Relationship between Sterol/Steroid Structure and Participation in Ordered Lipid Domains (Lipid Rafts): Implications for Lipid Raft Structure and Function

Jiwei Wang, Megha, and Erwin London*

Department of Biochemistry and Cell Biology and Department of Chemistry, Stony Brook University, State University of New York, Stony Brook, New York 11794-5215

Received September 19, 2003; Revised Manuscript Received November 20, 2003

ABSTRACT: The formation and stability of ordered lipid domains (rafts) in model membrane vesicles were studied using a series of sterols and steroids structurally similar to cholesterol. In one assay, insolubility in Triton X-100 was assessed in bilayers composed of sterol/steroid mixed with dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylcholine, or a 1:1 mixture of these phospholipids. In a second assay fluorescence quenching was used to determine the degree of ordered domain formation in bilayers containing sterol/steroid and a 1:1 mixture of DPPC and a quencher-carrying phosphatidylcholine. Both methods showed that several single modifications of the cholesterol structure weaken, but do not fully abolish, the ability of sterols and steroids to promote ordered domain formation when mixed with DPPC. Some of these modifications included a shift of the double bond from the 5−6 carbons (cholesterol) to 4−5 carbons (allocholesterol), derivatization of the 3-OH (cholesterol methyl ether, cholesteryl formate), and alteration of the 3-hydroxy to a keto group (cholestanone). An oxysterol involved in atherosclerosis, 7-ketocholesterol, formed domains with DPPC that were as thermally stable as those with cholesterol although not as tightly packed as judged by fluorescence anisotropy. It was also found that 7-ketocholesterol has fluorescence quenching properties making it a useful spectroscopic probe. Lathosterol, which has a 7−8 carbon double bond in place of the 5−6 double bond of cholesterol, formed rafts with DPPC that were at least as detergent-resistant as, and even more thermally stable than, rafts containing cholesterol. Because lathosterol is an intermediate in cholesterol biosynthesis, we conclude it is unlikely that sterol biosynthesis continues past lathosterol in order to create a raft-favoring lipid.

Many studies now indicate that ordered lipid domains (rafts) composed of relatively saturated lipids (sphingolipids) and sterol are likely to exist in eukaryotic cell membranes (*1*) and play important roles in a number of biological processes (*2*−*7*). The rules governing the participation of different lipids in rafts have been partly worked out. Lipid rafts exist in the liquid-ordered (L_o) state (*8*−*11*). This state is characterized by tight packing, similar to that in the more solidlike gel state, but also fast lateral motion, although slightly slower than that observed in the more disordered liquid-crystalline state (*12*−*16*).

In model membranes, the identification of lipid domains has been detected by detergent insolubility, spectroscopic, and microscopy methods (*9*, *14*, *17*−*21*). In this report, we have used detergent insolubility and spectroscopy techniques to look at the effect of sterol structure on lipid raft formation in model membrane vesicles. Our previous studies have shown that sterols having structures with an ability to pack tightly with saturated lipids appear to be necessary for raft formation (*22*, *23*). However, the range of sterol structures that possess this property has not been fully explored. This question is of interest because its answer may reveal sterols that can be used to distinguish biological processes dependent on cholesterol itself and those that can be supported by any raft environment. Methods of cholesterol depletion, used in some studies to identify raft-dependent processes, do not distinguish between these alternatives (see Discussion).

In this report, we examine a number of sterols and steroids that differ from cholesterol by one or two chemical features. The results indicate that most small modifications of cholesterol structure have moderately deleterious effects on the ability to support raft formation. However, the shifted double bond found in lathosterol, an intermediate in cholesterol biosynthesis, seems to allow it promote raft formation more strongly than cholesterol. In the case of 7-ketocholesterol, a sterol with a physiologically important role in atherosclerosis (*24*), raft-stabilizing abilities were maintained, although packing with DPPC[1] was not as tight as for cholesterol. The implications of these results for sterol function and studies of rafts are discussed.

EXPERIMENTAL PROCEDURES

Materials. Diphenylhexatriene (DPH) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylcholine (DOPC),

* Corresponding author. Phone: 631-632-8564. Fax: 631-632-8575. E-mail: Erwin.London@stonybrook.edu.

[1] Abbreviations: DHE, dehydroergosterol; DOPC, dioleoylphosphatidylcholine; DPH, diphenylhexatriene; DPPC, dipalmitoylphosphatidylcholine; DRM, detergent-resistant membranes; OD, optical density; 12SLPC, 1-palmitoyl-2-(12-doxyl)stearoylphosphatidylcholine; SM, brain sphingomyelin.

10.1021/bi035696y CCC: $27.50
Published on Web 01/09/2004

1-palmitoyl-2-(12-doxyl)stearoylphosphatidylcholine (12SLPC), and brain sphingomyelin were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol and dehydroergosterol (DHE) were purchased from Sigma Chemical Co. (St. Louis, MO). All other sterols and steroids were purchased from Steraloids (Newport, RI).

The poly(Leu) peptide acetyl-KKGL_9WL_9KKA-amide was obtained and purified as previously described (*25*, *26*).

Sterol melting points measured in a capillary apparatus generally agreed with the values reported by the manufacturer within a few degrees. Sterol purity was confirmed by thin-layer chromatography of about 40 nmol samples on silica gel plates, using the solvent systems 22:3 or 1:1 (v/v) hexane/ethyl acetate. After the plates were sprayed with 3% (w/v) cupric acetate:8% (v/v) phosphoric acid, the plates were charred. At most, only minor impurities were detected.

Fluorescence Measurements. Fluorescence intensities were measured on a Spex fluorometer using a semi-micro quartz cuvette with a 10 mm excitation path length and 4 mm emission path length. DPH fluorescence was measured at an excitation wavelength of 358 nm and emission wavelength of 429 nm. DHE fluorescence was measured at room temperature at an excitation wavelength of 324 nm and emission wavelength of 376 nm. Unless otherwise noted, fluorescence was measured at room temperature.

Fluorescence Quenching Experiments. For experiments to detect quenching by 12SLPC, ethanol dilution vesicles were prepared as described previously (*27*). Lipids were mixed, dried, redissolved in 20 μL of ethanol, diluted 40-fold with (800 μL) PBS buffer (10 mM sodium phosphate, 2 mM potassium phosphate, 137 mM NaCl, 3 mM KCl, pH 7.4) that had been heated to 70 °C, briefly vortexed, and finally cooled to room temperature. The samples contained 40 nmol of phospholipid with or without 10 nmol of sterol/steroid and 0.2 nmol of DPH. Samples with quencher (*F* samples) contained either 20 nmol of DPPC and 20 nmol of 12SLPC or contained 20 nmol of DOPC and 20 nmol of 12SLPC. In corresponding samples without quencher (F_0 samples) DOPC replaced 12SLPC, so that they contained 20 nmol of DPPC and 20 nmol of DOPC or contained 40 nmol of DOPC, respectively. In some experiments SM was substituted for DPPC. Background samples were also prepared in which DPH was omitted. Background values were subtracted to give the reported fluorescence intensities.

To perform quenching experiments, the DPPC-containing *F* samples and F_0 samples were prepared in quadruplicate. Their fluorescence and that of the background samples and samples containing DOPC in place of DPPC were measured at 23 °C. After ensuring that the fluorescence intensity values in the individual DPPC-containing samples were reproducible (commonly within 5−10%), each set of quadruplicates was pooled into one *F* sample and one F_0 sample, respectively. The temperature dependence of the DPPC-containing samples was then measured. Samples were heated continuously at a rate of about 1.5 deg/min. An electronic thermometer was used to check sample temperature, and at the desired temperature fluorescence was quickly measured.

A similar protocol was used for SM-containing samples. However, samples in which DOPC replace DPPC show quenching that is relatively temperature independent (*27*, *28*), and so their fluorescence was only measured at 23 °C.

Samples of ethanol dilution vesicles were prepared similarly for experiments in which quenching by 7-ketocholesterol was measured. Lipids dissolved in 20 μL of ethanol were diluted to 1 mL with 10 mM sodium phosphate and 150 mM NaCl, pH 7. Samples contained a total of 200 μM nonfluorescent lipid. Fluorophores were present at an additional 0.5−2 mol %. Samples with quencher (*F* samples) contained 20 mol % 7-ketocholesterol, while quencher-lacking samples (F_0 samples) contained 20 mol % cholesterol instead. Backgrounds without fluorophore were prepared, and background values were subtracted from reported values. Fluorescence quenching measurements were performed at room temperature.

Percent Solubilization Experiments. Multilamellar lipid vesicles containing 500 nmol of total lipid were prepared in 950 μL of PBS as previously described (*22*). Samples contained 375 nmol of phospholipid and 125 nmol of sterol with or (in background samples) without an additional 10 nmol of DHE. The phospholipid was DPPC, DOPC, or a 1:1 (mol/mol) mixture of DPPC and DOPC. The lipids dissolved in organic solvent were mixed, dried with nitrogen, dissolved in 200 μL of chloroform, redried under nitrogen and then for 30 min at high vacuum, and finally dispersed in PBS that was heated to 70 °C. The samples were then mixed continuously for 15 min at 70 °C using an automatic vortexing machine. After the samples were cooled to room temperature, optical density at 400 nm was measured using a Beckman 650 spectrophotometer, and DHE fluorescence (or background fluorescence) was measured. Next, 50 μL of a 10% (v/v) Triton X-100 solution was added to each sample. After incubation at room temperature for 2 h, OD and fluorescence were remeasured. Each sample was then subjected to centrifugation at high speed for 15 min at ambient temperature. Almost all of the supernatant (generally over 950 μL) was then removed, and the pellet was dispersed by vortexing in 900−950 μL of PBS. The OD and fluorescence in the pellets and supernatants were then measured.

The values for the amount of DHE in the insoluble fraction were roughly corrected for the difference between the fluorescence intensity of a DHE molecule within a DPPC-rich ordered domain and that for a DHE molecule in a detergent micelle. This was estimated from the ratio of fluorescence intensity (I_p/I_s) in samples containing DPPC and sterol/steroid after the addition of Triton X-100 to that in samples containing DOPC and sterol/steroid after the addition of Triton X-100. The latter samples dissolve completely, while the former samples were in most cases almost fully insoluble (see Results). (Because there was some solubilization in DPPC, the correction factors used are slight underestimates. Furthermore, no correction was made for 4-cholesten-3-one, which did not form an insoluble mixture with DPPC.)

The fraction of DHE fluorescence in the pellet relative to total fluorescence (fr_{pellet}) is given by $fr_{pellet} = F_p/(F_s + F_p)$, where F_s is the intensity of fluorescence in the supernatant and F_p is the intensity of fluorescence in the pellet. The fraction of DHE molecules in the pellet ($fr_{DHEpellet}$) is given by $fr_{DHEpellet} = (F_p/I_p)/(F_s/I_s + F_p/I_p) = F_p/(F_s[I_p/I_s] + F_p)$, where I_p is the intensity of fluorescence per DHE molecule in the insoluble pellet, and the intensity per DHE molecule in the supernatant is defined as I_s. Combining these equations gives $fr_{DHEpellet} = 1/(1 + (1/fr_{pellet} - 1)[I_p/I_s])$. Substitution

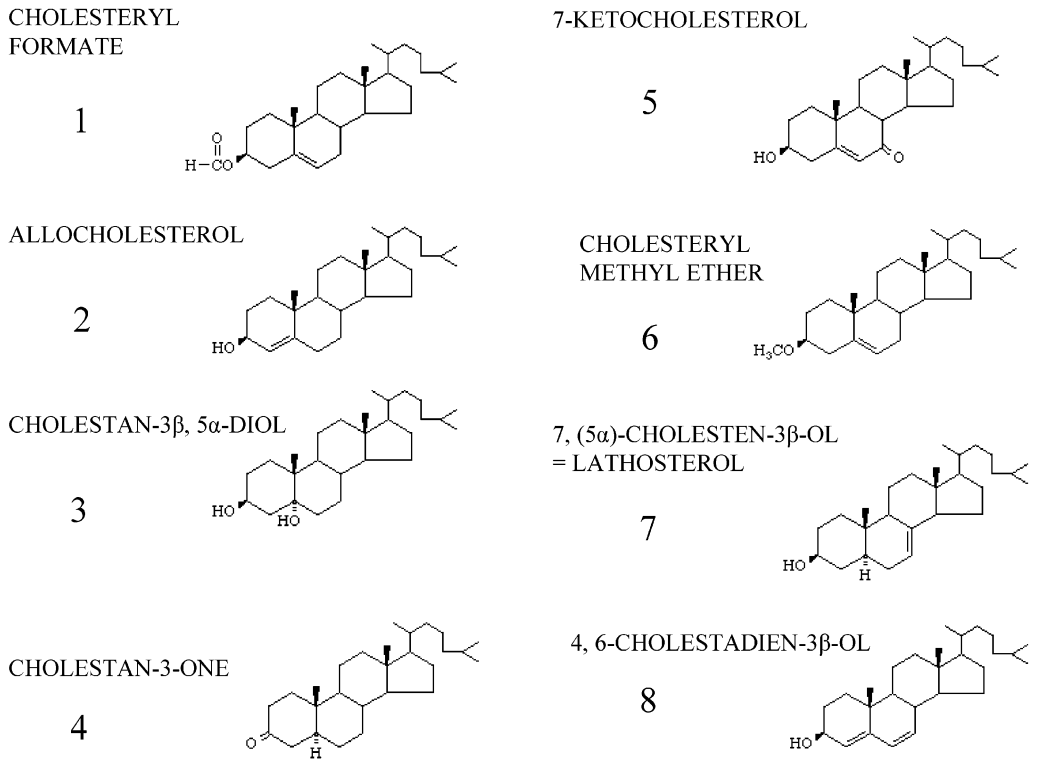

FIGURE 1: Chemical structures of sterols/steroids studied in this report. The numbers shown indicate the key for the *x*-axes in Figures 2 and 3.

of fr_{pellet} and I_p/I_s ratios can be used to solve this equation.

In most cases I_p/I_s values were in the range of 1.45−1.95. This is indicative of a higher intensity of DHE fluorescence in ordered bilayers relative to its intensity in micelles. The exceptions were 4-cholesten-3-one ($I_p/I_s = 0.98$), 7-ketocholesterol ($I_p/I_s = 0.66$), and 4,6-cholestadien-3β-ol ($I_p/I_s = 1.16$). These molecules must quench DHE fluorescence within bilayers. Upon solubilization in excess detergent, it appears that quenching is abolished due to dilution of the DHE and quenching sterol/steroid in the micelles.

Anisotropy Measurements. Fluorescence anisotropy measurements were made at 23 °C using a SPEX Glan-Thompson automated polarizer accessory. Samples were prepared as described for the insolubility experiments except samples were composed of 67 nmol of DPPC and 0.5 nmol of DPH with or without 33 nmol of sterol. The lipids were dispersed in 1 mL of PBS.

RESULTS

Choice of Cholesterol Analogues. The sterols and steroids used to study the relationship between molecular structure and raft formation are shown in Figure 1. Most of these molecules were chosen because they differ from cholesterol by a single chemical feature in the sterol ring portion of the molecule. The aim was to identify the features of the cholesterol molecule most and least critical for the stabilization of ordered lipid domains. Some molecules chosen had an altered polar group attached to the 3-position carbon atom (cholesterol methyl ether, cholesteryl formate, cholestanone), others had a shift in double bond position relative to that in cholesterol (lathosterol, allocholesterol), one had both an additional double bond and altered double bond positions (4,6-cholestadien-3β-ol), and two had an additional polar functional group attached to the sterol rings (7-ketocholesterol, cholestane-3β,5α-diol).

Effect of Sterol/Steroid Structure on the Level of Detergent-Resistant Membranes Isolated from Model Membrane Vesicles: Optical Density Experiments. The level of ordered domain (raft) formation in the presence of the cholesterol analogues was first studied using a detergent-insolubility assay. This method is based on the observation that ordered lipid domains tend to be insoluble in Triton X-100 while disordered fluid domains are dissolved by the detergent (*9*, *29*, *30*). As a result, the amount of detergent-resistant membrane (DRM) obtained from a preparation of model membrane vesicles after addition of Triton X-100 can closely reflect the amount of ordered domains that were present in the sample prior to addition of detergent. Previous studies have shown that this assumption is valid at 23 °C for model membrane vesicles containing the types of lipid mixtures used in this report (*9*, *23*).

The level of DRM obtained from model membrane vesicles was assayed by two methods. In the first, the light scattering (measured by optical density) remaining after the addition of detergent to a preparation of multilamellar vesicles was measured. Previous studies have shown that the percent optical density (% OD) remaining after detergent addition roughly approximates the percent DRM present for multilamellar vesicles (*23*).

Figure 2 shows the effect of Triton X-100 on percent OD values for mixtures of the sterols/steroids shown in Figure 1 with phospholipids. For comparison, these experiments were repeated with cholesterol and with 4-cholesten-3-one, a steroid that tends to destabilize ordered domain formation (*23*). The phospholipids examined were dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylcholine (DOPC), and a 1:1 (mol/mol) DPPC/DOPC mixture. The highest levels

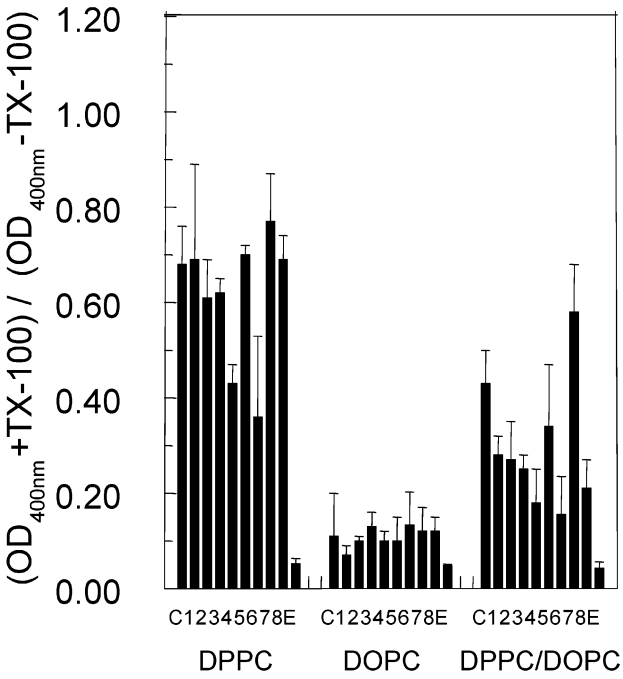

FIGURE 2: Insolubility of 3:1 (mol/mol) phospholipid/sterol or 3:1 (mol/mol) phospholipid/steroid mixtures as assayed by optical density (OD). *y*-axis shows the ratio of the OD after the addition of Triton X-100 to that prior to the addition of Triton X-100. Left series: Vesicles composed of DPPC and sterol/steroid. Middle series: Vesicles composed of DOPC and sterol/steroid. Right series: Vesicles composed of 1:1 DPPC/DOPC and sterol/steroid. The average from quadruplicate samples and standard deviation are shown. Values shown are not corrected for dilution with Triton X-100. Numbers along *x*-axis indicate sterol/steroid used as defined in Figure 1. C = cholesterol; E = 4-cholesten-3-one. Prior to solubilization samples (multilamellar vesicles) contained 500 nmol of total lipid. Vesicles were dispersed in 950 μL of PBS.

of insolubility were observed in mixtures of DPPC and the sterols/steroids (Figure 2, left). This insolubility indicates that the mixtures of DPPC and sterol/steroid were sufficiently tightly packed to resist solubilization by detergent and that an ordered state was present (*20*, *23*, *30*). This is not surprising because DPPC is a saturated lipid and at room temperature tends to form the tightly packed liquid-ordered state in the presence of high cholesterol concentrations. In contrast, vesicles composed of 4-cholesten-3-one mixed with DPPC were almost totally solubilized by detergent, in agreement with previous studies (*23*).

In contrast to the behavior of mixtures containing DPPC, only very low levels of insolubility were observed in mixtures of the unsaturated lipid DOPC with sterols/steroids (Figure 2, middle). This is as expected, because previous studies have demonstrated that mixtures of DOPC and cholesterol form the loosely packed liquid-disordered state and so are unable to resist solubilization by detergent (*10*).

The most interesting results were those in which sterols/steroids (25 mol %) were added to a 1:1 DPPC/DOPC mixture (Figure 2, right). In such cases, DPPC- (and sterol/steroid-) rich ordered domains can coexist with DOPC-rich disordered fluid domains (*9*, *22*, *23*). Under these conditions, insolubility should reflect the amount of ordered domains present (and to some degree the ability of such domains to resist solubilization by detergent). The highest percent OD values were observed for DPPC/DOPC mixtures containing lathosterol, and values were just slightly less for mixtures with cholesterol. This suggests that lathosterol has at least as strong an ability to support detergent-resistant ordered domain formation as does cholesterol. Mixtures with most of the other sterols/steroids exhibited insolubility that was significantly less than in the cholesterol-containing samples but more than in the samples lacking DPPC and/or containing 4-cholesten-3-one. This behavior indicates that, except for 4-cholesten-3-one, the sterols/steroids tested support the formation of separate DPPC-rich ordered domains to some degree.

We were concerned that percent OD would not exactly parallel insolubility (*30*, *31*). Therefore, a second insolubility assay was developed to try to confirm the percent OD results. To do this, 2 mol % dehydroergosterol (DHE) was added to the vesicles. Dehydroergosterol (DHE) is a fluorescent cholesterol analogue with properties that are fairly close to those of cholesterol (*32*, *33*). Like cholesterol, DHE should have a significant affinity for ordered domains (*33*), so that the amount of DHE isolated in the DRM fraction (as detected by DHE fluorescence) would roughly parallel DRM levels. (This assumes DHE affinity for ordered domains is not strongly dependent on the lipid composition of the domains.) Figure 3 shows the DRM levels in phospholipid mixtures with sterols/steroids as assessed by fluorescence. The values shown in Figure 3B have been roughly corrected for the apparent difference between the intensity of DHE molecules in ordered DPPC-rich domains and that of the same number of DHE molecules when dissolved in detergent (see Experimental Procedures for details). In general, the results closely parallel those obtained with percent OD. In particular, the fluorescence assay again showed that lathosterol and cholesterol were the sterols that most effectively promoted the formation of detergent-resistant ordered domains and that all of the other sterols/steroids except for 4-cholesten-3-one were able to support some degree of ordered domain formation.

Effect of Sterol/Steroid Structure on the Stability of Detergent-Resistant Membranes Isolated from Model Membrane Vesicles: Fluorescence Quenching Experiments. Despite the agreement between the two methods used to assay detergent insolubility, it still was possible that there might be significant detergent-induced perturbations of the equilibria between ordered and disordered domains when assessed by the amount of material that was detergent-insoluble (*30*, *34*). Therefore, the participation of sterols/steroids in domain formation by DPPC and the relative stability of the ordered domains formed in mixtures containing DPPC and sterol/steroid were assessed spectroscopically using intact vesicles. This was done by measuring the quenching of the fluorescence of diphenylhexatriene (DPH) incorporated into model membrane vesicles composed DPPC, 12SLPC [a fluorescence quenching lipid with behavior similar to that of DOPC (*9*)], and sterol/steroid. When DPPC and 12SLPC are mixed, they tend to form separated DPPC-rich ordered domains and 12SLPC-rich disordered fluid domains. Due to 12SLPC-induced quenching, fluorophores residing within 12SLPC-rich domains fluoresce weakly, whereas those in the DPPC-rich domains, which contain relatively little 12SLPC, fluoresce more strongly (*35*−*37*). Since DPH associates to a significant degree with DPPC-rich domains (*9*, *35*, *38*), it is quenched less in vesicles containing a mixture of DPPC-rich and 12SLPC-rich do-

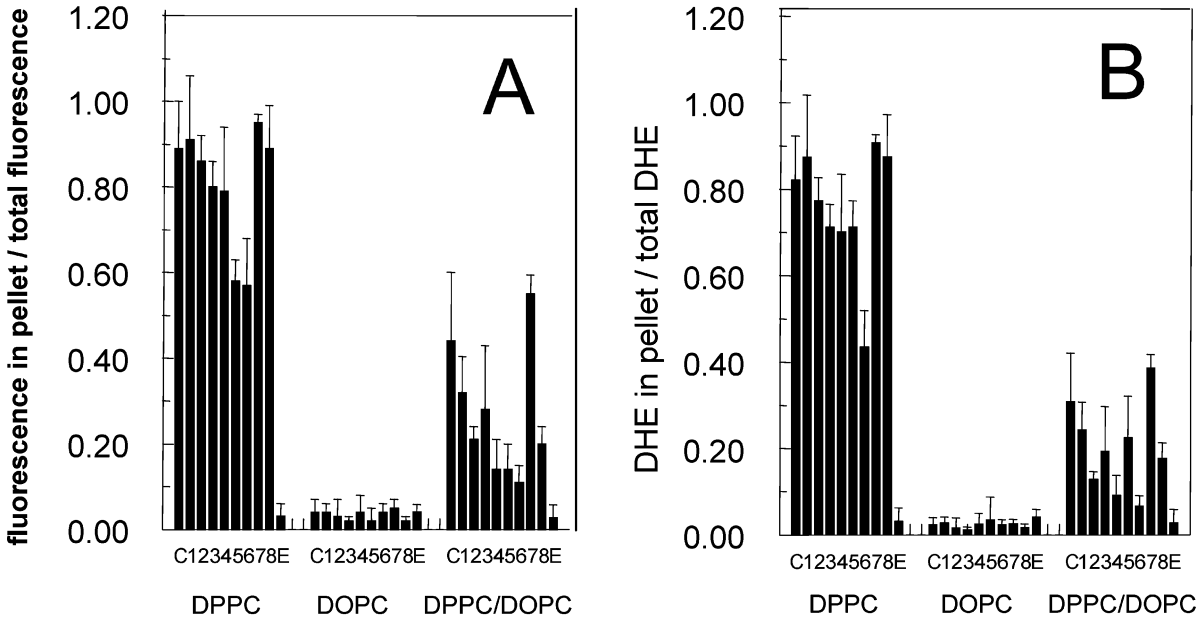

FIGURE 3: Insolubility of 3:1 (mol/mol) phospholipid/sterol or 3:1 (mol/mol) phospholipid/steroid mixtures in Triton X-100 as assayed by the fraction of DRM-associated DHE fluorescence. (A) Ratio of DHE fluorescence in the pellet collected from centrifugation to the total DHE fluorescence in the samples as a function of sterol/steroid. (B) Ratio of DHE in the pellet to total DHE in the samples after correction of fluorescence values for the difference between DHE fluorescence per DHE molecule in micelles and that in DRM. Left series: Vesicles composed of DPPC and sterol/steroid. Middle series: Vesicles composed of DOPC and sterol/steroid. Right series: Vesicles composed of 1:1 DPPC/DOPC and sterol/steroid. The average of quadruplicate experiments and standard deviation are shown. Numbers along *x*-axis indicate sterol/steroid used as defined in Figure 1. C = cholesterol; E = 4-cholesten-3-one. Samples are the same as in Figure 2.

mains than it is in homogeneous (domain-lacking) vesicles containing the same overall amount of 12SLPC (*9*, *39*).

In domain-forming lipid mixtures, the temperature dependence of quenching can reveal the thermal stability of ordered domains. When DPPC-rich domains are present, they bind DPH so that its fluorescence is relatively weakly quenched by 12SLPC. However, at temperatures above that at which DPPC-rich domains are stable all lipids mix in a relatively homogeneous fashion, and quenching increases because DPH molecules come into increased contact with 12SLPC molecules. The midpoint of a quenching vs temperature curve (the apparent "melting" temperature) is a measure of raft stability (*23*, *40*). We have previously used this method to assess the thermal stability of ordered domain formation by DPPC and sterols in a number of studies (*22*, *23*, *27*).

Figure 4A shows the thermal dependence of DPH fluorescence quenching for 1:1 (mol/mol) mixtures of DPPC and 12SLPC in the presence of various sterols/steroids. For comparison, samples with no sterol and cholesterol were also included. Even in the absence of sterols, there is some formation of DPPC-rich domains at 23 °C, but domain formation is abolished by 37 °C. [The melting temperature of these domains in the absence of sterol is significantly lower than that of pure DPPC (41 °C) because the ordered domains are not pure DPPC but rather a DPPC-rich mixture with some 12SLPC.] In agreement with our previous studies, the addition of cholesterol stabilizes domain formation significantly, and the midpoint of the melting of the DPPC- (and cholesterol-) rich domains increases by about 5 °C. Most of the sterols and steroids tested had a weak stabilizing effect on domain formation but less so than cholesterol. None fell within the ordered domain-destabilizing class we had previously identified (i.e., sterols/steroids that depress the domain melting temperature to values *below* those observed in the absence of sterol). Figure 4A also shows that in one case,

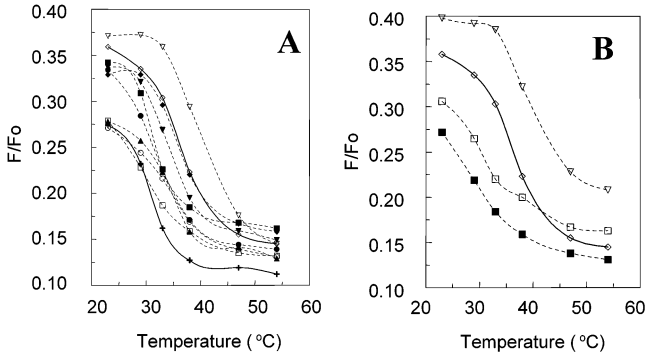

FIGURE 4: Assay of the thermal dependence of ordered domain formation via quenching of DPH fluorescence in lipid mixtures. (A) Ratio of DPH fluorescence in vesicles composed of a 1:1 (mol/mol) mixture of DPPC/12SLPC to DPH fluorescence in control vesicles composed of a 1:1 (mol/mol) mixture of DPPC/DOPC (F_0). Samples contained (+) no sterol or 20 mol % (◇) cholesterol, (●) cholesteryl formate, (○) allocholesterol, (△) cholestane-3β,5α-diol, (■) cholestan-3-one, (◆) 7-ketocholesterol, (▼) cholesterol methyl ether, (▽) lathosterol, and (□) 4,6-cholestadien-3β-ol. Solid lines are drawn through data for samples without sterol and with cholesterol for emphasis. (B) Ratio of DPH fluorescence in vesicles composed of a 1:1 (mol/mol) mixture of SM/12SLPC with or without 20 mol % sterol/steroid (F) to DPH fluorescence in control vesicles composed of a 1:1 (mol/mol) mixture of SM/DOPC with or without 20 mol % sterol/steroid (F_0). Symbols are as in panel A. F/F_0 values for samples in which DPPC or SM was replaced by DOPC (i.e., in cases in which ordered domains do not form) generally fell in the range 0.10−0.15. Samples (ethanol dilution vesicles) contained 40 nmol of phospholipid with or without 10 nmol of sterol/steroid. Vesicles were dispersed in 800 μL of PBS.

that of 7-ketocholesterol, DPPC-rich domains formed that were as stable as those in the presence of cholesterol. In another case, that of lathosterol, the DPPC-rich domains formed were even more stable than those formed in the presence of cholesterol. The quenching experiments with lathosterol and cholesterol were repeated in samples in which

Table 1: Fluorescence Anisotropy of 2:1 (mol/mol) DPPC/Sterol or DPPC/Steroid Vesicles Dispersed in PBS at 52 °C[a]

sterol/steroid	anisotropy
none (36 °C)	0.30 ± 0.011
none	0.08 ± 0.001
cholesterol	0.22 ± 0.007
cholesteryl formate	0.20 ± 0.006
allocholesterol	0.20 ± 0.003
cholestane-3β,5α-diol	0.18 ± 0.004
cholestan-3-one	0.20 ± 0.014
7-ketocholesterol	0.16 ± 0.007
cholesterol methyl ether	0.16 ± 0.003
lathosterol	0.23 ± 0.008
4,6-cholestadien-3β-ol	0.20 ± 0.011
4-cholesten-3-one	0.16 ± 0.005

[a] Average values and standard deviation for triplicate samples are shown.

sphingomyelin (SM) replaced DPPC. As shown in Figure 4B lathosterol also stabilizes SM-rich ordered domain formation better than cholesterol does. In contrast, as in the case of DPPC-rich domains, cholestan-3-one and 4,6-cholestadien-3β-ol stabilized SM-rich domains less than cholesterol.

It should also be noted that comparison of insolubility and quenching data shows that the ordered domains that form in the absence of sterol are not detergent insoluble. This reflects the sensitivity of gel state domains to solubilization at temperatures close to their melting temperatures. The reason for this behavior is discussed elsewhere (*30*).

Fluorescence Anisotropy Measurements in Mixtures of DPPC and Sterols/Steroids. Polarization/anisotropy measurements can be used to compare the level of order in lipid mixtures. Previous studies showed that those sterols able to promote the formation of separate DPPC-rich domains in lipid mixtures also tend to form more highly ordered mixtures with DPPC than sterols that do not promote the formation of separate domains (*23*). These experiments were repeated for mixtures of DPPC and the sterols/steroids examined in this study, measuring the fluorescence anisotropy of DPH incorporated into the lipid mixtures (Table 1). For comparison, anisotropy values are shown for DPPC by itself at 36 °C (gel state), DPPC by itself at 52 °C (liquid-disordered state), and DPPC/cholesterol mixtures at 52 °C (liquid-ordered state). As found in previous studies, anisotropy/polarization values are highest in the gel state, lowest in the liquid-disordered state, and intermediate in the liquid-ordered state (at 52 °C).

When mixed with DPPC, most of the sterols/steroids tested exhibited anisotropy values at 52 °C falling below those for DPPC/cholesterol mixtures (although significantly higher than the values in the absence of sterol/steroid). However, anisotropy values in DPPC/lathosterol mixtures were as high as those in DPPC/cholesterol mixtures, consistent with the ability of lathosterol to form mixtures with DPPC that are as tightly packed as those formed by DPPC and cholesterol. Overall, anisotropy is not very closely correlated with insolubility levels. This is probably due to two factors: (1) in addition to motion, steady-state anisotropy is dependent upon the lifetime of the DPH excited state; (2) anisotropy in these mixtures only reflects the interaction of sterols and DPPC whereas domain formation in the ternary lipid mixtures we studied is affected by the interaction of sterol with both DPPC and DOPC (or 12SLPC).

Table 2: Fluorescence Quenching in Vesicles Containing 7-Ketocholesterol[a]

lipid mixture containing quencher	F/F_0
8:2 DOPC/7-KC + 2 mol % DHE	0.37 ± 0.003
4:4:2 DPPC/DOPC/7-KC + 2 mol % DHE	0.29 ± 0.0
8:2 DOPC/7-KC + 2 mol % poly(Leu) peptide	0.59 ± 0.037
4:4:2 DPPC/DOPC/7-KC + 2 mol % poly(Leu) peptide	0.75 ± 0.024
8:2 DOPC/7-KC + 0.5 mol % DPH	0.99 ± 0.06
4:4:2 DPPC/DOPC/7-KC + 0.5 mol % DPH	0.92 ± 0.025

[a] Samples composed of 200 μM nonfluorescent lipid dispersed in 10 mM phosphate and 150 mM NaCl, pH 7. 7-KC = 7-ketocholesterol. F_0 samples contained cholesterol in place of 7-ketocholesterol. Average values and range of duplicates are shown.

Fluorescence Quenching Properties of 7-Ketocholesterol. In the course of control experiments we found that 7-ketocholesterol has fluorescence quenching properties (see Experimental Procedures). There are few good cholesterol analogues for spectroscopic studies of lipid rafts. Therefore, we decided to investigate the fluorescence quenching properties of 7-ketocholesterol in more detail.

Table 2 shows the effect of 7-ketocholesterol on the fluorescence of molecules incorporated into model membrane vesicles. Strong quenching of the fluorescence of DHE and of the Trp fluorescence of a transmembrane poly(Leu)-class polypeptide was observed in vesicles containing 20 mol % 7-ketocholesterol. DPH fluorescence was not quenched. This is consistent with the origin of quenching being energy transfer from the fluorophores to 7-ketocholesterol, which has a weak absorbance band (extinction coefficient at 320 nm = 70 M^{-1} cm^{-1}) that overlaps the emission bands of DHE and Trp but not that of DPH.

Quenching by 7-ketocholesterol in DPPC/DOPC mixtures tends to confirm its preferential location in DPPC-rich domains. The poly(Leu) peptide, which is excluded from DPPC-rich ordered domains (*27*), was more weakly quenched in a DPPC/DOPC/7-ketocholesterol mixture than in a DOPC/7-ketocholesterol mixture. This is expected if the 7-ketocholesterol is mainly located in the DPPC-rich domains. In contrast, DHE was more strongly quenched by 7-ketocholesterol in the DPPC/DOPC/7-ketocholesterol mixture than in a DOPC/7-ketocholesterol mixture. This is the predicted result if both DHE and 7-ketocholesterol are concentrated within DPPC- (and sterol-) rich domains, as expected for sterols that have cholesterol-like behavior.

DISCUSSION

Features of Sterols Important for Ordered Domain (Raft) Formation: Polar Group Structure. The hydroxyl group attached to the 3-position carbon of cholesterol has long been recognized as being critical to cholesterol function. We found that small modifications of the cholesterol hydroxyl group (adding a methyl or formate group or changing it to a keto function) had a deleterious effect on the ability to promote ordered domain formation. On the other hand, even with these changes these steroids promoted the formation of ordered domains to some degree. It should be noted that the nature of the change at the 3-OH position has a marked effect on the degree to which raft formation is altered. Our previous experiments showed that epicholesterol, in which the 3-OH

group has a α instead of a β configuration, has only a small effect on raft stability, while a charged sulfate group makes cholesterol sulfate a raft-destabilizing agent (23). We conclude that a free 3-OH group is important, but not absolutely necessary, for the promotion of ordered domain formation by steroids.

This conclusion is supported by the quenching data. The most striking example was cholesterol methyl ether. Despite its inability to support the formation of detergent-insoluble domains as well as cholesterol, or to form as highly ordered a state as cholesterol when mixed with DPPC (as judged by anisotropy), cholesterol methyl ether was able to stabilize the formation of DPPC-rich domains (as judged by fluorescence quenching) almost as well as cholesterol. In other words, domains rich in DPPC and cholesterol methyl ether form readily, presumably due to favorable DPPC−cholesterol methyl ether interactions, even though the insolubility data indicate that these domains do not seem to be as tightly packed as those formed by DPPC and cholesterol.

Features of Sterols Important for Ordered Domain Formation: Effect of Double Bond Position and Number. The position and number of double bonds within the sterol rings also affect raft formation to a significant degree. Ordered domain formation is promoted most strongly when the double bond is between carbons 7 and 8, as in lathosterol, rather than positions 5 and 6, as in cholesterol. Furthermore, the ability to promote raft formation was significantly weakened relative to cholesterol when the double bond spanned carbons 4 and 5 as in allocholesterol.

Another interesting observation is that 4,6-cholestadien-3β-ol, a sterol with double bonds spanning carbons 4−5 and carbons 6−7, did not promote raft formation as well as cholesterol even though the combination of these double bonds should flatten out the part of the B ring between carbon 5 and carbon 6 in a fashion similar to the 5−6 double bond of cholesterol. These results are in stark contrast to previous studies showing that two double bonds can be very strongly raft promoting when they span carbons 5−6 and 7−8 (*22*). They reinforce the idea that the ability of sterols to support ordered domain formation is very sensitive to double bond position. (One caveat should be mentioned: 4,6-cholestadien-3β-ol contained a minor impurity, and this may have had some effect on its ability to stabilize raft formation.)

The marked effect of the double bond position is somewhat surprising, because any double bonds in the rings should help to make sterols more planar, a feature believed to be important for imparting the properties critical for allowing sterols to pack tightly with saturated lipids. In addition, since the saturated and relatively flat cholestanol molecule, which lacks double bonds, promotes ordered domain formation as well as cholesterol (*23*), it might have been predicted that additional or shifted double bonds within the sterol rings would have little additional effect on the ability to form rafts. Since this is not the case, it appears that the fine details of ring conformation must be important for raft formation.

Features of Cholesterol Important for Ordered Domain Formation: Effect of Extra Polar Groups on Sterol Rings. An extra hydroxyl group added to the steroid ring at the 5-position also decreased the ability of sterol to form detergent-resistant ordered domains. This could involve a combination of steric effects that weaken packing with phospholipids and/or changes in orientation of the sterol rings within the bilayer. Such changes might be necessary to place both the 3-position and 5-position OH groups close to the bilayer surface.

In contrast, 7-ketocholesterol was able to stabilize the formation of DPPC-rich domains about as well as cholesterol, although DPPC/DOPC mixtures with 7-ketocholesterol did not exhibit quite as much insolubility as those with cholesterol, and 7-ketocholesterol was unable to form as tightly packed bilayers when mixed with DPPC, as judged by anisotropy. In this regard, its behavior was similar to that of cholesterol methyl ether (see above). The similarities and differences between 7-ketocholesterol and cholesterol are consistent with previous studies performed on 7-ketocholesterol/DOPC mixtures. A cholesterol-like condensing effect and cholesterol-like reduction in bilayer permeability due to 7-ketocholesterol were observed, although the change in permeability in egg phosphatidylcholine-containing vesicles was less than that observed with cholesterol (*41*).

Cumulative Effects of Multiple Modifications upon Raft Promotion Activity. Combining the results in this study with previous studies (*22*, *23*) suggests that (at least in some cases) when single modifications of cholesterol structure are combined, they can have nearly additive effects on the ability of sterols/steroids to promote the formation of ordered domains. For example, by themselves 3-keto and 4−5 double bonds both partly negate the ability of sterol to promote raft formation, but 4-cholesten-3-one, which contains both of these modifications, actually inhibits the ability of saturated lipid domains to form ordered domains. Another example is 7-dehydrocholesterol, which contains both the 5−6 and 7−8 double bonds and which [as shown previously (*22*)] promotes raft formation to a greater degree than either cholesterol or lathosterol, each of which has one of these double bonds. Such additive behavior is of interest because it may allow the design of various sterols with precisely engineered raft-promoting activities (see below).

Implications for Sterol Function in Cells. The observation in this study that lathosterol, a biosynthetic precursor of cholesterol, is able to stabilize both DPPC-rich and SM-rich rafts more efficiently than cholesterol is of particular interest. It should be noted that this conclusion is supported by a very recent study of binary mixtures of lathosterol and SM, in which an increase in melting temperature relative to cholesterol/SM mixtures was observed. It was concluded from that data that lathosterol would stabilize rafts more efficiently than cholesterol (*42*). In addition, another very recent study found that in mammalian cells the steady-state fraction of lathosterol isolated in DRM was similar to the fraction of cholesterol in DRM and that newly synthesized lathosterol reached the cellular compartments from which DRM arise even more quickly than cholesterol (*43*). These results suggest that lathosterol is at least as strongly raft associated as cholesterol in cells.

Combined with our previous observation that 7-dehydrocholesterol, a cholesterol precursor after lathosterol in the cholesterol biosynthesis pathway, also stabilizes rafts more efficiently than cholesterol (*22*), these results suggest that the reason biosynthesis proceeds all the way to cholesterol cannot be to synthesize a sterol that efficiently forms rafts. In other words, the importance of cholesterol structure is likely to involve more than its ability to form lipid rafts.

This is consistent with the observation that severe disease results in mammals when cellular sterol biosynthesis cannot proceed beyond lathosterol (*44*).

Use of 7-Ketocholesterol as a Fluorescence Quencher. The behavior of 7-ketocholesterol was of additional interest because it was found to be a fluorescence quencher. In model membrane vesicles, 7-ketocholesterol may be a good spectroscopic probe for association of at least some types of fluorescent molecules (including proteins) with rafts. It may be especially valuable because there are relatively few good cholesterol analogues (other than DHE) for spectroscopic studies. Most cholesterol-based probes have attached probe groups that are large enough to strongly perturb cholesterol properties (*33*). As shown in Results, it should be possible to use 7-ketocholesterol in conjunction with DHE (as an energy transfer donor) to look at sterol clustering in bilayers. It should also be noted that low 7-ketocholesterol concentrations are sufficient to obtain significant quenching levels. We estimate <10 mol % 7-ketocholesterol should be sufficient in many experiments. At this level it could be used in mixtures with cholesterol, further reducing any 7-ketocholesterol perturbation of raft properties. It is also noteworthy that both the weak 7-ketocholesterol absorbance band and the moderate quenching induced at high 7-ketocholesterol concentrations are consistent with quenching that is very short range (calculation not shown). This means that quenching by 7-ketocholesterol will require direct contact or near contact with fluorescent molecules. Such short-range quenching is useful because it can be used to evaluate the binding of fluorescent molecules to quenchers without interference from quenchers not in contact with fluorophore.

Implications for Strategies To Distinguish Cholesterol-Dependent from Raft-Dependent Phenomena. It would be useful to be able to distinguish biological processes dependent upon cholesterol itself from those that were raft-dependent. For example, there are some α virus−cell membrane fusion processes that require cholesterol, and although these fusion events may occur within rafts in cells (*45*), they do not seem to absolutely require rafts (*46*). The cholesterol depletion/sequestration methods widely used to disrupt rafts, such as the use of saponins, nystatin, or methyl-β-cyclodextrins, are not well suited for distinguishing between the effects of cholesterol and the effects of lipid rafts. Both cholesterol-dependent and raft-dependent processes should be inhibited by such methods.

One possible approach to this problem is to identify sterols that can maintain rafts but which differ sufficiently from cholesterol to disrupt highly specific protein−cholesterol interactions. Cholesterol-dependent biological processes that are not supported by such molecules are unlikely to be only raft-dependent. In part, the present study aimed at identification of the chemical features of sterols necessary for stabilizing raft formation in order to identify suitable candidates for such studies. Both lathosterol and 7-ketocholesterol may be useful in this regard. They have sufficient raft-promoting abilities to distinguish sterol−protein interactions that require sterol molecules having a 5−6 double bond, or that cannot tolerate substitutions at the 7-position, from those interactions only requiring raft formation. We are presently searching for other raft-promoting sterols useful for such experiments.

REFERENCES

1. Brown, D. A., and London, E. (2000) *J. Biol. Chem. 275*, 17221−17224.
2. Manes, S., del Real, G., and Martinez, A. C. (2003) *Nat. Rev. Immunol. 3*, 557−568.
3. Grassme, H., Jendrossek, V., Riehle, A., von Kurthy, G., Berger, J., Schwarz, H., Weller, M., Kolesnick, R., and Gulbins, E. (2003) *Nat. Med. 9*, 322−330.
4. Gulbins, E., and Grassme, H. (2002) *Biochim. Biophys. Acta 1585*, 139−145.
5. Simons, K., and Toomre, D. (2000) *Nat. Rev. Mol. Cell Biol. 1*, 31−39.
6. Ehehalt, R., Keller, P., Haass, C., Thiele, C., and Simons, K. (2003) *J. Cell Biol. 160*, 113−123.
7. Yamabhai, M., and Anderson, R. G. (2002) *J. Biol. Chem. 277*, 24843−24846.
8. Ge, M., Field, K. A., Aneja, R., Holowka, D., Baird, B., and Freed, J. H. (1999) *Biophys. J. 77*, 925−933.
9. Ahmed, S. N., Brown, D. A., and London, E. (1997) *Biochemistry 36*, 10944−10953.
10. Schroeder, R., London, E., and Brown, D. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12130−12134.
11. Brown, D. A., and London, E. (1998) *J. Membr. Biol. 164*, 103−114.
12. Almeida, P. F., Vaz, W. L., and Thompson, T. E. (1992) *Biochemistry 31*, 6739−6747.
13. Rubenstein, J. L., Smith, B. A., and McConnell, H. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 15−18.
14. Dietrich, C., Bagatolli, L. A., Volovyk, Z. N., Thompson, N. L., Levi, M., Jacobson, K., and Gratton, E. (2001) *Biophys. J. 80*, 1417−1428.
15. Kahya, N., Scherfeld, D., Bacia, K., Poolman, B., and Schwille, P. (2003) *J. Biol. Chem. 278*, 28109−28115.
16. Drevot, P., Langlet, C., Guo, X. J., Bernard, A. M., Colard, O., Chauvin, J. P., Lasserre, R., and He, H. T. (2002) *EMBO J. 21*, 1899−1908.
17. Schroeder, R. J., Ahmed, S. N., Zhu, Y., London, E., and Brown, D. A. (1998) *J. Biol. Chem. 273*, 1150−1157.
18. Feigenson, G. W., and Buboltz, J. T. (2001) *Biophys. J. 80*, 2775−2788.
19. Samsonov, A. V., Mihalyov, I., and Cohen, F. S. (2001) *Biophys. J. 81*, 1486−1500.
20. Li, X. M., Momsen, M. M., Smaby, J. M., Brockman, H. L., and Brown, R. E. (2001) *Biochemistry 40*, 5954−5963.
21. Veatch, S. L., and Keller, S. L. (2002) *Phys. Rev. Lett. 89*, 268101.
22. Xu, X., Bittman, R., Duportail, G., Heissler, D., Vilcheze, C., and London, E. (2001) *J. Biol. Chem. 276*, 33540−33546.
23. Xu, X., and London, E. (2000) *Biochemistry 39*, 843−849.
24. Jessup, W., Wilson, P., Gaus, K., and Kritharides, L. (2002) *Vasc. Pharmacol. 38*, 239−248.
25. Lew, S., and London, E. (1997) *Anal. Biochem. 251*, 113−116.
26. Ren, J., Lew, S., Wang, J., and London, E. (1999) *Biochemistry 38*, 5905−5912.
27. Fastenberg, M. E., Shogomori, H., Xu, X., Brown, D. A., and London, E. (2003) *Biochemistry 42*, 12376−12390.
28. London, E., Brown, D. A., and Xu, X. (2000) *Methods Enzymol. 312*, 272−290.
29. Brown, D. A., and Rose, J. K. (1992) *Cell 68*, 533−544.
30. London, E., and Brown, D. A. (2000) *Biochim. Biophys. Acta 1508*, 182−195.
31. Partearroyo, M. A., Urbaneja, M. A., and Goni, F. M. (1992) *FEBS Lett. 302*, 138−140.
32. Hale, J. E., and Schroeder, F. (1982) *Eur. J. Biochem. 122*, 649−661.
33. Mukherjee, S., Zha, X., Tabas, I., and Maxfield, F. R. (1998) *Biophys. J. 75*, 1915−1925.
34. Heerklotz, H., Szadkowska, H., Anderson, T., and Seelig, J. (2003) *J. Mol. Biol. 329*, 793−799.
35. London, E., and Feigenson, G. W. (1981) *Biochim. Biophys. Acta 649*, 89−97.
36. Dibble, A. R., Yeager, M. D., and Feigenson, G. W. (1993) *Biochim. Biophys. Acta 1153*, 155−162.
37. Feigenson, G. W. (1983) *Biochemistry 22*, 3106−3112.
38. Lentz, B. R., Barenholz, Y., and Thompson, T. E. (1976) *Biochemistry 15*, 4529−4537.
39. London, E. (2002) *Curr. Opin. Struct. Biol. 12*, 480−486.
40. London, E., Brown, D. A., and Xu, X. (2000) *Methods Enzymol. 312*, 272−290.

41. Theunissen, J. J., Jackson, R. L., Kempen, H. J., and Demel, R. A. (1986) *Biochim. Biophys. Acta 860*, 66−74.
42. Nyholm, T. K., Nylund, M., and Slotte, J. P. (2003) *Biophys. J. 84*, 3138−3146.
43. Lusa, S., Heino, S., and Ikonen, E. (2003) *J. Biol. Chem. 278*, 19844−19851.
44. Krakowiak, P. A., Wassif, C. A., Kratz, L., Cozma, D., Kovarova, M., Harris, G., Grinberg, A., Yang, Y., Hunter, A. G., Tsokos, M., Kelley, R. I., and Porter, F. D. (2003) *Hum. Mol. Genet. 12*, 1631−1641.
45. Ahn, A., Gibbons, D. L., and Kielian, M. (2002) *J. Virol. 76*, 3267−3275.
46. Waarts, B. L., Bittman, R., and Wilschut, J. (2002) *J. Biol. Chem. 277*, 38141−38147.

BI035696Y