### Phase behavior and the partitioning of caveolin-1 scaffolding domain peptides in model lipid bilayers

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<td><a href="http://dx.doi.org/10.1016/j.jcis.2006.08.057">http://dx.doi.org/10.1016/j.jcis.2006.08.057</a></td>
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<td>Publisher</td>
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<td>Final published version</td>
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Phase behavior and the partitioning of caveolin-1 scaffolding domain peptides in model lipid bilayers

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Received 3 July 2006; accepted 28 August 2006
Available online 4 October 2006

Abstract

The membrane binding and model lipid raft interaction of synthetic peptides derived from the caveolin scaffolding domain (CSD) of the protein caveolin-1 have been investigated. CSD peptides bind preferentially to liquid-disordered domains in model lipid bilayers composed of cholesterol and an equimolar ratio of dioleoylphosphatidylcholine (DOPC) and brain sphingomyelin. Three caveolin-1 peptides were studied: the scaffolding domain (residues 83–101), a water-insoluble construct containing residues 89–101, and a water-soluble construct containing residues 89–101. Confocal and fluorescence microscopy investigation shows that the caveolin-1 peptides bind to the more fluid cholesterol-poor phase. The binding of the water-soluble peptide to lipid bilayers was measured using fluorescence correlation spectroscopy (FCS). We measured molar partition coefficients of $10^4$ M$^{-1}$ between the soluble peptide and phase-separated lipid bilayers and $10^3$ M$^{-1}$ between the soluble peptide and bilayers with a single liquid phase. Partial phase diagrams for our phase-separating lipid mixture with added caveolin-1 peptides were measured using fluorescence microscopy. The water-soluble peptide did not change the phase morphology or the miscibility transition in giant unilamellar vesicles (GUVs); however, the water-insoluble and full-length CSD peptides lowered the liquid–liquid melting temperature.

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Keywords: Lipid rafts; Fluorescence microscopy; Fluorescence correlation spectroscopy; Cholesterol; Bilayers; Lipid domains; Membranes

1. Introduction

Lipid rafts, or detergent-insoluble domains of the plasma membrane enriched in cholesterol and sphingolipids, are thought to play a role in sequestering various molecules to facilitate cell signaling. Caveolae are a specialized type of lipid raft with flask-like invaginated morphology enriched in the protein caveolin-1 that participate in cell signaling and lipid metabolism [1–5]. Caveolin-1 has been shown to bind cholesterol [6] and associate with sphingolipids [7] and may have a structural role in the formation of caveolae [8,9].

Model lipid rafts in synthetic lipid bilayers have provided a basis for understanding cholesterol-enriched phases of the plasma membrane. Lipids extracted from cell membranes and reconstituted in giant unilamellar vesicles (GUVs) exhibit microscopic phase coexistence with lipid domains resembling ternary lipid mixtures of cholesterol, phosphatidylycholine, and sphingomyelin [10]. In this widely-studied model system, liquid-ordered (Lo) domains are formed from the packing among saturated lipid acyl chains and cholesterol and are immiscible with liquid-disordered (Ld) domains enriched in phosphatidylycholine [11]. These Lo lipid domains are considered models of lipid rafts in the plasma membrane. Various physical properties of model lipid rafts, including composition, morphology, and molecular mobility, have been studied [11,12]. There has also been an increasing effort to understand how proteins partition into either the Lo phase or the Ld phase [13]. What remains to be well studied, however, is how peptides and proteins influence the lipid phase behavior of these model lipid rafts.

Proteins are a significant part of the composition of cell membranes and it is therefore important to consider proteins in model studies of lipid rafts and caveolae. Studying the in-
terplay between proteins and lipid phase separation may give insight into the formation of lipid rafts, as proteins have been suggested to promote domain formation by associating with certain lipids [14]. It has also been suggested that lipid molecules can organize around proteins and modulate phase separation [15,16]. Using model lipid membranes, researchers have studied how proteins and peptides can cause lateral redistribution of lipids in bilayer membranes using differential scanning calorimetry (DSC) [14] and fluorescence microscopy [17]. An advantage of fluorescence microscopy is that it allows one to directly observe the lipid-phase partitioning of labeled molecules as well as microscopic phase separation.

In this study we investigated the partitioning and phase behavior of lipid bilayer membranes containing caveolin-1. We selected caveolin-1 because caveolae are enriched in the lipid raft components cholesterol and sphingomyelin and the membrane interaction of caveolin-1 is not well understood. Mutagenesis experiments have identified the caveolin scaffolding domain (CSD) as the region of caveolin-1 responsible for membrane binding and targeting the full-length protein to caveolae [18]. The CSD comprises amino acids 82–101 of the N-terminal domain of caveolin-1 and has been shown to associate with detergent-insoluble membrane fractions assayed in vivo [19]. We have selected model peptides derived from the CSD of caveolin-1 to study phase separation and the influence of cholesterol concentration on peptide–lipid interactions in lipid bilayers.

In previous model membrane experiments, the full-length CSD formed cholesterol-enriched domains in model membranes composed of DOPC, the acidic lipids phosphatidylserine and phosphatidylinositol-4,5-bisphosphate (PIP$_2$), and cholesterol [20]. Subregions of the CSD and their membrane interactions have also been previously investigated. In live-cell mutagenesis experiments, KYWFYR was shown to be the membrane-attachment sequence [19] of caveolin-1, and in recent model membrane experiments, authors have demonstrated, using DSC, that KYWFYR does not promote local high cholesterol concentrations, nor does it bind cholesterol in phosphatidylcholine membranes [21]. DSC analysis has been used to study the peptide N-acetyl-VTKYWFYR amide, which was shown to promote local cholesterol crystal formation and depletion from other domains, though this effect was more pronounced with the full-length CSD [22].

While the effect of acidic lipids [20,23] and cholesterol [20,22] on the spatial organization and binding of CSD peptides has been investigated, to our knowledge the interaction of CSD peptides with putative model lipid rafts containing sphingomyelin has not yet been investigated. Therefore, our primary goal was to study caveolin-1 in model membranes with defined L$_o$ and L$_d$ domains and to investigate how the CSD can impact the phase behavior of L$_o$ and L$_d$ phases. Sphingomyelin was recently shown to be a component of caveolae in vivo [7] and thus we focused on a membrane containing sphingomyelin in order to understand caveolin and lipid interactions.

2. Materials and methods

2.1. Commercial reagents

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), cholesterol, and brain sphingomyelin (BSM) were purchased from Avanti Polar Lipids (Alabaster, AL). Texas Red 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (TR-DPPE) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine 4-chloro-benzenesulfonate salt (DiD) were purchased from Invitrogen (Eugene, OR). Rhodamine 6G and Cy5 dyes in sugar solutions (Merck, Darmstadt, Germany) were used for FCS system calibration. Lipids were dissolved in high-performance liquid chromatography-grade chloroform and methanol from either Fluka (Switzerland) or Mallinckrodt (Phillipsburg, NJ). All other chemicals used were reagent grade. Fluorescently labeled synthetic peptides containing sequences derived from the caveolin scaffolding domain (CSD) were purchased from SynPep (Dublin, CA) and the MIT Biopolymers Laboratory. The CSD peptide labeled at the N terminus with fluorescein isothiocyanate is FITC-CGIWKASFTTFVTKYWFYR-acetyl (CSD). A shorter fluorescently labeled peptide containing the membrane-attachment segment amino acid sequence residues 89–101 is FITC-FTTFVTKYWFYR-acetyl (C-SOL). The soluble peptide containing these residues was synthesized with a FITC label at the N-terminus and the sequence SBSG between the FITC and CSD residues to improve peptide water solubility without adding net charge, resulting in a final peptide structure of FITC-SBSGFTTFVTKYWFYR-acetyl (C-SOL). All peptides were purified using HPLC.

2.2. Preparation of vesicles

We prepared giant unilamellar vesicles (GUVs) with the electroformation technique [25]. Approximately 40 µL of lipids

![Fig. 1. Schematic of fluorescently labeled synthetic peptides used in experiments. CAV-CSD contains the caveolin scaffolding domain (residues 83–101); CAV-SOL contains residues 89–101 and is solubilized at the N terminus by a serine–glycine–serine (SGS) sequence. CAV-INSOL contains the same residues as CAV-SOL but lacks the SGS sequence.](image-url)
dissolved in HPLC-grade chloroform and methanol at a concentration of approximately 10 mg/mL were spread onto conductive indium tin oxide plates and dried under vacuum. To this lipid mixture, we added 1 mol% of the water-insoluble caveolin-1 peptides CAV-CSD or CAV-INSOL. We visualized the lipid phases by adding 0.1 mol% of TR-DPPE to the lipid mixture. The GUVs were grown in a 100 mM sucrose and 5 mM KCl solution for 1.5–2 h at a temperature above the lipid miscibility transition temperature. We formed large unilamellar vesicles (LUVs) using the extrusion technique [26], and dried lipids were rehydrated in 100 mM glucose and 5 mM KCl. This solution was then passed 10 times through two 100-nm polycarbonate filters using the Avanti Mini-Extruder.

2.3. Microscopy

The miscibility transitions were observed using fluorescence microscopy [27]. The stock GUV solution was diluted approximately twofold with 100 mM glucose and 5 mM KCl to provide density contrast and was placed in a CoverWell imaging chamber (Grace Bio-Labs, Bend, OR) adhered to coverglass. A Nikon Diaphot inverted microscope with a 100× objective was used to visualize phase separation and domain morphology on the surface of the GUVs. Sample heating over a temperature range of 10–50°C was accomplished by a microscope heating stage unit (Instec, Boulder, CO) and an objective collar heater (Biophtechs, Butler, PA). The sample chamber temperature was measured with a thermocouple (Omega, Stamford, CT). The miscibility transition temperatures were measured by both heating and cooling and the error bars represent the range over which phase miscibility was observed. In order to observe the impact of the water-soluble CAV-SOL peptide on microscopic phase separation, the soluble peptide was dissolved in glucose buffer and added to GUVs in solution.

The fractional area of the GUV surface occupied by the DOPC-enriched liquid phase was calculated using ImageJ software (NIH, Bethesda, MA). As described elsewhere [28], the geometry of the system prevents the use of automated algorithms for calculating the relative fractional areas of each phase, so at least 20 different GUV surfaces were analyzed to determine the average fractional area of phases at each composition.

Fluorescence confocal microscopy experiments were performed at the W.M. Keck Microscopy Facility at the Whitehead Institute with a Zeiss laser scanning module (LSM) microscope with a Zeiss C-Apochromat 40× NA = 1.2 water immersion objective. For two-channel experiments, the excitation light from lasers at 488 and 543 nm was reflected by a dichroic mirror (HFT 488/543) and the emission was split by another dichroic mirror (NFT 490) into two channels and passed through a 505–719 emission filter in the first channel and a 558–719 emission filter in the second channel to detect the FITC-labeled peptide and TR-DPPE, respectively. For the one-channel control experiment to image the phase partitioning and membrane localization of the FITC-labeled peptide, the 488-nm laser and a LP 505 filter were used. For three-dimensional image projections of vesicles, z-scans were taken in 0.45-µm increments and projected using Zeiss LSM software.

2.4. Fluorescence correlation spectroscopy

Recently, fluorescence correlation spectroscopy (FCS) has been used to measure the binding between large unilamellar vesicles (LUVs) and water-soluble peptides in nanomolar concentrations [29]. We employed this FCS peptide–membrane assay to measure the binding of fluorescently labeled water-soluble CAV-SOL peptide to LUVs with only slight modifications. In order to prevent adhesion of the LUVs to the chamber surfaces, LabTek II chamber slides were filled with 1 mg/ml bovine serum albumin (Fluka) dissolved in water for at least 30 min and then air-dried before being filled with FCS samples. The incident laser power was 160 µW for all experiments; we verified that photobleaching was not affecting the measurements by measuring the same diffusion times at 480-µW laser power. A sugar buffer solution of 100 mM glucose with 5 mM KCl was used for all FCS measurements and calibrations. The focus volume was calibrated with Rhodamine 6G for experiments at 488 nm and with Cy5 at 633 nm. Attaching the water-soluble FITC peptide to the end of the caveolin scaffolding domain peptide away from the membrane attachment sequence should minimize the influence of the fluorophore on the peptide–membrane binding. As a control experiment, we verified that the binding of free FITC to membranes was negligible for the lipid compositions used.

Diffusion times for LUVs at each lipid composition were measured from LUVs containing 0.01 mol% DiD at 633 nm excitation. The number of peptides in the focus volume was measured over a range of CAV-SOL concentrations, to characterize signal/noise effects and the exact peptide concentration. Above a CAV-SOL concentration of 10−8 M, the number of CAV-SOL molecules detected per unit volume was proportional to CAV-SOL concentration. This characterization ensured that the peptide was soluble only as monomolecular units and guided our choice to study CAV-SOL concentrations greater than 10 nM.

The calculation of molar partition coefficients from FCS data is outlined [29]. The expression for the normalized time correlation function $G(\tau)$ is in Ref. [30],

$$G(\tau) = \frac{1}{N} \times g(\tau)$$

\[= \frac{1}{N} \times \left(1 + \frac{T}{1 - T} e^{-\tau/\tau_D}\right) \left(\frac{1}{1 + \tau/\tau_D}\right) \times \left(\frac{1}{1 + \tau/S^2\tau_D}\right)^{1/2}, \tag{1}\]

where the average number of fluorescent molecules counted in the laser focus is $N$ and $\tau_D$ is the diffusion time of the molecules. The fraction of fluorophores in the triplet state is $T$, the triplet lifetime is $\tau_T$, and the structural parameter, $S$, is the ratio of the radial to axial distances of the center of the laser beam to the edge of the focus volume. The triplet fraction of the FITC-labeled peptide was ∼0.7 and the triplet lifetime was ∼3.5 µs. From Rhodamine 6G calibration measurements, we determined that $S = 5.5$.

In our experiments, both bound and free peptides diffuse within the laser focus volume, so the autocorrelation function is
The molar partition coefficient of the peptide, \( K \), is a proportionality constant between the fraction of peptide bound to the membrane \([P]_{\text{mem}}\) and the molar concentrations of peptide \([P]\) and lipids \([L]\) in solution and is described by \([P]_{\text{mem}} = K[P][L]\) [29]. \( K \) was computed from a material balance on the free \([P]\) and membrane-bound peptide \([P]_{\text{mem}}\) [29],

\[
\frac{[P]_{\text{mem}}}{[P]_{\text{tot}}} = \frac{K[L]_{\text{acc}}}{1 + K[L]_{\text{acc}}} = 1 - A_P N_0, \tag{3}
\]

where \([P]_{\text{tot}}\) is the sum of \([P]_{\text{mem}}\) and \([P]\), \([L]_{\text{acc}}\) is 50% of the total lipid concentration, or the approximate concentration of lipids in the outer leaflet of the LUVs that is accessible to the peptide, \(A_P\) is determined from fitting Eq. (2), and \(N_0\) is the number of peptides in the focus volume counted in the absence of LUVs.

3. Results

3.1. Phase partitioning

All of the caveolin-1 peptides studied partition into the liquid-disordered or cholesterol-poor phase over a range of cholesterol concentrations. The evidence for this partitioning is twofold, demonstrated by (1) the binding of peptides to the majority phase and (2) the colocalization of peptides with the liquid-disordered phase marker, TR-DPPE. Fig. 2 demonstrates that the water-soluble CAV-SOL peptide binds the majority phase, or the cholesterol-poor phase at the cholesterol concentrations of 15 mol% (Fig. 2, I and II) and 20 mol% (Fig. 2IIIa). We also verified that the lipid raft marker, TR-DPPE, does not affect the partitioning of CAV-SOL in the cholesterol-poor phase (Fig. 2, I and II). The binding of the water-soluble CAV-SOL peptide to the liquid-disordered DOPC-enriched phase is further indicated by the colocalization of CAV-SOL and TR-DPPE, which partitions into the less-dense phase (Fig. 2III). The equatorial fluorescence confocal micrographs in Fig. 2 indicate that the CAV-SOL peptide is evenly distributed.
composition. Hydrodynamic radii (solution with LUVs of 100 µM accessible lipid concentration added with listed terol) fluorescence micrographs.

50% chol

DOPC/BSM ratio are shown in Table 1. An increase in cholesterol concentration corresponds to a decrease in $R_h$.

Fig. 5. The pseudo-ternary lipid phase diagrams for the DOPC/BSM/cholesterol system with and without the addition of the caveolin-1 peptides are shown in Fig. 5. The ratio of DOPC to BSM was fixed at 1:1 for all experiments. The miscibility transition temperature, $T_m$, was measured over a range of cholesterol concentrations with and without added peptides. Both liquid–liquid and liquid–solid phase coexistence were observed. Solid–liquid coexistence was observed only in GUVs with cholesterol concentrations of 10 mol% or less and solid domains were identified by their noncircular morphology, rigid body rotation, and inability to ripen into larger domains [27]. In contrast, liquid domains have round, fluctuating edges and can coalesce and form larger domains.

The nearly identical miscibility transition temperatures measured over a range of concentrations with and without CAV-SOL peptide indicate that the addition of CAV-SOL does not affect the phase diagram (Fig. 5a). By contrast, inclusion of the insoluble caveolin-1 peptides in the GUV membrane does depress $T_m$ for the liquid–liquid transition. As illustrated in Fig. 5b, the addition of 1 mol% CAV-INSOL and CAV-CSD to GUVs containing 25 and 30 mol% cholesterol caused a significant decrease ($>5^\circ$C) in $T_m$. At a composition of 30 mol% cholesterol, not all of the GUVs in the observation slide were phase-separated after reaching the lower limit of the microscope cooling stage. The addition of the CAV-INSOL and CAV-CSD to GUVs containing 10 mol% cholesterol did not cause phase separation.

To systematically investigate the effects of phase separation and cholesterol concentration on CAV-SOL–membrane interaction, we used FCS to study the binding of CAV-SOL to membranes in solution. The autocorrelation curves in Fig. 4 demonstrate the binding of CAV-SOL to LUVs. CAV-SOL binds more strongly to vesicles that have phase-separating lipid mixtures (Fig. 4, c and d) and lower cholesterol concentrations than those in a single phase region with higher cholesterol concentrations (Fig. 4, e and f). Molar partition coefficients were calculated based on FCS data and are listed in Table 1 and show how lower cholesterol concentration and phase separation increase the membrane–peptide interaction.

### 3.3. Phase diagram

#### Table 1

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<tr>
<th>Lipid composition</th>
<th>$R_h$ of LUVs (nm)</th>
<th>$K$ (M$^{-1}$)</th>
<th>Area% of L$_d$ phase</th>
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<tr>
<td>20% chol + 1:1 DOPC/BSM</td>
<td>91 ± 4</td>
<td>~6 × 10$^4$</td>
<td>43 ± 16</td>
</tr>
<tr>
<td>30% chol + 1:1 DOPC/BSM</td>
<td>89 ± 4</td>
<td>~3 × 10$^4$</td>
<td>22 ± 13</td>
</tr>
<tr>
<td>40% chol + 1:1 DOPC/BSM</td>
<td>78 ± 3</td>
<td>~1 × 10$^3$</td>
<td>No phase separation</td>
</tr>
<tr>
<td>50% chol + 1:1 DOPC/BSM</td>
<td>75 ± 3</td>
<td>~7 × 10$^2$</td>
<td>No phase separation</td>
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Note. $K$ calculated from measurement of 50 nM CAV-SOL peptide dissolved in solution with LUVs of 100 µM accessible lipid concentration added with listed composition. Hydrodynamic radii ($R_h$) of the LUVs calculated from measured diffusion times of DiD-labeled LUVs. Fractional area and standard deviation of the GUV surface occupied by the DOPC-rich L$_d$ phase calculated from digital image analysis of ($N = 26$, 20 mol% cholesterol; $N = 28$, 30 mol% cholesterol).

Throughout liquid-disordered phase, Fig. 3 illustrates how both CAV-INSOL and CAV-CSD similarly partition into the liquid-disordered phase marked by TR-DPPE. The control experiment in which TR-DPPE was omitted from the lipid mixture was also performed with the CAV-INSOL and CAV-CSD peptides (data not shown).

#### 3.2. Peptide–membrane binding

We further investigated the binding of CAV-SOL to lipid bilayers with a fluorescence correlation spectroscopy–peptide binding assay. Lipid bilayers with varying cholesterol concentration and a fixed 1:1 DOPC/BSM ratio were formed as extruded large unilamellar vesicles (LUVs). There was an approximately two-order-of-magnitude difference between the measured diffusion time of CAV-SOL (40 µs) and the average diffusion time of the LUVs (5000 µs), allowing us to fit the data as a sum of two autocorrelation functions. Increasing the cholesterol concentration decreases the diameter of LUVs extruded from mixtures of cholesterol, DOPC and BSM. The hydrodynamic radii ($R_h$) of LUVs composed of cholesterol and an equimolar DOPC/BSM ratio are shown in Table 1. An increase in cholesterol concentration corresponds to a decrease in $R_h$.

Fig. 3. CAV-INSOL and CAV-CSD bind the liquid-disordered phase. Fluorescence micrographs of GUVs with CAV-INSOL and CAV-CSD peptides added. The left image in each panel is viewed for TR-DPPE and the right image is the same GUV viewed for the FITC-labeled peptides: (a, c) 1 mol% of CAV-INSOL added to lipid mixture: (b) GUVs composed of 10 mol% cholesterol, 1:1 DOPC/BSM and 1 mol% CA V-INSOL, (c) GUVs composed of 20 mol% cholesterol, 1:1 DOPC/BSM and 1 mol% CAV-INSOL. (b, d) 1 mol% of CAV-CSD added to lipid mixture: (b) GUVs composed of 10 mol% cholesterol, 1:1 DOPC/BSM and 1 mol% CA V-CSD, (d) GUVs composed of 20 mol% cholesterol, 1:1 DOPC/BSM and 1 mol% CA V-CSD. All scale bars 20 µm.
significant change in $T_m$ at the solid–liquid to liquid–liquid phase transition.

Fig. 6 illustrates how the size and shape of the liquid-ordered domains are qualitatively the same with or without peptides present. The bright cholesterol-poor phase was labeled with TR-DPPE, which is excluded from the cholesterol-rich phase [10,27]. The circular shape of the cholesterol-rich domains indicates liquid–liquid phase coexistence with high line tension [27] both with and without peptides.

4. Discussion

We studied the phase partitioning behavior of peptides derived from caveolin-1, a protein known to reside in cell membrane fractions resembling lipid rafts, but whose exact lipid raft targeting mechanism is not well understood. We investigated the phase partitioning of caveolin-1 peptides in a model membrane system with defined lipid domains of differing compositions and densities. Cholesterol-rich liquid-ordered ($L_0$) phases
formed from ternary mixtures of BSM, DOPC, and cholesterol provide a model system for studying lipid rafts. The composition and morphology of model lipid domains can be studied through lipid phase diagrams [11], which may give insight into the physical properties of lipid rafts in cell membranes. Lipid domains in cells may serve as platforms to locally concentrate molecules such as proteins to enable cell signaling. The preference of a protein for either the \( L_o \) domain or the DOPC-enriched liquid-disordered \( (L_d) \) domain is dictated by the physical properties of both the protein and the lipid domain.

In our study of peptides derived from the scaffolding domain of caveolin-1, we found that both the soluble and insoluble caveolin-1 peptides partition into the liquid-disordered \( (L_d) \) phase at all studied lipid compositions. The fact that caveolin-1 peptides prefer the fluid \( L_d \) domains to the dense \( L_o \) domains may be due to their exclusion from the tightly packed \( L_o \) domains. \( L_o \) domains are more densely packed than \( L_d \) domains due to the alignment of the long and saturated fatty acid tails of the sphingolipid molecules and the intercalating cholesterol. The packing of the liquid-ordered phase may be due to hydrogen bonding between the cholesterol and saturated phospholipids or sphingomyelin [31]. Recently, Radhakrishnan and McConnell proposed a model accounting for cholesterol and lipid interactions using cholesterol-saturated lipid complexation and predicted the tie lines of a three-phase lipid diagram [32]. The tight molecular packing and acyl chain alignment within \( L_o \) domains may create a locally ordered environment that does not readily accommodate additional molecules. This phenomenon of model peptide exclusion from \( L_o \) domains has been studied previously experimentally. The linker for activation of T-cells protein is believed to associate with rafts in vivo, but it prefers the \( L_d \) phases in model membranes studied using both fluorescence microscopy and detergent resistance [33]. Detergent assays demonstrate that model peptides, including hydrophobic transmembrane peptides [34,35] and palmitoylated peptides [34], are excluded from detergent-insoluble fractions due to tight lipid packing in the detergent-resistant phase.

The exclusion of our caveolin-1 peptides from the \( L_o \) phase may also be due to our peptides’ lack of lipid anchor moieties and their inability to form oligomers. There are some general trends associated with proteins and peptides that have been shown to partition into \( L_o \) lipid phases [13]. In model phase-separated membranes, the cholesterol-binding protein NAP-22 is only targeted to \( L_o \) domains in its myristoylated form [36]. In detergent resistance studies, lipidated peptides with multiple acyl chains partition into detergent-insoluble \( L_o \) membrane phases [37,38]. While the C-terminus of caveolin-1 contains three palmitoylated residues, the scaffolding domain we studied does not contain such lipid anchors. An additional important feature of proteins and complexes that have been shown to parti-

Fig. 5. Influence of caveolin peptides on the partial lipid phase diagrams of GUVs composed of different cholesterol concentrations and a 1:1 fixed DOPC/BSM ratio; \( L_o + L_d \) liquid–liquid phase coexistence region shown. The curves are drawn to guide the eye and are not fit to any theory. (a) CAV-SOL does not have significant impact on phase diagram. \( T_m \) measured for GUVs lacking peptide at transitions from liquid–solid phase coexistence (diamonds) and liquid–liquid phase coexistence (circles, solid curve) to single liquid phase. \( T_m \) measured for liquid–solid (squares, dotted curve) transitions after addition of 50 nM of CAV-SOL peptide to GUVs in solution. (b) Insoluble peptides influence the liquid–liquid melting transition. \( T_m \) for the solid–liquid transition at 10 mol% cholesterol with no peptide (diamonds), 1 mol% CAV-INSOL (+), 1 mol% CAV-CSD (star), and 50 nM CAV-SOL ( ) added to GUVs. The liquid–liquid \( T_m \) was measured at 20, 25, and 30 mol% cholesterol with 1 mol% CAV-INSOL (triangle point up, dashed curve), 1 mol% CAV-CSD (triangle point down, dash–dotted curve), and 50 nM CAV-SOL (squares, dotted curve) added to GUVs.

Fig. 6. Fluorescence micrographs of lipid phase separation in GUVs with 20 mol% cholesterol, 1:1 DOPC/BSM, and 0.1 mol% TR-DPPE marking the \( L_d \) phase. (a) No peptide added, (b) 50 nM CAV-SOL added to GUVs, (c) 1 mol% CAV-INSOL included in lipid mixture, (d) 1 mol% CAV-CSD included in lipid mixture. All scale bars 20 µm.
tion into L_d phases is their ability to oligomerize or form higher-order assemblies. A well-studied example of this effect is the B subunit of the protein cholera toxin (CTB), which binds the ganglioside GM1 with pentameric symmetry and is localized in L_o domains [39]. A recent study suggests that the CTB–GM1 complex localizes to L_o domains only upon complex formation [40]. Using antibodies to cross-linked saturated phospholipid analogs causes the lipids to show increased affinity for L_o phases in model lipid membranes [39]. Human placental alkaline phosphatase (PLAP) has a glycosylphosphatidylinositol anchor and researchers have shown that cross-linking PLAP favors its partitioning into L_o domains [41]. The scaffolding domain of caveolin-1 alone is insufficient for oligomerization; in addition to the CSD, residues 61–101 of the N terminus [42] and residues 168–178 in the C-terminal domain are necessary for oligomerization of caveolin-1 constructs in vivo [43]. Recent work in live cells suggests that the oligomerization of caveolin-1 is important for the protein to exit the Golgi complex, to acquire detergent insolubility and to associate with the plasma membrane [44]. The oligomerization of caveolin-1 and the formation of caveolin filaments that are anchored into the membrane are responsible for the invaginated morphology of cellular caveolae [45–48]. We did not observe measurable changes in lipid curvature or domain morphology when we included caveolin-1 peptides in our lipid membranes. Our peptides’ inability to form oligomers and their lack of lipid anchor moieties also precludes the deformation of the membrane into highly curved invaginations.

The result that our caveolin-1 peptides are excluded from the L_o phase is less surprising for the shorter peptides CAV-SOL and CAV-INSOL, which lack the full-length CSD that is necessary to target caveolin-1 constructs to caveolae in vivo [18]. We also demonstrate, however, that CAV-CSD does not partition into L_o domains in our model membrane system. In the in vivo investigation of CSD constructs targeting to detergent-assayed raft domains, the CSD was targeted to rafts only 20% as efficiently as full-length caveolin-1 [18]. While detergent extraction is the standard assay for determining whether proteins prefer the L_d or L_o phase in cells [49], some question whether detergent resistance can be used to determine whether a protein resided in a domain prior to detergent extraction [50]. Another concern in comparing live cell membranes and their detergent extracts to model membrane systems is bilayer asymmetry. The two leaflets of the cell plasma membrane have asymmetric lipid compositions and densities, which are not preserved in the detergent extraction process [51]. Model membranes approximating the lipid composition of the inner leaflet of the plasma membrane, where caveolin-1 is thought to bind, do not phase separate into L_d and L_o phases [52]. Previous model membrane experiments have demonstrated that CSD peptides can reside in membranes regions enriched in cholesterol, PIP_2, and acidic lipids [20], yet our model membrane system is substantially different. The defined immiscible L_d and L_o lipid domains studied here differ in molecular density and contain sphingomyelin, a known component of cellular caveolae [7]. The tight molecular packing in the L_o domains excludes CAV-CSD from the L_o domains. Our results may also suggest limitations associated with using model lipid rafts to approximate cholesterol-rich domains of cellular membranes. Recent reviews highlight the gaps in our understanding of lipid rafts in controlled model systems and rafts in cellular membranes [50,51,53].

The tendency of our caveolin-1 peptides to associate with less-dense membrane domains is further demonstrated by FCS experiments with CAV-SOL. The water solubility of CAV-SOL allowed us to quantify the binding of this peptide to membranes with varying cholesterol concentrations using FCS. In our assay, the peptide is added to LUVs in solution and binds more strongly to LUVs with phase-separating lipid compositions (20 and 30 mol% cholesterol, 1:1 DOPC/BSM) than non-phase-separating lipid compositions (40 and 50 mol% cholesterol, 1:1 DOPC/BSM) (Table 1). The peptide’s enhanced binding to membranes containing the L_d phase over homogeneous membranes with high cholesterol content (40, 50 mol%) is consistent with the preference of CAV-SOL for less dense and more fluid membranes. Our measured molar partition coefficients for CAV-SOL binding to LUVs were ~10^4 M^-1 for phase-separated lipid mixtures and ~10^{-3} M^-1 for non-phase-separating lipid mixtures. We measured the relative area fraction of the L_d phase in GUVs (Table 1) and doubling the area fraction of the L_d phase to which CAV-SOL binds approximately doubles the molar partition coefficient. Our measured molar partition coefficients are similar to those measured with a shorter caveolin-1 peptide containing residues 92–101 bound to vesicles with low (1–10 mol%) acidic lipid compositions measured by succrose gradients and radiolabeling of peptides [23].

The observation of lipid phase separation in mixtures of cholesterol, sphingomyelin, and DOPC can give insight into the fluidity and ordering of membranes. In these model studies domains are defined as microscopic immiscible phases with simple morphologies [54]. The lipid miscibility transition temperatures of lipid phases can be influenced by the length of lipid acyl chains [55] and clustering of protein molecules [17]. We studied the miscibility transition of membranes containing cholesterol, BSM, and DOPC and caveolin-1 peptides to study how the peptides influence T_m and the morphology of the lipid phases. We show that the water-soluble peptide has negligible impact on the phase diagram and the insoluble peptides depress T_m at cholesterol concentrations above 20 mol%.

We demonstrate that the phase diagram and phase morphology of membranes with 1:1 DOPC/BSM ratio and different cholesterol compositions are unaffected by the addition of CAV-SOL peptide (Figs. 5 and 6). The identical miscibility transition temperatures measured over a range of lipid compositions with and without CAV-SOL (Fig. 5a) indicate that CAV-SOL does not moderate the relative amounts of cholesterol nor does it redistribute the lipid concentrations in the two phases and that CAV-SOL is unable to induce the formation of cholesterol-rich phase-separated domains in non-phase-separated lipid bilayers. We attribute this lack of impact on the partial phase diagram to the weak-to-moderate binding of the CAV-SOL peptide to membranes. We do not expect CAV-SOL to penetrate deeply into lipid bilayer membranes. The interaction of similar caveolin-1 peptides and lipid bilayer membranes has been previously studied both with model membrane systems and in vivo.
The membrane attachment sequence of caveolin-1 is KYW-FYR and was identified through mutagenesis experiments and posited to insert into inner membrane leaflet of cells [19]. The same sequence KYWFYR was subsequently investigated with NMR was less inserted into model membranes composed of cholesterol in 1-stearoyl-2-oleoylphosphatidylcholine (SOPC) membranes than a peptide comprising the well-characterized cholesterol-binding sequence LWYIK [21]. These same authors also demonstrated that longer caveolin-1 peptides with sequence VTKYWFYR and the full CSD do not penetrate into SOPC and cholesterol membranes as deeply as LWYIK [22].

Unlike CAV-SOL, the insoluble peptides CAV-INSOL and CAV-CSD decreased \( T_m \) at cholesterol concentrations greater than 20 mol\% (Fig. 5b). CAV-INSOL and CAV-CSD incorporated into cholesterol/BSM/DOPC membranes prevented the formation of \( L_0 \) phases at miscibility transition temperatures observed without the peptides. We expect that the mechanism of the insoluble peptides interacting with membranes in our experiments to be different than how the water-soluble peptide binds membranes. Unlike CAV-SOL experiments where peptides were added to preexisting lipid membranes in solution, CAV-INSOL and CAV-CSD were included in the lipid mixture prior to forming membranes and were therefore able to access the full depth of the membranes and interact with all molecules in the lipid mixture. Phase separation is thought to be driven by the tendency of sphingolipids to interact with cholesterol and form ordered domains [32,56,57] and our results suggest that CAV-SOL and CAV-INSOL disrupt this phase separation process. The decrease in \( T_m \) that we observed at the liquid–liquid to single-liquid phase transition suggests that the addition of the insoluble peptides to the membranes also increases the fluidity of the membrane by promoting a single homogeneous phase.

5. Summary

We studied how synthetic peptides derived from the scaffolding domain of caveolin-1 interact with phase-separated model lipid bilayer membranes. The widely used model lipid raft system of cholesterol, DOPC, and a saturated lipid or sphingolipid may give insight into lipid rafts in cells and the formation of liquid-ordered phases enriched in cholesterol. Studying the temperature and composition dependence of lipid phase separation may improve our understanding of how proteins affect lipid packing and mobility. The insoluble caveolin-1 peptides CAV-INSOL and CAV-CSD depressed the melting temperature of liquid lipid phases, suggesting that the insoluble caveolin-1 peptides prevent the lateral organization of lipids at certain temperatures and therefore promote membrane fluidity. This effect contrasts the result that CAV-SOL added to membranes in solution did not impact the partial phase diagram. Model liquid-ordered domains have a dense molecular environment that is unfavorable to caveolin-1 peptide insertion. The insoluble scaffolding domain was excluded from the liquid-ordered phase, despite its earlier demonstrated preference for cholesterol in model membranes [20] and its localization to detergent-insoluble fractions of cell membranes [18]. We attribute the partitioning of our caveolin-1 peptides into liquid-disordered domains to their exclusion from the tightly packed liquid-ordered domains and their preference for more fluid membranes. Overcoming the exclusion of molecules from the tightly packed liquid-ordered domains may be achieved by lipid anchor moieties or oligomerization. While the full-length protein both oligomerizes and has palmitoylation sites, our peptides lack these features.

Acknowledgments

We thank L. Rusu for assistance with FCS, N. Watson for assistance with confocal microscopy, and P. Matsudaira and S. Manley for useful discussions. This research was supported by funding from the Alexander von Humboldt Foundation (A.P.G.) and a National Science Foundation Graduate Research Fellowship (M.R.H.).

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