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Detergent-Free Domain Isolated from *Xenopus* Egg Plasma Membrane with Properties Similar to Those of Detergent-Resistant Membranes†

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ABSTRACT: Microdomains known as "rafts" have been isolated from many cell types as detergent-resistant membranes (DRMs) and are enriched in sphingolipids and cholesterol. However, there has been considerable controversy over whether such domains are found in native membranes or are artificially generated by the purification procedure. This controversy is based at least in part on the fact that raft membranes were first detected following detergent extraction in the cold. We isolated two plasma membrane fractions, without detergent treatment, using a discontinuous sucrose density gradient. One fraction was designated "light" and the other "heavy." These fractions were compared with DRMs, which were isolated in the presence of 1% Triton X-100. We found that *Xenopus* DRMs are enriched with sphingomyelin and cholesterol and exhibit a phase state similar to the liquid-ordered phase. Comparison of DRM complexes with the light and heavy plasma membrane fractions revealed some physical and biochemical similarities between the light fraction of the plasma membrane and the DRM complexes, based on (1) the phosphatidylcholine/sphingomyelin ratio and (2) the protein composition visualized on a two-dimensional gel. These two fractions are also quite similar in their thermotropic phase behavior, and their high levels of ganglioside GM1. We conclude that the light membrane fraction isolated in a detergent-free environment has many of the characteristics normally associated with DRMs.

Over the past few years, much research has been conducted on membrane specializations known as detergent-resistant membranes (DRMs)[1] or "rafts" (*1*). They have been found in all mammalian cell types that have been investigated (*2*). Normally, they are purified by ultracentrifugation, following extraction of detergent-soluble lipids at low temperatures (*3*). It has been proposed that because of their special acyl chain composition (long chains, nearly completely saturated), glycosphingolipid (GSL), sphingomyelin, and saturated phospholipids preferentially associate laterally with themselves, resulting in phase separation from the Triton-soluble lipids (*4*−*6*). Cholesterol is also found at a high concentration in DRMs and is thought to contribute to the formation of these domains in native membranes (*3*). DRMs have been found to contain certain proteins that have important roles in cell surface signaling, cell adhesion, and motility, such as GPI-anchored proteins, nonreceptor tyrosine kinases, G-protein subunits, and several transporters (*7*−*9*). As a result, it has been suggested that in native membranes these domains serve as signaling platforms in which receptors and their accessory proteins are organized (*2*). Investigation of rafts is important not only because of their unique protein and lipid content but also because of their unusual physical state (the L_o or liquid-ordered state), which may have biological significance in living cells. The L_o state displays a fluidity intermediate between that of the liquid-disordered state (L_d) and the gel state. Modeling with defined lipids in vitro has shown that at the relative concentrations found in the plasma membrane sphingolipids and cholesterol can indeed form a L_o phase in the lipid bilayer (*6*). Both the high melting temperature and the long, saturated acyl chains of the sphingolipids have been proposed to be critical parameters for the formation of the L_o phase, which is further facilitated by the presence of cholesterol (*6*, *10*). An important and controversial issue regarding sphingolipid−cholesterol rafts is whether the biochemical method used to isolate these structures actually harvests pre-existing microdomains or creates them artifactually (*11*). Here we present evidence for a detergent-free plasma membrane domain with properties similar to those of DRMs. The approach we have taken is to isolate membrane fractions with and without detergent extraction (*12*−*14*) and to compare the properties

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[1] Abbreviations: GSL, glycosphingolipid; DRMs, detergent-resistant membranes; MβCD, methyl-β-cyclodextrin; PI3K, phosphatidylinositol 3-kinase; PLCγ1, phospholipase Cγ1; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; LMs, light membranes; HMs, heavy membranes; PI, phosphatidylinositol; FFA, free fatty acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; MLVs, multilamellar vesicles; FTIR, Fourier transform infrared spectroscopy; CTX, cholera toxin B; SEM, standard error of the mean.

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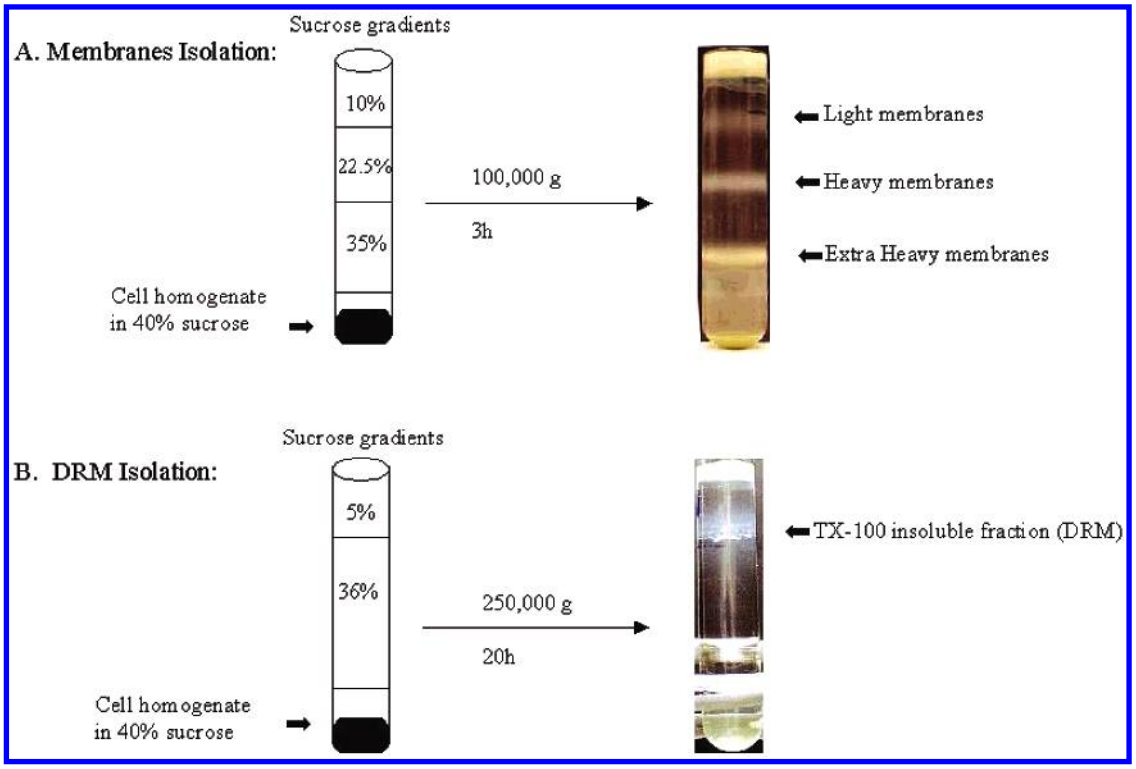

FIGURE 1: Method used to isolate membrane fractions (light, heavy, and DRMs). *Xenopus* eggs were homogenized in TNE buffer without (A) or with (B) Triton X-100 as described in Materials and Methods. (A) Two fractions of plasma membranes were isolated from detergent-free egg extract: light (10−22.5% sucrose interface) and heavy membranes (22.5−35% sucrose interface). (B) The DRM fraction was isolated from the 5−36% sucrose interface gradient centrifugation of the Triton X-100-treated egg extract.

of those fractions. We report that one fraction obtained from the membrane without detergent extraction is strikingly similar to DRMs with respect to phase behavior, lipid and protein composition, and ganglioside content.

MATERIALS AND METHODS

Materials. Polyclonal anti-Fyn, anti-Src, anti-PLCγ1, and rabbit IgG antibodies and mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-Na^+/K^+ ATPase was purchased from Upstate Biotechnology (Lake Placid, NY). Chloroform and methanol were from EM Science (Gibbstown, NJ). Acetic Acid and $AgNO_3$ were purchased from Fisher Scientific (Fair Lawn, NJ). All other products were from Sigma Chemical Co. (St. Louis, MO).

Preparation of Eggs. Xenopus laevis eggs were obtained by gentle abdominal massage of wild-type and albino frogs that had been primed with 50 IU of pregnant mare serum gonadotropin (PMSG) 48 h prior to hCG injection (500 IU). Then, 8−10 h after hCG injection, the eggs were dropped into a plastic dish filled with F1 medium [41.25 mM NaCl, 1.75 mM KCl, 0.5 mM $NaHPO_4$, 0.5 mM NaOH, 2.5 mM HEPES, 0.25 mM $CaCl_2$, and 0.06 mM $MgCl_2$ (pH 7.8)], and the eggs were dejellied by a short exposure to 0.3% β-mercaptoethanol in F1 medium (pH 9.0) followed by extensive washing with F1.

Subcellular Fractionation. The membrane fractions isolated and compared as described below are illustrated in Figure 1. We prepared membrane fractions by centrifugation (Figure 1A) and DRMs by detergent extraction (Figure 1B), as follows.

Isolation of Light and Heavy Membrane Fractions. One hundred or 300 eggs (as indicated in each of the figure legends) were lysed by repeatedly pipeting in ice-cold 2× TNE buffer [10 mM Tris-HCl, 150 mM NaCl, and 5 mM EDTA (pH 7.4)] with protease inhibitors (1 μM iodoacetamide, 2 μM leupeptin, 10 μM aprotinin, and 100 μM soybean trypsin inhibitor) along with 1 mM benzamidine and 1 mM Na_3VO_4. Light and heavy fractions were isolated according to the method of Monneron and d'Alayer (*12*) with the following modifications. Egg lysates were sonicated for 1 min in a cold bath at 50 Hz, 117 V, and 80 W (Branson 12-Smithkline Co.). The lysates were added to an equal volume of 80% sucrose to give a final equivalent density of 40% sucrose. They were then overlaid with 3 mL each of sucrose at concentrations of 35, 22.5, and 10%.

Isolation of DRMs. Either 100 or 300 eggs were lysed by repeatedly pipeting in an ice-cold TNE buffer containing 1% Triton X-100. DRMs were isolated according to the method of Brown and Rose (*3*).

Lipid Analysis. Lipids from 300 eggs of each fraction were isolated by chloroform/methanol extraction (2:1), following vigorous mixing. The solution was centrifuged in a tabletop centrifuge, and the organic phase was recovered and evaporated under nitrogen gas. Lipid extracts were dissolved with 20 μL of chloroform, and triplicate aliquots of 1.5 μL were loaded on silica gel-coated glass rods (Iatroscan Laboratories, Inc., Tokyo, Japan), as described elsewhere (*15*, *16*). The profiles were quantified by comparing them to standard curves of various lipid species using a National Instruments (Austin, TX) Virtual Bench Datalogger. Identification of the peaks was established by comparison of the retention data with known data using PeakFit software (Jandle Scientific, San Jose, CA).

Phospholipid Purification. The lipid sample (200 μL) from 300 eggs was injected on silica columns, and the phospho-

lipids were separated with high-pressure liquid chromatography (*17*).

Phase Transitions and Membrane Dynamics Measurements. Forty microliters of each fraction from 300 eggs was placed between two FTIR windows, and the phase transitions of light, heavy, and DRM fractions were determined by infrared spectroscopy (*18*, *19*).

Biotinylation of Raft Proteins. Dejellied eggs were split into two groups of 100 eggs and incubated in the presence or absence of 2 mg/mL Sulfo-NHS biotin (Pierce, Rockford, IL) in F1 medium at room temperature. After a 20 min incubation period, the eggs were washed six times in F1 medium. The DRM fraction was isolated as described above, and the egg lysates were loaded on a 10% SDS−PAGE gel for protein separation. The biotinylated proteins were detected by a specific Avidin HRP (Pierce) using an ECL kit (Santa Cruz Biotechnology).

SDS−PAGE and Western Blotting Analysis. Proteins were dissolved in a concentrated SDS sample buffer and boiled for 5 min (*20*). The SDS-treated proteins were separated by SDS−PAGE and then were transferred to a nitrocellulose immobilization membrane (Protan, Schleicher & Schuell). Membranes were blocked with T-TBS buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20] supplemented with 5% bovine serum albumin for 30 min and washed three times with T-TBS. Membranes were incubated with primary rabbit or mouse antibodies (1:3000 dilution) overnight at 4 °C. After being washed three times (10 min each) with T-TBS, the membranes were further incubated for 1 h with anti-rabbit IgG−HRP or with anti-mouse IgG−HRP antibodies (1:5000 dilution). Antibody binding was detected using the ECL kit (Santa Cruz Biotechnology).

Two-Dimensional (2D) Gel Electrophoresis. A Multiphor II electrophoresis unit, equipped with a multidrive 3500XL gradient power supply, was used to perform 2D isoelectric focusing (IEF)/SDS−PAGE, according to the manufacturer's instructions (Amersham-Pharmacia Biotech, Arlington, IL).

Computer Analysis of 2D Gels. Gel images were captured using a scanning densitometer (DUOSCAN, Agfa). Analysis of the images, spot finding, quantification, and matching were done using PDQuest software (Bio-Rad Laboratories, Hercules, CA).

Fluorescent Staining. Endogenous gangliosides GM1 were labeled in multilamellar vesicles (MLVs). Lipids were extracted from the DRM, light, or heavy fractions as described above. MLVs were formed by freezing and thawing the lipid and were placed on a polylysine-coated cover slide for 30 min. The MLVs were labeled with FITC-labeled cholera toxin B (10 μg/mL) for 30 min and washed three times with cold PBS. The FITC-labeled cholera toxin fluorescence was analyzed with a Zeiss LSM 410 inverted laser scan confocal microscope equipped with an argon/krypton excitation laser (Zeiss, 110 V, 25 mW, 488/586 nm). Transmission and fluorescence images were both taken for each preparation.

RESULTS

Assignment of the Source of Membrane Fractions. Plasma membrane fractions were separated by ultracentrifugation on a discontinuous sucrose gradient, forming "light", "heavy", and "extra heavy" fractions, following a procedure that was previously carried out on thymocytes (Figure 1A) (*12*, *21*). In this isolation procedure, the light and heavy fractions were found to be derived from the plasma membrane, while the extra heavy third fraction originated mainly from intracellular components (*12*, *21*). In addition, we isolated a detergent-resistant membrane (DRM) fraction (Figure 1B). We then examined the distribution of signaling molecules as well as plasma membrane markers in each fraction. We found that the plasma membrane transporter, Na^+/K^+ ATPase, was highly concentrated in heavy membranes, and was also clearly present in the light and DRMs (Figure 2A). The nonreceptor protein tyrosine kinase, Fyn, was clearly enriched in the heavy fraction, while Src kinase was found in all three fractions (Figure 2B,C). PLCγ1, which translocates from the cytosol to the plasma membrane upon activation, was not detected in any of the fractions, but was enriched in the cell lysate homogenate (Figure 2D). In the DRM fraction, we also observed several biotinylated protein bands (26, 36, 40, and 48 kDa), indicating the presence of surface proteins (Figure 2E, lane 1). Nonspecific avidin binding was observed in the control on a few bands (Figure 2E, lane 2). These results suggest that light, heavy, and DRM fractions are all derived in part from the plasma membrane, as has been clearly demonstrated in other cells (*12*, *21*).

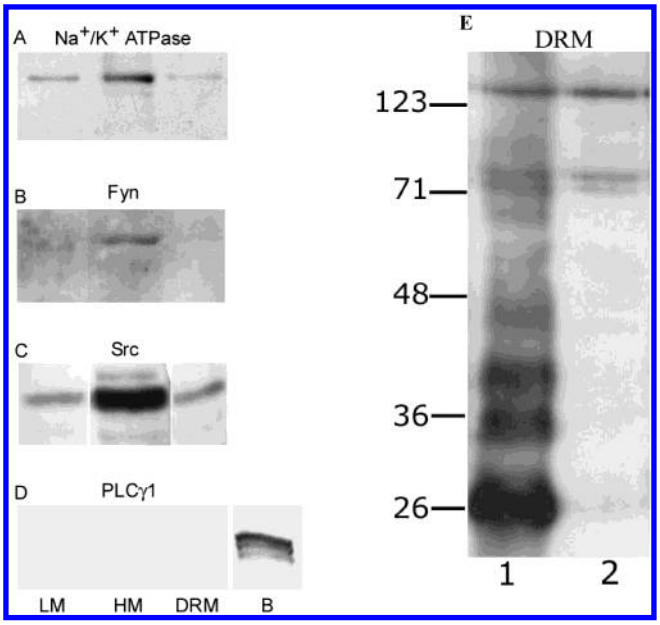

FIGURE 2: Labeling of the plasma membrane marker, Na^+/K^+ ATPase, and signaling molecules in light, heavy, and DRM fractions and cell surface proteins in the DRM fraction. Twenty-five microliters of the protein extract was subjected to PAGE and transferred to nitrocellulose, and analyzed by Western blot analysis for the distribution of Na^+/K^+ ATPase (A). Nonreceptor protein tyrosine kinase, Fyn (B), Src (C), and PLCγ1 (D), where panel B represents the bottom of the tube (40% cell lysate). (E) DRM-biotinylated proteins that were extracted and incubated in the presence (1) or absence (2) of Biotin-LH-NH_2, for 30 min at room temperature (E). These results are representative of three different experiments.

Localization of Ganglioside GM1. Ganglioside GM1 is commonly found in high concentrations in rafts (*22*), and it can be fluorescently labeled using FITC-conjugated cholera toxin B (*23*). CTB binds to the glycosphingolipid GM1 at the cell surface, and after internalization, this toxin subunit is delivered to the Golgi complex (*24*). Herein, we used CTB

Table 1: Summary of Experimental Results with FITC-Conjugated Cholera Toxin B-Labeled Multilamellar Vesicles from Figure 3[a]

		multilamellar vesicle intensity (av pixels ± SEM)	multilamellar vesicle area (av μm^2 ± SEM)	intensity/area
DRM	1	52 ± 14 ($N = 9$)	62 ± 17	0.8
	2	50 ± 6 ($N = 18$)	46 ± 7	1.1
	3	30 ± 3 ($N = 8$)	408 ± 94	0.1
	4	13 ± 2 ($N = 4$)	176 ± 130	0.1
	5	13 ± 3 ($N = 4$)	197 ± 93	0.1
	6	21 ± 4 ($N = 8$)	155 ± 36	0.1
	7	50 ± 16 ($N = 7$)	298 ± 46	0.2
	8	15 ± 2 ($N = 13$)	237 ± 57	0.1
av ± SEM ($N = 8$)				0.3 ± 0.1
light	1	74 ± 29 ($N = 6$)	44 ± 15	1.7
	2	33 ± 11 ($N = 4$)	22 ± 11	1.5
	3	38 ± 27 ($N = 3$)	53 ± 6	0.7
	4	50 ± 4 ($N = 17$)	40 ± 6	1.3
	5	41 ± 3 ($N = 13$)	68 ± 20	0.6
	6	56 ± 6 ($N = 19$)	29 ± 5	1.9
	7	67 ± 10 ($N = 16$)	56 ± 8	1.2
av ± SEM ($N = 7$)				1.3 ± 0.2
heavy	1	14 ± 1 ($N = 2$)	9419 ± 8032	0.001
	2	18 ± 10 ($N = 12$)	4998 ± 966	0.004
	3	22 ± 1 ($N = 12$)	7405 ± 2076	0.003
	4	17 ± 1 ($N = 11$)	10328 ± 3333	0.002
	5	24 ± 2 ($N = 14$)	9151 ± 3063	0.003
av ± SEM ($N = 5$)				0.002 ± 0.0004

[a] Intensity and area measurements from each vesicle were analyzed by confocal microscopy.

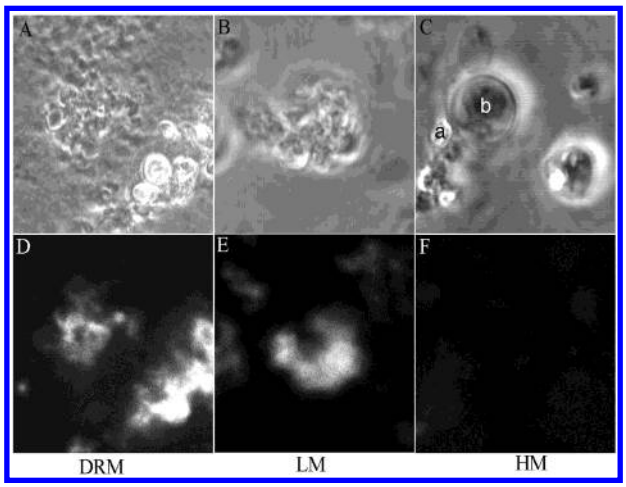

FIGURE 3: Ganglioside GM1 distribution in multilamellar vesicles. Ganglioside GM1 was visualized in multilamellar vesicles generated from lipid isolated from DRM, light membranes, or heavy membranes (Materials and Methods). Ganglioside GM1 was highly concentrated in DRM and light membranes, but not in the heavy fraction of the plasma membrane. Transmission pictures (A−C) or fluorescence pictures (D−F) were taken from each fraction: (a) small vesicles and (b) large vesicles (bar, 100 μm).

as a marker for raft localization in light or heavy plasma membrane fractions. We prepared multilamellar vesicles from each of our membrane fractions and exposed them to this label. Ganglioside GM1 was easily detected in vesicles originating from DRM and light fractions, while very little fluorescence was observed in vesicles from heavy membranes (Figure 3). Quantification of all vesicles from many different experiments revealed a significant difference between the amount of labeling in the DRM and light fractions and the amount in the heavy membrane vesicles. We normalized the labeling by dividing the mean fluorescence intensity by the total vesicle cross sectional area. The average intensity/area ratios in the DRM, light, and heavy fractions are 0.3, 1.3, and 0.002, respectively (Table 1). The prominent

Table 2: Protein and Lipid Concentrations in Light, Heavy, and DRM Fractions[a]

	lipid (μg/egg) (av ± SEM)	protein (μg/egg) (av ± SEM)	lipid/protein ratio	cholesterol (μg/egg) (av ± SEM)
DRM	1.66 ± 0.09	0.47 ± 0.02	3.6	0.27 ± 0.02
light	0.96 ± 0.03	0.11 ± 0.01	8.7	0.21 ± 0.01
heavy	1.48 ± 0.08	0.70 ± 0.06	2.1	0.16 ± 0.00

[a] Light, heavy, and DRM fractions were isolated as described in the legend of Figure 1. The total protein concentration was measured in each fraction using the Bradford reagent (Bio-Rad). Cholesterol from each fraction was extracted and analyzed by thin-layer chromatography. The total lipid concentration in one egg was calculated by adding the mass of each lipid component (fatty acids, cholesterol, PI/PS, PE, PC, and SM) that was analyzed by TLC and dividing by the number of the eggs in each of the fractions. Each value is the mean ± SEM of three different experiments.

presence of ganglioside GM1 in the DRM and light fractions suggests that DRMs originate from the light membrane fraction of the plasma membrane.

Content of Membrane Fractions. Analysis of the overall lipid and protein composition of light, heavy, and DRM fractions is described in Table 2. Heavy membranes have 67% more protein than the DRM fraction, and 7 times more than the light membranes. The highest concentration of lipids was found in the DRM fraction. Light membranes were found to have low concentrations of proteins and lipids. As a consequence of the high protein concentration, the ratio of lipid to protein is 2.1 in the heavy membrane and 3.6 in the DRM fraction, while in light membranes, the low concentration of lipids and proteins caused the ratio to be as high as 8.7. Cholesterol was found to be enriched in the DRM fraction followed by the light membrane fraction. The lowest concentration of cholesterol was observed in the heavy membrane fraction (Table 2).

Analysis of Lipid Content. The lipid content of each fraction was analyzed using both TLC and HPLC methods (Figures 4 and 5). Both methods were used because neither

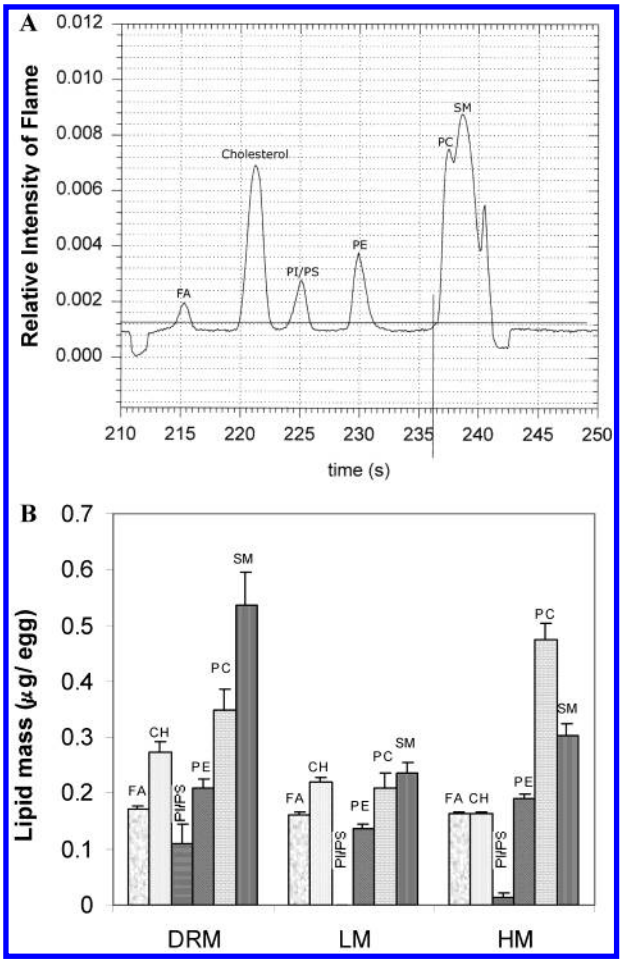

FIGURE 4: Lipid composition in light, heavy, and DRM fractions. Lipids were extracted with methanol and chloroform, and 1.5 μL samples were separated by thin-layer chromatography. Lipids were detected by a flame intensity recorder, Iatroscan TH-10 analyzer. (A) Analysis of lipids that were separated from the DRM fraction. The *X*-axis represents the time running of the flame (in seconds). The *Y*-axis represents the relative intensity of the flame as it is scanned over the TLC rod. (B) Mass values of lipid components from each fraction. Each value is an average of three different experiments ± SEM.

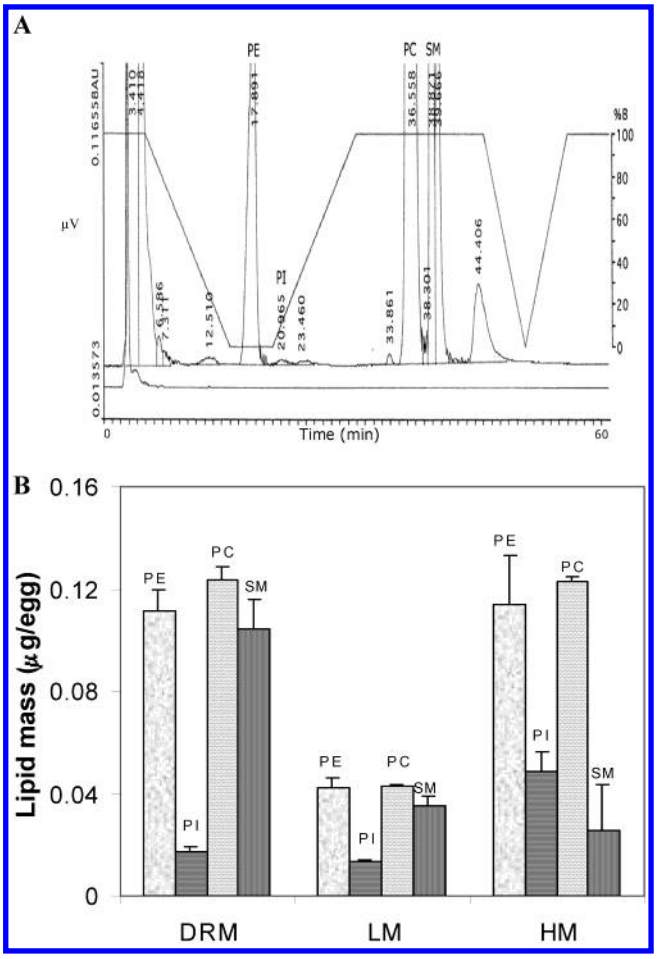

FIGURE 5: Phospholipid composition in light, heavy, and DRM fractions. Lipid samples (200 μL) were extracted with methanol and chloroform and separated with high-pressure liquid chromatography. (A) Analysis of lipids that were separated from the DRM fraction. The phospholipid elution times (0−60 min) are on the *x*-axis, and the ELSD response (microvolts) is on the *y*-axis. The solvent gradient is shown with straight lines. (B) Mass values of phospholipid components from each fraction. Each value is an average of three different experiments ± SEM.

could separate all of the lipid components. Separation of lipids by thin-layer chromatography is shown in Figure 4. The salient features obtained with TLC are as follows. (1) The major component in the DRM fraction is SM. The level of cholesterol is higher there than in other fractions, and PC levels are higher than in the light fraction. (2) In the light membranes, cholesterol, PC, and SM were all present in approximately equal amounts. (3) In heavy membranes, PC is the dominant component and the level of cholesterol is substantially lower. (4) The relative amounts of each lipid in the DRM and light fractions are nearly the same (Figure 4B).

Since PI and PS, and PC and SM, could not be cleanly separated into two different peaks by the TLC procedure (Figure 4A), we also separated the phospholipids in each fraction by HPLC. Using this method, each of the lipid components except cholesterol was eluted in a different peak (Figure 5A). By HPLC separation, PE, PC, and SM are the major components in the DRMs and the light membrane fraction (Figure 5B). However, in the heavy membranes, the dominant components are PC and PE, while the SM concentration is much lower (Figure 5B). PS could not be reliably detected by HPLC due to peak splitting and migration, but PI was found in all the fractions, with a slight enrichment in heavy membranes (Figure 5B). Analysis reveals that the phospholipids (PE, PI, PC, and SM) in the DRM and light fractions are present in different total amounts but exhibit the same relative proportions. Comparison of lipid components by TLC and HPLC revealed similar relative proportions of a given lipid in each fraction; however, the actual lipid mass per egg was quite different for the two methods (Figures 4 and 5). Due to the methodological difference, the HPLC method produced lower lipid estimates, while the TLC method produced higher values. Using fatty acid analysis on a gas chromatograph, correction factors for the HPLC method were independently generated (1.55 for PC, 1.24 for PE, 2.04 for PS, 2.04 for PI, and 2.82 for SM), and if these are applied, the values generated with this method are closer to the TLC results but still smaller. Figure 5 presents the raw data without the use of any correction factors.

Qualitative examination of Figures 4 and 5 suggests that the DRM and light fractions have similar compositions, both of which differ significantly from that of the heavy fraction. To test this suggestion more rigorously, the data were

Table 3: One-Way Analysis of Variance for Comparison between PC/SM Ratios in the Three Membrane Fractions[a]

	PC (%) (av ± SEM)	SM (%) (av ± SEM)	PC/SM (av ± SEM)
DRM	34.81 ± 1.1	29.16 ± 0.90	1.18 ± 0.07
light	32.07 ± 1.43	26.34 ± 1.26	1.20 ± 0.11
heavy	40.22 ± 1.91	8.56 ± 8.8	4.76 ± 0.14*

[a] PC and sphingomyelin values in each fraction were calculated as percent values. ANOVA was run using Sigmastat (Jandel Software, Inc.). There is a statistically significant difference ($P < 0.0001$) between the DRM and HM values (asterisk). All pairwise multiple-comparison procedures (Student−Newman−Keuls method).

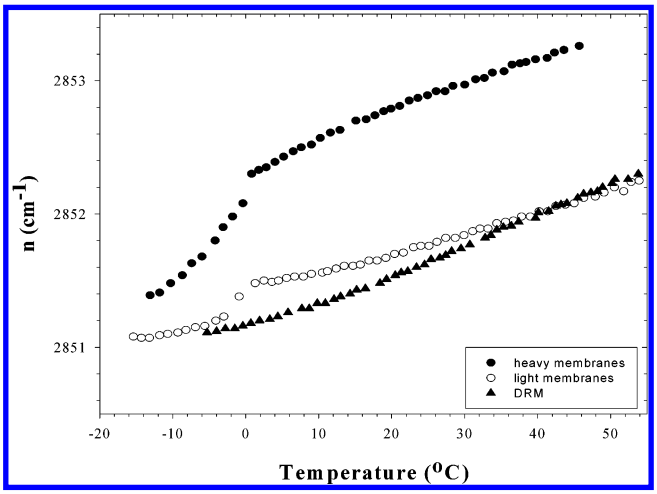

FIGURE 6: Comparison of thermotropic phase transition measurements between light (○), heavy (●), and DRM (▲) fractions. The CH_2 symmetric stretch (ranging from 2850 to 2854 cm^{-1}) was recorded as the temperature was varied between −20 and 50 °C. The thermotropic phase transition was determined by the temperature value at which the lipid fraction was changed from the gel to the liquid state. Results are representative of three different experiments.

subjected to analysis of variance. Since the HPLC analysis could not separate cholesterol, the data for phosphatidylcholine and SM were used. The ratios between PC/SM contents were calculated for each fraction, using the two analytical techniques, and those ratios were compared in a one-way analysis of variance. The results (Table 3) show that the DRMs and light membrane fraction were not significantly different in composition, while the DRMs and the light membrane fraction were both highly significantly different from the heavy membrane fraction.

Phase Behavior. We used FTIR spectroscopy on membrane fractions subjected to a steady temperature increase from −20 to 50 °C to measure phase behavior. We chose the vibrational frequency (wavenumber) of CH_2 groups because the vast majority of these are in the acyl chains in membrane lipids. The wavenumber in the heavy membranes starts at 2851.4 cm^{-1}, while in light and DRM fractions this point is lower (2851.1 cm^{-1}) (Figure 6). A higher wavenumber suggests increased disorder in the acyl chains. The wavenumber versus temperature plot of the heavy membranes showed a wavenumber range of approximately 1.8 cm^{-1}. In light membranes, the wavenumber range from −16 to 54 °C was 1.1 cm^{-1}, and in the DRM fraction, the wavenumber band from −5 to 54 °C was also 1.1 cm^{-1} (Figure 6). The wavenumber excursion in the first thermal transition (−14 to 2 °C) was 0.8 cm^{-1}/°C in heavy membranes, while in light membranes, it was 0.4 cm^{-1}/°C. In the DRM fraction, the main phase transition and the first transition excursion were hardly detected (0.012 cm^{-1}/°C), indicating greater conformational order in the DRM fraction (Figure 6). The similarity in lipid composition between the light and DRM fractions appears to be reflected in their thermotropic phase behavior.

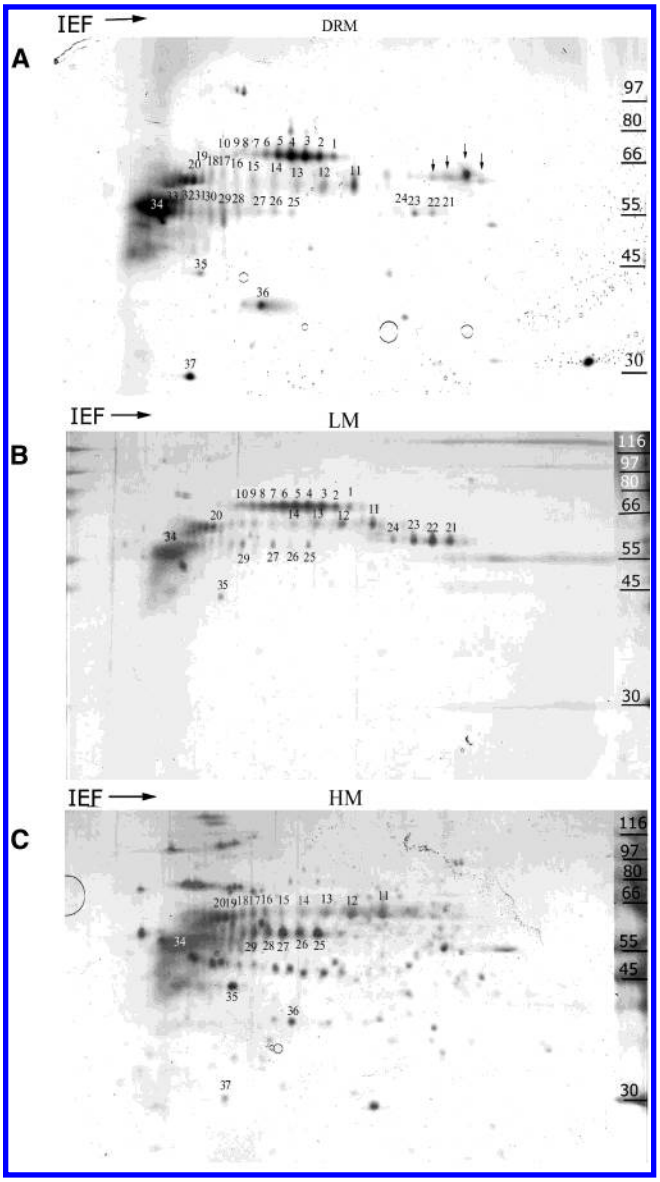

FIGURE 7: IEF2D gels of DRM (A), light (B), and heavy (C) fractions. Proteins were extracted from 300 eggs as described in Materials and Methods. The proteins were separated by 2D gel electrophoresis (acidic end on left) and analyzed with PDQuest (Bio-Rad). Major proteins were labeled with numbers. Numbers 25−33 in panel B and C are below the corresponding proteins. Proteins that were detected only in DRMs are labeled with an arrowhead.

Protein Composition of Membrane Fractions. Using two-dimensional gel electrophoresis, we analyzed the protein composition in each of the fractions (Figure 7). The distribution of the membrane proteins ranged between 21 and 116 kDa in the pH range of 4−9 (Figure 7). Most of the proteins fell into the molecular mass range of 50−70 kDa. A group of proteins with the same molecular mass range were detected at approximately 70 kDa (group 1−10 proteins in Figure 7A,B). These could be isoforms of one protein that were shifted as a result of glycosylation or phosphoryl-

ation. Comparison between the three membrane fractions shows a high degree of similarity in the protein composition in the DRM and light fractions (Figure 7A,B). Alignment of the gels allowed identification of major proteins in each fraction, labeled with a number on each figure (Figure 7A−C). Most of the proteins that were found in the light membranes are also localized in the DRM fraction (Figure 7A,B, proteins 1−8, 22, and 23). Some other proteins (like 11−20 and 25−34) were detected in all three fractions with different intensities (Figure 7A−C). Proteins labeled with arrowheads were found only in the DRM fraction (Figure 7A). The resemblance between the DRM and the light fractions is remarkable (nearly 60% congruity), while the heavy fraction is clearly different from the DRM and light fractions. In statistical analysis, it emerged that the DRMs and light membrane fractions are not significantly different, while both the light membranes and DRM fraction differ significantly from the heavy membrane fraction (data not shown).

DISCUSSION

New Findings. We have isolated DRMs from mature *X. laevis* egg plasma membrane and show that they are very similar to the detergent-free light membrane fraction from a discontinuous sucrose gradient. These fractions are not identical, but our attempts to compare them quantitatively indicate that these two membrane fractions are not significantly different in three aspects: (1) the relative amount of lipid in each fraction (Figures 4 and 5), (2) the PC/SM ratio (Table 3), and (3) and the number of common spots on 2D protein gels (Figure 7). In addition, both DRMs and the light membrane fraction exhibit high cholesterol and high ganglioside GM1 content as well as similar thermotropic phase behavior. While this paper was being reviewed, another paper was published describing DRMs in *Xenopus* eggs (*25*). They reported a similar enrichment in cholesterol and GM1 ganglioside in DRMs and also provided evidence that these plasma membrane domains may be involved in tyrosine kinase signaling during fertilization.

Assignment of the Source of Membrane Fractions. We are confident that the light, heavy, and DRM fractions isolated here are from the plasma membrane based on the results of cell surface biotinylation and the presence of several plasma membrane markers (Na^+/K^+ ATPase and several signaling molecules). In addition, when this discontinuous sucrose gradient technique was used on other cell types, plasma membrane fractions were also isolated (*12*, *21*, *26*). To compare lipid contents, membrane phase tansitions, and protein distributions from the three fractions, we used the same volume as previously suggested (*7*, *27*).

Localization of Gangliosides. The cholera toxin B marker was used as a probe for ganglioside GM1 (*23*), which is typically enriched in rafts. The relatively heavy labeling of GM1 in DRM and light membrane vesicles suggests that these two preparations were derived from the same source.

Similarities between Light Membrane and DRM Lipid Analysis. The similarity between the light fraction and the DRMs is clearly supported by lipid analysis (Figures 4 and 5). While the absolute amounts of the various lipids in the DRMs and the light fraction are different, the relative amount of each lipid is similar. This is not too surprising since the isolation procedures for each of these fractions use different conditions (centrifuge time and presence of detergent), so the absolute amount of purified lipid will differ. Similar limited amounts of core sphingolipid domains were recovered under detergent-free conditions in a discontinuous sucrose gradient as light membranes highly enriched for GPI-anchored proteins (*14*). We have shown that the light fraction and the DRM fraction have similar high cholesterol and SM contents. These make up 50% of the total membrane lipid in the DRM fraction and 43% in the light membrane fraction. These are the two components most critical for raft formation and the liquid-ordered state (*28*). A comparison with previous studies of light and heavy membrane fractions from other cell types indicates that this enrichment in cholesterol and SM is not universal. In T lymphocytes, the light membranes do not appear to be more enriched in cholesterol or SM than the heavy membranes (*13*, *26*). In neurons, light membranes were found to be enriched in cholesterol, SM, PC, and PE compared with heavy membranes (*29*). However, these results may be misleading since the lipids isolated from light and heavy membranes were compared on the basis of loading equal amounts of protein (*29*). Brown and London (*7*) commented on this study by stating, "every lipid would appear to be enriched in a light membrane sample for equal amounts of protein, just because the light membranes have a higher lipid:protein ratio, even if the lipid composition of the two fractions were identical".

Cholesterol and SM are critical in the formation of rafts as they enhance the order among the acyl chains and lead to the L_o state (*28*). Nevertheless, The relative amount of cholesterol in our DRM fraction is smaller than the amounts found in other cell types. For example, human platelet DRMs contain ~50% cholesterol by weight compared to our 15% (*30*). However, the SM levels are higher in frog egg DRMs (30%) than the levels in platelet DRMs (20%). Perhaps the higher SM levels can have a similar raft-inducing effect like a high cholesterol level would. We must also point out here that the frog egg plasma membrane faces an environment very different from that of the platelet. It develops in pond water where it must have much lower water permeability than platelets, and it faces much cooler temperatures than platelets do. These two major differences might influence the selection of SM over cholesterol. Despite that, *Xenopus* eggs are an excellent choice for the study of membrane function.

Similarities between Light Membrane and DRM FTIR Phase Behavior. The FTIR melting profiles of *Xenopus* plasma membrane fractions suggest that the DRM fraction is more similar to the light membrane fraction than the heavy membrane fraction. Examination of the thermal response of the CH_2 stretching mode revealed several parameters that support this hypothesis. First, in the heavy membranes, the vibrational frequency is higher than in the light membranes or the DRM fraction. Second, the vibrational excursion in the heavy membranes is higher than in the light fraction or the DRMs (1.8 vs 1.1 cm^{-1}, respectively), and third, the gel-to-liquid phase transition in heavy membranes is much sharper than in the light or DRM fractions (Figure 6). These data suggest that the average conformational state of the lipid acyl chains in the heavy membranes is more disordered than that of the light membranes and the DRMs. It has been demonstrated that high concentrations of cholesterol and SM

are necessary for the formation of liquid-ordered domains (*6*, *28*). Cholesterol is intercalated between the lipid acyl chains, and this alters the physical state of the membrane (*31*, *32*). Significant motional ordering of the acyl chains is seen in the liquid crystalline phase, and disordering occurs in the gel phase. As a result, the transition becomes broader and with the high cholesterol content becomes difficult to detect (*33*, *34*). The phase behavior seen with the DRM fraction and to a lesser extent with the light membranes (Figure 6) seems to be consistent with these known effects of cholesterol. The differences in the phase behavior of DRM and light fractions, which are fairly minor, might be due to the differences in preparative procedures. Nevertheless, the DRM and light membrane fractions are remarkably similar in this regard, and both are quite different from the heavy membrane fraction.

Similarities in Protein Composition. Careful comparison of 2D gels from each membrane fraction indicates that the protein composition of the DRM fraction is similar to that of the light membrane fraction (Figure 7). Identification of proteins from two-dimensional gels would be very useful, but we were unable to obtain sufficient amounts of protein for identification by mass spectroscopy; therefore, the identifications are necessarily limited to the availability of antibodies to known membrane proteins.

In many cell types, GPI-anchored proteins and Src-family kinases partition into DRMs and are used as raft markers (*9*, *22*, *35*−*37*). GPI-anchored proteins as well as Src-family kinases were found in the light membranes of T lymphocytes (*26*). However, in T lymphocytes, Src-family kinases were not restricted to the light membranes but were also found in the heavy membrane (*26*). In that paper, Fyn was found in the light membranes, but only when large amounts of protein were loaded on the gels (*26*). Since we are suggesting that the DRM fraction is a component of the light membrane fraction, we would expect proteins that are enriched in the DRM fraction to be present in the light membranes. That holds true for Src. However, Fyn and PLCγ1 are not present in either fraction.

DRMs Found in Other Eggs and Sperm. The very recent report of Sato et al. (*25*) also found that PLCγ1 was absent in the raft fractions of *Xenopus* eggs while the Src-family kinase Xyk was localized in rafts. DRMs were also found in sea urchin eggs (*38*) and mouse sperm membranes (*39*); depletion experiments suggested an involvement in egg activation and acrosomal exocytosis, respectively.

CONCLUSIONS

We conclude that the light membrane fraction is more similar to the detergent-resistant fraction, DRM, than to the heavy membrane fraction. However, we must note that all of our membrane extractions were performed in the cold to minimize enzymatic degradation and this might have lead to phase separation and could have artificially stabilized the raft aggregates (*11*). Recently, the laser trap technique and fluorescence resonance energy transfer measurements were used to study the presence of rafts at physiological temperatures (*36*, *40*). One of these detected the presence of lipid rafts ~26 nm in size in living cells and suggested that raft aggregation is functionally relevant in signal transduction. We propose that the light membrane fraction isolated from *Xenopus* eggs contains similar microdomains based on lipid and protein composition, the presence of phase-separated L_o domains, ganglioside GM1, and cholesterol and SM content.

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