-GPI did not insert spontaneously into liposomes. Furthermore, we analyzed the different steps during the reconstitution process of AP-GPI into liposomes. Finally, electron cryomicroscopy exhibited a heterogeneity in the AP-GPI distribution on the liposome surface with a high density of protein, suggesting formation of protein clusters at the liposome surface.

Materials and Methods

Materials

Egg yolk phosphatidylcholine (PtdCho), phosphatidic acid (EPA), CHAPS and n-octyl β-D-glucoside (OctGlc) were purchased from Sigma Chemicals. Triton X-100 and L-α-dipalmitoyl-glycerophospho (Me-^{3}H) choline (^{3}H-PtdCho) were obtained, respectively, from Fluka and NEN Products. Amberlite XAD-2 beads (Pharmacia) and Bio-Beads (Bio-Rad) were extensively washed before use, as described by Holloway (Holloway, 1973). Glycosylphosphatidylinositol-specific phospholipase C (PtdIns-PLC) from *Bacillus thuringensis* (250 U) was obtained from Oxford Glycosystem.

Preparation of AP with GPI-Anchor (AP-GPI)

AP-GPI was purified as described previously (Morandat et al., 2002). Briefly, the membrane fraction prepared from fresh bovine intestine mucosa in 0.1 M Tris-HCl, 1 mM $MgCl_2$, pH 8.5 buffer was washed twice with the same buffer. A first chloroform/methanol (1/2 by vol.) delipidation was carried out before AP-GPI was extracted by 50 mM CHAPS in above buffer. The solubilized fraction ($S_{AP\text{-}GPI}$) was subjected to a second delipidation under the same conditions. The lipid-free fraction was then suspended in (in mM) 20 Tris-HCl, 150 NaCl, 1 $MgCl_2$, 0.1 % Triton X-100, pH 7.4 buffer. After centrifugation (100.000 × *g*, 30 min) the supernatant was submitted to immuno-affinity chromatography and the enzyme fraction was dialyzed against (in mM) 10 Tris-HCl, 150 NaCl, pH 8.5 buffer (TBS) supplemented with 1 $MgCl_2$. The specific activity of purified enzyme was higher than 1.000 U/mg, with a high activity yield (75%) and the protein was homogeneous as evaluated by Laemmli PAGE with an apparent molecular mass of about 130.000 Daltons. The presence of the GPI anchor was evidenced by non-denaturing electrophoresis, with or without previous sample hydrolysis by PtdIns-PLC (Angrand et al., 1997).

AP Activity

Aliquots of enzyme solutions were taken and diluted in 10 mM glycine-NaOH buffer, pH 10.4 containing *p*-nitrophenyl phosphate as enzyme substrate (Cyboron & Wuthier, 1981). Activities were measured spectrophotometrically at 37°C, by monitoring the release of *p*-nitrophenolate at 420 nm (ε = 18.5 cm^{-1}. mM^{-1}). One unit of specific activity hydrolyzes one μmole of substrate per min per mg of protein.

Determination of Protein Concentration

The protein concentration was measured by the method of Bradford (Bradford, 1976), modified by Read and Northcote (Read & Northcote, 1981), using bovine serum albumin as standard.

Liposome Preparation

Large unilamellar liposomes were prepared by dialysis. A typical preparation contained 50 mg of PtdCho and 90 mg of OctGlc solubilized in 5 mL of chloroform. The organic solvent was removed by N_2 flow under atmospheric pressure. The lipid film was then dispersed in 5 mL of TBS buffer and dialyzed automatically in a "Liposomat" (Dianorm) against the same buffer for 90 minutes to remove the detergent. The PtdCho concentration of the resulting liposome suspension was 10 mg/mL - i.e. 13 mM. We used ^{3}H-PtdCho to label vesicles in proportion of 0.5 μCi/mL. The liposome size distribution was homogeneous, as analyzed by quasi-elastic light-scattering (QELS) and the mean vesicle diameter was 150 nm (Angrand et al., 1997).

For electron cryomicroscopy experiments, PtdCho/EPA unilamellar liposomes were prepared by reverse phase evaporation.

Preparation of AP-GPI Proteoliposomes

Proteoliposomes were reconstituted according to Angrand et al. (1997). Incorporation of AP-GPI was performed by incubation of 1 mg liposomes with 16 mM OctGlc for 15 min at 25°C, which just destabilizes the lipid bilayer to promote protein insertion, followed by addition of enzyme.

The detergent in excess was removed using Amberlite XAD-2, following the steps described by Angrand et al. (1997). In the

first step, 80 mg Amberlite XAD-2/mL were added in reconstitution medium and left to incubate for 2 h. In the second step, the first reconstitution medium was taken and mixed with 80 mg of fresh Amberlite XAD-2/mL and incubated for 12 hours. Then for the last step, the reconstitution medium was taken and incubated again with 80 mg of fresh Amberlite XAD-2/mL for 1 hour.

For electron cryomicroscopy experiments, a different procedure was applied. AP-GPI was incorporated into PtdCho/EPA 9:1 (mol/mol) liposomes according to Angrand et al. (1997). Briefly, 1 mL of PtdCho/EPA unilamellar liposomes prepared by reverse phase evaporation at 4 mg/mL in TBS buffer, was treated with 23.5 mM OctGlc, a detergent concentration corresponding to the saturation of liposomes. Then, AP-GPI was added at lipid/protein 10:1 (w/w) ratio and after 15 minutes incubation, OctGlc was removed by one addition of 40 mg Bio-Beads for one hour followed by a second addition of 40 mg Bio-Beads for one hour.

This procedure—i.e., insertion via OctGlc—provides maximum unidirectional incorporation of AP-GPI into the outer leaflet of the liposomes (Angrand et al., 1997).

Density-Gradient Analysis of Proteoliposomes

Proteoliposomes were subjected to ultracentrifugation in 2.5–30% (w/v) sucrose gradient to control the incorporation of AP-GPI (Angrand et al., 1997). The liposome mixture obtained after the incorporation process was supplemented with 30% sucrose and 0.05% (v/v) Triton X-100 (final concentrations) and then layered under the sucrose gradient. After centrifugation (2 h at 4°C and $160.000 \times g$), gradients were collected from the bottom of the tubes and analyzed for lipid and enzyme contents. The AP-GPI content was determined by measuring its enzymatic activity, while ^{3}H-PtdCho radioactivity was counted by liquid scintillation. The sucrose concentration was determined by refractometry on an RRA refractometer (PZO, Poland). All sucrose percentages were expressed in w/v. The incorporation yield was defined as the ratio of the activity recovered in the liposomal fraction over the total activity layered under the gradient. It corresponds to the percentage of AP-GPI associated with the liposomes—i.e., on the outer layer—and is accessible to the substrate used to measure the enzymatic activity (Angrand et al., 1997).

Calculation of the Number of AP-GPI Molecules/Liposome

The number of liposomes in 1 mg of PtdCho was calculated using the following equation:

$$N_L = \frac{\left(\frac{m_{PC}}{M_{PC}} \times N_A\right)}{\left(\frac{4\pi r_{out}^2}{0.71}\right) + \left(\frac{4\pi r_{in}^2}{0.71}\right)}$$

where m_{pc} is the PtdCho mass used (1 mg), M_{PC} is the PtdCho molar mass (765 g/mol), N_A is the Avogadro number, r_{out} and r_{in} correspond, respectively, to the outer (35 nm) and inner (31 nm) radii of the proteoliposomes and 0.71 to the area (in nm^2) of a PtdCho molecule in outer and inner leaflets of large unilamellar vesicles (Huang & Mason, 1978; Schreier, Moran & Caras, 1994). As the lipid loss on Amberlite XAD-2 was about 30%, after incorporation the 1 mg PtdCho media contained 142.10^{11} proteoliposomes of about 70 nm in diameter, as determined previously (Angrand et al., 1997). Different AP-GPI/liposome ratios were tested for the incorporation in order to calculate the maximal number of AP-GPI/liposome.

Electron Cryomicroscopy

Five microliters of the reconstituted proteoliposomes were deposited on a glow-discharged holey carbon grid. The excess solution was removed with a filter paper, and the grid was flash-frozen in a bath of liquid ethane cooled to -180°C with liquid nitrogen (Dubochet et al., 1988).

Results

AP-GPI Did not Insert Spontaneously into PtdCho Liposomes

Purified AP-GPI did not insert spontaneously into PtdCho liposomes (Angrand et al., 1997). To verify whether other lipids or proteins that co-localize with AP-GPI could promote the incorporation of AP-GPI into liposomes, the supernatant containing AP-GPI ($S_{AP\text{-}GPI}$) obtained during the first step of AP-GPI purification was incubated 1 hour in presence of ^{3}H-PtdCho-labelled liposomes, without any detergent for reconstitution.

Reconstitution medium was analyzed using a sucrose gradient centrifugation. The presence of protein was identified by measuring its activity, while the presence of radiolabelled liposomes was monitored by determination of radioactivity. The density gradient profile indicated that the AP-GPI was localized near the bottom (25–30 % sucrose), whereas the liposomes emerged at 15% sucrose (Fig. 1). Therefore, AP-GPI was not incorporated into liposomes, even in the presence of lipids or proteins obtained during the purification of AP-GPI. Higher incubation times did not improve the AP-GPI insertion (*data not shown*).

Kinetics of AP-GPI Reconstitution into Liposomes

To obtain proteoliposomes, it was necessary to add a detergent—i.e., OctGlc—to the solution containing liposomes and protein. To determine the kinetics of reconstitution of AP-GPI into liposomes, proteoliposomes were analyzed by sucrose density gradients during the reconstitution process and during the detergent elimination.

In the presence of OctGlc and before incubation with Amberlite XAD-2 (Fig. 2*A*), the flotation profile shows the co-distribution of AP-GPI and radiolabelled liposomes along the gradient. Two populations of liposomes emerged: the first one near 15% sucrose (indicated by arrow *1*) and the second one near the bottom of the tube (indicated by arrow *2*). To remove the detergent OctGlc, the proteoliposome solution was then subjected to 1 hour incubation in the presence of Amberlite XAD-2. In Fig. 2*B*, two localizations of liposomes and proteins were identi-

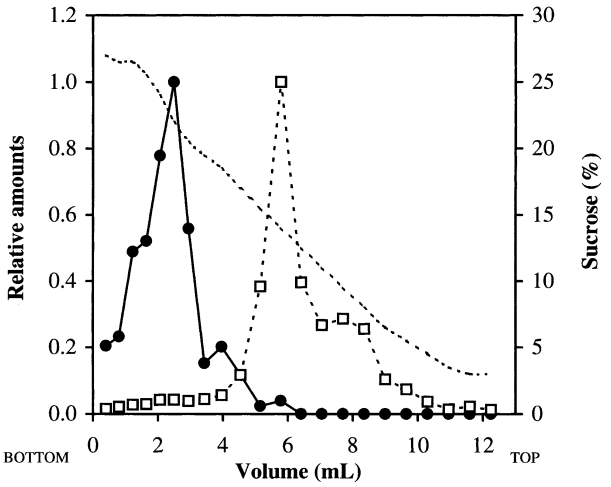

Fig. 1. Liposome flotation in presence of unpurified AP-GPI. Liposomes were incubated with the supernatant $S_{AP\text{-}GPI}$ containing unpurified and partially delipidated AP-GPI. OctGlc was omitted. Directly at the end of the incubation (1 hour), the medium was analyzed for AP-GPI activity (●), ^{3}H-PtdCho (□) and sucrose (- - -) by flotation in sucrose gradient, whose profile was shown. Fractions were collected from the bottom of the tube.

fied on the flotation profile, as previously observed in Fig. 2*A*. Longer incubation time up to two hours was not sufficient to obtain one population of proteoliposomes, as shown by the gradient profile in Fig. 2*C*. Therefore, to better remove detergent, we repeated the incubation procedure with fresh Amberlite XAD-2 for a longer incubation time up to 2 hours (total incubation time of 4 hours; Fig. 2*D*) and 12 hours (total incubation time of 14 hours; Fig. 2*E*). For both incubation times, the analysis of the gradient profile exhibited only one population of proteoliposomes located near 15% sucrose. Subsequent incubation with fresh Amberlite XAD-2 for 1 hour (total incubation time of 15 hours) gave the same distribution (Fig. 2*F*). For each incubation step, the AP-GPI incorporation yields were calculated from the proteoliposome population located near 15% sucrose on the different gradient profiles (Fig. 2). Incorporation yields were then plotted against incubation time (Fig. 3). The incorporation ratio of AP-GPI into liposomes increased from 45% (no Amberlite XAD-2 treatment) to 74% after successive Amberlite XAD-2 treatments. Higher incubation times with Amberlite XAD-2 did not improve the incorporation yield (*data not shown*).

Effect of the Initial AP-GPI/Lipid Ratio on the Protein Quantity Associated with Liposomes

AP-GPI was incorporated in the presence of OctGlc into PtdCho liposomes at different AP-GPI/lipid ratios (mg protein/mg PtdCho). The OctGlc was removed by Amberlite XAD-2 treatment in three steps as described in Methods—i.e., 15 h incubation. Incorporations were analyzed by density gradients. The fractions collected were assayed for ^{3}H-PtdCho and AP-GPI activity. The colocalization of the ^{3}H-PtdCho and AP-GPI in the sucrose gradient demonstrated the enzyme association with liposomes. The AP-GPI liposomal quantity was obtained from the total activity recovered in the vesicle fraction, converted into mg, using the specific AP-GPI activity (1.000 U/mg). The quantity was determined for each initial AP-GPI/lipid ratio in the incorporation medium. In Fig. 4, the AP-GPI liposomal quantity is plotted against the initial AP-GPI/lipid ratio. At the highest AP-GPI/lipid ratio tested, 800 μg of enzyme—i.e., 37×10^{14} molecules—were associated with liposomes, corresponding to about 260 AP-GPI molecules/proteoliposome.

Electron Cryomicroscopy of Proteoliposomes

The reconstituted proteoliposomes were observed by electron cryomicroscopy (Fig. 5*A*, *B* and *C*). Two dark circles were identified as the densities of both leaflets of the lipid membrane (indicated by arrows *1* and *2* respectively). An additional density was also present, forming an extra ring surrounding the liposomes (indicated by arrow *3*). Such density can be clearly attributed to the protein anchored into the outer leaflet of the liposome. The distance from the outer leaflet to the protein density is 2.5 ± 0.5 nm. Moreover, an inhomogeneous distribution of AP-GPI associated with the liposomes was observed. This distribution can be caused by the reconstitution process—i.e., AP-GPI being incorporated as clusters into liposomes—or by a reorganization of proteins after the incorporation. Both hypotheses imply protein-protein interactions, as shown by the important density of proteins in Fig. 5 and the number of molecules that can be associated with one liposome, as determined by calculation (Fig. 4).

Discussion

AP-GPI Does not Insert Spontaneously into PtdCho Liposomes

The AP-GPI present in the supernatant $S_{AP\text{-}GPI}$ obtained in the first step of the purification could not be incorporated into PtdCho liposomes without detergent, as probed by sucrose gradient analysis (Fig, 1). This suggests that residual lipidic components as well as proteins present in $S_{AP\text{-}GPI}$ did not allow a spontaneous enzyme insertion. This was in agreement with recent results on a Langmuir monolayer of distearoylphosphatidylcholine and dioleoylphosphatidylcholine (Ronzon et al., 2002c), in which the penetration of AP-GPI was measured by increase of

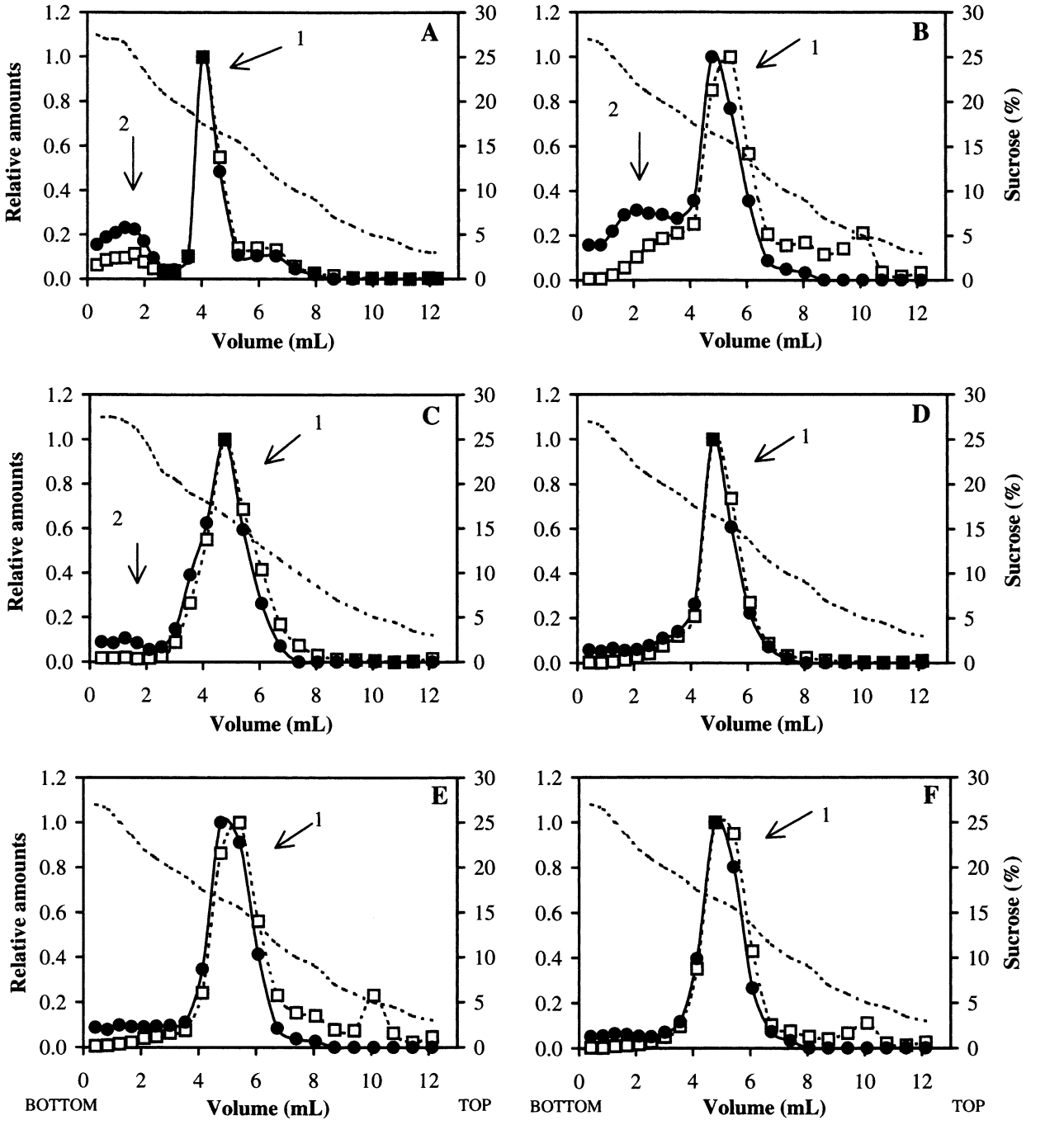

Fig. 2. Kinetics of proteoliposome reconstitution. Liposomes (1 mg) were treated with 16 mM OctGlc before addition of AP-GPI. After 15 min incubation, OctGlc was removed by Amberlite XAD-2 treatment in three steps: 80 mg hydrophobic resins/mL were added and left to incubate 2 h. The process was repeated twice, with incubations of 12 h and 1 h respectively. Solution was analyzed at different incubation times by ultracentrifugation in density gradients. After gradient fractionation ^{3}H-PtdCho (□), AP-GPI activity (●) and sucrose (- - -) were assayed. Fractions were collected from the bottom of the tube. The profiles shown correspond to incubation medium (*A*) before addition of Amberlite XAD-2 and after (*B*) 1 h; (*C*) 2 h; (*D*) 4 h; (*E*) 14 h; (*F*) 15 h incubation with Amberlite XAD-2. The two proteoliposome populations detected were indicated by arrows on the gradient profiles. 1 and 2 correspond to population 1 and 2 respectively, as described in the text.

the surface pressure. The exclusion surface pressure found was rather similar for both lipids, about 21 mN/m and was lower than the surface pressure range, 30 to 35 mN/m, which is believed to coincide with the packing density of biological membranes or lipid bilayers (Blume, 1979; Seelig, 1987; Marsh, 1996). At this pressure level we found no significant pressure change after addition of AP-GPI into the subphase.

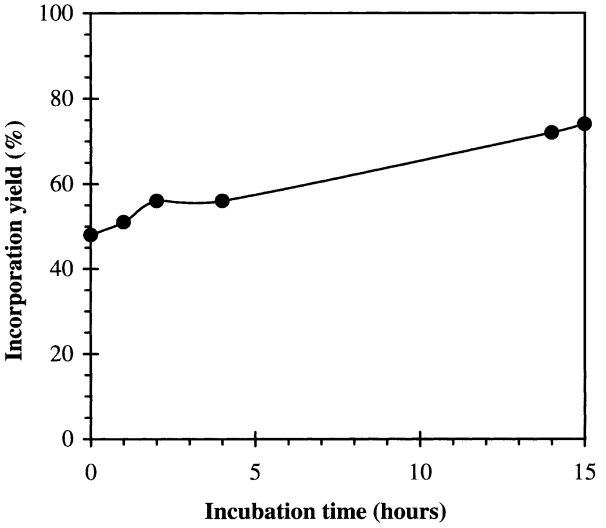

Fig. 3. Effect of incubation time on the AP-GPI incorporation yield. From Fig. 2, incorporation yield was plotted versus incubation time with Amberlite XAD-2. For each time, the incorporation yield was calculated as the ratio of the activity co-localized with liposomes/the total activity. The activity co-localized with liposomes corresponds to proteoliposome population 1 on Fig. 2.

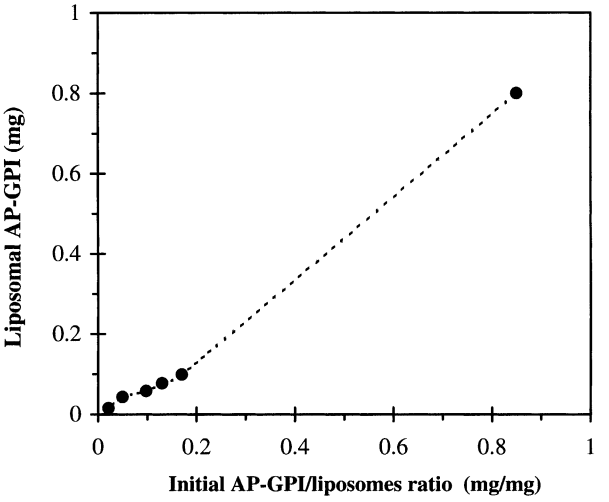

Fig. 4. Effect of the AP-GPI/lipid ratio on the liposome-associated protein quantity. AP-GPI was incorporated 15 min via OctGlc into PtdCho liposomes at different AP-GPI/lipid ratio (mg protein/mg PtdCho). The OctGlc was removed by three-step Amberlite XAD-2 treatment, as described in Methods (i.e., 15 h incubation). Proteoliposomes so obtained were analyzed by density gradient. After gradient fractionation, ^{3}H-PtdCho and enzyme activity were assayed. For each initial AP-GPI/lipid ratio, the AP-GPI liposomal quantity (mg)—i.e., the total enzyme activity recovered in liposomal fractions is converted in quantity, using the specific activity (1000 U/mg)—was determined and was plotted versus AP-GPI/lipid ratio.

However, when liposomes contain raft lipids—i.e., cholesterol and/or sphingolipids—spontaneous insertion of AP-GPI occurred (Milhiet et al., 2002; Morandat et al., 2002). This highlights the influence of lipid composition on protein insertion.

Reconstitution Kinetics of AP-GPI into Liposomes

The incorporation process used was based on a reconstitution method usually applied to transmembrane proteins (Rigaud, Pitard & Levy, 1995), which permits incorporation of AP, a GPI-anchored protein, into liposomes (Angrand et al., 1997; Morandat et al., 2002).

The different steps of the reconstitution of AP-GPI into liposomes were analyzed by density gradients (Fig. 2). The major part of the AP-GPI activity and of the radioactivity colocalized near 15% sucrose immediately after the incubation of detergent-saturated liposomes with AP-GPI. A mixture of AP-GPI and ^{3}H-PtdCho was observed for 25–30% sucrose only in the cases where detergent removal was not complete (Fig. 2*A*, *B* and *C*). This could correspond to another proteoliposome population. Thus, AP-GPI in the bottom of the tube might be inserted into vesicles with a high ratio of protein/lipids during the first step of removal of the detergent excess. This second proteoliposome population could result from some heterogeneity of AP-GPI molecular species in the bovine intestine, particularly in the hydrophobic part of the GPI-anchor (Bublitz et al., 1993; Armesto et al., 1996). Recently, acyl- and alkyl-chain compositions of GPI have been shown to influence the association of GPI-anchor protein with raft lipid-containing vesicles (Benting et al., 1999b), as previously suggested (Schroeder et al., 1998). The initial insertion of AP-GPI into liposomes could depend on the composition of the hydrophobic part of the anchor. However, only a small part of AP-GPI is inserted into these high-density vesicles. During the detergent elimination these vesicles disappeared and the protein activity was concentrated into a narrow peak. The percentage of AP-GPI inserted in liposomes increases during the reconstitution process up to 14% (Fig. 3). Consequently, all steps for detergent elimination are necessary to obtain a maximum of reconstituted protein.

Evaluation of the Number of AP-GPI Molecules Incorporated into Liposomes

The number of GPI-anchored molecules introduced into vesicles is usually low, whatever the reconstitution process. Schreier et al. (1994) engineered a chimeric protein with a GPI anchor and they incorporated only 1 to 2 protein molecules into PtdCho:cholesterol liposomes by simple incubation. In the case of intestinal AP-GPI, 34 molecules/liposome were incorporated in the presence of OctGlc into PtdCho liposomes (Angrand et al., 1997). In this

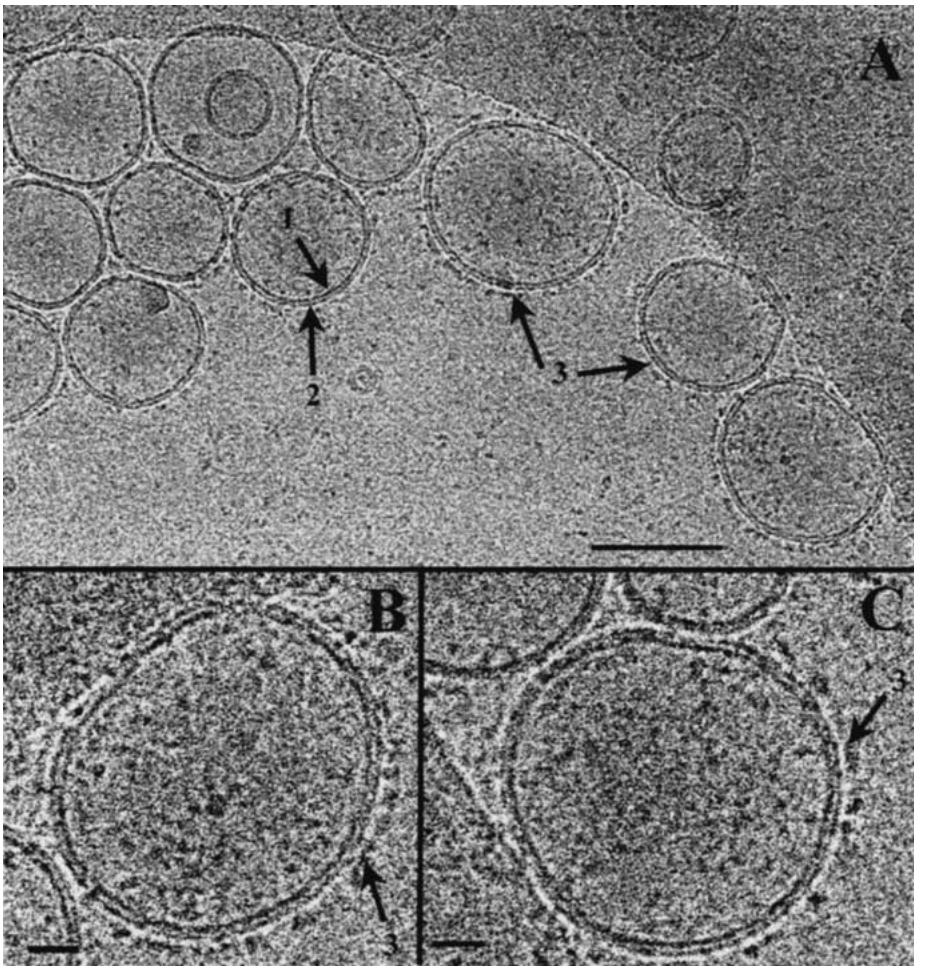

Fig. 5. Electron cryo-microscopy of reconstituted AP-GPI into liposomes. Arrows *1* and *2* indicate the two bilayer leaflets. The ring surrounding the liposomes corresponds to the density of the incorporated protein into the outer leaflet of the liposomes (indicated by arrow *3*). The distance between the densities of the outer leaflet and the protein is attributed to the length of the GPI part (*see* text). Bars correspond to 100 nm in *A* and 20 nm in *B* and *C*.

report, we show that the increase of the initial AP-GPI/lipid ratio in the incorporation medium led to 260 molecules associated with one proteoliposome. As previously reported, the enzyme activity measured in the proteoliposome fraction corresponded to the protein located in the outer leaflet of the vesicle (Angrand et al., 1997). Assuming that AP-GPI is a prolate ellipsoid of large diameter of 10 nm and smaller diameter of 5 nm, the total surface of 260 molecules was 100.10^2 nm^2, which is in accordance with the surface of a 70 nm diameter proteoliposome, i.e., 154.10^2 nm^2. Consequently, the vesicle coverage is about 65%, corresponding to a density of 16,900 molecules of AP-GPI per μm^2 (Fig. 4). It is likely that some steric hindrances or protein-protein interactions occurred.

Electron Microscopy on Proteoliposomes Containing AP-GPI

To characterize the organization of AP-GPI on liposomes, cryo-transmission electron microscopy was used (Fig. 5). It was obvious that the protein core did not interact with membrane, as shown by a 2.5 nm space between the liposome and the protein. In fact, electron microscopy photos of protein interacting with membranes, such as annexin, were very different, since the protein core can not be distinguished from the liposome membrane (Olivier Lambert, personal communication). Therefore, the AP-GPI was exclusively associated with liposomes via the GPI-anchor, as previously determined (Angrand et al., 1997; Morandat et al., 2002). Moreover, the distance—about 2.5 nm—between the protein core and the liposome membrane suggested that the interactions were due only to fatty-acid chains of the GPI-anchor with alkyl chains of phospholipids. However, recently Lento and Sharom (2002) have obtained results from fluorescence resonance energy transfer studies, which indicate that GPI-anchored proteins interact closely with the membrane surface. The distance between the protein core of placental AP and the lipid-water interfacial region of the bilayer was estimated to be smaller than 10–14 Å. Such a difference in distance could be explained by (i) the technique used, (ii) the modification of the protein with the fluorescent dye, (iii) the lipid composition of the vesicles. It should be noted that the cryo-transmission electron micro-

scopy gives a direct measure of the distance, unlike the fluorescence resonance energy transfer.

As described extensively (Friedrichson & Kurzchalia, 1998; Hooper, 1999), GPI-anchored proteins, are localized in membrane microdomains enriched in sphingolipids and cholesterol, where their density is up to 30,000 molecules per μm^2 for caveolar membrane (Rothberg, 1995). Our data obtained from proteoliposomes indicated a surface density of about 16,900 molecules per μm^2. The existence of interactions between AP-GPI molecules was inferred from electron microscopy (Fig. 5). Inhomogeneous distribution of AP-GPI proteins on the liposome surface was observed, suggesting that clusters of proteins occurred. The mechanism of information of the lipidic rafts is not well known, especially with respect to the interactions between proteins during their formation. The presence of interactions between AP-GPI molecules may contribute to the formation of protein domains into lipidic rafts.

In conclusion, two populations of vesicles containing AP-GPI were present during the first steps of the reconstitution. After detergent removal, only one population of proteoliposomes remained. We showed that the partially purified and partially lipid-free AP-GPI did not insert spontaneously into liposomes. The electron cryomicroscopy indicated that AP-GPI interacted with membrane exclusively with its GPI-anchor. A heterogeneous distribution of AP-GPI around liposomes was also observed.

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