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Cholesterol-dependent insertion of glycosylphosphatidylinositol-anchored enzyme

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Abstract

Evidence is now accumulating that the plasma membrane is organized in different lipid and protein subdomains. Thus, glycosylphosphatidylinositol (GPI)-anchored proteins are proposed to be clustered in membrane microdomains enriched in cholesterol and sphingolipids, called rafts.

By a detergent-mediated method, alkaline phosphatase, a GPI-anchored enzyme, was efficiently inserted into the membrane of sphingolipids- and cholesterol-rich liposomes as demonstrated by flotation in sucrose gradients. We have determined the enzyme extraluminal orientation. Using defined lipid components to assess the possible requirements for GPI-anchored protein insertion, we have demonstrated that insertion into membranes was cholesterol-dependent as the cholesterol addition increased the enzyme incorporation in simple phosphatidylcholine liposomes.

Keywords: Glycosylphosphatidylinositol anchor; Liposome; Cholesterol; Reconstitution; Alkaline phosphatase

1. Introduction

Membrane proteins could be associated to the membrane either by a transmembrane domain or a lipid binding domain or a lipid moiety covalently linked to the polypeptide chain. Among the latter, modification of protein with a glycosylphosphatidylinositol (GPI) anchor is a mode of membrane attachment for more than 200 eukaryotic cell-surface proteins [1]. They are functionally diverse, including hydrolytic enzymes, protozoan surface proteins, adhesion proteins, surface antigens, receptors and prion proteins [2]. In addition, to afford the protein association with the membrane, this lipid tail presents important biological functions especially in signal transduction, membrane addressing and in recognition processes [3]. Their participation in miscellaneous processes favours the probability of their being involved in pathological circumstances. Thus, several blood infections in mammals are due to the presence of parasites such as malaria, toxoplasmosis and others involving *Trypanosome* species. These infectious agents express GPI-linked molecules on their surface, which were involved in signalling functions, but most notably in the surface protection of the parasite. Some novel pathologic agents, the prion proteins, are the cause of several neurodegenerative disorders. The causative agent is a modified form of a normal constituent of nerve tissues, the GPI-anchored prion protein, which is converted into anchorless scrapie prion protein [3].

The detailed molecular structure of GPI tails has been determined for several proteins [1]. These complex anchors have a conserved basic core. A glycan moiety is bonded to phosphatidylinositol, mostly with two saturated acyl chains anchored into plasma membrane lipid bilayer. The anchor hydrolysis by specific phospholipases induces the selective release of these cell-surface proteins into the extracellular medium. This process may modulate the structure and the function of GPI-anchored proteins [2,3].

Evidence is now accumulating that the plasma membrane is organized in different lipid and protein subdomains [4,5]. Thus, GPI-anchored proteins are clustered into sphingolipid- and cholesterol-rich membrane domains, also called

Abbreviations: BIAP, alkaline phosphatase from bovine intestinal mucosa; Chol, cholesterol; GC, galactocerebroside; GPI, glycosylphosphatidylinositol; OctGlc, *n*-octyl β-D-glucoside; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PtdIns-PL C, phosphoinositol-specific phospholipase C; SL, sphingolipid; SM, sphingomyelin

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rafts, containing other proteins, of which several are lipid-modified proteins [6–8]. Rafts are postulated to act as moving relay stations in membrane trafficking and signal transduction [6,9,10]. Because of their insolubility in cold non-ionic detergents such as Triton X-100, these domains can be isolated as detergent-resistant membranes [6,11–15].

In order to better understand the mechanisms involved in the clustering of GPI-anchored proteins with sphingolipids and cholesterol, we have attempted to build a model of these peculiar membrane microdomains. The GPI-anchored protein selected is alkaline phosphatase (EC 3.1.3.1), a widely distributed enzyme isolated from eukaryote cells. It is one of the first proteins that were shown to be GPI-anchored in the plasma membrane of mammals [16]. We have prepared liposomes, enriched in cholesterol, sphingomyelin and cerebroside and alkaline phosphatase from bovine intestinal mucosa (BIAP) was inserted via a detergent-mediated method. Most of the proteins associated with liposomes were anchored in the outer leaflet of the bilayer, oriented towards the outside of the liposome. Moreover, to evidence the possible dependence of BIAP insertion towards raft typical lipids, BIAP was inserted into liposomes with different lipid composition. The results obtained showed for the first time the cholesterol-dependent insertion of a GPI-anchored enzyme.

2. Materials and methods

2.1. Materials

Phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), galactocerebroside (GC), cholesterol (Chol), *n*-octyl β-D-glucoside (OctGlc) were purchased from Sigma Chemicals. Dipalmitoyl-phosphatidyl-[*N-methyl*-^{3}H]-choline ([^{3}H]-PC) and [4-^{14}C]-cholesterol ([^{14}C]-Chol) were purchased from NEN Products. Amberlite XAD-2 beads were obtained from Pharmacia and extensively washed before use as previously described [17]. Phosphoinositol-specific phospholipase C (PtdIns-PL C) from *Bacillus thuringiensis* (250 U) was obtained from Oxford Glycosystem.

2.2. BIAP preparation

Alkaline phosphatase from fresh bovine intestine mucosa (BIAP) was prepared as previously described [17], with slight modifications to reduce the membrane protein and lipid conjugate contaminations. In summary, the membrane fraction, prepared 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, v/v) delipidation was carried out before BIAP was extracted by 50 mM (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) in the previous buffer. The solubilized fraction was subjected to a second delipidation (same conditions). The delipided fraction was then suspended in 20 mM Tris–HCl, 150 mM NaCl, 1 mM $MgCl_2$, 0.1% Triton X-100, pH 7.4 buffer. After centrifugation (100,000×*g*, 30 min) the supernatant contained most of the BIAP activity. The fraction obtained by immuno-affinity chromatography was dialysed against a 10 mM Tris–HCl, 150 mM NaCl, pH 8.5 buffer (TBS). Purified BIAP was homogenous as evaluated by Laemmli PAGE. The presence of GPI anchor was evidenced by non-denaturing electrophoresis, with or without previous sample hydrolysis by PtdIns-PL C.

2.3. Enzyme 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. Activities were measured spectrophotometrically at 37 °C, by monitoring the release of *p*-nitrophenolate at 420 nm (ε=18.5 cm^{2}/μmol). One unit of specific activity hydrolyses one micromole of substrate per minute per milligram of protein. Purified BIAP activity was typically 900–1000 U/mg protein.

2.4. Liposome preparation

Large unilamellar vesicules were prepared by freeze–thaw and extrusion procedure. A typical preparation contained 10 mg of lipids solubilized in 1 ml of chloroform. The organic solvent was removed by N_2 flow under atmospheric pressure. The lipid film was then dispersed in 500 μl TBS and shaken for 15 min. The hydrated lipid dispersion was exposed to six freeze–thaw cycles (−180 °C/+25 °C or −180 °C/+45 °C for SM- and GC-containing liposomes) and passed 39 times through a polycarbonate membrane (0.2 μm) with a Mini-Extruder (Avanti Polar Lipids). The total lipid concentration of the resulting liposome suspension was 20 mg/ml. Several kinds of liposomes were prepared: PC liposomes, PC/Chol (2:1) liposomes, PC/SM (5:1) liposomes, PC/GC (5:1) liposomes and GSC liposomes (PC/PE/GC/SM/Chol, 1:1:1:1:2). We used [^{3}H]-PC to label PC containing vesicles and [^{14}C]-Chol to label Chol-containing vesicles, in proportions of 0.1 μCi/ml for each one.

2.5. Preparation of BIAP proteoliposomes

Proteoliposomes were reconstituted according to Angrand et al. [17]. Incorporation of BIAP was performed by incubation of 1 mg/ml liposomes with 16 mM OctGlc 15 min at 25 °C, which just destabilises the lipid bilayer to promote protein insertion, followed by addition of aliquots of enzyme. The detergent was then removed by hydrophobic adsorption on Amberlite XAD-2 resin at 4 °C. All reconstituted proteoliposomes were prepared and used as suspensions of 1 mg lipids/ml in TBS, unless otherwise indicated. Incorporation of BIAP into liposomes was moni-

tored by flotation in sucrose density gradients. Control samples containing anchorless BIAP and liposomes were also subjected to incorporation process.

2.6. Density gradient analysis of proteoliposomes

Proteoliposomes were subjected to ultracentrifugation in 2.5–30% (w/v) sucrose gradient to control the incorporation of BIAP [17]. The liposome mixture obtained after incorporation process was supplemented with 30% sucrose and 0.05% (v/v) Triton X-100 (final concentrations) and then layered under the sucrose gradient in TBS. After centrifugation (2 h at 4 °C and 160,000×*g*), gradients were collected from the bottom of the tubes, and analysed for lipid and enzyme contents. The BIAP content was determined by measuring its enzymatic activity, while [^{3}H]-PC and [^{14}C]-Chol radioactivities were counted by liquid scintillation. The sucrose concentration (w/w) was determined by refractometry on a RRA refractometer (PZO, Poland). The incorporation yield was defined as the ratio of the activity recovered in liposomal fractions over the total activity layered under the gradient. It corresponds to the percentage of BIAP associated with the liposomes and accessible to the substrate used to measure the enzymatic activity. Control samples containing either BIAP alone or liposomes alone have been previously subjected to centrifugation and analysis. Each experiment was repeated three times.

2.7. Action of PtdIns-PL C on BIAP proteoliposomes

After the reconstitution process, the so-formed proteoliposomes were incubated with PtdIns-PL C for 2 h at 38 °C, with a BIAP/PtdIns-PL C ratio of 20 (U/U). Parallel controls were incubated without PtdIns-PL C, in the same conditions.

3. Results and discussion

3.1. BIAP was incorporated by GPI anchor into GSC liposomes

The proteoliposomes were prepared by detergent-mediated incorporation of BIAP (5 μg) in GSC liposomes (PC/PE/GC/SM/Chol, 1:1:1:1:2) (1 mg of lipids). The association of BIAP with the GSC liposomes was controlled by centrifugation in density gradient (Figs. 1 and 2). The co-localization of the [^{3}H]-PC, [^{14}C]-Chol and BIAP demonstrated the enzyme association with liposomes (Fig. 1A). The control density gradient of BIAP (5 μg) shows that the free enzyme remained at the bottom of the gradient (Fig. 1B). In order to prove that BIAP was GPI-anchored into the liposome bilayer, we have tested the detergent-mediated insertion of anchorless BIAP into GSC liposomes. Incorporation was monitored by flotation in sucrose gradient (Fig. 2A). The enzymatic activity was found at the bottom of the gradient, whereas the liposomes were banded into one population located at about 20% (w/w) sucrose. We have also tested the accessibility and the release of BIAP to the action of PtdIns-PL C before density centrifugation. BIAP was incorporated via OctGlc into GSC liposomes. Incorporation media were then incubated with PtdIns-PL C. The phospholipase incubation was conducted at 38 °C, but temperature was not responsible for the release of BIAP, as shown by the density gradient profile of the control (Fig. 2B). About 70% of the incorporated BIAP activity were released from the proteoliposomes by PtdIns-PL C treatment, and collected at the bottom of the gradient as free enzyme (Fig. 2C). Besides, we observed in these conditions that about 30% of activity remained associated with the

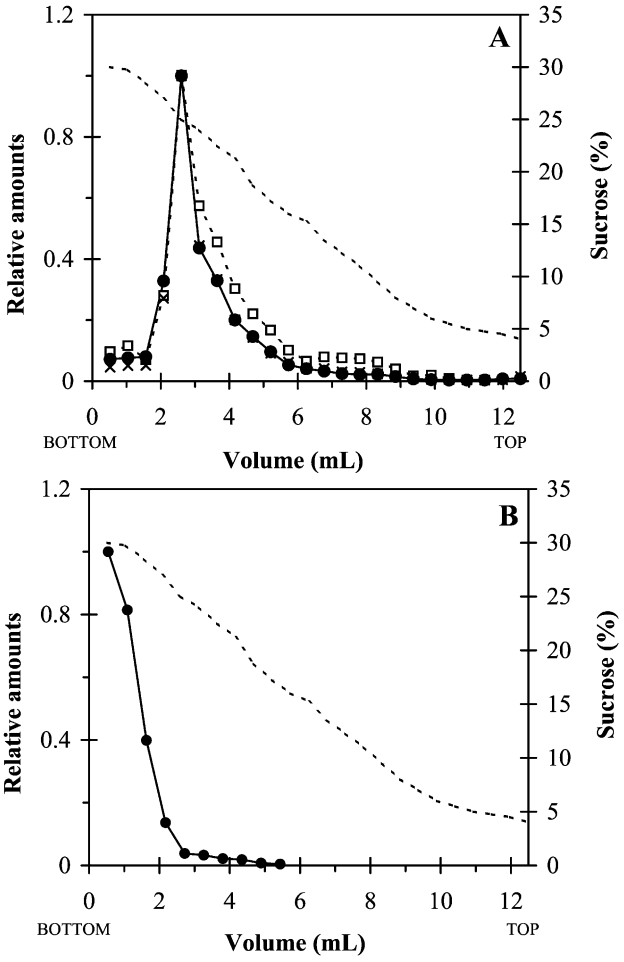

Fig. 1. Density gradients of alkaline phosphatase (BIAP) and of liposomes containing BIAP. (A) BIAP (5 μg) was incorporated into GSC liposomes (PC/PE/GC/SM/Chol, 1:1:1:1:2) (1 mg). Proteoliposomes were analysed by ultracentrifugation directly at the end of the reconstitution process. Fractions were collected from the bottom of the tube and analysed for BIAP activity (●), [^{3}H]-PC (□), [^{14}C]-Chol (×) and sucrose (- - -). (B) BIAP (5 μg) was analysed by ultracentrifugation. Fractions were collected from the bottom of the tube and analysed for BIAP activity (●). Abbreviations: Chol, cholesterol; GC, galactocerebroside; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin.

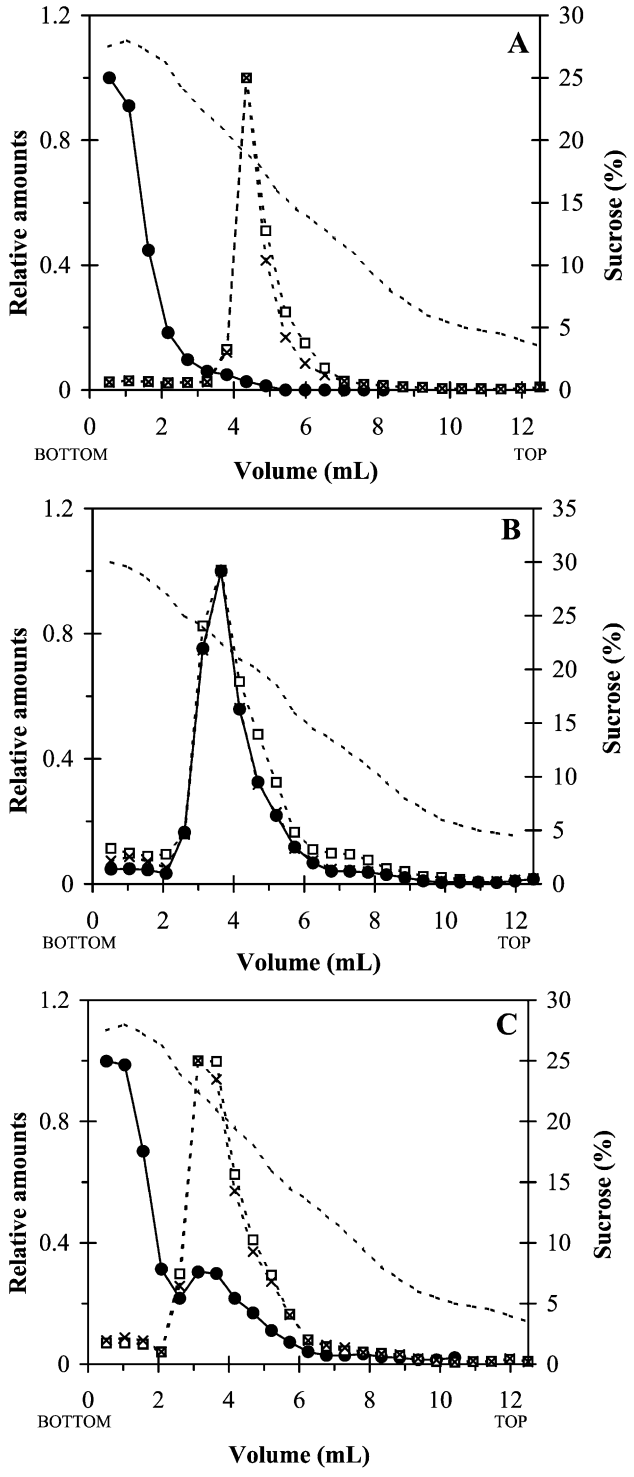

Fig. 2. The BIAP incorporation into liposomes involves the GPI anchor. (A) Anchorless BIAP (5 μg) was incorporated into GSC liposomes (PC/PE/GC/SM/Chol, 1:1:1:1:2) (1 mg). Incorporation medium was analysed by ultracentrifugation directly at the end of the reconstitution process. BIAP with GPI anchor (5 μg), was incorporated into GSC liposomes (1 mg). Proteoliposomes were analysed by ultracentrifugation directly after incubation for 2 h at 38 °C (B) or after incubation for 2 h at 38 °C with phosphoinositol-specific phospholipase C (C). All fractions were collected from the bottom of the tube and analysed for BIAP activity (●), [^{3}H]-PC (□), [^{14}C]-Chol (×) and sucrose (- - -). Abbreviations: Chol, cholesterol; GC, galactocerebroside; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin.

liposomes. Same results were obtained without Triton X-100 in the samples for flotation in sucrose gradient (data not shown). BIAP was essentially oriented to the outside of the vesicles through its GPI anchor. These data were in accordance with that observed previously for alkaline phosphatase insertion into PC liposomes [17]. However, only 70% of BIAP were released from liposomes by enzymatic hydrolysis: either the cleavage site became inaccessible after incorporation, or the anchorless BIAP remained associated through other associations. Moreover, in cellular membranes not all GPI anchors are PtdIns-PL C sensitive [18].

3.2. Incorporation of BIAP into liposomes was cholesterol-dependent

In order to evidence the possible dependence of BIAP insertion towards raft typical lipids (i.e. SM, GC and Chol), we have compared the incorporation yields in different kinds of liposomes: PC, GSC, PC/Chol (2:1), PC/SM (5:1) and PC/GC (5:1). Various amounts of BIAP were incorporated via OctGlc into the different liposomes (1 mg of lipids). Incorporation was monitored by flotation in sucrose gradients. The fractions collected were assayed for [^{3}H]-PC, [^{14}C]-Chol and BIAP activity. Control samples with anchorless BIAP exhibited the result shown previously (i.e. Fig. 2A). The incorporation yield was determined for each kind of liposomes and for each protein/lipid ratio (μg BIAP/mg liposomes). Fig. 3 shows the effect of lipid composition and the effect of BIAP quantity on the incorporation yields. For all the BIAP amounts inserted via

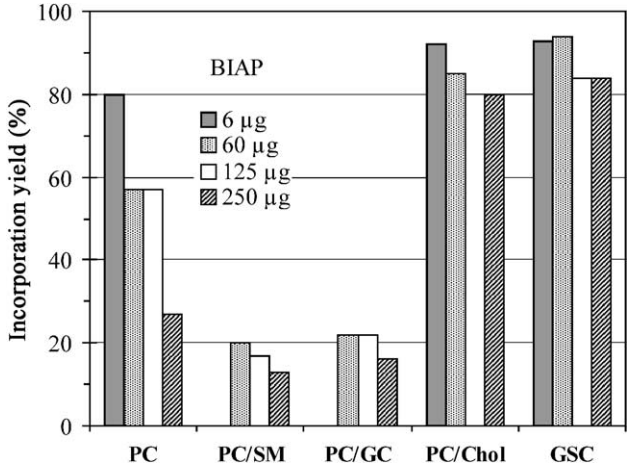

Fig. 3. Incorporation yields according to lipid composition of liposomes. Various amounts of BIAP (6–25 μg) were incorporated into different kinds of liposomes (1 mg). Proteoliposomes so obtained were analysed by ultracentrifugation on sucrose gradient. After gradient fractionation, [^{3}H]-PC, [^{14}C]-Chol and enzyme activity were assayed. For each amount of BIAP, the incorporation yield was calculated as the ratio of the total activity associated with liposomes/the total activity. Liposomes used were: PC liposomes, PC/SM (5:1) liposomes, PC/GC (5:1) liposomes, PC/Chol (2:1) liposomes and GSC liposomes (PC/PE/GC/SM/Chol, 1:1:1:1:2). Abbreviations: Chol, cholesterol; GC, galactocerebroside; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin.

OctGlc, the Chol-containing liposomes—i.e. PC/Chol and GSC—exhibited the highest incorporation yields, whereas SL-containing vesicules—i.e. PC/GC and PC/SM—exhibited the lowest ones. Values obtained for PC liposomes were intermediate. Whatever the liposomes composition, the incorporation yields decreased with increasing enzyme amounts. However, the yields varied from 80% to 27% in PC liposomes, whereas they varied only from 90% to 80% in Chol-containing liposomes. It is likely that high enzyme concentrations enhanced steric hindrances or protein–protein interactions, to the detriment of the BIAP insertion.

The highest incorporation yields obtained in Chol-containing liposome might be correlated to the phase state of the bilayer, but in the presence of 16 mM OctGlc, it is difficult to predict lipid phase behavior. Therefore, either Chol adjusted membrane fluidity to provide the proper lipid environment for insertion [19] or Chol interacted directly and specifically with acyl chains of GPI anchor. Interaction studies of BIAP with different lipids exhibited clearly the preferential binding with Chol but also with the other raft lipids, i.e. SM, PE and GC (data not shown). Such a dependence of protein insertion towards Chol has been described for caveolin [20,21], but this palmitoylated and transmembrane protein also binds to Chol [20]. The binding of Chol might change the caveolin conformation into an incorporation-competent state. It seems unlikely that lipid/protein interaction occurred in our case because (i) BIAP polypeptidic chain did not interact directly with the membrane, as caveolin did (Ronzon et al., submitted); (ii) Chol is postulated to fill the voids formed underneath the sphingolipid headgroups in membrane rafts [6,22], so it does not emerge at the bilayer surface.

3.3. High temperature promoted BIAP interaction with GSC liposomes

Proteoliposomes were prepared by incubation without OctGlc of GSC liposomes (1 mg of lipids) with BIAP (20 μg) in TBS, 10 min at 25, 38 and 50 °C. Control samples, i.e. PC liposomes with BIAP were incubated in the same conditions. When the sample was layered under the sucrose gradient, Triton X-100 was omitted. Incorporation was monitored by ultracentrifugation in density gradients. Table 1 summarised the results obtained. The temperature increase from 25 °C to 38 °C improved the incorporation yield for GSC liposomes as for PC liposomes. However, at 50 °C, this improvement continued only in GSC liposomes contrary to PC liposomes. The temperature increase induced lipid phase modifications that could favour insertion of GPI anchor into the bilayer, modifications appearing more markedly in GSC liposomes. Recent studies on GPI-linked proteins show that membrane transfer from erythrocytes to liposomes do not occur spontaneously but need some catalyst [23]. It should be noticed that (i) the transfer was membrane-to-membrane and not micelle-to-membrane, as in our study, (ii) liposomes did not mimic raft lipid composition as only dimyristoyl-PC and dipalmitoyl-PC were used.

Table 1
Incorporation yields according to incubation temperature and lipid composition of liposomes

Temperature (°C)	Incorporation yield (%)	
	PC liposomes	GSC liposomes
25	0	0
38	24	35
50	27	49

BIAP (20 μg) was incorporated without OctGlc into PC or GSC liposomes (PC/PE/GC/SM/Chol, 1:1:1:1:2) (1 mg) at 25, 38 or 50 °C. Proteoliposomes so obtained were analysed by ultracentrifugation on sucrose gradient. The incorporation yields were calculated as the ratio of the total activity associated with liposomes/the total activity layered under the gradient. Abbreviations: Chol, cholesterol; GC, galactocerebroside; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin.

In conclusion, our work again highlights the importance of Chol in membrane microdomains. As a raft component, Chol has a functional significance in membrane trafficking [24,25]. In addition, Chol is a prerequisite for signal transduction via GPI-anchored proteins in T cells [26]. Several GPI-anchored proteins with widely different functions have been shown to cluster on the cell surface and to associate with caveolae [27]. It would appear that Chol plays a critical role in maintaining the caveolae domain and modulates the interaction of GPI-anchored membrane proteins via their phospholipid anchors, as shown for the clustering of the folate receptor [28]. The clustering is controlled by lipid–lipid interactions between the fatty acyl chains of the bilayer, and Chol plays an essential role in this phenomenon. In the same way, in living cells, GPI-anchored proteins are clustered in rafts and that is dependent on the level of Chol in the cell [7]. It would be interesting to determine if other GPI-anchored protein(s) exhibit a similar insertion dependence towards Chol or other typical raft lipid(s).

The understanding of raft formation and the interactions existing between proteins and lipids will become important in the following years as rafts play a central role in the metabolism of prion proteins as well as in the membrane budding of influenza and human immunodeficiency viruses [29–31].

The membrane model we described here will now be used to study the possible surface aggregation of BIAP and Chol and/or GC. It would also be interesting to define the role of lipid composition and phase state of the membrane on enzyme activity and therefore a possible regulation of the enzyme. Surface localization of proteins on liposomes will be achieved by atomic force electron microscopy.

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