Bilayer Asymmetry Influences Integrin Sequestering in Raft-Mimicking Lipid Mixtures

Noor F. Hussain, † Amanda P. Siegel, † Yifan Ge, † Rainer Jordan, ‡ and Christoph A. Naumann†*

†Department of Chemistry and Chemical Biology, Indiana University-Purdue University Indianapolis, Indiana; and ‡Department of Chemistry and Chemical Biology, Indiana University-Purdue University Indianapolis, Indiana; and  § Makromolekulare Chemie, Dresden University of Technology, Dresden, Germany

ABSTRACT There is growing recognition that lipid heterogeneities in cellular membranes play an important role in the distribution and functionality of membrane proteins. However, the detection and characterization of such heterogeneities at the cellular level remains challenging. Here we report on the poorly understood relationship between lipid bilayer asymmetry and membrane protein sequestering in raft-mimicking model membrane mixtures using a powerful experimental platform comprised of confocal spectroscopy XY-scan and photon-counting histogram analyses. This experimental approach is utilized to probe the domain-specific sequestering and oligomerization state of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins in bilayers, which contain coexisting liquid-disordered/liquid-ordered ($l_d/l_o$) phase regions exclusively in the top leaflet of the bilayer (bottom leaflet contains $l_d$ phase). Comparison with previously reported integrin sequestering data in bilayer-spanning $l_d/l_o$ phase separations demonstrates that bilayer asymmetry has a profound influence on $\alpha_v\beta_3$ and $\alpha_5\beta_1$ sequestering behavior. For example, both integrins sequester preferentially to the $l_o$ phase in asymmetric bilayers, but to the $l_d$ phase in their symmetric counterparts. Furthermore, our data show that bilayer asymmetry significantly influences the role of native ligands in integrin sequestering.

INTRODUCTION

The plasma membrane is a complex supramolecular system in which the composition and heterogeneous distribution of lipids in the lipid bilayer matrix may have a profound influence on membrane protein distribution and functionality (1). Lipid rafts prominently reflect the functional importance of lipid heterogeneities, as they have been linked with several important membrane-associated biological processes, including transmembrane (TM) signaling (2), pathogenesis (3), cell adhesion, cell morphology, and angiogenesis (4). These processes are largely associated with the ability of lipid rafts to regulate the sequestration of membrane proteins in the plasma membrane (5). Several molecular processes have been identified as factors determining protein sequestration, including protein acylation, receptor clustering, ligand addition, and other specific protein-protein interactions (6–9). Yet, the underlying factors of protein sequestration and their interaction remain somewhat elusive, largely due to the small size and transient character of raft domains in the plasma membrane (10). Furthermore, common procedures of lipid raft analysis in plasma membranes, such as detergent extraction, cholesterol (CHOL) depletion, and utilization of crosslinking agents, have been shown to be prone to artifacts (11,12).

Therefore, model studies on membrane proteins in well-defined raft-mimicking lipid mixtures with larger size liquid-ordered ($l_o$)-liquid-disordered ($l_d$) phase separations have emerged as an attractive complementary tool for the characterization of lipid-raft-associated membrane processes. For example, model membrane experiments confirmed the phase separation of lipids into stable CHOL-rich $l_o$ and CHOL-deficient $l_d$ domains (13,14) and provided insight into the sequestration of membrane proteins in the absence and presence of cross-linking agents in coexisting $l_o$ and $l_d$ domains (15–17). A particularly attractive feature of model membrane experiments is that raft-associated membrane protein sequestering processes can be studied in the absence of artificial cross-linking agents. Recently, our group applied this concept and explored the role of native ligands in integrin sequestering in the presence of raft-mimicking lipid mixtures without artificial cross-linkers (18). To conduct these experiments, we developed a powerful experimental platform comprised of confocal spectroscopy XY-scan, epifluorescence microscopy, and photon-counting histogram (PCH) analyses, in which the sequestration and oligomerization state of TM proteins can be investigated in a planar model membrane system with single-molecule sensitivity. By using this experimental approach, we showed that native ligands alter $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin sequestering but not oligomerization in a polymer-tethered lipid bilayer in the presence of bilayer-spanning, coexisting $l_o$ ($Bl_o$) and $l_d$ ($Bl_d$) domains. Polymer-tethered membranes were employed because they enable the functional reconstitution of TM proteins (19–21).

In this article, we expand our model membrane experiments on integrins and report on the fascinating but poorly understood relationship between bilayer asymmetry and membrane protein sequestering in raft-mimicking lipid mixtures. It is well established that the plasma membrane of eukaryotic cells is characterized by an asymmetric
distribution of lipids and membrane proteins. One important consequence of the bilayer asymmetry is that sphingolipidand CHOL-enriched lipid rafts are typically limited to theexoplasmic leaflet of the membrane. At the same time, however,we observed co-clustering of raft-associated GPI-anchored proteins in the exoplasmic leaflet and Src-kinase in thecytoplasmic leaflet also suggest a potentially importantrole of raft domains in transbilayer signaling (22), presumably due to either mediation by TM proteins or a lipid-based transbilayer coupling mechanism (23). To explore therelationship between bilayer asymmetry and integrinsequestering, here we determine the distribution of $\alpha_\beta_3$and $\alpha_\beta_1$ integrins in a polymer-tethered lipid bilayer ofasymmetric lipid composition with coexisting $l_o$-$l_d$ lipidregions and compare these experiments with our previouswork on symmetric bilayers (18).

As illustrated in Fig. 1, $l_o$-$l_d$ phase separations in the asymmetric bilayer system are exclusively located in the top leaflet of the bilayer (bottom leaflet contains a homo-geneous $l_d$ phase), whereas those in the symmetric bilayer system exist in both leaflets and are bilayer-spanning. To distinguish monolayer-associated and bilayer-spanning $l_o$ and $l_d$ lipid regions, the following abbreviations are introduced: $Ml_o$, monolayer-associated $l_o$ region; $Ml_d$, mono-layer-associated $l_d$ region; $Bl_o$, bilayer-spanning $l_o$region; and $Bl_d$, bilayer-spanning $l_d$ region. According tothis terminology, the symmetric bilayer exhibits coexisting$Bl_o$ and $Bl_d$ regions, whereas the asymmetric bilayer system shows $Ml_o/Ml_d$ and $Bl_d$ regions. Because the bottomleaflet of the asymmetric bilayer system contains a homo-geneous $l_d$ phase, in the following we refer to the lipid mixing behavior in this bilayer system as the coexistence of $Ml_o$ and $Bl_d$ domains. Integrins were chosen because they not only represent important signaling molecules, but are also linked to several raft-associated processes, including cell adhesion, motility, and angiogenesis (24). Polymer-tethered lipid bilayers of asymmetric lipid composition are built layer-by-layer using subsequent Langmuir-Blodgett (LB) and Langmuir-Schaefer (LS) transfers with $l_d$-$l_d$ phase separating and homogeneous $l_d$ forming lipid mixtures being in the LS and LB layers, respectively.

A fluorescence-based assay enables the distinction betweensubstrate-spanning ($Bl_o$) and monolayer $l_o$ ($Ml_o$) do-mains in the planar membrane system. Importantly, we experimentally showed that reconstituted $\alpha_\beta_3$ and $\alpha_\beta_1$ integrins show a preference for $Ml_o$ domains, which is in marked contrast to the previously observed $l_d$ phase preference in corresponding bilayer systems with coexisting bilayer-spanning $l_o$ ($Bl_o$) and $l_d$ ($Bl_d$) regions (18). Furthermore, introduction of reconstituted native ligands ($\alpha_\beta_3$: vitronectin (VN) and $\alpha_\beta_1$: fibronectin (FN)) has a different impact on integrin sequestering in lipid mixtures with $Ml_o$ versus $Bl_d$ domains. Corresponding PCH analysis confirms that ligand binding does not alter the integrin oligomerization state. Our findings are significant because they highlight the potentially important role of membrane asymmetry in membrane protein sequestering and function. We hypothe-size that bilayer asymmetry in cellular membranes may influence TM protein distribution and functionality in a similar way.

**MATERIALS AND METHODS**

**Materials**

The phospholipids DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), and CHOL were purchased from Avanti Polar Lipids (Alabaster, AL). The procedure for synthesizing the lipopolymer $diC_{18M50}$ (1,2-dioctadecyl-sn-glycero-3-n-poly(2-methyl-2-oxazoline)50) has been described previously in Lüdtke et al. (25). The dye-labeled lipids NBD-DHPE (n-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyloxy-sn-glycero-3-phosphoethanolamine), TRITC-DHPE (n-(6-tetramethylrhodamine-thiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt), DID (1,1’-diodocetyl-3,3’,3’-tetramethylindodicarbocyanine; 4-chlorobenzenesulfonate salt), and Dil (1,1’-diodotacadecanoyl-3,3’,3’-tetramethylindocarbocyanine perchlorate), as well as the kits for fluorescently labeling antibodies with Alexa-555 were obtained from Invitrogen (Carlsbad, CA).

Chloroform (HPLC grade; Fisher Scientific, Pittsburgh, PA) was used as the spreading solvent for the lipid monolayer at the air-water interface in the trough. Milli-Q water (pH = 5.5, 18 MQ-cm resistivity; Millipore, Billerica, MA) was employed as the subphase material in the trough. Glass cover slips were prepared by first baking them for 3 h at 515°C in a kiln followed by subsequent sonication steps in a bath sonicator using solutions of 1% sodium dodecyl sulfate for 45 min, MeOH saturated with NaOH, and 0.1% HCl (Fisher Scientific). The slides were rinsed with Milli-Q water in between sonication steps for 10 min. Human integrin $\alpha_\beta_3$ and $\alpha_\beta_1$, octyl-$\beta$-D-glucopyranoside formulation, the monoclonal antibodies (MAbs) anti-integrin $\alpha_\beta_3$ and anti-integrin $\alpha_\beta_1$, human purified VN, and human purified FN were purchased from Millipore. Rhodamin6G was obtained from Sigma-Aldrich (St. Louis, MO). The surfactant OX (n-octyl-$\beta$-D-glucopyranoside) was obtained from Fisher BioReagents (Fairlawn, NJ).
Construction of polymer-tethered phospholipid bilayers containing $M_{lo}$ domains

Polymer-tethered phospholipid bilayers of asymmetric lipid composition were prepared layer-by-layer using the LB/LS technique, thereby adapting procedures reported recently for those of symmetric lipid compositions (18,20,26,27). To build the LB and LS monolayers, chloroform solutions of the corresponding lipid/lipopolymer and lipid mixtures were spread at the air-water interface of a film balance with a dipper (Labcon, Darlington, UK). Each monolayer was compressed to 30 mN/m and kept at this pressure for 40 min before monolayer transfer to a glass cover slide. LB monolayer transfers were conducted using the dipper of the film balance. LS transfers were accomplished by positioning a depression slide underneath the air-water interface and gently pushing the cover glass with the LB layer through the LS monolayer onto the depression slide. The depression slide was removed using a transfer dish and the glass substrate with the LB/LS bilayer was transferred to a petri dish, where Milli-Q water was replaced by phosphate-buffered saline (PBS) (Fisher Scientific) 10× concentrations, diluted in Milli-Q water. To obtain a polymer-tethered lipid bilayer without $B_{lo}$ domains, but with $M_{lo}$ domains in its top leaflet (LS layer), the following LB and LS compositions were employed:

- LB layer: (2:1) (DOPC/CHOL) with 5 mol % dIC18M50; and
- LS layer: (1:1:1) (DOPC/DPPC/CHOL).

Alternatively, experiments were conducted with an LS composition of (2:1:1:2:1) (DOPC/DPPC/CHOL). The LS compositions contain 0.4 mol % NBD-DHPE to confirm $l_a$, $l_d$, and $l_{pd}$ phase separations in the asymmetric bilayer system. Furthermore, 0.1 mol % DID, which is less prone to flip-flop, was included in the LB composition to assure that coexisting $l_a$ and $l_d$ phases only form in the top leaflet (LS layer) of the bilayer. To determine the influence of bilayer asymmetry on protein sequestration, experimental findings obtained on the asymmetric bilayer system were compared with those reported on a corresponding symmetric bilayer system (18). In the latter case, the LB and LS compositions were as follows (both monolayers typically contain 0.2 mol % NBD-DHPE):

- LB layer: (1:1:1) (DOPC/DPPC/CHOL) with 5 mol % dIC18M50; and
- LS layer: (1:1:1) (DOPC/DPPC/CHOL).

Protein incorporation into bilayers

The incorporation of integrins in polymer-tethered lipid bilayers was accomplished using a modified Rigaud technique (direct protein incorporation method) as described previously in Siegel et al. (18). Briefly, micelle-stabilized membrane proteins ($1.3 \times 10^{11}$ mol leading to a bilayer concentration of $10^{-3}$ mol %) were added to the bilayer sample together with 2 mL of 0.08 mg/mL of OG and incubated for 1.5–2 h. This corresponds to a surfactant concentration in the bilayer sample of ~0.002 critical micelle concentration. To remove the surfactants from the bilayer, a single layer of SM-2 Bio-Beads (Bio-Rad, Hercules, CA) was put on the bilayer sample with the reconstituted integrins for 15 min followed by their removal through extensive rinsing with PBS. Next, Alexa-555 labeled anti-integrin MAbs were added and incubated for 3–4 h at room temperature followed by a washing step with PBS to remove excess antibodies. The MAAb labeling strategy is well suited for background in CS-XY scans. DHPE but without Alexa-555-labeled anti-integrin MAbs were done to correct for background in CS-XY scans.

FCS data were collected in 50-s runs on a bilayer or in a solution to enable fluorescence correlation spectroscopy (FCS) and PCH analysis with the same pinhole size. FCS autocorrelation analysis was used to identify the average brightness of dye-labeled MAbs in solution and to determine the domain-specific lateral mobility of dye-labeled lipids (TRITC-DHPE) in asymmetric and symmetric bilayers with coexisting $l_a$, $l_d$, and $l_{pd}$ phases. Regions. Due to uncertainties about the exact shape of the Gaussian intensity profile in the confocal laser spot at the glass-water interface in standard FCS studies, the laser focus dimension, knowledge of the shape of which is required for the determination of diffusion coefficients, was derived by reference to the diffusion coefficient of TRITC-DHPE in a one-component, solid-supported fluid lipid bilayer obtained using the more accurate widefield single-molecule fluorescence microscopy method described previously (20). The FFS data acquisition for PCH analysis on the bilayer sample was accomplished by maximizing the photon-count rate of laterally mobile, bilayer-based single molecules in the confocal volume.

Microscopy techniques

A confocal ConfoCor 2 system (Carl Zeiss, Jena, Germany) equipped with an Axiovert 200M inverted optical microscope (Carl Zeiss) and C-Apochromat objective (water immersion, $40\times$, NA = 1.2) was employed to explore the lipid domain-specific distribution and oligomerization state of integrins in the polymer-tethered lipid bilayer using fluorescence fluctuation spectroscopy (FFS) and epifluorescence microscopy (EPI). EPI image acquisition and analysis was done using an Axiocam MRm monochrome digital camera (Carl Zeiss) and Axiowision 4.8 software (Carl Zeiss). EPI was employed to confirm the presence of coexisting $l_m$ and $l_d$ domains in the lipid bilayer. FFS experiments were conducted by confocal spectroscopy XY (CS-XY) scans (maximum scan size: $10 \times 10$ μm; step size: 0.5 μm) using a 1.0-mW HeNe laser (543 nm) with a 560–615-nm emission filter (the Alexa-555 channel), a 5.0-mW HeNe laser (633 nm) with a 650–710-nm long-pass filter (the DID channel), and a 30-mW Argon laser (488 nm) with 505–530-nm emission filter (the NBD channel). The 488 and 633 HeNe lasers were utilized to investigate the distribution of the dye lipids NBD-DHPE and DID, respectively. The 543-nm laser was employed to probe the corresponding distribution of Alexa-555-labeled anti-integrin MAbs bound to integrins. Control experiments with DID and NBD-DHPE but without Alexa-555-labeled anti-integrin MAbs were done to correct for background in CS-XY scans.

Data analysis

The domain-specific distribution and oligomerization state of integrins in the presence of $M_{lo}$ domains was determined by adapting procedures described by Siegel et al. (18) for coexisting bilayer-spanning $l_m$ and $l_d$ domains. Raw data of the integrin distribution were obtained from confocal XY scans of the NBD-DHPE, DID, and integrin distributions. Each raw data set was corrected for NBD-DHPE and DID channel bleed-through as well as background. A set of separate experiments showed that the NBD bleedthrough in the Alexa-555 channel represents ~6% of the total background, whereas the DID bleedthrough and background without dye contributions are ~40 and 54%, respectively. To correct for background, control experiments were conducted without integrins and dye-labeled anti-integrin MAbs. Typical signal/background in the presence of integrins with Alexa-555-labeled MAbs was identified to be ~4:1. Bilayers were also constructed with integrins and dye-labeled anti-integrin MAbs with no DID-labeled lipids in the bottom leaflet to determine whether there was an effect on the integrin distribution in the presence of DID lipids. None was evident. The integrin distribution in the bilayer can be quantified in terms of a partition coefficient $K_c(M_{lo}/B_{lo})$, defined as $I_{M_{lo}}/I_{B_{lo}}$ where $I_{M_{lo}}$ and $I_{B_{lo}}$ are the intensities from the coexisting $M_{lo}$ and $B_{lo}$ regions, respectively. The background was subtracted from the intensities obtained from the control experiments without integrins and dye-labeled anti-integrin MAbs. Changes in $K_c(M_{lo}/B_{lo})$ can be expressed by the parameter $E_{ads}$, which is defined as...
A parameter $X_{\text{migrate}}$ can be defined, which provides quantitative information about changes in $E_{\text{raft}}$ due to addition of ligands:

$$X_{\text{migrate}} = \left[ \frac{E_{\text{raft}}(+\text{ligand}) - E_{\text{raft}}(-\text{ligand})}{2} \right].$$

PCHs of the avalanche photodiode photon counts were used to study the behavior of the oligomerization status of integrins in the bilayer. The PCH method represents a powerful tool to determine the average number and brightness ($N_{\text{avg}}, \epsilon$) of monomers and dimers/multimers of dye-labeled molecules from the analysis of confocal photon count rates (28,29). Using this method, we determined the number and brightness of integrin monomers ($N_{\text{avg}}, \epsilon$) and dimers ($N_{\text{avg, dimmer}}$, ($2\epsilon$)) in the $M_l^d$ and $B_l^d$ phases of the bilayer. These values were used to calculate the mole fraction of the dimers ($X_{\text{dimmer}}$) for each species. Corresponding model fits with monomers and tetramers provided comparable results (data not shown). The algorithm that was used to fit the PCH data (the PCH model) was initially tested for its ability to distinguish changes in number and brightness using CdSe/ZnS quantum dots (QDs) in solution and on a bilayer as well as monomeric fluorescent species in solution (Rhodamine 6G and dye-labeled MAbs) and in a planar, glass-supported lipid bilayer (TRITC-DHPE) (data not shown). Pre-incubation of species in solution (Rhodamine 6G and dye-labeled MAbs) and in a planar, glass-supported lipid bilayer (TRITC-DHPE) (data not shown). Pre-incubation of species in solution (Rhodamine 6G and dye-labeled MAbs) and in a planar, glass-supported lipid bilayer (TRITC-DHPE) (data not shown). Pre-incubation of species in solution (Rhodamine 6G and dye-labeled MAbs) and in a planar, glass-supported lipid bilayer (TRITC-DHPE) (data not shown). Pre-incubation of species in solution (Rhodamine 6G and dye-labeled MAbs) and in a planar, glass-supported lipid bilayer (TRITC-DHPE) (data not shown). Pre-incubation of species in solution (Rhodamine 6G and dye-labeled MAbs) and in a planar, glass-supported lipid bilayer (TRITC-DHPE) (data not shown).

### RESULTS AND DISCUSSION

**Design and characterization of polymer-tethered lipid bilayers with $M_l^d$ domains**

Planar polymer-tethered lipid bilayers with $M_l^d$ domains in their top leaflet were constructed as described in the Materials and Methods. Fig. 2 shows representative EPI micrographs of such a model membrane in which the raftophobic membrane marker DID located exclusively in the bottom leaflet shows a homogeneous distribution whereas the raftophilic membrane marker NBD-DHPE spread in the top leaflet shows distinct evidence of $l_{c,l_d}$ phase separations:

- **LS composition**: DOPC/DPPC/CHOL (2.1:1.2:1.0) + 0.5 mol % NBD-DHPE; and
- **LB composition**: DOPC/CHOL 2:1 + 5% mol diC18M50 + 0.1 mol % DID.

Probe molecules of the DiI family, such as DID, are well suited for this fluorescence assay because their rate of flip-flop is very slow (31), thus providing reliable information about the lipid mixing behavior in the bottom (LB) leaflet of the bilayer. Consequently, in the absence of phase separations in the DID channel, those observed through the NBD channel must be attributed to the presence of $M_l^d$ domains in the top (LS) leaflet of the membrane. (Note: Control experiments on symmetric bilayers with raft-mimicking 1:1 DPPC/CHOL/DOPC lipid mixtures confirmed that DID shows preference for the $l_d$ phase in the presence of coexisting $l_c$ and $l_d$ lipid phases; data not shown.) To confirm the stability of $M_l^d$ domains in the polymer-tethered lipid bilayer for membrane protein studies, we conducted EPI microscopy studies on these model membranes over time. Specifically, the stability of the $M_l^d$ domains was confirmed by determining the presence and absence of $l_{c,l_d}$ phase separations in the NBD and DID channels, respectively, both shortly after construction and 12 h later. To mimic integrin reconstitution conditions, the asymmetric bilayer was incubated in the presence of 0.55 µM OG for 2 h, rinsed, and additionally incubated for 10 h.

As the EPI micrographs of such a bilayer sample in Fig. 1 in the Supporting Material illustrate, the presence and absence of $l_{c,l_d}$ phase separations through the NBD and DID channels, respectively, assure the feasibility of the experimental assay. Next, we compared the lipid mixing behavior of the asymmetric lipid composition in Fig. 2 with a symmetric lipid composition of (1.5:0.5:1) (DOPC/DPPC/CHOL) (see Fig. S2). The latter represents the scenario of a hypothetical flip-flop-mediated disappearance of DOPC and DPPC concentration gradients across the bilayer. As Fig. S2, A and B, illustrates, the presence and absence of $l_{c,l_d}$ phase separations in these asymmetric and symmetric lipid compositions demonstrates the stability of $M_l^d$ domains as employed in this study. Our results are in good agreement with previous findings on model membranes of asymmetric composition with raft-mimicking lipid mixtures, which seem to support a relatively slow spontaneous flip-flop process of phospholipids (26,33).

To characterize $l_{c,l_d}$ phase separations in asymmetric and symmetric bilayers, we also determined the domain-specific brightness, concentration, and lateral mobility of 0.002 mol % TRITC-DHPE in both types of bilayers using confocal fluorescence intensity and FCS analyses. The results from these lipid domain characterization experiments

**FIGURE 2** EPI-micrographs showing the lipid mixing behavior in the top (A) and bottom leaflets (B) of the asymmetric lipid bilayer, as observed through the NBD and DiI channels of the microscope. Lipid composition: top (LS) monolayer DOPC/DPPC/CHOL (2.1:1.2:1.0) 0.5 mol % NBD-DHPE, and bottom (LB) monolayer (DOPC/CHOL) 2:1 (5% mol diC18M50 and 0.1 mol % DiI).
are summarized in Table 1 together with corresponding control experiments on DOPC, and the binary mixtures DOPC/CHOL (4:1) and DOPC/CHOL (2:1). As Table 1 illustrates, the control experiments show that substantial increases in cholesterol content (lipid packing density) are associated with reduced TRITC-DHPE lateral mobility and brightness. In contrast, the TRITC-DHPE brightness and lateral mobility data determined in asymmetric and symmetric bilayers with monolayer-associated and bilayer-spanning \( l_o \) and \( l_d \) regions do not show comparable changes, thus excluding substantial differences in lipid packing between both membrane systems. Similarly, domain-specific analysis of the normalized fluorescence intensity, \( I = I_{hl} = I_{lo} \) obtained using CS-XY scans suggests rather moderate lipid-packing differences between monolayer and bilayer-spanning \( l_o \) and \( l_d \) regions. The observed 47 and 64% reductions (relative to DOPC) of TRITC-DHPE lateral mobility in DOPC/CHOL (4:1) and DOPC/CHOL (2:1), respectively, and the largely indistinguishable lipid lateral mobility of these probe molecules in \( l_o \) and \( l_d \) regions are in excellent agreement with fluorescence-recovery-after-photobleaching data on comparable lipid mixtures reported recently (34,35).

### Table 1

<table>
<thead>
<tr>
<th>Bilayer type</th>
<th>Diffusion time (ms)</th>
<th>Diffusion coefficient (( \mu m^2/s ))</th>
<th>Brightness (PCH analysis)</th>
<th>Normalized fluorescence intensity (CS-XY analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC only</td>
<td>3.16 ± 0.34</td>
<td>1.68 ± 0.20</td>
<td>6.25 ± 0.71</td>
<td>—</td>
</tr>
<tr>
<td>DOPC:CHOL (4:1)</td>
<td>5.89 ± 0.99</td>
<td>0.89 ± 0.15</td>
<td>4.57 ± 0.79</td>
<td>—</td>
</tr>
<tr>
<td>DOPC:CHOL (2:1)</td>
<td>8.69 ± 1.50</td>
<td>0.61 ± 0.13</td>
<td>3.45 ± 0.36</td>
<td>—</td>
</tr>
<tr>
<td>Asymmetric-( l_o )</td>
<td>5.87 ± 1.65</td>
<td>0.90 ± 0.21</td>
<td>3.97 ± 0.57</td>
<td>0.60 ± 0.03</td>
</tr>
<tr>
<td>Asymmetric-( l_d )</td>
<td>6.19 ± 1.17</td>
<td>0.86 ± 0.15</td>
<td>3.77 ± 0.73</td>
<td>0.40 ± 0.02</td>
</tr>
<tr>
<td>Symmetric-( l_d )</td>
<td>6.21 ± 1.17</td>
<td>0.90 ± 0.14</td>
<td>4.10 ± 0.40</td>
<td>0.70 ± 0.03</td>
</tr>
<tr>
<td>Symmetric-( l_o )</td>
<td>6.34 ± 1.34</td>
<td>0.83 ± 0.15</td>
<td>3.57 ± 0.44</td>
<td>0.30 ± 0.02</td>
</tr>
</tbody>
</table>

FCS analysis provides information about the lateral mobility (diffusion time and diffusion coefficient) of TRITC-DHPE in the different lipid environments. PCH analysis gives complementary insight into the average brightness per TRITC-DHPE molecule. The normalized fluorescence intensity values, \( I = I_{lo} \) obtained using CS-XY scans were corrected for background and CHOL-induced intensity changes (determined by PCH-based TRITC-DHPE brightness analysis). Together, the different data in Table 1 indicate that there are no substantial differences in lipid packing between \( l_o-l_d \) regions in both types of bilayer systems.

Integrin sequestration in bilayers with \( M_{lo} \) domains before and after ligand addition

Integrins were added to a polymer-tethered lipid bilayer containing coexisting \( M_{lo} \) and \( B_{ld} \) domains as described in the Materials and Methods, and their domain-specific distribution was determined using confocal CS-XY scans. The methodology has been applied previously to characterize integrins in raft-mimicking lipid mixtures of asymmetric composition (\( B_{lo} \) and \( B_{ld} \)) (18). Fig. 3, A–J, compares CS-XY scans of dye-lipid and antibody-labeled integrin (\( \alpha_x \beta_3 \)) distributions in representative bilayer regions of asymmetric and symmetric lipid compositions before (top row) and after addition of native ligands (VN) (bottom row). In the case of the asymmetric bilayer system, the homogeneous \( l_d \) phase in the bottom leaflet and the presence of coexisting \( l_o-l_d \) phase separations in the top leaflet of the bilayer are confirmed by the corresponding CS-XY scan data obtained through the DID (bottom leaflet, Fig. 3, A and F) and NBD channels (top leaflet, Fig. 3, B and G). In the symmetric bilayer system, which contains NBD-DHPE in both of its monolayers, bilayer-spanning \( l_o-l_d \) phase separations can be observed through the NBD channel (Fig. 3, D and J). In both types of membrane systems, the corresponding integrin distribution in the same bilayer region is depicted through the Alexa-555 channel (Fig. 3, C, E, H, and J). Examination of the CS-XY scans in Fig. 3 provides valuable qualitative information about the role of bilayer asymmetry on \( \alpha_x \beta_3 \) sequestration. Specifically, comparison of the NBD and Alexa-555 channel data from both types of bilayer systems provides two intriguing results. Although \( \alpha_x \beta_3 \) receptors without ligands have a preference for \( B_{ld} \) regions of the symmetric bilayer (Fig. 3, D and E, demonstrates opposite preferences), they exhibit a preference for \( M_{lo} \) regions in the asymmetric bilayer system under comparable ligand-free conditions (Fig. 3, B and C, shows similar preferences). Furthermore, addition of native ligands (VN) has a different impact on integrin distribution in both types of bilayers, as exemplified by the observed ligand-mediated \( \alpha_x \beta_3 \) net translocation from \( B_{ld} \) to \( B_{lo} \) regions in symmetric bilayers (Fig. 3, D and E, versus Fig. 3, I and J) and the largely unchanged \( M_{lo} \) affinity in their asymmetric counterparts (Fig. 3, B and C, versus Fig. 3, G and H). Comparable qualitative results were obtained from corresponding CS-XY scans of \( \alpha_x \beta_3 \) integrin (data not shown).

As outlined in the Materials and Methods, the CS-XY data can be analyzed in terms of the parameter \( E_{raft} \), which provides a quantitative measure of protein sequestering in the planar model membrane environment. Fig. 4 summarizes the \( E_{raft} \) values of \( \alpha_x \beta_3 \) and \( \alpha_x \beta_1 \) obtained in the presence of coexisting \( M_{lo} \) and \( B_{ld} \) domains together with corresponding \( E_{raft} \) data on bilayer systems with coexisting bilayer-spanning \( B_{lo} \) and \( B_{ld} \) domains, reported recently in Siegel et al. (18). Most notably, in the absence of native ligands, the \( E_{raft} \) data of \( \alpha_x \beta_3 \) and \( \alpha_x \beta_1 \) illustrate a
moderately higher affinity for the $M_{lo}$ over coexisting $Bld$ domains. In contrast, these integrins exhibit a preference for the $Bld$-phase in the symmetric bilayer system (18). A comparison of integrin affinity data from both types of membrane compositions sets a hierarchy: $M_{lo}$ is the preferred state, followed by $Bld$, followed by $Blo$.

Several partially competing factors are known to contribute to the specific lipid domain affinity of membrane proteins (36), the three most important of which are bilayer compressibility, bilayer width, and interactions between the extracellular integrin headgroup and the bilayer itself. The $ld$ phase preference of TM $\alpha$-helical structures of membrane proteins is considered as one important factor of protein sequestering in heterogeneous lipid environments that can be related to bilayer compressibility. For example, it has been shown that $\alpha$-helices of TM domains, when constructed as simple peptides, associate strongly with $ld$ regions (37). This $ld$ phase preference of TM domains can be rationalized in terms of differences in bilayer compressibility modulus (lipid-packing density) in $ld$ and $lo$ domains, which affects the energy to incorporate membrane proteins into the lipid bilayer (36).

$E_{raft}$ analysis of dye-labeled lipids provides some insight into lipid-packing conditions in monolayer-associated and bilayer-spanning $ld$-$lo$ domains:

1. TRITC-DHPE distribution in the presence of coexisting $ld$-$lo$ regions in asymmetric and symmetric bilayers provides $E_{raft}$ values of $-0.2$ and $-0.46$, respectively. These $E_{raft}$ data of TRITC-DHPE suggest that the $M_{lo}$ phase has a slightly lower lipid-packing density and greater compressibility than the $Blo$ phase. In other words, the observed $M_{lo}$ phase preference of integrins in asymmetric bilayer systems can be rationalized, at least in part, in terms of the more favorable energetics of protein incorporation in these membrane regions relative to their bilayer-spanning counterparts.

2. Hydrophobic matching of protein TM and bilayer hydrophobic regions represents another significant factor that determines the affinity of membrane proteins for particular lipid environments. Previously reported bilayer x-ray diffraction data of DOPC-CHOL and CHOL-sphingolipid mixtures, and DOPC indicate hydrophobic thicknesses values of the bilayer for the $Bld$, $M_{lo}$, and $Blo$ domains of $\approx 33 \pm 1 \,\text{Å}$ ($Bld$), $35.5 \pm 1 \,\text{Å}$ ($M_{lo}$), and $38 \pm 1 \,\text{Å}$ ($Blo$) (38). Interestingly, the hydrophobic thickness value of $Bld$ domains best matches those reported for TM $\alpha$-helices of integrin $\alpha$- and $\beta$-subunits, which are $31.6 \pm 3.4 \,\text{Å}$ and $30.0 \pm 3.6 \,\text{Å}$, respectively (39,40). Therefore, the previously reported $ld$-$lo$ phase preference of $\alpha_3\beta_3$ and $\alpha_2\beta_1$ in the presence of coexisting $Bld$ and $Blo$ domains in the symmetric bilayer compositions appears plausible on the basis of hydrophobic matching arguments (available hydrophobic thickness data), as a hypothetical $lo$ phase association of integrins would be accompanied by a substantial hydrophobic thickness mismatch of $\approx 7 \,\text{Å}$.

![Image](image-url)
3. The situation is less obvious in the asymmetric membrane system because the reported error margin of the integrin hydrophobic thickness of ~3.5 Å does not exclude a scenario of similar hydrophobic mismatch conditions in Mlo and Bld membrane regions. Therefore, the observed $M_l$ phase preference in asymmetric bilayers with coexisting $M_l$ and $B_l$ domains may indicate contributions from another regulatory factor affecting protein affinity for lipid domains. This third factor is most likely linked to the interaction between the lipid bilayer and the extracellular integrin headgroups or ectodomains. Indeed, contributions from ectodomain-lipid interactions can be expected if one considers the close vicinity of bent ectodomains and lipid bilayer in resting, inactive integrins. The observed hierarchical preferences suggest that these interactions lead to a moderate $l_o$ phase preference in asymmetric, raft-mimicking lipid compositions. Interestingly, a comparison of integrin $E_{raft}$ data without ligands in Fig. 4 shows a slightly higher $l_o$ phase preference of $\alpha_5\beta_3$ relative to $\alpha_\beta$. This enhanced $l_o$ phase preference, which suggests subtle differences in lipid-protein interactions, can be observed for both symmetric and asymmetric lipid compositions.

4. The next significant finding from Fig. 4 is the relatively minor change in integrin sequestering upon addition of ligands in the asymmetric bilayer systems, which is in stark contrast to the substantial translocation from $B_{ld}$ to $B_{lo}$ phases in symmetric bilayer systems. For $\alpha_5\beta_3$, in the $M_{lo}$ system, there was very little change in raftphilicity ($X_{migrate}(\alpha_5\beta_3) = -5.5 \pm 6\%$), whereas in the $B_{lo}$ system more than half of the proteins translocated from $B_{ld}$ to $B_{lo}$ domains (18). This difference becomes understandable if one considers the reported large change in integrin ectodomain orientation that has been reported to occur upon the addition of the extracellular ligands and cytosolic integrin-binding proteins (41,42). The conformation becomes more stretched upon ligand binding, and this change is accompanied by a substantial reorganization of the TM $\alpha$-helical structures and cytosolic domains of the $\alpha$- and $\beta$-subunits, including a change in the tilt angle. For example, fluorescence resonance energy transfer experiments on integrins in the plasma membrane have shown that ligand-mediated conformational changes of integrin ectodomains can be propagated across the plasma membrane, thereby leading to a significant separation of $\alpha$- and $\beta$-integrin tails (43). Furthermore, multiscale simulations on integrin $\alpha_{\text{Iib}/\beta_3}$ TM helix dimer in the presence of a POPC/POPG lipid bilayer showed that $\alpha_{\text{Iib}}$ mutations, which were found to have a significant effect on integrin activation (44), lead to a perturbation of TM helix packing and changing crossing angles of the two integrin TM helices from $35^\circ$ (wild-type) to $10^\circ$ (mutation) (45). Interestingly, upon integrin activation, the tilt angle of $\beta_3$ remained largely constant at $30^\circ$, whereas that of $\alpha_{\text{Iib}}$ changed from $5^\circ$ to $20^\circ$. Based on these structural data, one can expect that ligand binding to $\alpha_5\beta_3$ and $\alpha_5\beta_1$ leads to a rather stretched conformation of integrin ectodomains, which should reduce the interactions between ectodomain and lipid bilayer. Furthermore, we hypothesize that the quite reproducible $l_o$ phase preference upon ligand binding in symmetric and asymmetric bilayer compositions can be attributed to the parallel reorganization of the integrin TM region, which includes the opening up of the tight association and the tilting of the two integrin TM helices in the $B_{ld}$ phase region.

**PCH analysis to determine degree of integrin oligomerization**

Ligands and crosslinking agents are known to alter the sequestering of membrane proteins in coexisting $l_d$ and $l_o$ lipid phases (46,47). Therefore, we next determined the oligomerization state of $\alpha_5\beta_3$ and $\alpha_5\beta_1$ in $M_{lo}$-containing bilayers with and without their respective ligands using PCH analysis. As reported before for integrins in bilayers with bilayer-spanning $l_d$ and $l_o$ lipid domains, the PCH data were analyzed in terms of a model, which provides insight into the domain-specific number and brightness of integrin monomers ($N_{avg}$, $\epsilon$) and dimers ($N_{avgdimer}, \epsilon_{dimer} (= 2\epsilon)$) (18). Fig. 5 summarizes the results of the PCH analysis of $\alpha_5\beta_3$ and $\alpha_5\beta_1$ in bilayers with coexisting $M_{lo}$ and $B_{ld}$ domains. It includes experimentally determined PCH curves (markers) and best model fits (dashed line) (Fig. 5, A–D), as well as the results of the PCH model-derived molecular brightness, $\epsilon$ (Fig. 5 E), and fraction of dimers, $X_{\text{dimer}}$ (Fig. 5 F). Fig. 5 E shows that the ratio of $\epsilon$ of Alexa-555 labeled anti-integrin MAbs bound to bilayer-incorporated integrins and in solution remains in the range of 83 ± 1% regardless of the addition of ligands.

This is in excellent agreement with our previous findings on integrins in bilayers with bilayer-spanning $l_o$-$l_d$ phase separations (18). Fig. 5 F suggests that $\alpha_5\beta_3$ and $\alpha_5\beta_1$ in the lipid bilayer primarily exist as monomers with and without VN and FN, respectively, again in good agreement with recent results on bilayers with membrane-spanning domains (18). The results in Fig. 5, E and F, are significant because they imply that ligand addition does not cause any significant integrin oligomerization in the model membrane environment. These model membrane results are mirrored by comparable findings on integrins in OG-containing solution (48) and in plasma membranes in the absence of cytosolic integrin linkages (49). In the first case, any notable ligand-mediated integrin oligomerization could be excluded because the molecular weight of integrins determined by centrifugation remained within 10% the same before and after ligand addition. In the second case,
no integrin clustering was observed after ligand addition to integrins without cytosolic linkage.

**CONCLUSION**

This work provides direct experimental evidence that bilayer asymmetry has a significant impact on the sequestering of integrins in raft-mimicking lipid mixtures. Unlike in bilayers with bilayer-spanning $l_o$ and $l_d$ lipid phases, $\alpha_5\beta_3$ and $\alpha_5\beta_1$ integrins are deficient in $B_l$ domains of bilayers, in which $l_o$ and $l_d$ phase separations are limited to the top leaflet of the bilayer. Moreover, comparing CS-XY and PCH experiments on these integrins in bilayers with $Ml_o$ versus $B_l$ domains in the absence and presence of their respective ligands demonstrate the different influence of ligand binding on integrin sequestering in bilayers of symmetric and asymmetric lipid compositions. The observed $l_d$ and $l_o$ phase preferences of $\alpha_5\beta_3$ and $\alpha_5\beta_1$ in bilayers with $B_l$ and $Ml_o$ domains suggests two potential mechanisms of integrin sequestering regulation—predominantly sequestration regulation by changing hydrophobic matching conditions and, to a lesser extent, sequestration regulation by alterations in lipid composition affecting lipid packing.

The detected changes of integrin sequestering after ligand addition are likely associated with distinct ligand-induced conformational changes of integrins influencing hydrophobic matching and integrin-lipid interactions. Our experimental results are significant because they highlight the
potential importance of bilayer asymmetry in protein sequestration and function in biological membranes. They support a mechanism of protein sequestration, which is based on the subtle interplay of different molecular interactions. Our findings are particularly exciting in light of the postulated functional importance of some TM proteins in the assembly of raft-mediated transbilayer platforms (50–52). They are also intriguing with regard to recent findings that $\alpha_5\beta_1$ seems to recruit CHOL to the exoplasmic leaflet of plasma membranes (53). Furthermore, the observed ability of integrins to affiliate with the $L_0$ phase is interesting if one considers that several integrin-related cellular processes, such as cell adhesion, migration, and angiogenesis, are considered raft-associated (46,47,54,55). Importantly, the described methodology, which enables the parallel analysis of membrane protein sequestration and oligomerization under well-defined conditions with single-molecule sensitivity, can be conducted in the absence of artificial crosslinking agents. In that sense, it provides valuable complementary information to corresponding protein sequestration studies at the cellular level.

SUPPORTING MATERIAL

Two figures are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(13)00453-0.

This work was supported in part by the National Science Foundation (grant No. MCB-0920134) and the Indiana University-Purdue University Indianapolis Nanoscale Imaging Center.

REFERENCES

32. Reference deleted in proof.
41. Shattil, S. J., C. Kim, and M. H. Ginsberg. 2010. The final steps of integrin activation: the end game. *Nat. Rev. Mol. Cell Biol.* 11:288–300.