Placental Alkaline Phosphatase Is Efficiently Targeted to Rafts in Supported Lipid Bilayers

Evidence is growing that biological membranes contain lipid microdomains or “rafts” that may be involved in processes such as cellular signaling and protein trafficking. In this study, we have used atomic force microscopy to examine the behavior of rafts in supported lipid bilayers. We show that bilayers composed of equimolar dioleoylphosphatidylcholine and sphingomyelin spontaneously form rafts, which are detectable as raised features. A comparison of the extents of protrusion of the rafts in monolayers and bilayers indicates that the rafts in the two leaflets of the bilayer coincide. The rafts were observed both in the absence and presence of cholesterol (35 mol%). Cholesterol reduced raft protrusion presumably by increasing the thickness of the non-raft bilayer. PLAP (glycosylphosphatidylinositol-anchored protein placental alkaline phosphatase) was purified and shown to exist as a dimer. Following its incorporation into supported lipid bilayers, PLAP was found to be targeted efficiently to rafts, both in the absence and presence of cholesterol. We suggest that atomic force microscopy provides a powerful tool for the study of raft structure and properties.

It is well known that different lipids tend to coalesce in model membranes to form microdomains or “rafts” (1–3). The possibility that these rafts are present in biological membranes remains controversial, although evidence for their existence is growing (4, 5). For example, the treatment of membranes with non-ionic detergents leads to the production of detergent-resistant membrane fragments that are enriched in cholesterol and sphingolipids and that are believed to represent the biochemical equivalents of rafts (6, 7). The observation that the detergent-resistant membranes also contain particular proteins has led to the proposal that rafts are involved in processes such as cellular signaling and protein trafficking (4, 5).

In mixed lipid bilayers, sphingomyelin/cholesterol-enriched rafts are thought to exist in a liquid-ordered state in which the lipid molecules are tightly packed but still able to diffuse laterally (1). These liquid-ordered phase domains are surrounded by a liquid-disordered phase (composed principally of phospholipids with unsaturated and, therefore, kinked fatty acyl chains), which allows for a greater degree of lateral diffusion. The kinked structure of the fatty acyl chains of phospholipids also results in a shorter molecular length relative to the straight sphingomyelin molecules. For instance, the thickness of a bilayer composed of C18:1 phosphatidylcholine (PC) is 35 Å, whereas a bilayer composed of C18:0 sphingomyelin is 46 Å thick (5). Additionally, cholesterol is known to increase the length of the PC molecule so that the thickness of a C18:1 PC bilayer increases to 40 Å (5). In contrast, cholesterol does not affect the molecular length of sphingomyelin.

In this study, we have examined the sorting of the protein placental alkaline phosphatase (PLAP) between the raft and non-raft phases of supported lipid bilayers using atomic force microscopy (AFM), a technique that provides high resolution imaging under physiological conditions (8, 9). PLAP is a glycosylphosphatidylinositol (GPI)-anchored protein that is known to be enriched in detergent-resistant membrane fragments of biological membranes (6, 10). We show that PLAP is efficiently targeted to rafts in vitro.

EXPERIMENTAL PROCEDURES

Formation of Supported Lipid Monolayers and Bilayers—Dioleoylphosphatidylcholine (DOPC), brain sphingomyelin (almost exclusively C18:0), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. For monolayer formation, chloroform solutions of DOPC and sphingomyelin were combined at an equimolar ratio and floated on the surface of a Langmuir-Blodgett trough whereupon the chloroform evaporated. A lipid monolayer was produced at the air-water interface at a surface pressure of 40 mN/m and transferred to freshly cleaved mica (Goodfellow, Cambridge, United Kingdom). For bilayer formation, chloroform solutions of DOPC and sphingomyelin with or without cholesterol were dried down under a stream of nitrogen. The lipids were rehydrated overnight in distilled water to a final concentration of 2 mg/ml. The lipid mixture was vortexed to produce large multi-lamellar vesicles, and small unilamellar vesicles were then produced by sonication in a bath sonicator (Decon, Hove, United Kingdom) for 30 min. Supported bilayers were formed by deposition of 10 µl of vesicle suspension onto mica immediately followed by 50 µl of Hepes-buffered saline, pH 7.6 (HBS). After a 3-min incubation at room temperature, the sample was gently rinsed with the same buffer and transferred to the atomic force microscope.

Atomic Force Microscopy—Samples were imaged either in air (monolayers) or in HBS containing 2 mM CaCl2 (bilayers) using a Multimode atomic force microscope with a Nanoscope IIIa controller (Digital Instruments, Santa Barbara, CA) equipped with a J-scanner. Oxide-sharpened silicon nitride tips mounted on cantilevers with spring constants of 0.32 newton/m were used in intermittent tapping mode. The

* This work was supported by Grant B12816 from the Biotechnology and Biological Sciences Research Council (to R. M. H. and J. M. E.) and Grant GM47987 from the National Institutes of Health (to D. A. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

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The abbreviations used are: PC, phosphatidylcholine; PLAP, placental alkaline phosphatase; AFM, atomic force microscopy; GPI, glycosylphosphatidylinositol; DOPC, dioleoylphosphatidylcholine; HBS, Hepes-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
Preparation, Analysis, and AFM Imaging of PLAP—Partially pure PLAP was obtained commercially (Sigma) and further purified as described previously (11). Protein quantitation was carried out using a detergent-compatible protein assay kit (Bio-Rad). Protein purity was assessed by SDS-PAGE with silver staining. PLAP was further analyzed by continuous sucrose density gradient centrifugation. Purified PLAP was layered onto a linear 5–15% sucrose gradient containing 0.5% octylglucoside (total volume 5 ml), and the gradient was centrifuged at 140,000 × g for 18 h at 4 °C. Fractions (500 µl) were collected from the top of the gradient and subjected to SDS-PAGE. Separated proteins were electrophoretically transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) by semi-dry blotting. The blots were probed with a mouse monoclonal anti-PLAP antibody (Sigma) at a dilution of 1:1500. Immunoreactive bands were visualized using a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:10,000, Bio-Rad) and enhanced chemiluminescence (Pierce & Warriner, Chester, United Kingdom). Molecular mass markers for cytochrome c (12.4 kDa) and alcohol dehydrogenase (150 kDa) were run in an identical gradient, and their positions were determined by Coomassie Blue staining of an SDS gel.

For AFM imaging of PLAP on mica, 30 µl of protein at a concentration of 0.2 µg/ml in HBs containing 2% CHAPS was deposited onto mica. The sample was washed three times with 1 ml of HBs-CHAPS and imaged under fluid. The incorporation of PLAP into lipid vesicles was achieved by adding protein (60 µl of a 90 µg/ml solution in phosphate-buffered saline, pH 7.4, containing 2% CHAPS) to a lipid solution (140 µl of a 2 mg/ml solution in 2% CHAPS) and subsequently dialyzing against HBs, pH 7.6, for 4 days at room temperature with two changes of buffer solution each day. Protein-free liposomes were produced in the same way with the exception of the addition of PLAP. The supported bilayers were produced as described above.

Association of PLAP with Liposomes—PLAP association with liposomes was determined by flotation of the liposomes on a discontinuous sucrose density gradient. Samples (500 µl) of liposomes prepared in the presence of PLAP were added to 1.5 ml of 80% sucrose in HBs, pH 7.6. On this suspension (now 60% sucrose) was layered 1.5 ml of 50% sucrose in buffer and 1.5 ml of buffer alone. The gradient was centrifuged at 140,000 × g for 2 h at 4 °C. Fractions (500 µl) were taken from the top of the gradient and analyzed by SDS-PAGE and immunoblotting as described above.

Assay of PLAP Activity in Supported Lipid Bilayers—PLAP activity in supported lipid bilayers was measured by depositing PLAP-containing liposomes onto freshly cleaved mica as described for AFM imaging. The substrate (p-nitrophenyl phosphate) buffer (100 µl, Bio-Rad) was added to the mica and allowed to incubate for 5 min. The reaction was stopped by the addition of 100 µl of 0.4 M NaOH, and the absorbance of the resulting solution at 405 nm (A405) was determined. The control samples lacked PLAP and contained either buffer or lipids only.

Molecular Volume Calculation—The molecular volume of the protein particles was determined from particle dimensions derived from AFM images. The height and half-height diameters were measured from multiple cross-sections of the same particle, and the molecular volume was calculated using Equation 1, which treats the particle as a spherical cap,

\[ V_n = \frac{\pi h^2}{6} (3r^2 + h) \]  

(Eq. 1)

where \( h \) is the particle height and \( r \) is the radius at half-height (12). Molecular volume based on molecular weight was calculated using Equation 2

\[ V = \frac{2M_pN_A}{M_r} (V_1 + dV_2) \]  

(Eq. 2)

where \( M_p \) is the molecular mass of the protein, \( N_A \) is Avogadro’s number, \( V_1 \) and \( V_2 \) are the partial specific volumes of protein and water (0.74 and 1 cm³/g, respectively), and \( d \) is the extent of protein hydration (0.4 mol water/mol protein) (13).

Statistical Analysis—The statistical significance of differences between values was assessed using Student’s t test for unpaired data.

RESULTS

We began our experiments by imaging lipid monolayers composed of an equimolar mixture of DOPC (C18:1) and sphingomyelin (C18:0). The monolayers were formed on the surface of a Langmuir-Blodgett trough and then transferred to mica. This procedure results in supported monolayers with the polar headgroups of the lipids attached to the mica. A typical image of a supported monolayer obtained using tapping-mode AFM in air is shown in Fig. 1a. Rafts of approximate diameter of 200 nm can be seen protruding from the background monolayer. Sections through the monolayers reveal a mean step in height between the two phases of 5.5 ± 0.1 Å (mean ± S.E., \( n = 25 \)), which is the expected difference in length between DOPC and sphingomyelin molecules. We next used tapping-mode AFM

![Fig. 1. Visualization of rafts in lipid monolayers and bilayers.](image-url)
under fluid to image a bilayer composed of the same lipid mixture prepared by allowing liposomes to attach and then collapse onto the mica support. Protruding rafts were again seen (Fig. 1b), and the height of the steps was significantly greater than that in the monolayers (mean value $8.9 \pm 0.1 \text{ Å}$, $n = 25$, $p < 0.0001$). This result indicates that transbilayer coupling between the sphingomyelin rafts is occurring as has been reported previously (14). The observation that the height of the rafts in bilayers is somewhat less than double their height in monolayers probably reflects the fact that monolayers were imaged in air, whereas the bilayers were imaged under fluid. In other words, we are comparing the dimensions of dehydrated and hydrated lipids. In addition, there is probably an interdigitation among the tails of the sphingomyelin molecules in the bilayers that will tend to reduce the observed raft height. When cholesterol was added to the lipid mixture (equimolar DOPC/cholesterol/sphingomyelin), the supported bilayers again contained rafts (Fig. 1c), but the step in height between the two domains was now smaller ($6.6 \pm 0.1 \text{ Å}$, $n = 25$, $p < 0.0001$) as predicted from the ability of cholesterol to cause an extension of the DOPC molecule. Occasional imperfections in the bilayer were seen, and sections through these features (for example, see Fig. 1d) gave values for the bilayer thickness of $57 \pm 1 \text{ Å}$ ($n = 25$) in the absence of cholesterol and $58 \pm 2 \text{ Å}$ ($n = 25$) in the presence of cholesterol. These values are larger than the expected bilayer thickness of around 46 Å (as described above). The presence of a 10-Å hydration layer between a bilayer and its support has been reported previously (15, 16). Such a hydration layer would account for the discrepancy between expected and observed bilayer thickness in our experiments and might also contribute to the relatively large errors in the values of these parameters (compared with the errors on the values for raft heights), which mask the change in thickness that would be expected to occur upon the addition of cholesterol.

The principal aim of this study was to test whether PLAP, a GPI-anchored protein that is enriched in detergent-resistant fragments of biological membranes (6, 10), is targeted to rafts in supported lipid bilayers. PLAP was obtained commercially and purified until only a single band was seen on a silver-stained gel (Fig. 2a). When PLAP was run on a linear (5–15%) sucrose gradient, it migrated at a slightly lower size than the 150-kDa protein alcohol dehydrogenase (Fig. 2b). This result is consistent with previous reports that PLAP in solution exists as a dimer of 70-kDa subunits (17). When PLAP in solution was allowed to attach to a mica support and then imaged by AFM under fluid, a reasonably homogeneous spread of particles was observed (Fig. 2c). Sections through PLAP particles were used to calculate a molecular volume of $405 \pm 36 \text{ nm}^3$ ($n = 25$). This is somewhat larger than the volume of $260 \text{ nm}^3$ that is expected of a protein with a molecular mass of 140 kDa and may indicate a degree of aggregation of the protein.

Detergent-solubilized mixtures of PLAP and lipids were dialyzed to produce proteoliposomes. To confirm that the liposomes contained PLAP, they were floated on a discontinuous sucrose density gradient (buffer, 50% sucrose, 60% sucrose). It was found that 10% of the total PLAP floated with the DOPC/sphingomyelin liposomes above the 50% sucrose layer. This percentage was slightly greater (14%) for the DOPC/cholesterol/sphingomyelin liposomes (Fig. 3a). Hence, the efficiency of association of PLAP with liposomes was not significantly affected by the presence of cholesterol. When the proteoliposomes were allowed to collapse onto mica supports, PLAP activity could be recovered from the mica, demonstrating that the protein had attached to the support along with the lipids and also that its activity persists after extensive dialysis (Fig. 3b).

**DISCUSSION**

Rafts have been observed previously in supported lipid monolayers (18) and also in both supported (2, 19) and unsupported (3) bilayers. They have usually been visualized using...
fluorescence microscopy of the distribution of a labeled lipid (2, 3, 18), although some AFM studies of rafts have recently been reported (see Ref. 19). The fluorescence studies have typically reported raft sizes on the micrometer scale, i.e. considerably larger than the raft size reported here (~200 nm), which is similar to the size believed to occur in cell membranes (4). Contrasting effects of manipulation of cholesterol content have also been seen. For instance, rafts were found to disappear from monolayers in response to cholesterol depletion (18), although both phase separation and detergent insolubility of PLAP persisted in cholesterol-free sphingomyelin/PC liposomes (20, 21). Rafts in bilayers were also reported to disappear after the addition of cholesterol at 33 mol % (19), although others have shown that phase separation persists under these conditions (20). In this study, we found that rafts in bilayers remained clearly visible either in the absence or presence of cholesterol (also at 33 mol %). One potential problem with fluorescence-based assays in contrast to detection by AFM is that the resolution achieved might not be sufficient to discriminate between raft disappearance and reduction in size; however, it should be noted that in our study, we found no significant change in raft size with changing cholesterol content.

Several GPI-anchored proteins including PLAP (6, 7, 10), Thy-1 (4), and GP-2 (22), are recovered from detergent-resistant membrane fractions. Here, we found that PLAP was almost exclusively localized in rafts both in the presence and absence of cholesterol, thus ruling out the requirement not only for interactions with other proteins but also for cholesterol. Like PLAP, Thy-1 has recently been found to be enriched in rafts in supported lipid monolayers, although the targeting efficiency was lower than that for PLAP (18). The difference in efficiency of targeting of PLAP and Thy-1 to rafts might relate to the use of lipid bilayers and monolayers in the respective assays. Furthermore, the oligomerization state of the two proteins, known to be an important determinant of raft association (23, 24), might be different. In our assay, PLAP was shown to dimeric, but the oligomerization state of Thy-1 in the previous study (18) was not determined. Finally, there might be genuine differences in the efficiency of targeting of the various GPI-linked proteins to rafts. In support of this suggestion, it has been shown previously that a number of GPI-linked proteins present in the kidney microvillar membrane showed significant differences in the extent of their detergent insolubility (25). In addition, there is evidence that PLAP might be especially well targeted to rafts. For instance, PLAP at the plasma membrane was found to be 95% detergent-insoluble (6), and its insolubility after reconstitution into liposomes was also very high (21).

We suggest that the use of AFM imaging of protein-containing supported lipid bilayers could shed considerable light on the processes underlying the control of the distribution of proteins within the plane of a biological membrane.

**Fig. 3.** PLAP is efficiently targeted to rafts in lipid bilayers. **a**, liposomes composed of either DOPC/sphingomyelin (D/S) or DOPC/cholesterol/sphingomyelin (D/C/S) and containing PLAP were floated on a discontinuous sucrose density gradient. Fractions (1–10) were taken from the top of the gradient, and PLAP was detected by SDS-PAGE and immunoblotting using a mouse monoclonal anti-PLAP antibody. The majority of the lipids was recovered in fraction 3 (data not shown). **b**, liposomes composed of either D/S or D/C/S with or without PLAP were allowed to collapse onto mica. The mica was then washed, and the PLAP activity associated with the support was determined enzymatically. \( A_{405} \) is expressed in arbitrary units. **c**, PLAP-free DOPC/sphingomyelin. **d**, DOPC/sphingomyelin containing PLAP. **e**, PLAP-free DOPC/cholesterol/sphingomyelin. **f**, DOPC/cholesterol/sphingomyelin containing PLAP. A shade-height scale is shown at the bottom right. Scale bar = 200 nm.
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J. Biol. Chem. 2002, 277:26966-26970.
doi: 10.1074/jbc.M204669200 originally published online May 14, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M204669200

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