Characterization of Cholesterol-Sphingomyelin Domains and Their Dynamics in Bilayer Membranes

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ABSTRACT Lipids segregate with each other into small domains in biological membranes, which can facilitate the associations of particular proteins. The segregation of cholesterol and sphingomyelin (SPM) into domains known as rafts is thought to be especially important. The formation of rafts was studied by using planar bilayer membranes that contained rhodamine-phosphatidylethanolamine (rho-DOPE) as a fluorescent probe, and wide-field fluorescence microscopy was used to detect phase separation of the probe. A fluorescently labeled GM_1, known to preferentially partition into rafts, verified that rho-DOPE faithfully reported the rafts. SPM-cholesterol domains did not form at high temperatures but spontaneously formed when temperature was lowered to below the melting temperature of the SPM. Saturated acyl chains on SPMs therefore promote the formation of rafts. The domains were circular (resolution ≥ 0.5 μm), quickly reassumed their circular shape after they were deformed, and merged with each other to create larger domains, all phenomena consistent with liquid-ordered (l_o) rather than solid-ordered (s_o) domains. A saturated phosphatidylcholine (PC), disteoryl-PC, could substitute for SPM to complex with cholesterol into a l_o-domain. But in the presence of cholesterol, a saturated phosphatidylethanolamine or phosphatidylserine yielded s_o-domains of irregular shape. Lipids with saturated acyl chains can therefore pack well among each other and with cholesterol to form l_o-domains, but domain formation is dependent on the polar headgroup of the lipid. An individual raft always extended through both monolayers. Degrading cholesterol in one monolayer with cholesterol oxidase first caused the boundary of the raft to become irregular; then the raft gradually disappeared. The fluid nature of rafts, demonstrated in this study, may be important for permitting dynamic interactions between proteins localized within rafts.

INTRODUCTION

Interactions between all lipids are not equal. Lipids that attract and/or pack with each other more effectively should naturally separate into domains within membranes. The significance of this physical chemical phenomenon for cell biological membranes is increasingly appreciated. Rather than expect that lipids remain homogeneously distributed within biological membranes, one should expect that domains spontaneously form. Domains rich in cholesterol and sphingomyelin have been a subject of great interest recently in cell biology because some important integral membrane proteins may be preferentially located within them. Such domains that form around the protein caveolin, referred to as caveolae, have been unambiguously shown to exist (Anderson, 1998). When caveolin is not present, the domains are known as rafts; their existence is controversial.

It was suggested a half-century ago, based on x-ray diffraction and polarized light studies of myelin sheath of nerve, that cholesterol molecules complex with phospholipids and/or cerobrosides (Finean, 1953). By the early 1970s it had been shown that cholesterol and sphingomyelin (SPM) do, in fact, preferentially interact with each other in model membranes (Oldfield and Chapman, 1971, 1972; Long et al., 1971). This was followed by explorations of whether ordered domains of cholesterol and SPM exist in biological membranes (Goodsaid-Zalduondo et al., 1982), the demonstration that cholesterol must be present for capping of surface immunoglobulins on lymphocytes (Hoover et al., 1983), and the realization that some proteins might concentrate into microscopic domains rich in glycosphingolipids (Thompson and Tillack, 1985). The properties of phase-separated cholesterol-sphingolipid-rich domains that form in model lipid systems has continued to be investigated intensively by various biophysical techniques (e.g., Calhoun and Shipley, 1979; Estep et al., 1979, 1981; McIntosh et al., 1992; Smaby et al., 1994; Maulik and Shipley, 1996; Veiga et al., 2001).

The existence of rafts in plasma membranes is routinely inferred from the fact that at low temperature (4°C), the solubilization of cell membranes by non-ionic detergents such as Triton X-100 yields two fractions: the detergent-resistant membranes (DRMs) rich in sphingolipids and cholesterol as well as the detergent-soluble fraction (Simons and Ikonen, 1997). Some membrane proteins, such as GPI-anchored proteins and doubly acylated kinases of the Src family, are preferentially found in the DRMs (Low, 1989; Brown and London, 1997; Scheiffele et al., 1997; Schroeder et al., 1998; Zhang et al., 2000). If these proteins preferentially reside in rafts in the natural state, it would mean that rafts promote protein-protein interactions, and this in turn would be important for cellular processes such as cell signaling, transduction, and intracellular trafficking (Brown and London, 1998; Baird et al., 1999). But DRMs are not observed when the detergent solubilization is performed at room or physiological temperatures (Hoessli and Rungger-Brandle, 1985; Brown and Rose, 1992). Because the

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existence of a phase at one temperature does not imply its existence at another, it is still an open question as to whether rafts exist in biological membranes. However, several experimental approaches have been used to suggest that they do exist (Simons and Toomre, 2000), but they are probably spatially small, dynamic entities controlled by the principle of mass action (Kenworthy and Edidin, 1998; Kenworthy et al., 2000; Pralle et al., 2000).

By studying domains in model membrane systems, their physical chemistry can be isolated from a multitude of other processes. The occurrence and properties of domains can be monitored with lipid probes. The use of fluorescent lipid probes is particularly convenient because they allow direct visualization of domains large enough to be resolved microscopically. Heterogeneous distributions of phospholipids, sphingomyelin (SPM), and cholesterol have been extensively studied by microscopy for lipid monolayers (von Tscharner and McConnell, 1981; Hagen and McConnell, 1996; Worthman et al., 1997). In these monolayers, in the absence of compression, large domains are observed consisting of cholesterol and SPM or alternatively cholesterol and phosphatidylcholine (PC) that has saturated acyl chains. But these domains often disperse when the monolayers are compressed (Mattjus et al., 1995) to pressures that are still well below those of model phospholipid bilayers and biological membranes (Demel et al., 1975; Israelachvili et al., 1980; MacDonald and Simon, 1987). Therefore, the behavior of domains in monolayers may not be the same as that in bilayer membranes. While this paper was under review, another appeared that shows that microscopically observable rafts of cholesterol and SPM form not only in monolayers but in lipid bilayer membranes as well (Dietrich et al., 2001).

In this study, we investigated the nature of rafts by microscopy to determine their basic physical-chemical properties. We used planar bilayer membranes and wide-field fluorescence microscopy to study domain formation for membranes containing SPM and cholesterol in a background of the phospholipids dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylethanolamine (DOPE). Rhodamine-DOPE (rho-DOPE) was chosen as probe because its acyl chains match those of the phospholipids, and thus it should be freely miscible in them. Phase separation was explored for different SPMs, at temperatures above and below the melting temperature (T_m) of the SPM. Rafts occurred for temperatures below, but not above, the melting temperature of the SPM. We subjected these rafts to a series of tests to determine their phase, requirements for formation, and interactions between monolayers.

MATERIALS AND METHODS

Materials

Cholesterol, phospholipids, egg-SPM (e-SPM), and rho-DOPE were purchased from Avanti Polar Lipids (Birmingham, AL). Squalene was obtained from ICN Biomedicals (Aurora, OH). Hexane, the hemisynthetic *N*-stearoyl-sphingomyelin, *N*-oleoyl-SPM, and cholesterol oxidase (from *Streptomyces sp.*) were purchased from Sigma (St. Louis, MO).

Synthesis of BODIPY-GM_1

N-(BODIPY-FL-propionyl)-neuraminosyl-GM_1 (referred to as BODIPY-GM_1) was synthesized by *N*-acylating de-acetyl-GM_1. De-acetyl-GM_1 was obtained by alkaline hydrolysis using 1 M tetramethylammonium hydroxide in *n*-butanol-water, 9:1 at 100°C as described (Sonnino et al., 1985). De-acetyl-GM_1 was *N*-acylated by the method of mixed anhydride (Acquotti et al., 1986). Briefly, 10 μmol of BODIPY-FL-propionic acid (Molecular Probes, Eugene, OR) was dissolved in dried chloroform, and 10 μmol of triethylamine and 10 μmol of *iso*-butylchlorformate were then added. The reaction mixture was stirred for 1 h at room temperature, then evaporated, dissolved in 3 ml of ethylacetate, and washed with 2 ml of distilled water followed by a wash in a saturated NaCl solution. It was then stored for 2 h over anhydrous sodium sulfate. The water was evaporated, and the remaining mixed anhydride of BODIPY-FL-pentanoic acid was dissolved in 1 ml of tetrahydrofuran. This solution was added to the solution of 4 μmol of de-acetyl-GM1 dissolved in 2 ml of tetrahydrofuran/water (10:1) with 5 μmol of triethylamine. The reaction mixture was stirred overnight at room temperature. The BODIPY-GM_1 was isolated and purified by silica gel 100 column chromatography in chloroform/methanol/water, 65:25:2 (v/v/v). The yield of the final compound was 1.2 μmol (30%); it was a red powder. The ^{1}H-NMR spectra verified that it was BODIPY-GM_1.

^{1}H-NMR spectra (500 MHz) of the final compound were recorded in $^2H_4$-methanol at 300 K on a Bruker DRX-500 pulse spectrometer operating in the Fourier transform mode. The pulse width was 9 μs, the acquisition time was 1.3 s, and the number of transients was 128. Signals were assigned by placing the central signal of methanol at 3.47 ppm. The ^{1}H NMR signals (CD_3OD) were δ: 2.44 (s, 3H), 2.66 (s, 3H), 4.46 (d, J = 8 Hz, ^{1}H), 4.57 (d, J = 8 Hz, ^{1}H), 4.60 (d, J = 8 Hz, ^{1}H), 5.08 (d, J = 8 Hz, ^{1}H), 5.61 (m, J = 8 Hz, ^{1}H), 5.84 (m, J = 8 Hz, ^{1}H), 6.34 (s, ^{1}H), 6.53 (d, J = 4 Hz, ^{1}H), 7.19 (d, J = 4 Hz, ^{1}H), 7.56 (s, ^{1}H). We thus conclude that the final compound has one BODIPY-FL-C3 residue per molecule of the ganglioside GM_1.

Planar membrane formation

Horizontal bilayers were formed from a solution of DOPC/DOPE (2:1) and cholesterol/SPM (1:1) in the range of 10–25 mol % each and 5 mol % rho-DOPE in squalene. Any impurities contained by the squalene were removed by passing it through a column of activated aluminum oxide. Planar membranes were formed by a brush technique in a 150–200-μm diameter hole in Teflon film and were bathed by symmetrical solutions of 140 mM NaCl, 2.5 mM KCl, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM HEPES, pH 7. The temperature of the solution was maintained with a temperature controller (20/20 Technology, Wilmington, NC). The membranes were prepared above the T_m of the SPM employed: 41°C for e-SPM, 54°C for *N*-18:0-SPM, and room temperature for *N*-18:1-SPM (Boggs and Koshy, 1994; Cevc and Marsh, 1987; Ramstedt and Slotte, 1999a). The bilayers were voltage-clamped, and capacitance was determined from admittance measurements (Ratinov et al., 1998).

Fluorescence microscopy and physical manipulation of domains

The horizontal bilayer chamber was mounted on a stage of an inverted fluorescence microscope (Diaphot, Nikon, Garden City, NY). A neutral density filter attenuated the excitation light to minimize photobleaching. A standard filter set was used to monitor the fluorescence of rho-DOPE

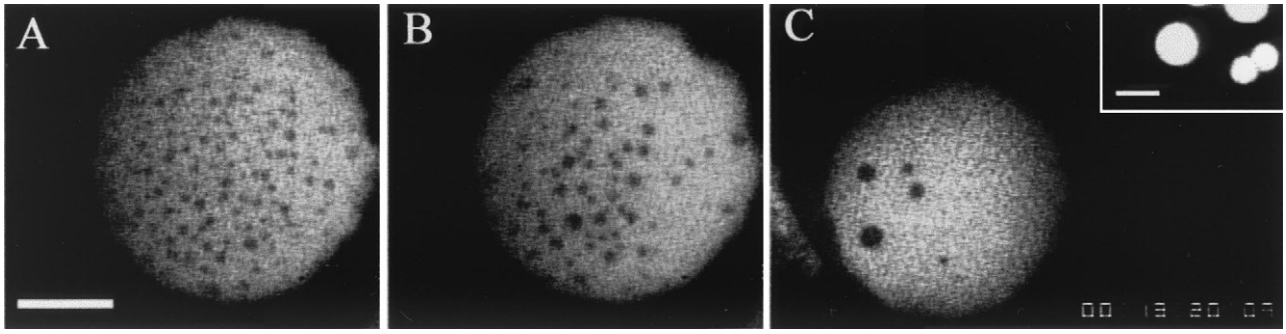

FIGURE 1 The formation of rafts in DOPC/DOPE (2:1) planar bilayer membranes containing e-SPM/cholesterol (15 mol % each) and rho-DOPE (5 mol %). A field diaphragm was narrowed so that only the central portion of the bilayer was illuminated. A. The rho-DOPE was excluded from small domains after the temperature was lowered to 25°C, below the T_m of e-SPM. Scale bar, 50 μm. B. Dark domains merged, and at a later time the domains were larger but fewer in number. C. Circular domains at higher magnification. The inset shows, at the same magnification, 2.5 and 4 μm fluorescent microspheres. Scale bar, 4 μm.

(excitation 510–560 nm, dichroic mirror 580 nm, emission > 590 nm). Suitable filter sets were used to monitor BODIPY and 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD). Fluorescence was monitored with a video camera (SIT-66; Dage MTI, Indianapolis, IN) set at maximum gain, and images were continuously recorded to videotape. We used a long-working-distance objective (25×, NA 0.4; Nikon) for forming the planar bilayers and following the evolution of sufficiently large domains. An oil immersion objective lens (63×, NA 1.25; Carl Zeiss) was used to detect and/or observe small (sub-micron) domains. The field diaphragm aperture was generally reduced to its minimum size to prevent light from the highly fluorescent Gibbs-Plateau border (the torus supporting the bilayer to the Teflon partition) from reaching the camera. The entire bilayer is thus generally not seen in the figures; the surrounding areas are black because of the minimized diaphragms. The sizes of domains were estimated by comparing their spatial extent with those of standard fluorescent microspheres that were 2.5 and 4 μm in diameter (Molecular Probes, Eugene, OR).

Domains were physically deformed in one of two ways. In the first, a solution with the same composition as that bathing the membrane was ejected, for ~1 s, from a small pipette. The pipette was positioned with a micromanipulator so that the fluid would be ejected directly across the domain of interest. This caused the domain to both deform and move relative to the bilayer. In the second, a patch pipette (with electrode connection to an amplifier) was brought into contact at the boundary of a large domain. After a giga-seal was established, the pipette was moved horizontally. This deformed the boundary without translating the domain with respect to the remainder of the bilayer.

RESULTS

Low temperatures promoted formation of domains that were in a liquid-ordered phase

A SPM was included in a membrane-forming solution containing rho-DOPE with the cholesterol/SPM ratio fixed at 1:1 (mol:mol). The particular SPM used depended on the experiment as stipulated below. The lipid rho-DOPE was chosen as the fluorescent probe for several reasons: it has the same acyl chains as DOPC and DOPE; it partitions preferentially into expanded liquid-disordered (l_d) bilayers; and it is largely excluded from condensed solid-ordered (s_o) and liquid-ordered (l_o) domains (Hagen and McConnell, 1996; Radhakrishnan and McConnell, 1999, 2000; Radhakrishnan et al., 2000; Mattjus and Slotte, 1996). Planar bilayer membranes were formed at temperatures above the T_m of the SPM employed; the fluorescence of rho-DOPE was uniform over the membrane (data not shown). After the bilayer reached a quasi-equilibrium as determined by a stable membrane capacitance, the temperature of the solutions bathing the bilayer was lowered to promote phase separations. We did not systematically explore what temperature the membrane had to be lowered to for domains to form; although we lowered temperature to below T_m, there is no theoretical basis for assuming that phase separation should occur precisely at that temperature. With less than 10 mol % cholesterol (plus 10 mol % SPM), phase separation was not observed (the fluorescence of the bilayer remained uniformly bright) after lowering of temperature. For 15 mol % cholesterol/SPM or greater, dark circular domains appeared against the uniform membrane brightness when the solutions were cooled to below T_m (Fig. 1). When 20 mol % cholesterol was included in the bilayer in the absence of SPM or 20 mol % SPM was included but cholesterol omitted, dark domains did not form when the same temperature-lowering protocol was followed. Domain formation clearly required the presence of both cholesterol and SPM. The dark domains moved faster than other portions of the bilayer when the bathing solutions were stirred. This suggests that the domains extended into the aqueous solutions (i.e., into the unstirred layers). Any added friction between the protruding domain and water should be negligible compared with the resistance to domain movement within the bilayer (Chizmadzhev et al., 1999) because the viscosity of the bilayer is about two orders of magnitude greater than that of water. Therefore, the viscous force against motion should be the same for a protruding and non-protruding domain, but the applied stirring force should be greater for the protruding one.

How were we to distinguish whether the domains excluding the rho-DOPE (i.e., the dark domains) were in

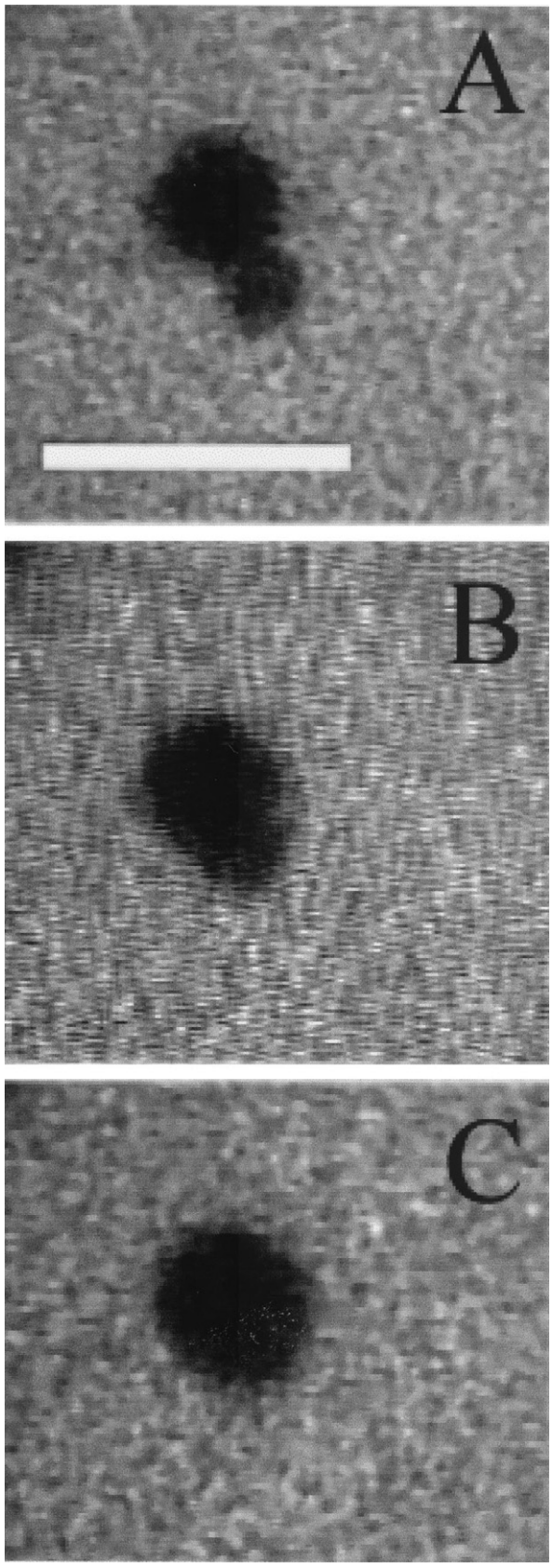

FIGURE 2 The merger of two dark domains. The region of the bilayer in which two domains merge is shown. (*A*) Domains have contacted each other. (*B*) Domains have merged. (*C*) The merged domain has assumed a circular shape. *B* is three video frames (100 ms) after *A*; *C* is two video frames (70 ms) after *B*. Scale bar, 50 μm.

liquid phase or in solid phase? We devised four means to determine domain phase, and the results of these tests suggest that the domains were in a l_o phase rather than in a s_o phase. First, the very fact that the shapes of the domains were circular indicates that they are liquid rather than in a frozen phase. (Circular domains have been observed in giant unilamellar vesicles (GUVs) (Dietrich et al., 2001). They appear oval due to the projection of a spherical liposome onto a flat surface.) Second is their ability to interact: the domains readily merged with each other (Fig. 2), with 30 ms (one video frame) to 100 ms between contact of the domains and their merger. (The merger of liquid lipid domains was previously demonstrated for lipid monolayers (Lee et al., 1994).) The time constant for two ~10-μm merged domains to relax from a figure eight to a circle was on the order of tens of milliseconds. Third, the elastic deformation properties were characteristic of a liquid rather than a solid: when a stream of solution was ejected from a small pipette to pass over a circular domain, the domain moved along with the stream while deforming, leaving a tail behind (Fig. 3 *A*). When the stream was stopped, the tail quickly withdrew back into the head of the domain (Fig. 3 *B*), which returned to its circular shape. This reveals that the line tension at the boundary minimized the perimeter of the dark domain. The minimization of perimeter and merger of area is expected of fluid, but not of solid domains (Weis and McConnell, 1984; Dietrich et al., 2001). Fourth was a more controlled test of elastic deformation: after small domains merged, a glass patch-pipette was used to deform the large domain. A tight seal between the pipette and the domain was established at its boundary and the pipette was displaced within the plane of the membrane so as to deform the domain without translating it along the bilayer (Fig. 4 *A*, the bright pipette is pulling the large dark domain at the top of the panel toward the bottom). The shape of the deformed portion of the boundary was observed to be two smooth arcs curving into the interior of the domain and joining at the tip of the pipette. When the domain detached from the pipette (Fig. 4 *B*), the dark area reassumed its circular form (Fig. 4 *C*). This behavior is a visco-elastic characteristic of a liquid, not a solid. All four tests indicate that the dark domain was a l_o phase. It is possible, however, that small, undetectable s_o phases coexisted within the l_o phase.

The partitioning of GM_1 into the dark domains verifies that these domains are rafts

How can we be certain that the dark domains from which rho-DOPE was excluded were in fact rafts? The ganglioside

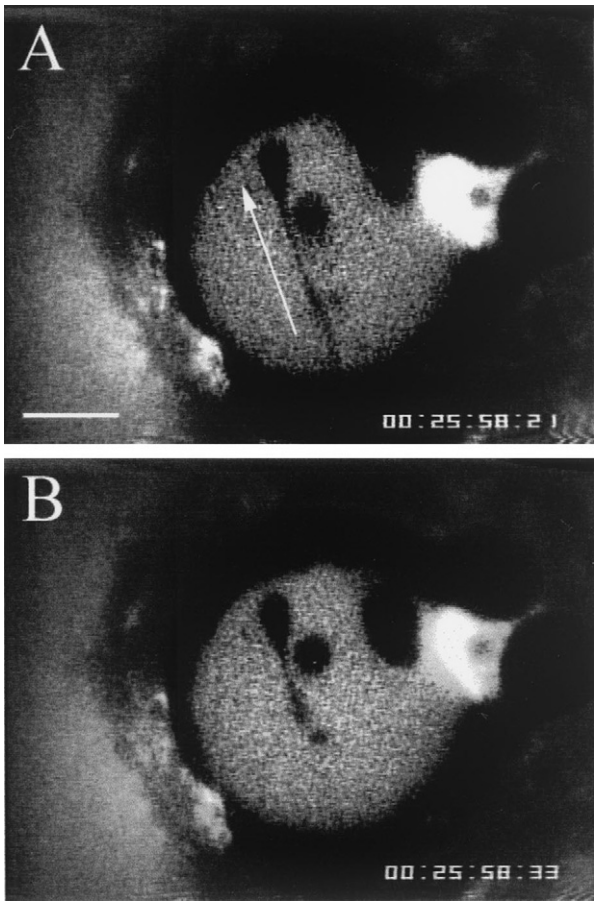

FIGURE 3 Domains are fluid. (*A*) A stream of solution, direction shown by arrow, was passed over a bilayer at the location of a domain for ~1 s. The circular domain moved within the bilayer as it was swept along with the stream. The domain did not move as a rigid body, but rather left behind a tethered tail. Scale bar, 50 μm. (*B*) When the flow of solution was halted, the tail was drawn back into the head of the domain, and the domain eventually reassumed a circular shape. The line tension minimized the perimeter of the domain of constant area.

GM_1 preferentially partitions into domains of cholesterol and sphingolipids that form in liposomes (Dietrich et al., 2001) and is found in DRMs isolated from cells (Harder et al., 1998). We determined whether GM_1 preferentially partitioned into the dark domains. We attached the fluorescent probe BODIPY to the headgroup of GM_1 (see Materials and Methods). DOPC/DOPE/cholesterol/SPM bilayers were formed that contained both rho-DOPE and BODIPY-GM_1 as probes. The two probes could be viewed independently. When viewing the BODIPY fluorescence, bright circular domains were observed, demonstrating accumulation of GM_1 (Fig. 5 *A*). These same domains were dark when viewing rho-DOPE (Fig. 5 *B*). The complementary dark and bright areas observed with the two dyes show that the dark domains observed with rho-DOPE were SPM/cholesterol rafts.

Choosing a probe to routinely study rafts

One might expect to be able to use a SPM with its acyl chain fluorescently labeled to probe for rafts. But this is not the case because the label alters the acyl chain. Both NBD-labeled C_6-SPM and BODIPY-labeled C_5-SPM yielded dark domains, showing that these SPM probes did not partition into rafts. (The sphingosine of various SPMs do not vary, and therefore SPMs have only one varying acyl chain, the labeled chain.) This is in agreement with recent findings of others (Wang and Silvius, 2000) that these probes do not partition into rafts. The melting temperatures of SPMs with the probes attached are not known. If their melting temperatures were well above those of the experiments, they would be expected to be excluded from rafts containing a SPM below its T_m. We found that, similarly, cholesterol with NBD attached to the terminal side chain was also excluded from rafts (data not shown). Thus, attaching bulky fluorophores to the acyl chains of SPM or to the side chain of cholesterol can prevent them from tightly interacting with the SPM and cholesterol within rafts. We could thus use either rho-DOPE or BODIPY-GM_1 to identify rafts. Because rho-DOPE was brighter than BODIPY-GM_1 and commercially available, we used it routinely to characterize the occurrence and properties of rafts.

Because the rafts form only at low temperatures, we performed the reverse procedure to see whether they would disappear above T_m (the temperature below which we could create rafts). When the temperature was increased to above T_m (i.e., to 46°–48°C for a membrane containing e-SPM) after the rafts were created, the smaller (less than 10–20 μm in diameter) rafts disappeared within minutes of raising the temperature (Fig. 6, *A–C*), showing that the SPM and cholesterol dissolved into the bilayer. Some of the large SPM/cholesterol rafts (>50 μm diameter) became smaller (none became larger), but they did not disappear during the life of the membrane (as long as 2 h) (Fig. 6, *D* and *E*). The overall effect of raising the temperature was thus to reduce the percentage of membrane area composed of SPM/cholesterol rafts.

Raft formation is facilitated by saturated acyl chains

Rafts occurred for e-SPM or *N*-stearoyl-SPM within the bilayer. *N*-stearoyl-SPM has a saturated tail; e-SPMs have heterogeneous tails, but the overwhelming majority are saturated (16:0) (Avanti Polar Lipids 1995 catalog). In contrast, membranes containing *N*-oleoyl-SPM (N-18:1-SPM) with an unsaturated acyl chain did not yield rafts even when the temperature was lowered to 5°C. (This may not be a low enough temperature to cause phase separation; *N*-oleoyl-SPM is still in the liquid state at 10°C (Li et al., 2000).) The sphingolipid GM_1 from the source we used (bovine brain) has predominantly saturated acyl chains (both 16:0 and 18:0 (Cherayil, 1968)), and it partitions into rafts. But when we

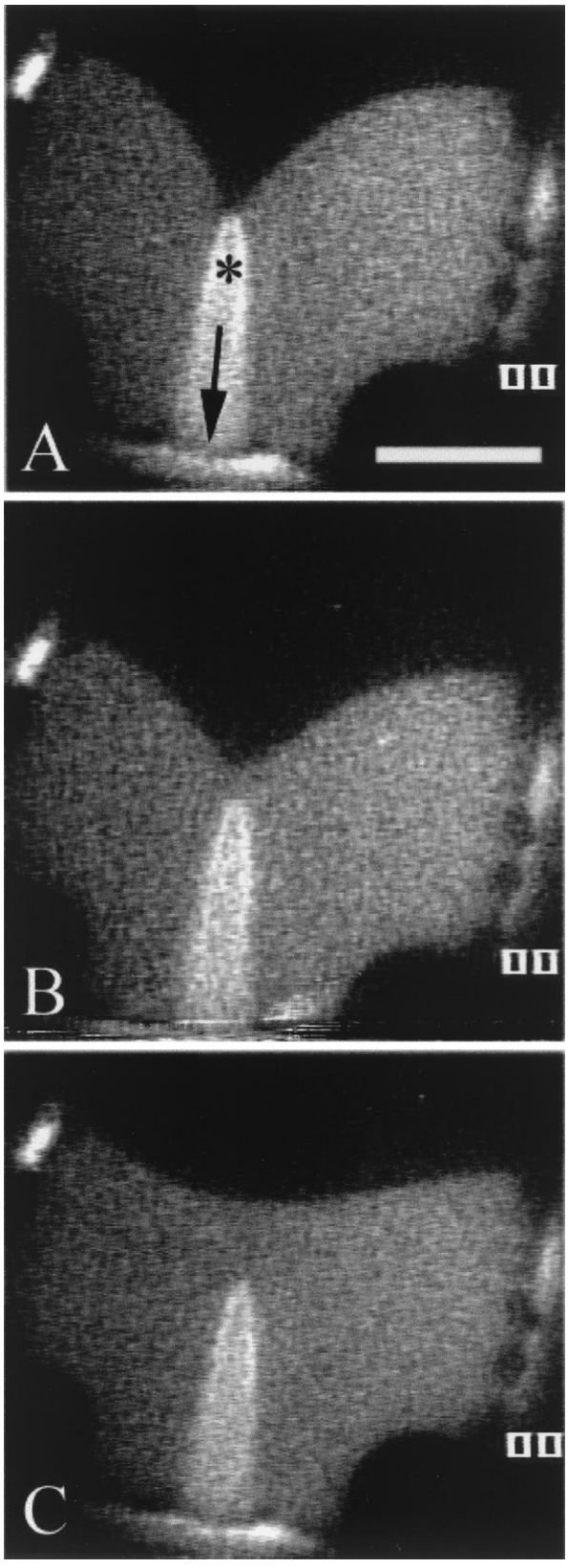

FIGURE 4 Domains are deformable. (*A*) A glass pipette (the bright object marked by an asterisk) was adhered to the edge of a large circular domain (the black region above the pipette) and pulled at 3–5 μm/s in the direction indicated by the arrow. (Only a segment of the domain can be seen in the image.) The domain distorted from its formerly circular shape. Scale bar, 50 μm. (*B*) The pipette was withdrawn from the bilayer, and the domain started to reassume its circular shape. The images of *A* and *B* were separated by five video images (165 ms). (*C*) At 300 ms after image *B*, the domain has again become circular.

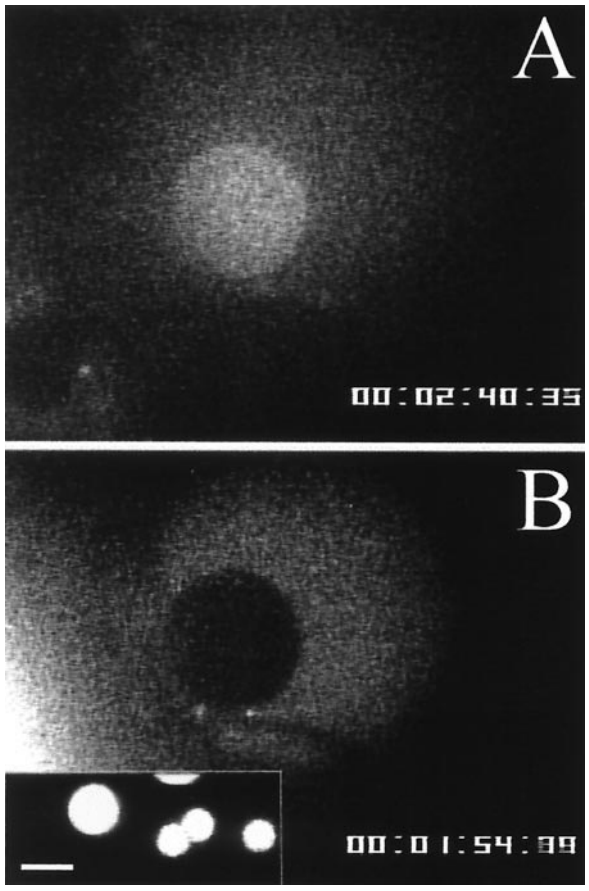

FIGURE 5 The simultaneous measurement of two fluorescent probes identifies the domains to be rafts. (*A*) The bright BODIPY-GM_1 fluorescence shows that the circular domain is a raft. (*B*) The probe rho-DOPE is excluded from the raft; (*Inset*) The 2.5- and 4.0-μm fluorescent microspheres are shown with the same objective (63×, 1.25 N.A.) as *A* and *B*. The weakly fluorescent BODIPY-GM_1 was observable with this objective but could not be readily observed with the 25×, 0.4 N.A. objective used when observing the entire bilayer. Scale bar, 4 μm.

used it alone as the sphingolipid rather than as the probe, it did not form separate domains with cholesterol: when 15 mol % GM_1 (and cholesterol) was included in the membrane formation solution, the fluorescence of both BODIPY-GM_1 and rho-DOPE was uniform. The ability of SPM to complex with sterols into domains may depend on several chemical features of the sterols: epicholesterol complexed with e-SPM to form l_o domains, but coprostanol did not (data not shown).

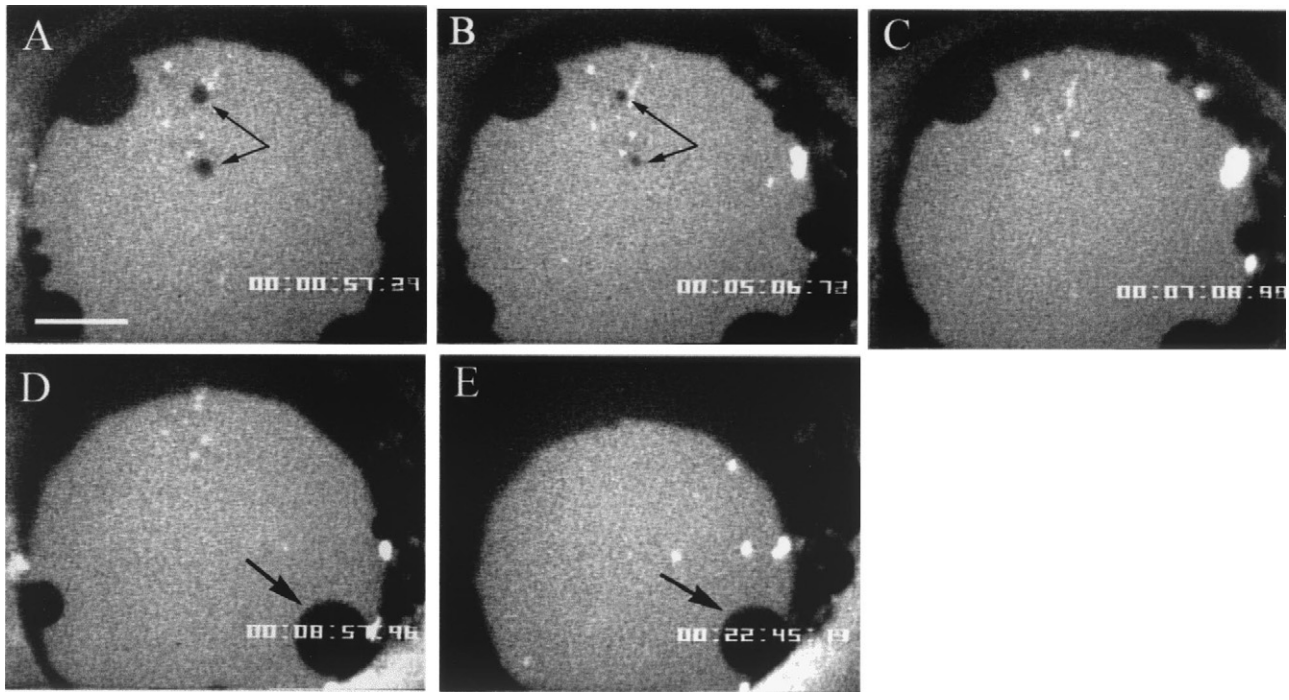

FIGURE 6 Rafts can dissolve at temperatures above the T_m of the SPM. (*A–C*) Two rafts, shown by arrows in *A*, have become smaller in *B* and have disappeared by *C*. (*D* and *E*) The large domains tended to accumulate at the Gibbs-Plateau border. The microscope stage supporting the bilayer chamber was moved at *D* to bring the large dark raft marked by the arrow into better view. The other large dark domain (upper left of *A*, *B*, and *C*) remained but cannot be seen in the illustrated field of view. The times after raising temperature were 59 s (*A*), 5 min (*B*), 7 min (*C*), 9 min (*D*), and 23 min (*E*). Scale bar, 50 μm.

High concentrations of SPM and cholesterol yield a more dynamic system

At 15 and 20 mol % SPM and cholesterol concentrations, the rafts enlarged by merging with each other. When the concentrations were increased in the membrane-forming mixture to 25 mol % each, the enlargement of rafts became more dynamic. The rafts could spontaneously enlarge without merger and they could become so large as to surround phospholipid regions. For temperatures above T_m, the membranes were still uniformly bright as occurred for lower SPM concentrations, and upon lowering of temperature, dark domains formed in the same manner (Fig. 7 *A*). But dark domains tended to continue to form with time. Dark domains became larger by merging with each other but were also observed to spontaneously enlarge without merger (Fig. 7 *B*), suggesting that SPM and cholesterol in the background continually partitioned into the rafts. The enlargement of the dark domains could become so extensive that regions of the membrane appeared as bright circular domains within a dark background (Fig. 7, *C–F*). That is, rather than dark rafts within a bright phospholipid bilayer, one could observe domains of lipid containing rho-DOPE surrounded by dark domains of rafts. The bright domains merged with each other, although contacting bright domains took longer to do so than did contacting dark domains. Immediately after merger, the boundary of the bright domain moved to assume a circular shape. This time was a factor of 2–3 greater than for merged dark domains to assume a circular shape. Eventually, the exceptionally large bright regions coexisted with massive dark regions (Fig. 7, *C* and *D*).

Cholesterol and a saturated PC form l_o phase domains

Cholesterol interacts not only with sphingolipids, but also with saturated PCs to form a l_o phase (Sankaram and Thompson, 1991; Slotte and Mattjus, 1995; Xu and London, 2000). As we found for rafts (Fig. 6), the l_o phase can continue to exist and remain separate from the l_d phase at temperatures above T_m for a DPPC membrane rich in cholesterol (Sankaram and Thompson, 1991). Because of these and other similarities (e.g., the same headgroups), we explored the ability of cholesterol and PCs to form domains.

Bilayers containing 30 mol % cholesterol in a DOPC/DOPE background yielded uniform fluorescence at all temperatures; there were no phase separations. The T_m of DOPC is low (−12°C); the absence of domain formation may have been due to the lack of sufficient reduction of temperature or because DOPC is an unsaturated lipid. We included 15 mol % of the saturated PC distearoylphospatidylcholine (DSPC), with its high T_m (55°C), along with 15

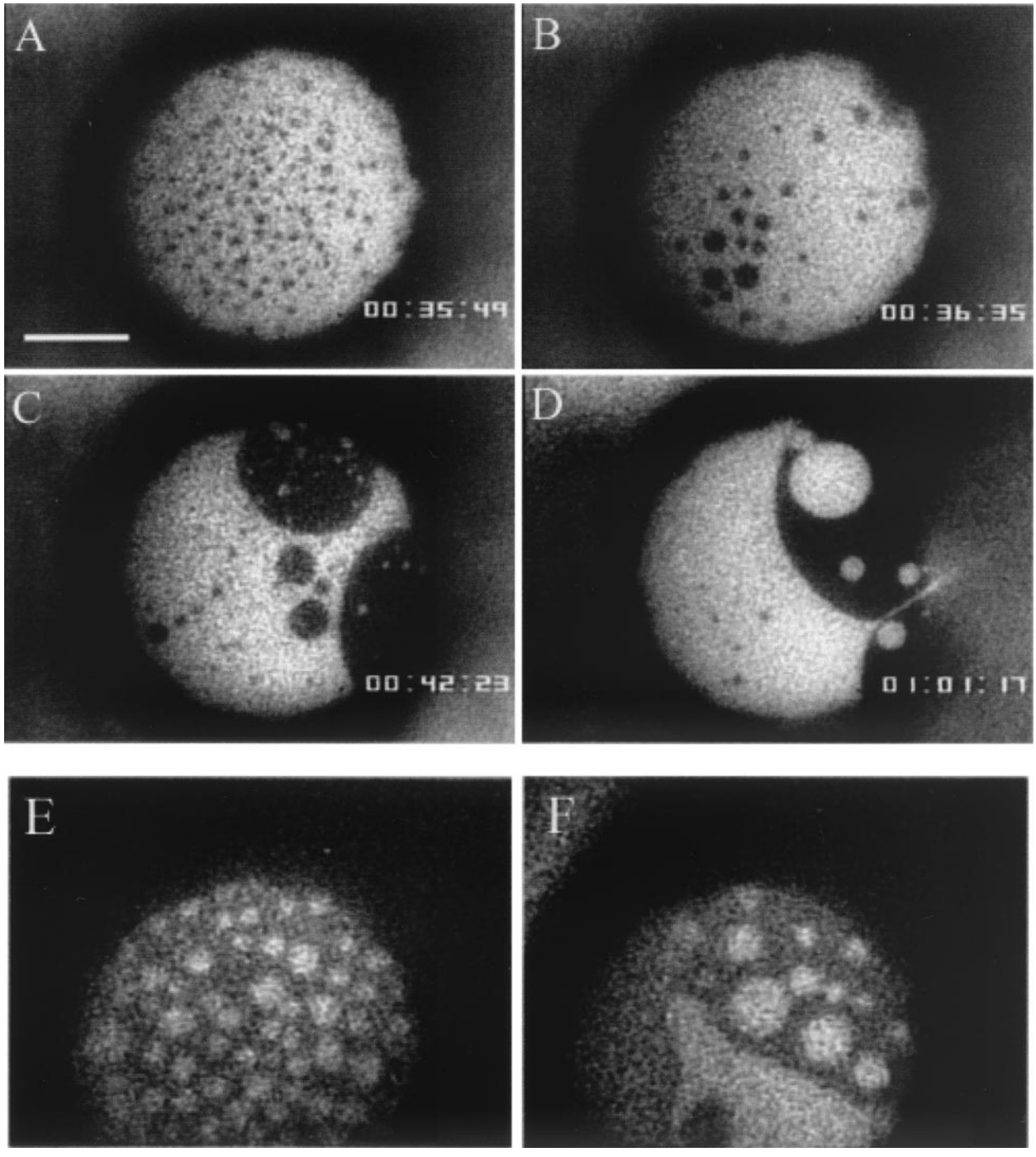

FIGURE 7 High concentrations of SPM and cholesterol induce massive-sized rafts. SPM and cholesterol each comprised 25 mol % of the membrane. (*A*) After lowering the temperature to below T_m of the SPM, dark domains form within the bright bilayer. (*B*) With time, the domains merged to become larger, but fewer in number. (*C*) The merger of the dark domains became so great that in places the background has become dark (i.e., enriched in SPM/cholesterol), and bright domains containing rho-DOPE are contained within them. (*D*) The bright domains merged over time; large bright circular domains were observed. *A–D* are the same membrane at the same magnification; scale bar, 50 μm. (*E*) In a different membrane, the appearance of many bright domains against a dark background can be seen. (*F*) With time, the bright domains merge.

mol % cholesterol in the DOPC/DOPE membrane to investigate whether saturated PC/cholesterol phases could form. After lowering the temperature to below T_m, dark circular domains formed in the same manner as described for SPM (Fig. 8). Thus, with cholesterol present, microscopically discernable lipid domains can form spontaneously within a l_d phase in the absence of SPM, but only below some temperature that correlates with the T_m of the saturated PC.

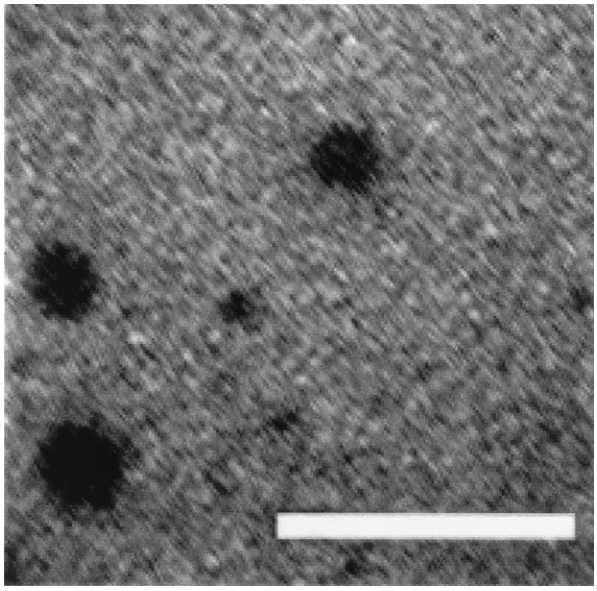

FIGURE 8 Domains excluding rho-DOPE form with a saturated PC and cholesterol. 15 mol % DSPC and 15 mol % cholesterol were included in the bilayer. Dark circular domains formed after lowering the temperature from above the T_m (55°C) of DSPC to 25°C. Scale bar, 50 μm.

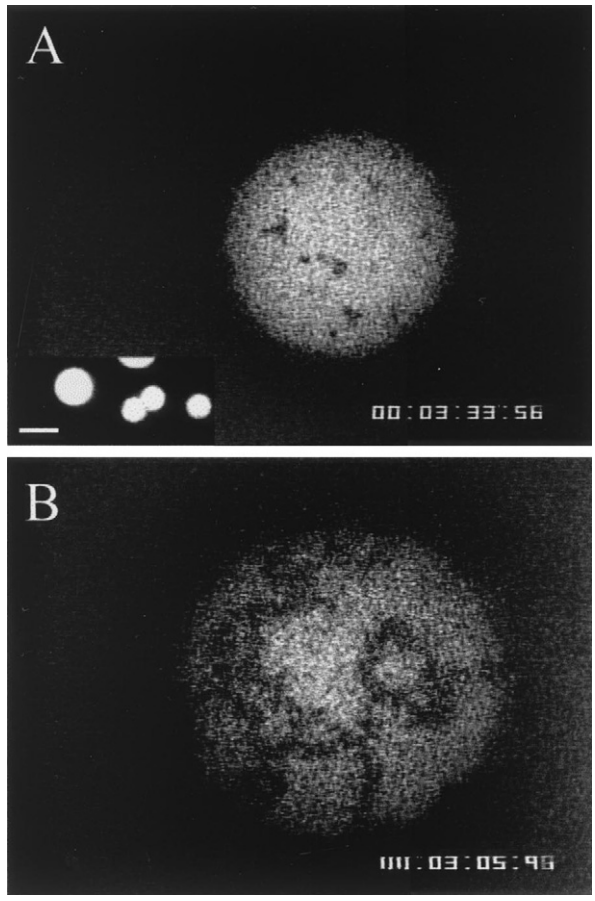

FIGURE 9 Domains in the s_o solid-gel phase are noncircular. Membranes were formed above the T_m of the saturated lipid, either DPPS or DMPE. Irregularly shaped domains (excluding rho-DOPE) formed after temperature was lowered to 25°C, below the T_m of both saturated lipids. (*A*) 25 mol % DPPS and 25 mol % cholesterol were included in the planar membrane. (*B*) 25 mol % DMPE and cholesterol were included in the membrane. Scale bar, 4 μm.

The domains were circular and merged with each other, indicating that they were in a l_o rather than a s_o phase. These results support the use of a saturated PC with cholesterol to model rafts, in agreement with previous findings (Ahmed et al., 1997).

We explicitly tested the expectation that s_o domains would be irregular by including either a saturated PE or a saturated PS in a DOPC/DOPE bilayer containing cholesterol. Cholesterol does not partition well into phase-separated domains of either saturated PS (Bach and Wachtel, 1989; McMullen et al., 1999) or saturated PE (McMullen and McElhaney, 1997). When 25 mol % of the saturated lipid DMPE (T_m = 50°C) and 25 mol % cholesterol was included, phase separation occurred when temperature was lowered from above 50°C to 25°C. But rather than circular, the dark domains exhibited an irregular, mesh-like appearance (Fig. 9 *B*). Similarly, including DPPS in the bilayer (Fig. 9 *A*) led to irregular domains when the temperature was lowered from 60°C to below the T_m of DPPS (54°C). Although cholesterol eliminates gel-to-fluid phase transitions (Estep et al., 1978), its presence did not prevent the phase separation of the saturated PE or PS into frozen s_o domains at temperatures below their T_m.

Interactions between lipids from the two monolayers in domain formation

An important physical aspect of rafts is the relationship between the raft of one monolayer and the other. Solid domains of saturated PC in lipid bilayer membranes co-localize within both monolayers, indicating that interactions between monolayers are part of domain integrity in bilayers (Korlach et al., 1999). In other words, the solid domain extends through the entire bilayer. This is the case for cholesterol/SPM rafts in GUVs as well (Dietrich et al., 2001). Visual observations directly provide two lines of evidence to show that in planar bilayers the rafts also spanned both monolayers. First, if rafts formed within individual monolayers, we should have observed overlap of dark circular domains. But we did not: circular domains were always isolated and when they met, they merged. Second, large rafts tend to accumulate near the Gibbs-Plateau border (the torus). But neither they nor the smaller dark domains merged with the torus. The torus consists of bulk hydrocarbon (squalene in the present study) with dissolved lipids and lipid monolayers at the hydrocarbon-water interface. The bilayer ends at the torus, but each monolayer extends on and becomes a

monolayer of the torus. If a raft existed only within an individual monolayer, it should be able to freely move into the torus. Because the rafts could not do this, we conclude that they extended through the entire bilayer as a unit that could not separate into monolayer rafts at the torus. However, the plane of a monolayer of the torus is at a non-zero angle relative to the plane of the bilayer (the contact angle) (Needham and Haydon, 1983). It remains formally possible that a raft within an individual monolayer could not enter the torus because the acyl chains of all its lipids could not reorient at the boundary. Taking the two results together, we conclude that the domains within the two monolayers interact to form a coherent unit. It is perhaps surprising that a SPM/cholesterol domain in one monolayer required the presence of a co-localized domain in the other monolayer as rafts in monolayers have been observed (Radhakrishnan et al., 2000).

We determined whether once a raft formed, the domain was retained for appreciable times after destroying its cholesterol. We applied cholesterol oxidase (COase) with a micropipette (5 U/ml in the pipette) to a membrane at the site of a large raft. COase converts cholesterol to cholestenone (i.e., 4-cholesten-3-one). Because cholestenone does not possess a 3β-hydroxyl group, it does not interact with SPM. It has been demonstrated that the addition of COase to lipid monolayers containing SPM and cholesterol immediately results in monolayer expansion, indicating that cholestenone and SPM do not interact (Grönberg and Slotte, 1990); also, 4-cholestenone inhibits domain formation for DPPC/cholesterol bilayers (Xu and London, 2000). It is controversial whether the rate of cholesterol flip-flop across membranes is slow (Brasaemle et al., 1988; Raggers et al., 2000) or fast (Lange et al., 1981; Backer and Dawidowicz, 1981). If flip-flop is slow, cholesterol lost through oxidation could only be replaced slowly (i.e., by diffusion out of the torus). The addition of COase would then be a means to test whether rafts could exist within an individual monolayer and, if it could not, whether the elimination of a SPM/cholesterol domain in one monolayer destroys the domain in the other monolayer. If flip-flop were fast, addition of COase would destroy cholesterol in both monolayers and thereby test whether SPM that had segregated with cholesterol continued to reside in a domain for significant times.

Using a pipette with a small orifice, COase was applied locally to a raft. The circular border distorted and the domain brightened, starting from the border and spreading inward (Fig. 10). This shows that oxidation of the cholesterol permitted the rho-DOPE to diffuse into the domain. For membranes that did not break after several minutes of this treatment, the raft disappeared, with only a faint trace of its prior existence. This is in accord with cholestenone's ineffectiveness in supporting raft formation (Xu and London, 2000). It also shows that a raft is not maintained when cholesterol is converted into cholestenone, illustrating that rafts can be dynamically controlled.

DISCUSSION

The advantages and disadvantages of planar bilayers in the study of lipid domains

We have studied the formation of lipid domains in unsupported planar bilayer membranes. This is, to our knowledge, the first study that has used planar bilayers to investigate lipid phase separation. Planar bilayer membranes offer some practical advantages for studying dynamic properties of lipid phases. As we have shown, large domains can be conveniently deformed by micromechanical manipulation and the relaxation of deformation accurately measured microscopically for a flat membrane. Utilization of this method would allow viscoelastic properties of rafts to be quantitatively determined. With a single flat membrane, there is no out-of-plane fluorescence so a standard wide-field fluorescence microscope (rather than a scanning confocal or scanning two-photon microscope) can be routinely used for studies. Because the planar membrane does not have to be optically scanned (whereas GUVs, for example, have to), we were able to continuously monitor domain formation and growth at video rates (30 frames/s). This time resolution allowed us to see that large domains formed by the merger of smaller ones.

In the planar bilayer system, organic solvent is used to dissolve the lipids, and some will naturally partition into the bilayer. In studying phase separation, the question arises as to whether the solvent could be the agent of separation. We chose squalene as our solvent because it has been shown to have an immeasurably small partition coefficient into liposomes and monolayers composed of a saturated phosphatidylcholine (Simon et al., 1977). Planar membranes formed with squalene have specific capacitances expected of a solvent-free bilayer when either a single lipid (White, 1978) or a highly heterogeneous biological mix of lipids (Niles et al., 1988) is used. Because it is highly unsaturated (with six double bonds), any squalene that was in the bilayer should preferentially reside in the DOPC/DOPE region of the bilayer and be absent from rafts with saturated acyl chains. It might still be argued that the presence of squalene, however small, could be the cause of our observed rafts. But the recent demonstration that large rafts also form in supported planar bilayers and in GUVs (Dietrich et al., 2001) where no solvent is present and that the properties of these rafts are the same as those of the unsupported squalene-based planar membranes show that this is not the case. If there is any amount of squalene located within the membrane, it is simply another lipid component. All biological membranes contain many lipid components, and some contain as much as 6 mol % squalene (Fleisler et al., 1997).

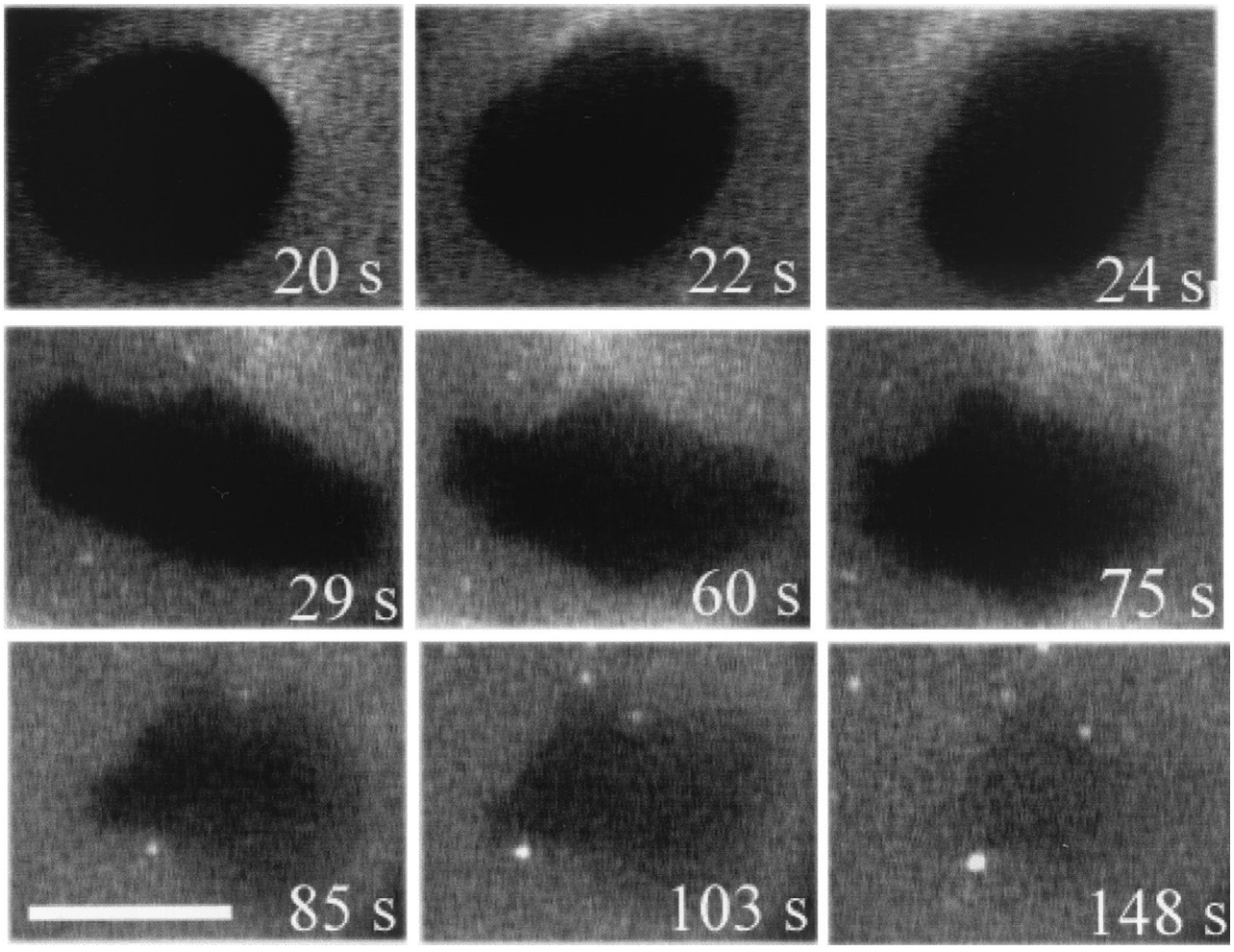

FIGURE 10 The addition of cholesterol oxidase to one side of a SPM/cholesterol raft affected lipid clustering in both monolayers. The times after locally applying the enzyme over a large raft are shown. The rafts became smaller and less regular and eventually disappeared. The raft moved as a result of applying the enzyme; the area of interest shown was set by the position of the raft. Scale bar, 50 μm.

Planar bilayer membranes are open systems, continuous with the Gibbs-Plateau border with which it continuously exchanges lipids. A liposome, in contrast, no matter how large, is a closed system. Liposomes readily reach equilibrium whereas planar bilayers do not. Cellular membranes are in this way closer to the situation of the planar bilayer than that of the liposome: they are not in equilibrium and lipid is continuously exchanged between plasma membranes and intracellular compartments via granule fusion and endocytosis with half-times for recycling of plasma membrane lipids on the order of 5–10 min (Hao and Maxfield, 2000). As with cellular membranes, the precise lipid composition of planar membranes is not known and they cannot be used to determine phase diagrams. Nevertheless, the system is clearly useful for the observation of rafts and their dynamics.

Ultimately, of course, one wants to relate the formation and properties of lipid domains as elucidated in model systems to those of biological membranes. Methodologies that could be used on both would greatly facilitate direct comparisons. In this study we have developed one new method and extended another, both of which can be used on biological membranes as well. We developed GM_1 with a fluorescently labeled headgroup; it should accurately reflect the location of GM_1 itself, without causing perturbation. Other procedures, such as binding cholera toxin subunit B (tagged, for example, with a fluorescent probe) can seriously alter the spatial distribution of GM_1 because the toxin is multivalent (Fishman et al., 1978) and can therefore itself create clusters of GM_1 (Goins and Freire, 1985). COase has often been used to destroy cholesterol (Pal et al., 1980); we extended this method to show that application of COase eliminated rafts in bilayer membranes. Treating cells with methyl-β-cyclodextrin to deplete them of cholesterol is the common method to eliminate biological rafts, but this is a harsh treatment. The use of COase treatment appears to be a gentler procedure.

Rafts are liquid ordered

Differential thermal calorimetry and x-ray diffraction studies showed that complexes of cholesterol and SPM form a phase that has structural characteristics (e.g., bilayer thickness, chain packing) between those of l_d and s_o phases (Maulik and Shipley, 1996). The circular shape of rafts in liposomes (Dietrich et al., 2001) and planar bilayers indicate that they are in a l_o rather than s_o phase. Our demonstration for the fast resumption of the circular shape after deformation and the ability to merge also indicate that rafts are l_o rather than s_o domains. But the l_d, l_o, and s_o phases can coexist for membrane mixtures containing phospholipids and cholesterol if the mole fraction ratio of cholesterol is not too high (Silvius et al., 1996). Small s_o phases may therefore reside within the l_o domains for lipid mixtures containing 15 mol % cholesterol, but these phases should be less significant at the higher (e.g., 25 mol %) cholesterol concentrations. Based on measured diffusion coefficients, a l_o cholesterol-sphingolipid domain has a two- to three-fold greater viscosity than that of the surrounding l_d phase (Dietrich et al., 2001). This would account for the greater time constant for a merged bright domain (Fig. 7 *D*) than a comparably sized dark domain (Fig. 2) to assume a circular shape; the movement of the boundary of a domain is opposed by the viscosity of the surrounding region. It could be biologically important that rafts are in a l_o phase, because proteins localized within a liquid state could more readily interact and associate with each other than would be possible within a solid state.

It is notable that the rafts are essentially liquid-ordered rather than solid-ordered (the SPM within the raft has not frozen into a solid (gel) phase) even though the membranes are at temperatures below the T_m of the SPM. Based on their mobility within the bilayer, rafts are thicker than the background portion of the bilayer. It is well known that the presence of cholesterol condenses phospholipid bilayer membranes, increases their thickness, and at high enough concentrations eliminates the gel-liquid phase transition. Cholesterol is thought to cause these effects by intercalating between acyl chains of phospholipids, possible because of its inverted cone shape. The intercalation would order the acyl chains of the lipids and reduce their ability to tilt. The reduction in tilting causes the membrane to thicken, and because lipids cooperatively tilt in tandem in the gel state (Cevc and Marsh, 1987), the presence of cholesterol eliminates the liquid-gel transition. In a similar manner, cholesterol may intercalate between the chains of SPM, prevent their freezing into a gel phase, and thicken the rafts. Or the greater thickness in rafts could be due in part to a l_o phase inherently thicker than the l_d phase: the thickness of a bilayer increases with lowering of temperature (Das and Rand, 1986).

Both acyl chain and headgroup interactions participate in raft formation

The lipid composition of DRMs has been analyzed and shown to be rich in sphingomyelin, cholesterol, and saturated phospholipids (Fridriksson et al., 1999; Zhang et al., 2000). The rafts of the present study contain less phospholipid than the surrounding bright regions. We do not know the precise amount of phospholipid, if any, that partitions into the rafts of the planar membrane. Because SPM, PC, cerobrosides, and gangliosides are located in the outer leaflets of cell membranes whereas PE and PS are preferentially located within the inner leaflets, DRMs consist of lipids of both leaflets. But rafts containing sphingolipids could reside only in the outer leaflet of biological membranes, whereas in phospholipid bilayers the rafts extend through both monolayers as a unit. Factors, such as cytoskeleton, may be important for formation and size of biological rafts. For example, well before the raft concept, it was shown that the Triton-X-100-insoluble fraction of red blood cell membranes contained cytoskeleton that bound to plaques of membrane that included more than 80% of the cells' SPM (Yu et al., 1973). We have found that rafts occurred at temperatures below the T_m of SPMs with saturated acyl chains. Therefore, the standard biochemical assay measuring DRMs at 4°C may not correlate with the ability of rafts to form at room temperature or 37°C.

Two prominent explanations have been advanced for how cholesterol-sphingolipid rafts form. One centers on the role of headgroup interactions and hydrogen bonding (Schmidt et al., 1977; Boggs, 1980. In one form (Simons and Ikonen, 1997), it is posited that sphingolipids interact with each other through their headgroups and through the interaction of the amide of the sphingosine base of one sphingolipid with hydroxyls or carboxyls of an adjacent sphingolipid. In that case, many sphingolipids would associate through the formation of a network of bonds (Simons and van Meer, 1988). The cholesterol would effectively pack into the space between the sphingolipids in a manner analogous to the way it fills space between phospholipids. Hydrogen bonding between the 3-OH group of cholesterol and the amide of the sphingosine would stabilize this localization of cholesterol. The other model, historically the first to be proposed (Finean, 1953; Vandenheuvel, 1963), considers the interactions between the chains as the primary determinant. This model places emphasis on the fact that saturated acyl chains are more extended than unsaturated ones and pack well with each other into liquid-ordered phases (London and Brown, 2000). Cholesterol may interact more favorably with a saturated than an unsaturated sphingolipid because cholesterol is a flat, rigid molecule. The interactions between acyl chains of the sphingolipids and cholesterol would be the critical factor in creating rafts. As evidence that cholesterol interacts more strongly with saturated than unsaturated acyl chains, methyl-β-cyclodextrin more readily removes cho-

lesterol from *N*-18:1-SPM/cholesterol monolayers than from monolayers containing cholesterol and SPM with saturated tails (Ramstedt and Slotte, 1999b). The demonstration that saturated PCs and cholesterol form l_o domains provided support for the model (Brown and London, 1998). We have confirmed this and provided further evidence for this model by showing that rafts of SPM and cholesterol are in a l_o phase, that saturated SPMs more effectively induce rafts than do unsaturated SPMs, and that raft formation occurs at temperatures below the T_m of the SPM. The need for lower temperature to form microscopically observable rafts is also found for liposome membranes (Dietrich et al., 2001), as expected from the phase diagrams (Maulik and Shipley, 1996). The low water permeability of bilayer membranes rich in cholesterol and SPM (Finkelstein, 1976) would be expected (without any other assumptions) if the acyl chains of SPM and cholesterol interacted over an extended length. But we also found that the headgroups were of consequence in raft formation: saturated PE or PS did not substitute for PC or SPM. Perhaps the headgroups of PC and SPM, which are the same, participate in raft formation. GM_1 may not have formed domains with cholesterol because of its unfavorable headgroup interactions (e.g., because of its negative charge). Perhaps epicholesterol could substitute for cholesterol in forming rafts whereas coprostanol could not because the four-ring portion of epicholesterol (and cholesterol) is flat but that of coprostanol is not. We are thus led to the view that cholesterol preferentially packs with saturated acyl chains of lipids. When interactions between headgroups stabilize the associations between saturated sphingolipids and cholesterol, liquid-ordered rafts are created (Simons and Ikonen, 2000).

Saturated acyl chains on proteins may promote partitioning into rafts

The mechanism by which specific proteins accumulate in domains is poorly understood. But many proteins found in DRMs and therefore thought to reside in rafts contain saturated acyl chains. For example, the lipid anchor of GPI-coupled proteins usually has two saturated fatty acyl chains (Casey, 1995); kinases of the Src family are also acylated with saturated chains. For influenza virus hemagglutinin to accumulate into rafts, it must be palmitoylated in the membrane-spanning and cytoplasmic domains (Melkonian et al., 1999). The ability of covalently linked saturated acyl chains to partition into a l_o phase rich in lipids with saturated tails may be important for targeting proteins to rafts (Moffett et al., 2000). The *cis*-double bond of an unsaturated acyl chain may hinder packing into a lipid-ordered environment because the cross-sectional area of a hydrocarbon chain would be increased, as would freedom of motion. But it is unlikely that the state of acylation of a protein is the only factor. For example, influenza virus neuraminidase is not palmitoylated but associates with rafts (Barman and Nayak, 2000). Perhaps interactions of specific amino acids with sphingomyelin and cholesterol are also important: point mutations of the transmembrane domain of neuraminidase within either the exoplasmic or cytoplasmic lipid leaflets affect whether the protein localizes into DRMs (Zhang et al., 2000).

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