Spontaneous insertion and partitioning of alkaline phosphatase into model lipid rafts

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Several cell surface eukaryotic proteins have a glycosyl-phosphatidylinositol (GPI) modification at the C-terminal end that serves as an anchor to the plasma membrane and could be responsible for the presence of GPI proteins in rafts, a type of functionally important membrane microdomain enriched in sphingolipids and cholesterol. In order to understand better how GPI proteins partition into rafts, the insertion of the GPI-anchored alkaline phosphatase (AP) was studied in real-time using atomic force microscopy. Supported phospholipid bilayers made of a mixture of sphingomyelin–dioleoylphosphatidylcholine containing cholesterol (Chl+) or not (Chl–) were used to mimic the fluid-ordered lipid phase separation in biological membranes. Spontaneous insertion of AP through its GPI anchor was observed inside both Chl+ and Chl– lipid ordered domains, but AP insertion was markedly increased by the presence of cholesterol.

INTRODUCTION

The organization of biological membranes into microdomains is now believed to play a key role in several cellular processes such as protein targeting and signal transduction. The existence of these microdomains, also named ‘rafts’, is explained mainly by the lateral phase separation of membrane lipids in a fluid liquid crystalline phase (Lα) and a liquid ordered phase (Lo) rich in cholesterol (Chl) and sphingolipids (Brown and London, 2000; Simons and Toomre, 2000). Experiments suggesting the presence of raft-like microdomains in eukaryotic cells are based generally on the resistance of membrane fragments to the solubilization at 4°C by non-ionic detergents, such as Triton X-100, leading to the formation of detergent-resistant membranes (DRMs) (Brown and Rose, 1992). DRMs contain few transmembrane proteins, but they are enriched in certain classes of lipid-anchored proteins, mainly glycosylphosphatidylinositol (GPI) and acylated proteins (for a review, see Simons and Toomre, 2000). GPI proteins are consequently used as a positive control to characterize DRMs, where their presence is explained by a preference of their anchor composed of saturated fatty acyl chains for a less fluid membrane in the Lα phase (Schroeder et al., 1994; Benting et al., 1999). Nevertheless, it seems that functionally different GPI-anchored proteins associated within DRMs could be organized in different domains at the plasma membrane (Madore et al., 1999).

Although the existence of rafts and, more generally, membrane microdomains is now well established, how proteins partition into these domains is documented poorly due to the complexity of cell membrane organization. This prompted several groups, including ours, to use membrane models to study the lipid phase separation process. Recent progress occurred in this field when Jacobson’s group studied, using fluorescence microscopy, the partition of the raft-associated protein Thy-1 in raft model supported monolayers (Dietrich et al., 2001b). Thanks to its capacity to image structures in aqueous media with a resolution that extends from the molecular to the microscopic level (Radmacher et al., 1992; Shao and Zhang, 1996; Engel et al., 1997), atomic force microscopy (AFM) appears to be a useful tool to investigate membrane microdomains. AFM has therefore been used to probe the mesoscopic lateral organization of lipid mixtures in supported monolayers and bilayers in order to test the raft hypothesis (McKiernan et al., 2000; Milhiet et al., 2001a, 2002). AFM also represents a useful tool to explore the structure of non-crystallized membrane

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proteins inserted or associated in lipid bilayers (Milhiet et al., 2001b; Vié et al., 2001).

Here, AFM was used to explore the real-time spontaneous insertion of the raft-associated alkaline phosphatase (AP, EC 3.1.3.1) in supported bilayers made of an equimolar ratio of dioleolphosphatidylcholine (DOPC) and sphingomyelin (SM), either with or without Chl. Such lipid mixtures separate into liquid crystalline and ordered phases at room temperature and can be considered as a simple model to study the lipid microdomains with the properties expected of rafts (Dietrich et al., 2001b; Rinia et al., 2001).

RESULTS

When DOPC–SM (1:1) bilayers supported on a freshly cleaved mica disk were observed in contact mode, round-shaped protruding domains of varying size from the mesoscopic to the microscopic scale were visualized (Figure 1A). The lighter (thicker) phase protruded from the liquid crystal matrix by 1 nm (Figure 1C) and certainly corresponded to gel phase domains, as suggested recently by differential scanning calorimetry and AFM experiments using a 1-palmitoyl-2-oleylphosphatidylcholine (POPC)–SM mixture (Milhiet et al., 2002). A few vesicles, which had not fused with the bilayer, appeared as brighter dots (see black arrows in A). The vertical color scale corresponds to a height of 10 nm.

Using a DOPC–SM–Chl (1:1:0.35) mixture, a phase separation was still observed (Figure 1B), but the domains were frequently connected and formed an extended network. The height difference (δh) between the lighter and the darker domains decreased to ~0.65 nm (Figure 1D).

**Insertion and distribution of AP in lipid ordered domains**

Supported DOPC–SM (1:1) bilayers were first incubated in the presence of 30 µg/ml purified bovine intestinal AP proteins (Angrand et al., 1997). After 30 min incubation, bright dots could be detected (Figure 2B). They were localized mainly at the periphery of the SM-enriched gel phase domains, which suggested the insertion of AP into the supported bilayer through its GPI anchor. Imaging at a higher magnification confirmed the presence of AP at the boundary of gel-fluid domains (Figure 2C), even if a few proteins could also be detected towards the center of the ordered lipid domains (black arrow in Figure 2C). In these experiments, the diameter of bright dots ranged from 15 to 50 nm, and these dots protruded from the SM-enriched phase by 2–5 nm (see the 2.5 µm × 2.5 µm AFM scan in Figure 2C). The maximal area and height of bright dots were obtained mainly by long incubation and could correspond to AP aggregates.

When DOPC–SM–Chl (1:1:0.35) bilayers were incubated with AP, insertion of the GPI-anchored protein was observed in the
Lo phase, confirming the preference of AP for lipid ordered domains. In this case, AP was homogeneously distributed (Figure 2E and F) and not confined to the periphery of the domains as observed with DOPC–SM bilayers. The number of inserted AP molecules in the Lo phase was also markedly increased. In contrast with the experiments in the absence of Chl, the shape of Lo domains was found to vary between successive scans of the same zone. During our experiments, a few bright dots have been visualized occasionally in the liquid phase, but their density was always very low when compared with the ordered lipid phase. In both mixtures, AP was detected only if very low forces (<300 pN) were applied during the scan (data not shown).

Our results suggested strongly that AP distribution in the lipid ordered domains was modified by the presence of Chl in the bilayer. To assess further this Chl effect, AP-incubated DOPC–SM bilayers were treated with 0.4 mM methyl-β-cyclodextrin (MβCD)–Chl complexes (10:1). Such treatment was performed to deliver Chl to the DOPC–SM bilayer (Christian et al., 1997). Bilayers were first incubated in the presence of AP, and insertion similar to that described above was observed (Figure 3A). Treatment with MβCD–Chl induced a time-dependent profound modification of the amount of AP inserted and of the shape of the ordered phase domains. After 20 min incubation, a large number of AP molecules were inserted, essentially at the periphery of the domains (white arrows in Figure 3B). Forty minutes later, the smallest domains were completely covered by AP, whereas bright dots, less patchy than in the gel phase, were observed at the periphery of the largest domains, which had started to fuse (arrow in Figure 3C). After 100 min incubation, Lo domains were still expanding and branching and, as observed previously for the DOPC–SM–Chl bilayer, the apparent number of inserted AP molecules increased further and was homogeneously distributed in the lipid ordered domains (Figure 3D). Taken together, these results suggest strongly that AP insertion is greatly enhanced in an Lo phase when compared with a gel phase.

During all experiments, the specificity of the in situ insertion of AP through its lipid anchor was controlled using the protein digested with phosphatidylinositol-specific phospholipase C (PtdIns-PLC) to remove the GPI part. Both DOPC–SM and DOPC–SM–Chl bilayers were then incubated with 150 µg/ml modified AP and, under these conditions, no AP insertion could be observed after 3 h incubation (data not shown).

**DISCUSSION**

**DOPC–SM supported bilayers as a model of microdomains**

In order to understand better the lipid phase separation into rafts, model supported bilayers, which preserve interactions between the two membrane leaflets, were prepared from a mixture of DOPC, SM and Chl. Phase separation was observed at room temperature for the DOPC–SM–Chl (1:1:0.35) lipid mixture, and...
DOPC fluid domains coexist with SM/Chl L_o phase domains. This assumption is supported mainly by the interconnection of the domains, when Chl was added in the lipid mixture, associated with the decrease in the apparent difference in thickness (δh) between the different domains, as compared with the DOPC–SM mixture. Both the interconnection of lipid ordered domains and the decrease in δh were reported to be associated with the formation of an L_o phase in DOPC–SM–Chl mixtures (Rinia et al., 2001) and in POPC–SM–Chl bilayers (Milhiet et al., 2002). Preferential interactions between SM and Chl are certainly responsible for the L_o phase formation, since Chl interacts poorly with DOPC (Demel et al., 1977). Moreover, control differential scanning calorimetry experiments indicated that the enthalpy of the upper peak in the DOPC–SM thermogram, corresponding to the SM melting, was reduced strongly in the DOPC–SM–Chl (1:1:0.35) mixture (data not shown). The size of L_o domains, from ~200 nm to several micrometers, is in the range of that reported from experiments either on plane bilayers or giant liposomes (Dietrich et al., 2001a; Milhiet et al., 2001a).

Partitioning of AP into raft-like microdomains

First described as a protein present in glycolipid-enriched membrane microdomains (Brown and Rose, 1992), AP was chosen as a model of GPI-anchored proteins to study the partition of such molecules. Our data indicate clearly that exogenously added AP could spontaneously insert into lipid ordered domains (L_o and gel phases) and support the idea of a preferential insertion of AP and, more generally, GPI-anchored proteins for lipid bilayers in which lipid packing is tight (Schroeder et al., 1994). Our results paralleled the spontaneous insertion of three different GPI-anchored proteins into Triton-X-100-resistant domains of HeLa and CHO plasma membranes (Premkumar et al., 2001). The composition of the AP anchor in saturated fatty acids (palmitic and stearic acids) also supports this hypothesis (Takami et al., 1988).

Due to the lateral resolution obtained with AFM, a major advantage when compared with other single molecule microscopy techniques, we have observed the differential insertion of AP in the two model supported bilayers. The presence of Chl, inducing the formation of L_o phase, clearly favored the insertion of AP in the lipid bilayers. This was observed with DOPC–SM–Chl bilayers when compared with DOPC–SM supported membranes and by treatment of the DOPC–SM bilayer with MβCD–Chl allowing an increase in the Chl content of the membrane (Christian et al., 1997). With the DOPC–SM–Chl bilayer, AP was inserted uniformly in the L_o domains, whereas its insertion occurred predominantly at the boundary of the gel phase domain of the DOPC–SM bilayer. The homogeneous distribution of AP in the L_o domains can be explained by the lateral diffusion properties of this phase. In the gel phase, the existence of a local heterogeneity in the composition of individual domains and/or a decrease in the interfacial tension at the boundary of the lipid phases could explain this preferential insertion of both hydrophobic chains of the GPI anchor at the periphery of the domains. This second hypothesis is in good agreement with previous theoretical studies from Mouritsen’s group (Mouritsen and Jorgensen, 1997), and a similar patchy localization has also been observed for Gα_M in DOPC–DPPC (1,2-dipalmitoylphosphatidylcholine) monolayers (Vié et al., 1998). It should also be
pointed out that, whatever the time of incubation, no significant modifications of the surface and shape of gel phase domains was observed during AP insertion. On the other hand, when Chl was present, the shape of domains was found to vary between successive scans of the same area, both in the absence and presence of inserted AP molecules. This provided additional support to the L<sub>o</sub> nature of the lipid ordered phase observed under these conditions.

In summary, using AFM, we have visualized the spontaneous insertion of AP in lipid ordered domains, which supports the preference of GPI-anchored proteins for the lipid bilayer area in which lipid packing is tight (L<sub>o</sub> and L<sub>o</sub>). However, a clear preference of AP for the L<sub>o</sub> phase was observed.

**METHODS**

**Preparation of AP and MβCD–Chl.** The GPI-linked AP was purified using an immuno-affinity chromatography from bovine intestine as described previously (Nosjean and Roux, 1999). When necessary, purified AP was treated with PtdIns-PLC to hydrolyze the diacylglycerol of the glycosyl-PLdIns moieties and purified using anion-exchange chromatography. MβCD–Chl was prepared according to the previously described protocol using an MβCD–Chl ratio of 10:1 (Christian et al., 1997).

**Preparation of small unilamellar vesicles.** Multilamellar vesicles (MLV) were prepared at 60°C in phosphate-buffered saline (PBS) under argon from 10 mM stock solutions of DOPC, SM and Chl (Sigma-Aldrich, Saint Quentin, France) in chloroform/methanol 2:1 (v/v). Purity of the phospholipids was checked by thin-layer chromatography, and the phospholipid concentration was determined according to Mrsny et al. (1986). Small unilamellar vesicles (SUV) were prepared at 60°C under argon by sonication of MLV (Giocconi et al., 2001). SUV were deposited on a freshly cleaved mica disk (0.5 inch diameter), inserted in a 13 mm holder for a swiney syringe (Millipore, Bedford, MA) and incubated at 60°C for 2 h in a water bath. The bilayers, always maintained in an aqueous environment, were carefully rinsed with PBS (pH 7.4) to remove excess SUV and observed under a microscope in the same buffer.

**Atomic force microscopy (AFM).** AFM imaging was performed in contact mode using a NanoScope IIIa microscope equipped with a fluid cell (Digital Instruments, Santa Barbara, CA) under ambient conditions and using a J scanner. Height images were acquired in constant-force mode using standard or sharpened silicon nitride tips on integral cantilevers with a nominal spring constant of 0.01 N/m. Scan rates varied from 1 to 2.5 Hz. Images were obtained from at least three different samples prepared on different days, with at least five macroscopically separated areas on each sample.

**AP insertion and Chl-loaded MβCD treatment.** When necessary, AP was injected directly into the fluid cell, and this step was taken as time zero for the kinetics of protein insertion. In order to visualize the transition from the L<sub>o</sub> to the L<sub>o</sub> phase, DOPC-SM bilayers were first incubated with AP until the protein was detected; 0.4 mM MβCD–Chl was then added.

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