Sterol Carrier Protein-2 Selectively Alters Lipid Composition and Cholesterol Dynamics of Caveolae/Lipid Raft vs Nonraft Domains in L-Cell Fibroblast Plasma Membranes†

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ABSTRACT: Although the functional significance of caveolae/lipid rafts in cellular signaling and cholesterol transfer is increasingly recognized, almost nothing is known regarding the lipids, cholesterol dynamics, and factors regulating these properties in caveolae/lipid rafts as opposed to nonlipid raft domains of the plasma membrane. The present findings demonstrate the utility of con-A affinity chromatography for simultaneous isolation of caveolae/lipid raft and nonlipid raft domains from plasma membranes of L-cell fibroblasts. These domains differed markedly in both protein and lipid constituents. Although caveolae/lipid rafts were enriched in total lipid, cholesterol, and phospholipid as well as other markers for these domains, the cholesterol/phospholipid ratio of caveolae/lipid rafts did not differ from that of nonlipid rafts. Nevertheless, spontaneous sterol transfer was 7−12-fold faster from caveolae/lipid raft than nonlipid raft domains of the plasma membrane. This was largely due to the near absence of exchangeable sterol in the nonlipid rafts. SCP-2 dramatically and selectively enhanced sterol transfer from caveolae/lipid rafts, but not from nonlipid rafts. Finally, overexpression of SCP-2 significantly altered the sterol dynamics of caveolae/lipid rafts to facilitate retention of cholesterol within the cell. These results established for the first time that (i) caveolae/lipid rafts, rather than the nonlipid raft domains, contain significant levels of rapidly transferable sterol, consistent with their role in spontaneous sterol transfer from and through the plasma membrane, and (ii) SCP-2 selectively regulates how caveolae/lipid rafts, but not nonlipid raft domains, mediate cholesterol trafficking through the plasma membrane.

Plasma membrane cholesterol is essential not only for maintaining cell permeability and structure, but also for regulating cholesterol uptake/efflux, receptor−effector coupling (insulin receptor), cell signaling (eNOS), immune function, transcytosis, and cell recognition (reviewed in refs *1*−*8*). Until recently almost nothing was known as to how plasma membrane cholesterol contributes to compartmentalizing and regulating these functions within the cell membrane. An important breakthrough was the observation that cholesterol self-assembles into lateral (reviewed in refs *5* and *9*) and transbilayer (reviewed in ref *10*) cholesterol-rich domains in model membranes. The functional significance of these cholesterol-rich microdomains observations became apparent when similar cholesterol-rich microdomains (termed caveolae/lipid rafts) were discovered in the plasma membranes of essentially all cells studied (reviewed in refs *6*−*8*). A review of the literature now reveals >2000 publications in the past decade that ascribe a growing variety of functions to plasma membrane caveolae/lipid rafts. Particularly significant is the finding that the high-density lipoprotein (HDL) scavenger receptor B1 (SR−B1) resides in caveolae/lipid rafts (reviewed in ref *8*). While HDL-mediated cholesterol uptake/efflux via caveolae/lipid rafts is very rapid, it is as yet unclear how cholesterol moves in and out of these highly cholesterol-rich domains. Finally, adding further interest in caveolae/lipid rafts is the fact that potential bioterror agents including bacterial (anthrax toxin, cholera toxin, Shiga toxin, enterohemorrhagic *Escherichia coli* Shiga-like toxin) and plant (ricin) toxins (*11*, *12*), viruses (Ebola, Marburg, Echovirus, Norwalk, Influenza) (*13*−*18*), and parasites (malaria) (reviewed in ref *19*) also use these cholesterol-rich microdomains as cell entry portals (*20*). Although caveolin-free cells do not have caveolae, such cells still contain lipid rafts (*21*). Because biochemical studies show that lipid rafts readily copurify with caveolae and exhibit many similar properties (*21*), caveolae may represent a subfraction of lipid rafts (reviewed in refs *6* and *22*).

Formation of caveolae/lipid rafts in membranes appears based on the unique properties of lipids such as cholesterol (reviewed in refs *5*, *6*, and *9*) and sphingolipids (*23*), both of which spontaneously and independently form lateral and transbilayer domains in model membranes (*24*). However,

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when both lipids are enriched in a membrane (e.g., plasma membranes), cholesterol has high affinity for the saturated acyl chains of sphingolipids and thereby synergistically facilitates formation of mixed cholesterol/sphingolipid domains (*23*, *24*). Furthermore, the high sphingolipid content of microdomains confers resistance to detergent solubilization, an effect not requiring cholesterol (*25*) but enhanced by cholesterol (*24*, *26*). The importance of cholesterol in plasma membrane caveolae/lipid rafts is underscored by the fact that cholesterol depletion or disruption abolishes many functions associated with caveolae/lipid rafts (reviewed in ref *8*).

Despite the contributions of caveolae/lipid rafts in both normal cell physiology, relatively little is known regarding their lipid composition (*8*, *27*−*29*), structure, cholesterol dynamics, or factors that regulate these features of caveolae/lipid rafts in living cells. This paucity of information is due at least in part to the fact that, in the majority of biochemical studies of caveolae/lipid rafts, detergents were used to isolate these microdomains from whole cells, not plasma membranes. Depending on the method used, estimates of the amount of plasma membrane comprised of caveolae/lipid rafts range from a few % (determined from nondetergent isolation) to the majority (determined from detergent-resistant isolation) of membrane lipids (*22*). In view of the scarcity of lipid compositional and cholesterol dynamic data for caveolae/lipid rafts, it is not surprising that even less is known about these parameters in the nonlipid raft domains of plasma membranes.

To begin to address these issues, a new method based on concanavalin-A affinity chromatography was developed to separate large quantities of caveolae/lipid rafts from nonlipid domains in the plasma membrane and to further characterize these fractions. Although this method does not resolve noncaveolar lipid rafts from caveolar lipid rafts, for the first time it allows comparisons of the properties of nonraft vs lipid raft domains. It was shown in the present work that (i) Caveolae/lipid rafts were enriched in known caveolae/lipid raft markers including flotillin, caveolin, SR−B1, ganglioside, and cholesterol. In contrast, nonlipid raft domains were deficient in these markers, but enriched in a nonraft marker (Na^+K^+-ATPase); (ii) Caveolae/lipid rafts were enriched in total lipid, total phospholipid, and select phospholipid classes. In contrast, nonlipid raft domains were relatively lipid poor. (iii) Caveolae/lipid rafts exhibited significantly faster spontaneous sterol transfer than nonlipid raft plasma membrane domains in vitro; (iv) Caveolae/lipid rafts, but not nonlipid rafts, were responsive to the action of sterol carrier protein (SCP-2) in accelerating sterol transfer in vitro; (v) SCP-2 overexpression in transfected cells dramatically altered the lipid composition and cholesterol dynamics of caveolae/lipid rafts and much less so the nonlipid raft domains of the plasma membrane. (vi) SCP-2 overexpression in transfected cells reduced the responsiveness of cholesterol dynamics in caveolae/lipid rafts to addition of SCP-2 in vitro.

MATERIALS AND METHODS

Materials. Concanavalin A-Sepharose (con-A sepharose) resin was purchased from Pharmacia (Piscataway, NJ). EDTA, tris-base, sucrose, PBS, and PMS-F were purchased from Sigma Chemical (St. Louis, MO). Rabbit polyclonal antisera to recombinant human SCP-2 was prepared as described previously (*30*). Rabbit polyclonal anti-sera to caveolin-1 and flotillin were purchased from BD Transduction Laboratories (Palo Alto, CA). Rabbit anti-Na^+ K^+-ATPase, and anti-SR-B1 were purchased from Novus Biologicals (Littleton, CO). Rabbit anti-transferrin receptor (CD71) was purchased from Zymed Lab Inc. (San Francisco, CA). Rabbit anti-clathrin was purchased from Oncogene Research Products (San Diego, CA). Rabbit anti-cholera toxin B was purchased from Sigma (St Louis, MO). Silica Gel G and Silica Gel 60 thin-layer chromatography plates were from Analtech (Newark, DE) and EM Industries, Inc. (Darmstadt, Germany), respectively. Neutral lipid and fatty acid standards were purchased from Nu-Chek Prep, Inc. (Elysian, MN), while phospholipid standards were obtained from Avanti (Alabasta, AL). Dehydroergosterol (DHE)[1] was synthesized and highly purified (>99%) as described earlier (*31*). All reagents and solvents used were of the highest grade available and were cell culture tested.

L-Cell Culture. Murine L-cells were grown to confluency at 37 °C and 5% CO_2 in Higuchi medium (*32*), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), as described earlier (*30*). For measurements of sterol transfer between isolated membrane fractions, dehydroergosterol (DHE, a naturally fluorescent sterol) was bioincorporated into caveolae/lipid raft and nonlipid raft domains as previously described for DHE incorporation into plasma membranes of L-cells (*33*). Control (mock transfected) L-cells and L-cells transfected with the 15-kDa pro-SCP-2 cDNA were prepared as described earlier (*34*). Western blot analysis performed on the SCP-2 expression cell line revealed that levels of SCP-2 (0.036% cytosol protein) were in the physiological range found in animal tissues (reviewed in refs *35* and *36*). In untransfected and control/mock transfected cells, levels of these proteins were below the level of detection.

Preparation of Caveolae/Lipid Rafts and Nonraft Domains from Isolated L-Cell Plasma Membranes. For in-depth biochemical characterization and comparison of caveolae/lipid rafts to nonraft microdomains, a two-stage method was developed based on (i) isolation of plasma membranes without the use of detergents and (ii) separation of caveolae/lipid rafts and nonraft domains by concanavalin-A (con-A) sepharose 4B affinity chromatography on the plasma membrane fraction. Briefly, cells were grown on 12 culture trays (200 × 200 mm, Nalge-Nunc, Milwaukee, WI) for 96 h. Before isolation, cells were washed with phosphate-buffered saline (PBS) and cultured overnight in serum-free medium. To harvest, cells were rinsed twice with PBS, scraped into PBS with protease inhibitor (0.2 μM PMS-F), and centrifuged for 5 min at 1000*g*. The pellet was resuspended in buffer A (0.25 M sucrose/5 mM TRIS-HCL, pH 7.8). The sample was homogenized in a N_2 Bomb Cell disrupter (Parr Instrument Co., Moline, IL) with 40 psi of N_2 for 13 min. The postnuclear supernatant was collected following centrifugation

[1] Abbreviations: SCP-2, sterol carrier protein-2; PL, phospholipid; PE, ethanolamine glycerophospholipid; PI, phosphatidylinositol; PS, phosphatidylserine; PC, choline glyerophospholipid; SM, sphingomyelin; PA, phosphatidic acid; TG, triacylglycerides; DG, diacylglycerides; MG, monoacylglycerides; CE, cholesteryl ester; C, cholesterol; FFA, free fatty acid; DHE, dehydroergosterol; DRM, detergent resistant membrane; PBS, phosphate buffered saline.

at 1000*g* for 10 min. The postnuclear supernatant was placed on top of 30% Percoll in sucrose/TRIS and centrifuged at 70000*g* for 30 min in an SW40Ti rotor on a model XL90 ultracentrifuge (Beckman Instruments, Fullerton, CA). The plasma membrane enriched fraction located at the interphase between the Percoll and sample layer was collected and sonicated briefly (15 s pulse 3 times over 3 min) using a Fisher 550 Sonic dismembrator (Fisher Scientific, Pittsburgh, PA). The caveolae/lipid raft fraction was isolated from the plasma membranes by a modification of the procedure of Schroeder et al. (*37*). Briefly, the plasma membrane fraction was added to 100 mL of prepared Con A-Sepharose resin (Pharmacia, Piscataway, NJ) in buffer X (10 mM Hepes, pH 7.4, 140 mM KCL, 1 mM $MgCl_2$, 1 mM $MnCl_2$), incubated for 5 min under bubbling N_2, and then allowed to stand undisturbed for 15 min. The solution was poured into a column and left to stand a further 15 min. The column was eluted under bubbling N_2 at a rate of 2 mL/min and 10 mL fractions were collected until the OD_{280} was close to zero. These fractions were designated "nonlipid raft fractions". Caveolae/lipid rafts were eluted under bubbling N_2 using buffer Y (buffer X plus 0.5 M α-methylmannoside) and were collected in 10 mL fractions at 2 mL/min until the OD_{280} leveled off. The caveolae/lipid raft enriched fractions were pooled and, along with the nonlipid raft fractions, were centrifuged at 70000*g* for 15 h. The pellets were resuspended in minimal amount of wash buffer (buffer X with 1 mM EDTA without $MgCl_2$ or $MnCl_2$). In some preparations, excess Mn^{2+} resulted in formation of a brown precipitate which was removed by briefly centrifuging the sample at 10 000 rpm for 2 min.

Western Blotting of Caveolae/Lipid Raft and Nonlipid Raft Markers. The level of caveolae/lipid raft markers (caveolin-1, flotillin, SR−B1) and nonlipid raft marker (Na^+,K^+-ATPase) was determined in both the caveolae/lipid raft and nonlipid raft fractions by Western blot analysis. Samples (0.5 μg) were run on tricine gels (12%) and transferred to nitrocellulose membranes. The blots were blocked in 3% gelatin in TBST (10 mM Tris-HCL, pH 8, 100 mM NaCl, 0.05% Tween-20) before incubation with the polyclonal rabbit antibodies against either caveolin, flotillin, SR−B1, or Na^+,K^+-ATPase. Alkaline-phosphatase conjugates of goat anti-rabbit IgG and Sigma Fast 5-bromo-4chloro-3-indolyl phosphate/nitro blue tetrazolim tablets (Sigma, St. Louis, MO) were used to visualize bands of interest. Proteins were quantitated by densitometric analysis after image acquisition using a single-chip CCD (charge couple device) video camera and a computer workstation (IS-500 system from Alpha Innotech, San Leandro, CA). Image files were analyzed (mean 8-bit gray scale density) using NIH Image, available by anonymous FTP from zippy.nimh.nih.gov.

Analysis of Ganglioside GM1 Levels in Caveolae/Lipid Rafts and Nonlipid Raft Plasma Membrane Domains. GM1 levels in each fraction were determined by a dot-blot technique as described elsewhere (*38*). Briefly, 2 μL of each fraction was spotted onto nitrocellulose membrane blots and washed with PBS. The blots were blocked in 3% (w/v) BSA in PBS and incubated with cholera toxin B (1 μg/mL) for 30 min at 4 °C. Membranes were washed and incubated with rabbit anti-cholera toxin B for 1 h, followed by incubation with alkaline phosphatase-conjugated swine anti-rabbit Ig for an additional 30 min. Sigma Fast 5-bromo-4chloro-3-indolyl phosphate/nitro blue tetrazolim tablets (Sigma, St. Louis, MO) were used to visualize spots. Proteins were quantitated by densitometric analysis as described above.

Lipid Extraction and Distribution. Lipids from the caveolae/lipid raft and nonlipid raft fractions were extracted with *n*-hexane-2-propanol 3:2 (v/v) and resolved into individual lipid classes using Silica gel G thin-layer chromatography plates developed in petroleum ether-diethyl ether-methanol-acetic acid 90:7:2:0.5 (*39*, *40*). Total cholesterol (C), free fatty acid (FFA), triacylglyceride (TG), and cholesteryl ester (CE) content were determined by the method of Marzo et al (*41*). Total phospholipids (PL) including phosphatidic acid (PA), ethanolamine glycerophospholipid (PE), phosphatidylinositol (PI), phosphatidylserine (PS), choline glyerophospholipid (PC), and sphingomyelin (SM) were scraped from the Silica gel G TLC plates and eluted from the resin using chloroform/methanol/HCL (100:50:0.375, v/v/v). The samples were dried under N_2 and resuspended in chloroform and then applied to silica gel 60 TLC plates where individual phospholipids and SM were resolved using chloroform/methanol/water/acetic acid (150:112.5:6:10.5, v/v/v/v) and quantitated densiometrically as described in ref *28*). Lipid spots were visualized by iodine vapor and were identified by comparison to known standards (*39*). Protein concentration was determined by the method of Bradford from the dried protein extract residue digested overnight in 0.2 M KOH (*42*). Lipids were stored under an atmosphere of N_2 to limit oxidation, and all glassware was washed with sulfuric acid-chromate before use.

Phospholipid Isolation and Fatty Acid Distribution. Acid-catalyzed transesterification was performed on total phospholipid samples isolated from caveolae/lipid raft and nonlipid raft fractions to convert the lipid acyl chains to fatty acid methyl esters. Fatty acid methyl esters were then extracted into *n*-hexane and separated by gas−liquid chromatography on a Gas Liquid chromatograph-14A (Shimadzu Kyoto, Japan) equipped with a RTX-2330 capillary column (0.32 mm i.d. × 30 m length, Restek, Bellefonte, PA). The injector and detector temperatures were set at 260 °C with the temperature program of 100 °C for 1 min, 10 °C/min to 140 °C, then 2 °C/min to 220 °C, hold 1 min, then ramp 10 °C /min to 260 °C. A Waters SAT/IN analytical-to-digital interface was used to collect peak area data using Millenium^{32} 3.2 software.

Determination of DHE Steady-State Polarization in Caveolae/Lipid Raft and Nonlipid Raft Domains Isolated from L-Cell Fibroblast Plasma Membranes. A PC1 spectrofluorometer with photon-counting electronics (ISS Instruments, Inc., Champaign, IL) was used to obtain steady-state fluorescence polarization of the fluorescent (membrane) probe molecules. The excitation source was a 300 W Xe arc lamp in conjunction with a monochromator (16-nm spectral slit width) set at 324 nm. Polarization was measured in the T-format with the emission monochromator set at 375 nm, with 16-nm spectral slit widths. Samples were stirred continuously with a magnetic stir bar below the level of the light beam. Neutral density filters and narrow (0.5 mm) entrance and exit slits on the excitation monochromator were used to minimize light intensity in the excitation light path. Emission was detected through low-fluorescence cutoff filters (Schott Glass Technologies, Inc., Duryea, PA) to reduce light scatter. DHE was incorporated into caveolae/lipid raft and

nonlipid raft domains as described in the cell culture section above. DHE excitation was at 324 nm, while emission was at 375 nm (monitored through KV-389 filters from Schott Glass Technologies, Inc., Duryea, PA). Residual light scatter from membranes contributing to polarization measurements was corrected by converting polarization to anisotropy according to $r = 2P/(3 - P)$ followed by subtraction of residual fluorescence anisotropy of both donor and acceptor membranes from all experimental data. To avoid inner filter artifacts, the absorbance of sample solutions at the excitation wavelength was maintained <0.15 absorbance units. All samples (2 mL) were resuspended in filtered (0.2 micron) 10 mM PIPES buffer solution at pH = 7.4 and placed in thermostated (37 ± 0.3 °C) quartz cuvettes with temperature maintained through use of a water heating bath (Fisher Scientific, Pittsburgh, PA).

Determination of Sterol Transfer in Caveolae/Lipid Raft and Nonlipid Raft Domains of Plasma Membranes. DHE is a naturally fluorescent sterol used to determine sterol transfer between the isolated membrane fractions according to a fluorescence polarization exchange assay previously described by our laboratory (*33*, *43*−*45*). In most exchange assays, DHE polarization in donor membranes (1.5 μg of protein/mL) was first measured for 30 min to ensure a stable signal baseline and obtain an initial value for the fluorescence polarization. This was followed by addition of a 10-fold excess of acceptor membrane fraction (i.e., 15 μg of protein/mL). Alternately, the concentration of the donor and acceptor membranes were both reduced 3-fold when sample concentration or size were limiting. This did not, however, affect sterol transfer kinetics. After addition of acceptor membranes, the dehydroergosterol polarization was subsequently recorded every 20 s for 3−4 h to continuously monitor sterol transfer between membranes.

Standard Curves for the Sterol Exchange Assay. Standard curves used to determine molecular sterol transfer from DHE polarization changes in caveolae/lipid rafts or nonlipid rafts (purified from L-cell plasma membranes) during the exchange were the same as those derived previously for L-cell plasma membranes (*33*, *43*, *44*). The standard curve used to calculate the fraction of dehydroergosterol remaining in the donor membranes during an exchange was based on a polynomial equation involving polarization P during the exchange:

$$P = \Sigma\, b_n X_d^{\,n} \tag{1}$$

For sterol transfer between membrane donors and membrane acceptors, a polynomial with two terms yielded a fit with $r^2 = 0.9999$, i.e., eq 2:

$$P(x) = b_0 + b_2 X_d^{\,2} \tag{2}$$

where $b_0 = 0.3155$ and $b_2 = -0.131$.

Determination of the Initial Rate of Molecular Sterol Transfer from Caveolae/Lipid Raft and Nonlipid Raft Domains. The initial rate of DHE exchange between caveolae was calculated based on the first 10 min of exchange data by using the standard curve described above in eq 2 (*46*, *47*). The time derivative of eq 2 yields:

$$(dP/dt) = 2b_2 X_d (dX_d/dt) \tag{3}$$

As $t \rightarrow 0$, $X_d \rightarrow 1$ (i.e., initial rate criteria) and rearranging eq 3 yields:

$$(1/2b_2)(dP/dt)|_{t\rightarrow 0} = (dX_d/dt)|_{t\rightarrow 0} \tag{4}$$

To obtain the molecular DHE transfer rate (d[DHE]/dt) from donor to acceptor membranes, dX_d/dt was transformed into d[DHE]/dt by factoring in the initial donor membrane concentration, the total sterol concentration in the membrane donor, the mole percent of DHE in the donor membrane, and the value of b_2 (*46*). This information was combined with eq 12 to yield eq 5 (*46*):

$$(d[DHE]/dt)|_{t\rightarrow 0} = -463\ \text{pmol}(dP/dt)|_{t\rightarrow 0} \tag{5}$$

The initial rate of dehydroergosterol transfer was directly estimated by substituting the initial measured rate of fluorescence polarization change for $(dP/dt)|_{t\rightarrow 0}$.

Derivation of Kinetic Parameters for Molecular Sterol Transfer from Caveolae/Lipid Raft and Nonlipid Raft Domains. To determine kinetic parameters of molecular sterol transfer, eq 2 was used in conjunction with the following expression:

$$X_d = f_1 \exp(-kt) + f_2 \tag{6}$$

where f_1 is the exchangeable fraction and f_2 is the nonexchangeable fraction of membrane sterol present in the exchange. By substituting the definition of X from eq 6 into eq 2, the following expression was obtained:

$$P(x) = b_0 + b_2[f_1 \exp(-kt) + f_2]^2 \tag{7}$$

Equation 7 was then used to fit the experimental curves and resolve kinetic parameters of exchange (*46*). The half-time of the exchange was then extracted by use of the following expression (*46*):

$$t_{1/2} = (\ln 2)/k \tag{8}$$

Statistics. All values were expressed as the mean ± SEM with n and P indicated in Results. Statistical analysis was performed using analysis of variance (ANOVA) combined with the Newman-Keuls multiple comparisons test (GraphPad Prism, San Diego, CA) or student's T test. Values with $P < 0.05$ were considered statistically significant.

RESULTS

L-Cell Fibroblast Plasma Membrane Isolation. Plasma membranes were isolated from cultured L-cells using a previously established nondetergent, Percoll gradient technique (*27*, *28*) as indicated in Materials and Methods. About 1 mg of purified plasma membrane was obtained per 34 mg of L-cell homogenate (Table 1). On a protein basis, the plasma membrane fraction accounted for about 3% of total protein in the cell homogenate (Table 1). Western blot analysis was performed to determine the distribution of several marker proteins typically found in caveolae/lipid raft (caveolin-1, flotillin, SR−B1) and nonlipid raft (Na^+,K^+-ATPase) domains of the plasma membrane as compared to the cell homogenate. As expected, the plasma membrane fraction (Figure 1B, lane 2) appeared enriched severalfold

Table 1: Distribution of Caveolae/Lipid Rafts and Nonraft Domains in L-Cell Plasma Membranes: Effect of SCP-2 Expression[a]

	control L-cells		SCP-2 expressing L-cells	
cell fraction	mg of protein	%	mg of protein	%
cell homogenate	33.5 ± 13.5	100	25.5 ± 7.0	100
plasma membranes	0.96 ± 0.40	2.93 ± 0.46	0.93 ± 0.31	3.66 ± 0.53
caveolae/lipid rafts	0.27 ± 0.06	1.00 ± 0.30	0.24 ± 0.04	1.03 ± 0.14
nonlipid raft	0.69 ± 0.34	1.96 ± 0.28	0.69 ± 0.29	2.63 ± 0.55

[a] Plasma membranes, caveolae/lipid raft, and nonraft domains were isolated as described in Materials and Methods. Each data point represents pooled cells from 8 to 10 dishes of control (mock transfected) L-cells and SCP-2 overexpressing L-cells cultured as described in Materials and Methods. Values represent the mean ± SEM, $n = 3$.

in all four markers, especially flotillin and Na^+,K^+-ATPase, as compared to the cell homogenate (Figure 1B, lane 1).

Resolution of L-Cell Fibroblast Plasma Membranes into Two Fractions by Concanavalin-A Sepharose Affinity Chromatography. Plasma membranes obtained in the preceding section were resolved by concanavalin-A (con-A) sepharose affinity chromatography as described in Materials and Methods. When the plasma membrane fraction (1 mg of protein) was briefly sonicated and passed over the affinity column two fractions were obtained: a nonadherent and an adherent fraction eluted with buffer containing the displacing sugar α-methylmannoside (Figure 1A). The nonadherent fraction was eluted from the con-A affinity column within the first 100 mL eluant as a large peak (Figure 1, nonadherent fraction). The nonadherent fraction contained about 0.7 mg of protein, representing 67.7 ± 5.5% of plasma membrane (Figure 2A) and 1.96 ± 0.28% of cell homogenate (Table 1) protein, respectively. To obtain the plasma membrane fraction that adhered to con-A, the column was eluted with a displacing sugar, i.e., α-methylmannoside (Figure 1A). The resulting con-A adherent fraction eluted contained about 0.3 mg of protein, representing about 31.9 ± 5.7% of plasma membrane (Figure 2A) and 1.0 ± 0.3% of cell homogenate (Table 1) protein, respectively.

Distribution of Nonlipid Raft and Caveolae/Lipid Raft Marker Proteins in the Nonadherent Fraction of Plasma Membranes Eluting from Concanavalin-A Sepharose Affinity Columns. Western blot analysis was performed on the nonadherent fraction obtained by con-A affinity chromatography to determine the presence of known protein markers for plasma membrane microdomains: (i) nonlipid raft markers (Na^+,K^+-ATPase, transferrin receptor (CD71), and clathrin; (ii) caveolae/lipid raft markers (caveolin-1, flotillin, and SR−B1). Since Na^+,K^+-ATPase is an enzyme widely distributed in almost all cell plasma membranes and is a well-known nonlipid raft marker (*28*), the nonadherent fraction was first examined by Western blotting with anti-Na^+,K^+-ATPase to reveal a 10-fold enrichment of Na^+,K^+-ATPase in the nonadherent fraction (Figure 1B, lane 3) as compared to cell homogenate (Figure 1B, lane 1). The nonadherent fraction was also tested for the presence of two other known nonlipid raft marker proteins, transferrin receptor and clathrin. While the transferrin receptor is an excellent protein marker for nonlipid rafts (*48*), it is almost exclusively found in endothelial cells. Neither the fibroblast homogenate, plasma membrane, or either con-A fraction contained detectable amounts of transferrin receptor (data not shown). In addition, clathrin, another protein associated with nonlipid rafts (reviewed in ref *49*), was detected in fibroblast homogenate and plasma membranes, but not in either con-A affinity chromatography eluting fraction. This was likely because clathrin is known to dissociate/reassociate from

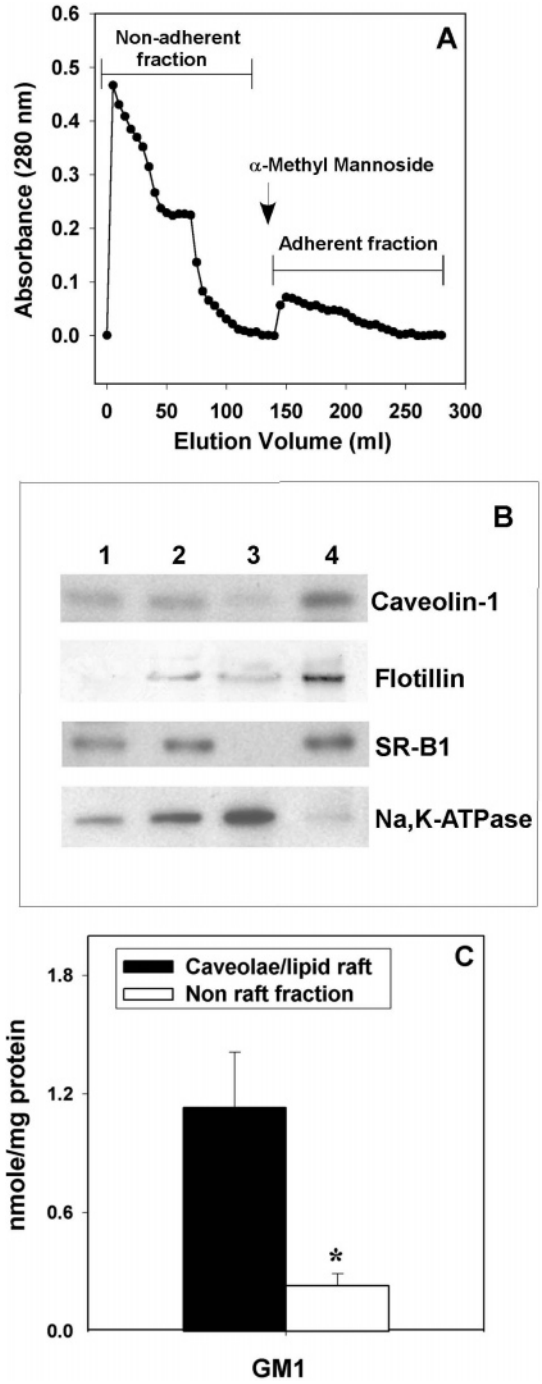

FIGURE 1: Separation and characterization of caveolae/lipid raft and nonlipid raft fractions by concanavalin-A affinity chromatography of plasma membranes from L-cells and SCP-2 expressing cells. (A) L-cell plasma membrane elution profile. (B) Western analysis of homogenate (lane 1), plasma membrane (lane 2), nonraft (lane 3), and caveolae/lipid raft fractions (lane 4). Antibodies against caveolin-1, flotillin, SR−B1, and Na^+, K^+-ATPase were used to identify proteins in each fraction. (C) Ganglioside GM1 quantitation in caveolae/lipid raft and nonlipid raft fractions of plasma membranes from L-cells. Values represent means + SEM. (*) indicates significance $P < 0.01$ ($n = 3-4$) as compared to the nonlipid raft fraction isolated from L-cells.

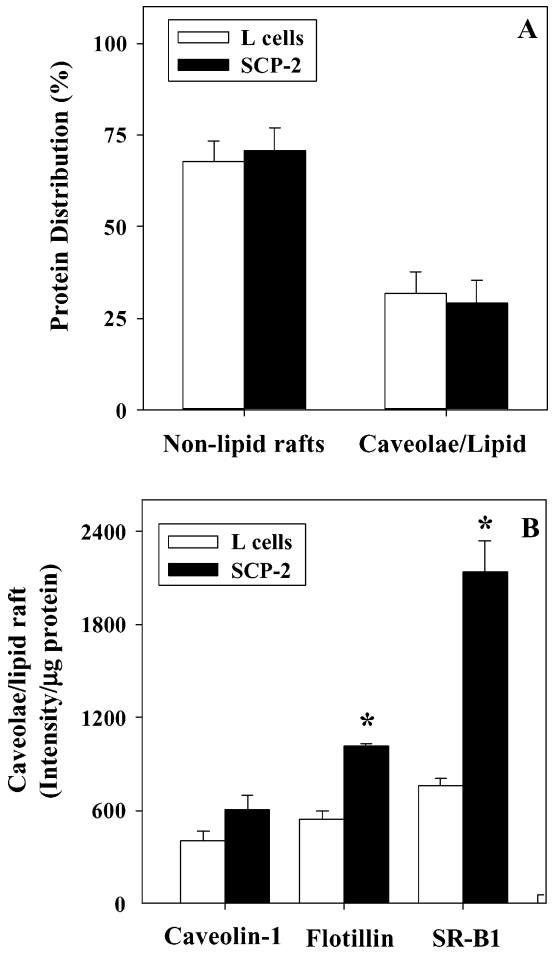

FIGURE 2: Distribution of protein and select protein markers in caveolae/lipid rafts from plasma membranes of L-cells and SCP-2 expressing cells. (A) Distribution (%) of plasma membrane protein between caveolae/lipid rafts and nonlipid rafts. (B) Caveolin-1, flotillin, SR−B1, and Na^+, K^+-ATPase levels were determined in caveolae/lipid raft fraction. Open bars refer to control (mock transfected) L-cells while solid bars refer to SCP-2 expressing cells. Values represent means ± SEM. (*) indicates significance $P < 0.05$ ($n = 3-4$) as compared to L-cells.

nonlipid raft regions of the plasma membrane as well as from internalized vesicles (*50*, *51*) and the clathrin remained associated with the column gel filtration matrix.

While these studies suggested that the nonadherent fraction contained nonlipid rafts, it was also essential to demonstrate that this fraction did not contain caveolae/lipid rafts. To distinguish this possibility, Western blotting of known caveolae/lipid raft protein markers (flotillin, SR−B1, caveolin) was performed. The nonadherent fraction was essentially devoid of SR−B1 and significantly reduced in caveolin and flotillin (Figure 1B, lane 3) as compared to plasma membrane (Figure 1B, lane 2), cell homogenate (Figure 1B, lane 1), and the con-A adherent fraction (Figure 1B, lane 4).

In summary, con-A sepharose affinity chromatography resolved a nonadherent fraction from the L-cell plasma membrane. Together with Western blotting to detect protein markers of plasma membrane microdomains, the data showed that the nonadherent fraction comprised about two-thirds of plasma membrane protein was highly enriched in the nonlipid raft protein marker Na^+,K^+-ATPase, and was either devoid (SR−B1) or 3−10-fold reduced (caveolin-1, flotillin) in caveolae/lipid raft protein markers. This was consistent with the con-A nonadherent plasma membrane fraction being enriched in nonlipid raft domains.

Distribution of Caveolae/Lipid Raft and Nonlipid Raft Marker Proteins in the Adherent Fraction of Plasma Membranes Eluted from Concanavalin-A Sepharose Affinity Columns by Displacing Sugar. Western blot analysis was also performed on the con-A affinity column adherent fraction which was eluted by the displacing sugar α-methyl-mannoside as described in Materials and Methods. The presence of known marker proteins for plasma membrane microdomains was determined: (i) caveolae/lipid raft markers (caveolin-1, flotillin, and SR−B1); (ii) nonlipid raft markers (Na^+,K^+-ATPase, transferrin receptor (CD71), and clathrin). Western blot analysis was performed to determine the distribution of caveolae/lipid raft (caveolin-1, flotillin, SR−B1) and nonlipid raft (Na^+,K^+-ATPase, transferrin receptor, clathrin) markers in the con-A sepharose adherent fraction (Figure 1B, lane 4). The con-A adherent fraction was almost completely devoid of the nonlipid raft marker Na^+,K^+-ATPase as compared to the nonadherent fraction (Figure 1B, lane 3), plasma membranes (Figure 1B, lane 2), or cell homogenate (Figure 1B, lane 1). For reasons detailed in the preceding section, neither transferrin nor clathrin was detectable in the adherent fraction (not shown).

Since the absence of nonlipid raft markers suggested that the adherent fraction might be enriched in caveolae/lipid rafts, additional Western blotting was performed to distinguish this point. The con-A sepharose adherent fraction was highly enriched >10-fold in SR−B1, 5-fold in flotillin, and 4-fold in caveolin-1 as compared to the nonadherent fraction (Figure 1B, lane 3) and/or cell homogenate (Figure 1B, lane 1).

In summary, con-A sepharose affinity chromatography also resolved a con-A adherent fractions from the L-cell plasma membrane. The data showed that the con-A sepharose adherent fraction comprising about one-third of total plasma membrane protein was highly enriched in caveolae/lipid raft protein markers (SR−B1, caveolin-1, flotillin), while essentially being devoid of nonlipid raft protein marker Na^+,K^+-ATPase. This suggested that the con-A adherent fraction of the plasma membrane fraction was enriched in caveolae/lipid rafts.

Distribution of Lipid Markers in L-Cell Plasma Membrane Nonadherent Fraction Resolved by Concanavalin-A Sepharose Affinity Chromatography. Since nonlipid raft regions of the plasma membrane are considered to be lipid poor, especially in gangliosides, sphingomyelin, and cholesterol (*28*, *29*, *52*, *53*), the lipids were extracted from the nonadherent fraction and analyzed for total lipid content. As compared to the to the con-A affinity column adherent fraction, the nonadherent fraction was lipid poor as evidenced by nearly 3-fold lower total lipid/protein ratio (Figure 3A). Examination of individual lipid components as described in Materials and Methods revealed that the nonadherent fraction had up to 5-, 3-, and 2.5-fold less ganglioside GM1 (Figure 1C), total lipid (Figure 3A), and cholesterol (Figure 3B), respectively ($P < 0.004$, $n = 4$), as compared to the adherent fraction. Interestingly, since the phospholipid content of the con-A nonadherent fraction was also 5-fold lower than that of the adherent fraction (Figure 3C, $P < 0.004$, $n = 4$), the molar ratio of cholesterol/phospholipid was not significantly different in the nonadherent as compared to the adherent fraction (Figure 3D). Analysis of the individual phospholipids classes

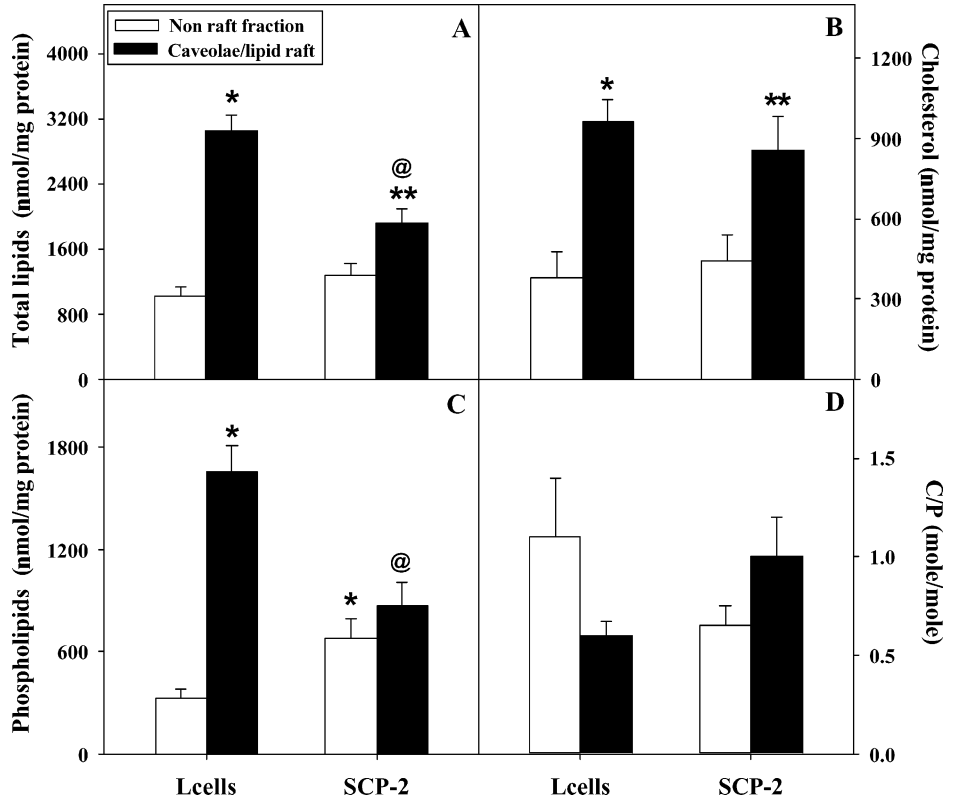

FIGURE 3: Lipid analyses of caveolae/lipid raft and nonlipid raft fractions isolated from plasma membranes of L-cells and SCP-2 expressing cells. Total lipid (panel A), cholesterol (panel B), total phospholipids (panel C), and the C/P ratio (panel D) were determined in the caveolae/lipid raft and nonlipid raft fractions isolated from L-cells and SCP-2 expressing cells. Values represent means ± SEM. Asterisk (*) indicates significance $P < 0.03$ ($n = 3-4$) as compared to the nonlipid raft fraction isolated from L-cells. Double asterisk (**) indicates significance $P < 0.03$ ($n = 3-4$) as compared to the nonlipid raft fraction isolated from SCP-2 cells. (@) indicates significance $P < 0.05$ ($n = 3-4$) as compared to L-cell caveolae/lipid raft fractions.

Table 2: Phospholipid Content in Caveolae/Lipid Raft and Nonraft Fractions Isolated from L-Cells and SCP-2 Expressing Cells[a]

	L-cells		SCP-2	
phospholipid	nonlipid raft	caveolae/lipid raft	nonlipid raft	caveolae/lipid raft
phosphatidylcholine	60.0 ± 22.1	910 ± 133*	84.8 ± 34*	343 ± 116@
	[18.1 ± 7]	[55 ± 9]*	[12.4 ± 5.4]	[39.3 ± 14]
phosphatidic acid	79.9 ± 39	137.7 ± 27.4	148 ± 53	251.8 ± 59
	[24 ± 12]	[8.2 ± 1.7]	[21.7 ± 8.6]	[28.9 ± 8.1]@
phosphatidylinositol	56.2 ± 25.5	171.9 ± 37*	112.8 ± 33.2	66.3 ± 27
	[17 ± 8.2]	[10.3 ± 2.4]	[16.5 ± 5.6]	[7.7 ± 3.3]
phosphatidylserine	41.7 ± 5.9	115.4 ± 32	86 ± 18	32.2 ± 15.5
	[12.6 ± 2.8]	[6.9 ± 2]	[12.6 ± 3.4]	[3.7 ± 1.8]
phosphatidylethanolamine	60.8 ± 11.8	61.6 ± 12.7	102.2 ± 8.7*	43.6 ± 4**
	[18.4 ± 4.7]	[3.7 ± 0.8]*	[15.0 ± 2.8]	[5.0 ± 0.9]**
sphingomyelin	50.8 ± 13.1	264.8 ± 38*	147.1 ± 34.6*	135 ± 28.6@
	[15.4 ± 4.7]	[15.9 ± 2.7]	[21.6 ± 6.2]	[15.4 ± 4]

[a] Phospholipids were isolated from caveolae/lipid rafts and nonlipid rafts purified from plasma membranes of L-cells and SCP-2 expressing cells and resolved into individual phospholipid classes as described in Materials and Methods. Quantities are expressed as (nmol/mg of protein) or as [mol %]. Values reflect the mean ± SEM of 3−4 separate experiments. Asterisk (*) indicates significance $P < 0.03$, as compared to L-cell nonlipid samples. Double asterisk (**) indicates significance $P < 0.03$, as compared to SCP-2 nonlipid rafts. (@) indicates significance $P < 0.01$, as compared to L-cell caveolae/lipid rafts.

including PC (phosphatidylcholine), PA (phosphatidic acid), PI (phosphatidylinositol), PS (phosphatidylserine), PE (phosphatidylethanolamine), and SM (sphingomyelin) revealed that several of these phospholipid classes were selectively depleted in the con-A adherent as compared to nonadherent fractions. The content (nmol/mg protein) of PC, PI, and SM was 15.2-, 3.1-, and 5.2-fold, respectively, lower in the nonlipid raft fraction as compared to the caveolae/lipid raft fraction (Table 2, $P < 0.04$, $n = 4$). While smaller decreases were noted in the contents (nmol/mg of protein) of individual anionic phospholipids (PA and PS), these increases were just below the level of detection (Table 2). When expressed on a % basis, the proportion of anionic phospholipids (PA + PI + PS) was 2.1-higher in the nonlipid raft vs caveolae/lipid raft fraction. In summary, the low lipid/protein ratio as well as the relative depletion of sphingolipids (ganglioside

GM1 and sphingomyelin), cholesterol, and phospholipids were in agreement with the protein markers (preceding section) indicating that the nonadherent fraction obtained by con-A affinity chromatography of L-cell plasma membranes represented nonlipid raft domains.

Distribution of Lipid Markers in L-Cell Plasma Membrane Adherent Fraction Resolved by Concanavalin-A Sepharose Affinity Chromatography. Lipid rafts/caveolae domains of plasma membrane are rich in lipids, especially ganglioside, sphingomyelin, and cholesterol (*28*, *29*, *52*, *53*). As compared to the nonadherent fraction eluting from the con-A affinity column, the adherent fraction was lipid-rich and exhibited nearly 3-fold higher total lipid/protein ratios (Figure 3A). In addition, the levels of ganglioside GM1 (Figure 1C), and cholesterol (Figure 3B) were 5- and 2.5-fold higher, respectively ($P < 0.004$, $n = 4$) in the adherent fraction. Analysis of the individual phospholipids classes revealed that the content (nmol/mg of protein) of PC, PI, and SM were 15.2-, 3.1-, and 5.2-fold, respectively, enriched in the caveolae/lipid raft fraction as compared to nonlipid raft fraction (Table 2, $P < 0.04$, $n = 4$). Thus, the enrichment in total lipid, especially sphingolipids (ganglioside GM1 and sphingomyelin) and cholesterol, was in agreement with the protein markers (preceding sections) indicating that the adherent fractions obtained by con-A affinity chromatography of L-cell plasma membranes was enriched in caveolae/lipid rafts.

Effect of SCP-2 Expression on the Proportion of Caveolae/Lipid Rafts and Nonlipid Rafts in L-Cell Plasma Membranes. Expression of SCP-2 in transfected L-cell fibroblasts did not significantly alter the distribution of caveolae/lipid raft and nonlipid raft domains in the plasma membrane (Table 1). In SCP-2 expressing cell plasma membranes, the caveolae/lipid raft fraction comprised about 70.6 ± 6.2% of the plasma membrane, not significantly different from control (mock-transfected) L-cells (Figure 2A). Likewise, caveolin-1 (Figure 2B) content/mg of protein was similarly enriched in caveolae/lipid rafts of SCP-2 expressing cells as in control (mock transfected) L-cells. Nevertheless, Western blotting of caveolae/lipid raft markers demonstrated that SCP-2 altered the caveolae/lipid raft domains by selective 1.9- and 2.8-fold enrichment of flotillin and SR−B1, respectively (Figure 2B). Thus, while SCP-2 expression did not alter the proportion of caveolae/lipid rafts in the plasma membrane, the content of several caveolae/lipid raft protein markers (flotillin, SR−B1) was increased severalfold in the caveolae/lipid raft fraction.

Effect of SCP-2 Expression on the Lipid Distribution in Caveolae/Lipid Rafts and Nonlipid Rafts in L-Cell Plasma Membranes. Although lipid markers for caveolae/lipid rafts [i.e., cholesterol (Figure 3B), ganglioside GM1 (not shown)] were enriched in caveolae/lipid rafts relative to nonlipid raft domains in SCP-2 expressing cells, comparison of the lipid compositions of caveolae/lipid rafts and nonlipid rafts isolated from SCP-2 expressing cells versus control (mock transfected) L-cells revealed that caveolae/lipid rafts and nonlipid rafts also differed significantly in the absolute level of other lipid components.

While SCP-2 expression did not alter the caveolae/lipid raft content (nmol/mg of protein) of cholesterol, the content of ganglioside GM1 (not shown) and sphingomyelin (Table 2) was decreased 5.4- and 2-fold, respectively. SCP-2 expression also decreased the total lipid (Figure 3A), total phospholipid (Figure 3C), and anionic phospholipid (PA + PI + PS, Table 2) contents of caveolae/lipid rafts by 1.6-, 1.9-, and 1.2-fold, respectively, as compared to those from control (mock transfected) L-cells. As a result the proportion (%) of anionic phospholipid increased from 25 to 40% in caveolae/lipid rafts from control cells vs SCP-2 expressing cells. Further analysis of the individual phospholipid classes revealed that the content (nmol/mg of protein) of PC was 2.7-fold less in the caveolae/lipid raft fraction of SCP-2 expressing vs control cells (Table 2, $P < 0.001$, $n = 4$). With the exception of PA all the content (nmol/mg of protein) of other phospholipid classes also trended to decrease in the caveolae/lipid rafts of SCP-2 expressing vs control cells (Table 2). When expressed on a % basis, the proportion of anionic phospholipids (PA + PI + PS) was 1.6-fold higher in the caveolae/lipid raft fraction from SCP-2 expressing vs control cells. Interestingly, while SCP-2 expression primarily affected the lipid composition of caveolae/lipid rafts, the lipid composition of nonlipid rafts was also affected as evidence

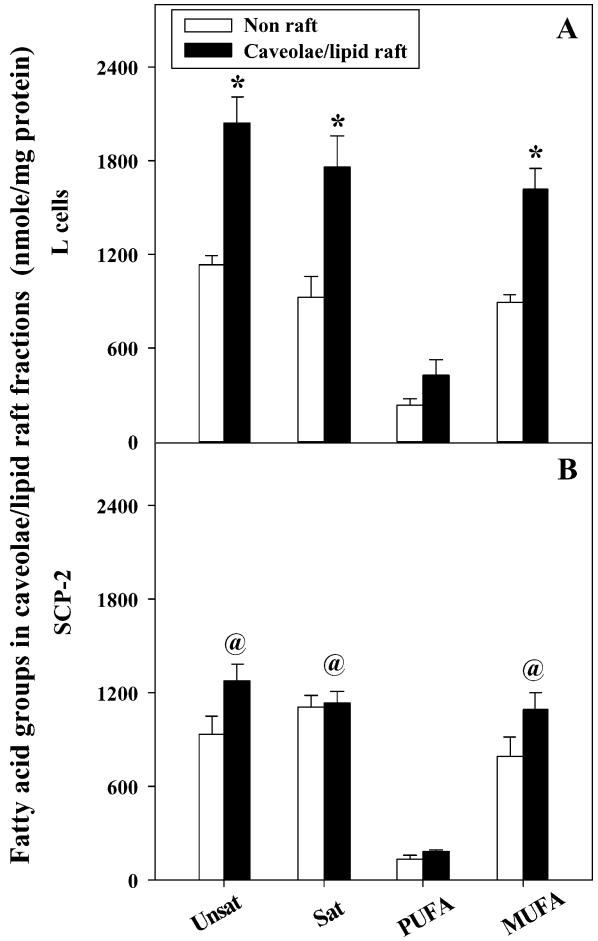

FIGURE 4: Fatty acids esterified to phospholipids isolated from caveolae/lipid raft and nonlipid raft fractions of plasma membranes from L-cells and SCP-2 expressing cells. Levels of unsaturated fatty acids (unsat), saturated fatty acids (sat), polyunsaturated fatty acids (PUFA), and monounsaturated fatty acids (MUFA) were quantitated in caveolae/lipid rafts and nonlipid raft fractions isolated from L-cells (panel A) and SCP-2 expressing cells (panel B). Values represent means ± SEM. Asterisk (*) indicates significance $P < 0.01$ ($n = 3-4$) as compared to the nonlipid raft fraction isolated from L-cells. ($_{@}$) indicates significance $P < 0.04$ ($n = 3-4$) as compared to L-cell caveolae/lipid raft fractions.

Table 3: Fatty Acid Content in Total Phospholipid Fractions from Caveolae/Lipid Raft and Nonraft Samples Isolated from L-Cells and SCP-2 Expressing Cells[a]

	L-cells		SCP-2	
fatty acid	nonlipid raft	caveolae/lipid raft	nonlipid raft	caveolae/lipid raft
8:0	ND	23.7 ± 0.7*	12.3 ± 0.9	25.9 ± 6.7
10:0	ND	31.5 ± 7.5*	20.1 ± 6.2	23.1 ± 4.9
12:0	ND	35.6 ± 15.7*	13.6 ± 0.1	21.7 ± 4.8
14:0	23.7 ± 6.3	71.1 ± 26.9	35.6 ± 12	44.1 ± 7.0
16:0	359.9 ± 89	683.3 ± 151	404.5 ± 42	420.1 ± 60.9
18:0	394 ± 89	704.9 ± 120	465.3 ± 52	469 ± 3.5
20:0	18.9 ± 1.4	30.7 ± 3.2*	19.0 ± 3.3	24.9 ± 4.1
22:0	31.9 ± 3.2	42.7 ± 6.8	31.1 ± 7.2	33.7 ± 4.9
24:0	101.5 ± 33	135.2 ± 43.1	108.4 ± 19.5	74.6 ± 14.7
12:1	ND	31.8 ± 9.1*	ND	ND[@]
14:1	12.9 ± 1.7	22.8 ± 6.5	13.1 ± 0.3	22.1 ± 5.4
16:1	46.5 ± 16	108.8 ± 38.4	37.27 ± 9	66.7 ± 11.9
18:1−9	636.9 ± 37	1081.3 ± 132*	525.5 ± 115	741.8 ± 104
18:1−11	54.4 ± 10	136.9 ± 9.9*	59.3 ± 9.6	102.1 ± 14
20:1	ND	15.6 ± 0.6*	11.3 ± 3.0	ND**,[@]
20:1−8	15.6 ± 0.6	28.8 ± 2.1*	13.6 ± 2.6	22.4 ± 3.2
20:1−11	52.9 ± 8.9	91.9 ± 5.9*	52.2 ± 7.5	63.7 ± 3.2[@]
22:1−13	15.1 ± 1.1	22.3 ± 1.1*	16.5 ± 3	19.2 ± 3.0
24:1	61.4 ± 14.5	77.2 ± 5.7	68.9 ± 14.2	54.9 ± 4.2[@]
18:2−9,12	28.1 ± 5.1	58.4 ± 5.4*	27.8 ± 10.2	35.2 ± 5.6[@]
18:3-γ−6,9,12	82.7 ± 39	104.8 ± 83	9.9 ± 0.5	23.7 ± 5.6
18:3-α−6,9,12	11.3 ± 1.1	19.9 ± 5	ND	ND[@]
20:2−11,14	11.9 ± 2.2	21.4 ± 2*	16.9 ± 2.7	16.2 ± 3.2
20:3−8,11,14	11.3 ± 2.8	85.7 ± 50	21.2 ± 11	24.8 ± 3.1
20:4	20.2 ± 4	40.3 ± 1.0*	16.8 ± 4.7	21.8 ± 2.1[@]
22:2	9.7 ± 2.4	15.2 ± 2.9	8.4 ± 1.4	15.3 ± 1.1**
22:3	14.6 ± 8.3	ND	ND	15.3 ± 2.0**,[@]
22:4	25.6 ± 11	39.9 ± 21	21.9 ± 8.8	14.0 ± 1.9
22:6	21.9 ± 7.1	37.9 ± 3.7	15.8 ± 4.7	15.7 ± 1.6[@]

[a] Caveolae/lipid raft and nonraft fractions were isolated from purified plasma membranes of L-cells and SCP-2 expressing cells, lipids were extracted, phospholipids were resolved, and fatty acid distribution in total phospholipids (nmol/mg protein) was determined as described in Materials and Methods. Values reflect the mean ± SEM of 3−4 separate experiments. Asterisk (*) indicates significance $P < 0.05$, as compared to L-cell nonlipid samples. Double asterisk (**) indicates significance $P < 0.01$, as compared to SCP-2 nonlipid samples. (@) indicates significance $P < 0.05$, as compared to L-cell caveolae/lipid raft samples. ND indicates levels were below the level of detectability.

by 2.1- and 2.9-fold increased total phospholipid (Figure 3C) and sphingomyelin (Table 2) contents, respectively.

In summary, SCP-2 expression significantly depleted the caveolae/lipid raft domains in terms of total lipid content (primarily phospholipids). While the decreased phospholipid content appeared to result in increased cholesterol/phospholipid molar ratio, this effect was not statistically significant. In contrast, SCP-2 expression significantly increased the nonlipid raft domains in terms of total phospholipid, phosphatidylethanolamine, and sphingomyelin. Thus, SCP-2 expression not only altered the lipid distribution in caveolae/lipid rafts, but appeared to reduce some of the differences between the caveolae/lipid raft vs nonlipid raft domains.

Effect of SCP-2 Expression on Phospholipid Fatty Acid Content of Caveolae/Lipid Rafts and Nonlipid Rafts from L-Cell Fibroblasts. Since SCP-2 expression substantially altered the content and distribution of phospholipids in caveolae/lipid rafts, mass data on the types of fatty acids esterified in the phospholipids isolated from caveolae/lipid raft and nonlipid raft fractions were also determined (Figure 4 and Table 3).

In control (mock transfected) L-cells, levels (nmol/mg of protein) of unsaturated and saturated fatty acids were significantly higher (up to 1.9-fold, Figure 4A, $P < 0.04$, $n = 3$) in the phospholipids isolated from caveolae/lipid rafts versus those from phospholipids of nonlipid raft domains. The higher content of unsaturated fatty acids was directly related to an increase in monounsaturated fatty acids (MUFA). Levels of MUFA were 3.8-fold higher than polyunsaturated fatty acids (PUFA) in the caveolae/lipid rafts, resulting from significant increases (Table 3) in 12:1 (32-fold), 18:1−9 (1.7-fold), 18:1−11 (2.5-fold), 20:1 (15.6-fold), 20:1−8 (1.8-fold), 20:1−11 (1.7-fold), and 22:1−13 (1.5-fold). Several PUFAs were also enriched in the L-cell caveolae/lipid raft fraction including 18:2−9,12; 20:2−11,14; and 20:4 (arachidonic acid) up to 2.0-fold (Table 3, $P < 0.05$, $n = 3$).

In contrast to control L-cells, for SCP-2 expressing cells the levels of PUFA and MUFA esterified to phospholipids in caveolae/lipid raft were overall similar to those in the nonlipid raft domains. However, comparison of phospholipid fatty acid composition in caveolae/lipid rafts from SCP-2 expressing versus control cells revealed important differences. Only one phospholipid fatty acid (22:3) was increased from nondetectable levels to 15.3 ± 2.0 nmol/mg in the caveolae/lipid raft fraction isolated from SCP-2 expressing cells (Table 3, $P < 0.05$, $n = 3$). Further comparison revealed caveolar/lipid rafts from SCP-2 expressing cells were decreased 1.6-, 1.5-, and 1.5-fold in the unsaturated, saturated, and monounsaturated fatty acid (MUFA) content as compared to control cells. This was reflected in the decrease in 20:1 and 18:3-α-6,9,12 to levels below detection, 20:1−11 (down 1.4-fold), 24:1 (down 1.4-fold), 18:2−9,12 (down 1.7-fold), 20:4 (down 1.8-fold), and 22:6 (down 2.4-fold).

In summary, the fatty acid composition of phospholipids from caveolae/lipid rafts differed significantly from those

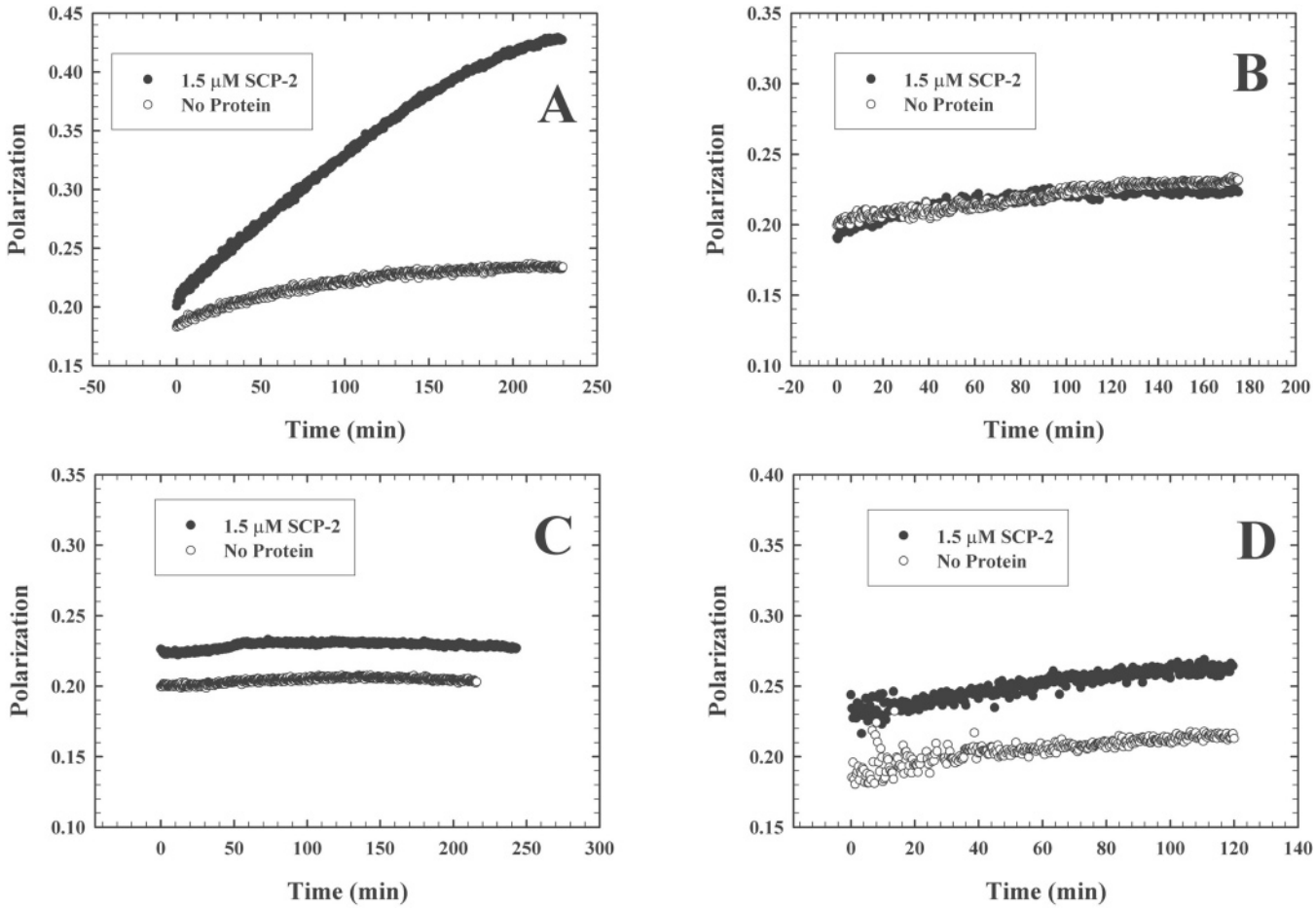

FIGURE 5: Sterol transfer from caveolae/lipid rafts and nonlipid raft domains of plasma membranes derived from control L-cell fibroblasts. Panel A depicts sterol transfer between caveolae/lipid raft donors and caveolae/lipid raft acceptors. Panel B shows sterol transfer between caveolae/lipid raft donors and nonlipid raft acceptors. Panel C shows sterol transfer between nonlipid raft donors and nonlipid raft acceptors. Panel D depicts sterol transfer between nonlipid raft donors and caveolae/lipid raft acceptors. For each type of assay, $n = 5$ runs were tabulated. Closed circles depict sterol transfer in the presence of 1.5 μM SCP-2. Open circles depict sterol transfer in the absence of added SCP-2.

of nonlipid raft domains. The phospholipids of caveolae/lipid rafts were relatively enriched in unsaturated (primarily monounsaturated) fatty acid content (nmol/mg protein). Specifically large increases were noted in 12:1, 18:1−9, 18:1−11, 18:2−9,12, 20:1−11, 20:2−11,14, and 20:4. Nevertheless, when expressed as the ratio of unsaturated/saturated fatty acids or as the double bond index, there were no significant differences. SCP-2 expression significantly reduced the contents of most of these unsaturated fatty acids in phospholipids from caveolae/lipid rafts such that differences in phospholipid fatty acid composition between caveolae/raft and nonlipid raft phospholipids were essentially abolished or significantly reduced.

Spontaneous Sterol Transfer from Caveolae/Lipid Rafts and Nonlipid Raft Domains of L-Cell Fibroblast Plasma Membranes. On the basis of the above differences in lipid composition between caveolae/lipid raft and nonlipid raft domains of plasma membranes isolated from control (mock-transfected) L-cells, the possibility that cholesterol dynamics would also differ between these membrane domains was examined using a fluorescent sterol (DHE) transfer polarization assay as described in Materials and Methods. Spontaneous sterol transfer from caveolae/lipid raft donors (containing DHE) to caveolae/lipid raft acceptors (containing cholesterol, but no DHE) was determined. Initially, the DHE polarization in donor caveolae/lipid rafts was very low, near 0.18 (Figure 5A, open circles), due to DHE self-quenching. However, upon addition of 10-fold excess acceptor caveolae/lipid raft membranes the DHE spontaneously transfers from the donor membrane to the acceptor membranes (while cholesterol transfers 1 for 1 in the reverse direction) such that DHE self-quenching is released and DHE fluorescence polarization significantly increased with time (Figure 5A, open circles). The initial rate of molecular sterol transfer from caveolae/lipid rafts, calculated as described in Materials and Methods, was 0.346 ± 0.041 pmol/min (Table 4). In contrast, when spontaneous sterol transfer was from nonlipid raft donors to nonlipid raft acceptors, the DHE fluorescence polarization changed very little over time (Figure 5C, open circles). Comparison of the initial rates revealed that spontaneous molecular sterol transfer from nonlipid rafts (0.045 ± 0.029 pmol/min, Table 4) was 7.7-fold slower from nonlipid rafts than caveolae/lipid rafts. When heterogeneous donor acceptor pairs were compared sterol transfer from caveolae/lipid raft donors to nonlipid raft acceptors resulted in modest increases in DHE polarization (Figure 5B, open circles) and an initial rate of spontaneous molecular sterol transfer (0.168 ± 0.039 pmol/min, Table 4) that was only 2-fold slower than that for homogeneous sterol transfer between caveolae/lipid rafts donors and acceptors (Table 4). In contrast, in the reverse

Table 4: Initial Rates of Molecular Sterol Transfer from Caveolae/Lipid Rafts and Nonlipid Rafts Isolated from Plasma Membranes Purified from Control L-Cell Fibroblasts: Effect of Sterol Carrier Protein-2[a]

donor	acceptor	SCP-2	initial rate (pmol/min)
caveolae/lipid rafts	caveolae/lipid rafts	−	0.346 ± 0.041
		+	1.115 ± 0.035*
caveolae/lipid raft	nonlipid rafts	−	0.168 ± 0.039
		+	0.197 ± 0.032
nonlipid rafts	nonlipid rafts	−	0.045 ± 0.029^{+}
		+	0.053 ± 0.027^{+}
nonlipid rafts	caveolae/lipid rafts	−	0.028 ± 0.022
		+	0.031 ± 0.020

[a] Initial rates of DHE transfer from caveolae/lipid rafts and nonlipid raft donors to acceptors were measured in the absence or presence of SCP-2 (1.5 μM) as described in Materials and Methods. Values represent the mean ± SD ($n = 3-4$). Asterisk (*) refers to $P < 0.05$ vs no SCP-2, while ($^{+}$) refers to $P < 0.05$ vs caveolae/lipid rafts.

direction (i.e., from nonlipid raft donors to caveolae/lipid raft acceptors) increased DHE polarization very little (Figure 5C, open circles) and an initial rate of spontaneous molecular sterol transfer (0.028 ± 0.022 pmol/min, Table 4) that was 12-fold slower than between caveolae/lipid raft donors and acceptors.

When the above DHE polarization changes during sterol transfer between membrane domains were kinetically analyzed as described in Materials and Methods, two kinetic domains were resolved in caveolae/lipid rafts: (i) A rapidly exchangeable sterol domain ($t_{1/2} = 64.4 \pm 15$ min) comprised 23.4% of the total sterol; (ii) A very slowly exchangeable sterol domain ($t_{1/2}$ = days or so slow as not to be resolvable) comprised the majority, 76.6% of sterol in the caveolae/lipid rafts (Table 5). In contrast, while nonlipid rafts also contained two kinetically resolvable sterol domains, these domains differed markedly from those of caveolae/lipid rafts: (i) The rapidly exchangeable sterol domain had a $t_{1/2}$ of 650 ± 51 min, 10-fold slower than from caveolae/lipid rafts, and comprised only 4.8% of the total sterol; (ii) The very slowly exchangeable sterol domain ($t_{1/2}$ = days or so slow as not to be resolvable) comprised even more, 90% of sterol in the nonlipid rafts as compared to the 76% in caveolae/lipid rafts (Table 5).

In summary, sterol transfer from caveolae/lipid raft donors was much faster (initial rate, $t_{1/2}$) than from nonlipid raft donors regardless of acceptors. Furthermore, kinetic analysis revealed that nonlipid rafts had 4.9-fold less exchangeable sterol and were comprised primarily (>90%) of nonexchangeable sterol.

SCP-2 Added in Vitro Selectively Enhances Sterol Transfer from Caveolae/Lipid Raft, but not Nonlipid Raft, Domains of L-Cell Fibroblast Plasma Membranes. The effect of SCP-2 on sterol transfer from caveolae/lipid raft donors (containing DHE) to caveolae/lipid raft acceptors (containing cholesterol, but no DHE) was determined. SCP-2 dramatically increased the DHE polarization in donor caveolae/lipid rafts over time (Figure 5A, closed circles) such that the initial rate of molecular sterol transfer from caveolae/lipid rafts was increased 3.2-fold to 1.115 ± 0.035 pmol/min (Table 4) and the $t_{1/2}$ of molecular sterol transfer was shortened 3.2-fold to 20.3 ± 8.0 min (Table 5). Concomitantly, the size of the exchangeable sterol domain was increased 4.2-fold such that the nonexchangeable sterol domain was almost completely abolished (Table 5). SCP-2 also increased the DHE polarization (Figure 5A, closed circles), initial rate of molecular sterol transfer (Table 4) and decreased the $t_{1/2}$ of molecular sterol transfer (Table 5) from caveolae/lipid rafts to nonlipid rafts (but not in the reverse direction), slightly less well by 2.1- and 5.8-fold, respectively. In contrast, while SCP-2 did not significantly affect the DHE polarization over time (Figure 5B,D), initial rates (Table 4) or $t_{1/2}$'s (Table 5) of sterol transfer from nonlipid rafts, regardless of acceptor, size of the exchangeable sterol domain was increased slightly, by about 2-fold (Table 5).

Effect of SCP-2 Overexpression in Transfected L-Cells on Spontaneous Sterol Transfer from Caveolae/Lipid Raft and Nonlipid Raft Domains of Plasma Membranes. The effect of SCP-2 overexpression in intact L-cells on spontaneous sterol transfer from purified caveolae/lipid rafts and nonlipid rafts was determined. Spontaneous sterol transfer from caveolae/lipid rafts was evident as a measurable increase in DHE polarization, regardless of acceptor (Figure 6A and 6B, open circles) as well as from nonlipid rafts, regardless of acceptor (Figure 6C,D, closed circles). Initial rates (Table 6), $t_{1/2}$ (Table 7), and nonexchangeable domain size (Table 7) of spontaneous sterol transfer from caveolae/lipid rafts were reduced about 2−3-, 1.3−1.9-, and 1.3-fold, respectively, as compared to those in caveolae/lipid rafts from plasma membranes of control (mock transfected) cells (Tables 4 and 5). In contrast, SCP-2 overexpression did not significantly alter the initial rates, $t_{1/2}$, or domain size of spontaneous sterol transfer from nonlipid rafts (Tables 6 and 7). Thus, SCP-2 overexpression significantly altered the

Table 5: Kinetic Analysis of Sterol Transfer from Caveolae/Lipid Rafts and Nonlipid Rafts Isolated from Plasma Membranes Purified from Control L-Cell Fibroblasts: Effect of SCP-2[a]

donor	acceptor	SCP-2	$t_{1/2}$ (min)	f_1	f_2
caveolae/lipid rafts	caveolae/lipid rafts	−	64 ± 15	0.234 ± 0.031	0.766 ± 0.034
		+	20 ± 8*	0.981 ± 0.042*	0.019 ± 0.009*
caveolae/lipid rafts	nonlipid rafts	−	607 ± 36	0.164 ± 0.024	0.836 ± 0.047
		+	125 ± 22*	0.397 ± 0.039*	0.603 ± 0.036*
nonlipid rafts	nonlipid rafts	−	650 ± 51^{+}	0.048 ± 0.025+	0.952 ± 0.044^{+}
		+	540 ± 60^{+}	0.101 ± 0.019^{+}	0.899 ± 0.022^{+}
nonlipid rafts	caveolae/lipid rafts	−	145 ± 21	0.209 ± 0.026	0.791 ± 0.031
		+	111 ± 11	0.427 ± 0.032	0.573 ± 0.028

[a] Fluorescence polarization exchange curves for DHE sterol transfer from caveolae/lipid rafts and nonlipid raft donors to acceptors were measured in the absence or presence of SCP-2 (1.5 μM) followed by kinetic analysis as described in Materials and Methods. Units for half times $t_{1/2}$ were in minutes, while f_1 and f_2 represent the fractions due to the exchangeable and nonexchangeable components, respectively. Values represent the mean ± SD ($n = 3-4$). Asterisk (*) refers to $P < 0.05$ vs no SCP-2 added; a (+) refers to $P < 0.05$ vs caveolae/lipid rafts.

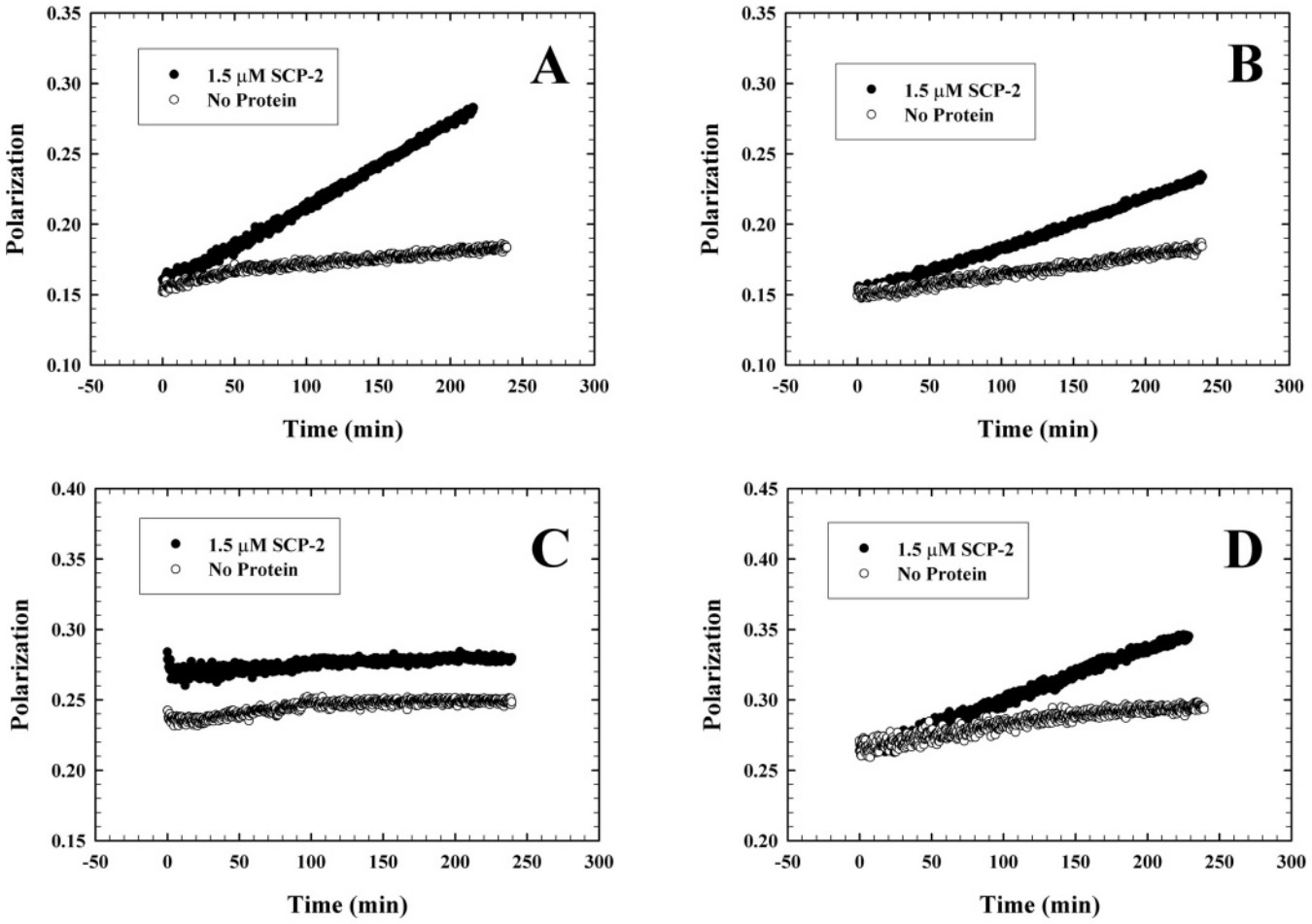

FIGURE 6: Sterol transfer from caveolae/lipid rafts and nonlipid raft domains of plasma membranes derived from SCP-2 overexpressing L-cell fibroblasts. Panel A depicts sterol transfer between caveolae/lipid raft donors and caveolae/lipid raft acceptors. Panel B shows sterol transfer between caveolae/lipid raft donors and nonlipid raft acceptors. Panel C shows sterol transfer between nonlipid raft donors and nonlipid raft acceptors. Panel D depicts sterol transfer between nonlipid raft donors and caveolae/lipid raft acceptors. For each type of assay, $n = 5$ runs were tabulated. Closed circles depict sterol transfer in the presence of 1.5 μM SCP-2. Open circles depict sterol transfer in the absence of added SCP-2.

Table 6: Initial Rates of Molecular Sterol Transfer from Caveolae/Lipid Rafts and Nonlipid Rafts from Plasma Membranes of L-Cell Fibroblasts Overexpressing SCP-2[a]

donor	acceptor	protein added	initial rate (pmol/min)
caveolae/lipid raft	caveolae/lipid rafts	none	0.174 ± 0.031
		SCP-2	0.249 ± 0.028
caveolae/lipid rafts	nonlipid rafts	none	0.064 ± 0.023
		SCP-2	0.137 ± 0.036
nonlipid rafts	nonlipid rafts	none	$0.045 \pm 0.019^{+}$
		SCP-2	$0.048 \pm 0.021^{+}$
nonlipid rafts	caveolae/lipid raft	none	0.031 ± 0.014
		SCP-2	0.035 ± 0.018

[a] Initial rates of DHE transfer from caveolae/lipid rafts and nonlipid raft donors to acceptors were measured in the absence or presence of SCP-2 (1.5 μM) as described in Materials and Methods. Values represent the mean $\pm$ SD ($n = 3-4$). Asterisk (*) refers to the $P < 0.05$ vs no SCP-2, while (+) refers to $P < 0.05$ vs caveolae/lipid rafts.

spontaneous sterol dynamics in caveolae/lipid rafts to increase the spontaneous sterol transfer rates, decrease $t_{1/2}$, and increase exchangeable domain size without altering these properties in nonlipid rafts.

Effect of SCP-2 Overexpression in Transfected L-Cells on Responsiveness of Sterol Transfer from Caveolae/Lipid Raft and Nonlipid Raft Domains to in Vitro Addition of More SCP-2. The effect of in vitro addition of SCP-2 on sterol transfer from caveolae/lipid rafts and nonlipid rafts isolated from plasma membranes of SCP-2 overexpressing L-cells was determined. SCP-2 added in vitro measurably increased DHE polarization over time for all donor/acceptor pairs (Figure 6A,B,D closed circles), except nonlipid rafts/nonlipid rafts (Figure 6C, closed circles). SCP-2 added in vitro significantly increased the initial rate (Table 6), decreased the $t_{1/2}$ (Table 7), and increased the exchangeable domain size (Table 7) of sterol transfer in caveolae/lipid rafts, but these effects were much smaller than those observed with caveolae/lipid rafts isolated from control (mock transfected) cells (Tables 4 and 5). SCP-2 added in vitro also increased the exchangeable domain size of nonlipid rafts by about 1.3-fold (Table 7). Thus, SCP-2 overexpression significantly reduced the responsiveness of caveolae/lipid rafts to the actions of additional SCP-2 added in vitro. Concomitantly, SCP-2 overexpression modestly increased the responsiveness of nonlipid rafts to the actions of additional SCP-2 added in vitro, largely by increasing the size of the exchangeable sterol domain rather than increasing initial rates or decreasing half-times of sterol transfer.

Table 7: Kinetic Analysis of Sterol Transfer from Caveolae/Lipid Rafts and Nonlipid Rafts Isolated from Plasma Membranes Purified from SCP-2 Overexpressing L-Cell Fibroblasts[a]

donor	acceptor	$t_{1/2}$ (min)	f_1	f_2
caveolae/lipid raft	caveolae/lipid rafts	116 ± 10	0.112 ± 0.019	0.888 ± 0.046
		88 ± 15*	0.325 ± 0.031*	0.675 ± 0.021*
caveolae/lipid rafts	nonlipid rafts	350 ± 61	0.236 ± 0.027	0.764 ± 0.038
		184 ± 24	0.301 ± 0.024	0.699 ± 0.061
nonlipid rafts	nonlipid rafts	306 ± 56$^+$	0.118 ± 0.022	0.882 ± 0.053
		172 ± 30$^+$	0.156 ± 0.036	0.844 ± 0.048
nonlipid rafts	caveolae/lipid rafts	203 ± 25	0.315 ± 0.040	0.685 ± 0.059
		181 ± 23	0.420 ± 0.039	0.580 ± 0.037

[a] Fluorescence polarization exchange curves for DHE sterol transfer from caveolae/lipid rafts and nonlipid raft donors to acceptors were measured in the absence or presence of SCP-2 (1.5 μM) followed by kinetic analysis as described in Materials and Methods. Units for half times $t_{1/2}$ were in minutes, while f_1 and f_2 represent the fractions due to the exchangeable and nonexchangeable components, respectively. Values represent the mean ± SD ($n = 3-4$). Asterisk (*) refers to $P < 0.05$ vs no SCP-2 added; a (+) refers to $P < 0.05$ vs caveolae/lipid rafts.

DISCUSSION

Despite growing interest in membrane microdomains (caveolae/lipid rafts) and the role of such domains in cholesterol transport (reviewed in refs *8*, *23*, and *53*−*57*), major unanswered questions remain about the lipid properties, cholesterol dynamics, and factors that regulate these parameters in plasma membrane caveolae/lipid raft microdomains. There is uncertainty regarding the relative proportion of caveolae/lipid raft vs nonlipid raft domains in membranes. For example, methods for isolating lipid rafts that use detergents (i.e., detergent resistant membranes) yield proportions varying from almost none to essentially the entire membrane, depending on the temperature used during the isolation procedure (*52*, *56*). In contrast, an earlier nondetergent method suggests that caveolae/lipid rafts comprise very low levels (a few %) of the plasma membrane (*27*). There is uncertainty whether the properties of detergent resistant membranes reflect those of caveolae/lipid rafts. For example, direct comparison of the lipids comprising detergent resistant membranes versus nondetergent isolated caveolae/lipid rafts suggests that detergents preferentially extract certain lipids (*28*, *29*). Finally, to our knowledge there are no data regarding purification and characterization of nonlipid raft domains. Thus, it has not been possible to directly determine how caveolae/lipid rafts and nonlipid rafts may differ in lipid or protein composition, much less cholesterol dynamics.

To begin to address the above issues, the present work applied concanavalin-A (con-A) sepharose 4B affinity chromatography to simultaneously fractionate purified plasma membranes into two fractions whose properties were consistent with those of caveolae/lipid rafts and nonlipid rafts, respectively. Con-A was chosen based on its specificity for certain carbohydrates: (i) Simple sugars and oligosaccharides that have D-manno- or D-glucopyranoside configuration with unmodified hydroxyl groups at C-3, C-4, and C-6 of the α anomer, which is preferred over the β anomer (reviewed in ref *58*). (ii) Glycopeptides with a core structure of Manα1 → 3[Manα1 → 6]Manβ1 → 4GlcNAcβ1 → 4GlcNAc → Asn (*58*). Such carbohydrate receptor structures are present in several lipid raft/caveolae components such as SR−B1 (*59*) and glucosylceramide found in L-cells [personal communication from J. Jefferson, Luther College, Decorah, IA]. Indeed, Western blotting and lipid analysis revealed that the con-A adherent fraction was enriched in a variety of markers for caveolae/lipid rafts (enriched in caveolin-1, flotillin, SR−B1, ganglioside GM1, sphingomyelin, total lipid, phospholipid, and cholesterol) while the nonadherent fraction was enriched in markers known for nonlipid rafts (enriched in NaK-ATPase, relatively lipid poor). These data, together with the fact that the con-A adherent and nonadherent fractions of L-cell plasma membranes are oriented right-side-out (i.e., exofacial leaflet on the outside of the vesicle), suggest that these fractions represent different regions of the plasma membrane (*37*). While it is beyond the scope of the present work to separate and resolve noncaveolar lipid rafts from caveolar lipid rafts, advances in simultaneous isolation of caveolae/lipid rafts and nonraft microdomains from the same preparation of L-cell plasma membranes yielded the following new insights.

First, it was shown that caveolae/lipid rafts represent about 30% of the plasma membrane protein, while nonlipid rafts represent 70% of the plasma membrane proteins. Furthermore, since caveolae/lipid rafts are nearly 3-fold enriched in lipids as compared to nonlipid rafts, the caveolae/lipid rafts contain 56% of plasma membrane lipid. Thus, caveolae/lipid rafts are not a minor component of plasma membranes. These lipid-rich microdomains contain half- and nearly one-third of plasma membrane lipid and protein, respectively.

Second, the lipid composition of caveolae/lipid rafts differed markedly from nonlipid rafts. Ganglioside GM1, unsaturated (especially monounsaturated) fatty acids, phosphatidylcholine, and sphingomyelin were much more enriched in caveolae/lipid raft than nonlipid raft domains. Interestingly, while caveolae/lipid rafts were enriched severalfold with phospholipid and cholesterol, these components were enriched nearly in parallel such that the sterol/phospholipid ratio did not significantly differ between caveolae/lipid rafts and nonlipid rafts. Thus, enrichment of cholesterol (nmol/mg of protein) in caveolae/lipid rafts did not equate with a higher cholesterol/phospholipid ratio. In support of this finding, a recent publication showed that the sterol/phospholipid ratio in caveolae/lipid rafts, isolated without the use of detergents from brain synaptosomal plasma membranes (*28*), did not significantly differ from that of the parent synaptosomal plasma membrane (*60*). Likewise, the cholesterol/phospholipid molar ratio of caveolae/lipid rafts (*29*), isolated from cultured kidney cell plasma membranes without the use of detergents, was similar to that typical of plasma membranes from many other cell types (reviewed in refs *6* and *54*). In contrast, the cholesterol/phospholipid ratio was significantly higher in detergent

resistant membranes than in caveolae/lipid rafts isolated from the same plasma membranes without the use of detergents (*28*, *29*). These data suggest that the use of detergents either results in preferential loss of certain phospholipids and/or detergent resistant membranes reflect a different population of caveolae/lipid rafts than those isolated by nondetergent methods (*27*−*29*).

Third, the cholesterol dynamics of caveolae/lipid rafts differed substantially from those of nonlipid raft domains of the plasma membrane. The initial rates and half-times of spontaneous molecular sterol transfer from caveolae/lipid rafts were 7.7-fold faster and 9.4-fold shorter, respectively, than those of sterol transfer from nonlipid rafts. The rapid spontaneous flux of sterol through caveolae/lipid rafts rather than nonlipid rafts was consistent with the enrichment in caveolae/lipid rafts of several proteins essential for rapid movement of cholesterol through the plasma membrane (caveolin-1, HDL receptor SRB1, P-glycoprotein) as follows: (i) Caveolin-1 binds cholesterol (*61*). (ii) SR−B1 binds high-density lipoprotein (HDL) and mediates the nonendocytic "selective" uptake (*8*, *59*, *62*, *63*) and efflux (i.e., "reverse cholesterol transport") (*64*) of cholesterol from/through the plasma membrane (*8*, *65*). (iii) P-glycoprotein (P-gp) translocates cholesterol from cytofacial to exofacial leaflets of the plasma membrane to facilitate availability of cholesterol for efflux to HDL at the exofacial leaflet of the membrane (reviewed in refs *1* and *2*). The importance of such caveolae/lipid raft proteins in spontaneous sterol transfer is evidenced by the fact that model membranes rich in the types of lipids present in caveolae (i.e., cholesterol and sphingomyelin) exhibit slow cholesterol dynamics (*5*, *10*, *66*).

Interestingly, kinetic analysis of spontaneous molecular sterol transfer revealed that caveolae/lipid rafts, but not nonlipid rafts, contained a substantial portion of sterol (i.e., 23%) as a rapidly exchangeable sterol pool. The nonexchangeable sterol does not reflect crystalline sterol since cholesterol in the plasma membrane and caveolae/lipid rafts is primarily monomeric (i.e., $\geq$99.7%) (*31*). Because of the rapid transbilayer migration rate of cholesterol across the plasma membrane (*10*, *60*, *67*), the rapidly (min-h) and nonexchangeable sterol pools are both thought to reflect lateral cholesterol domains (reviewed in refs *5*, *6*, *33*, and *36*). While it has been established that plasma membrane cholesterol is distributed into at least two kinetically (*33*, *43*, *44*) and structurally (*68*) resolvable sterol domains, molecular details of whether this represents cholesterol within caveolae/lipid rafts vs nonlipid rafts have not been reported (*43*). Thus, the present data show for the first time that both types of sterol domains are present, but in markedly different amounts, in caveolae/lipid rafts versus nonlipid rafts. The data further suggest that rapid cholesterol uptake and efflux via caveolae/lipid rafts (*59*) is due at least in part to the presence of the rapidly transferable sterol domain in caveolae/lipid rafts but not nonlipid rafts (*8*, *64*, *65*).

Fourth, spontaneous sterol transfer between the caveolae/lipid raft and nonlipid raft microdomains of the plasma membrane was slow. Sterol transfer from caveolae/lipid raft to nonlipid raft domains was 2-fold slower, while that from nonlipid rafts to caveolae/lipid rafts was 12-fold slower than that observed between caveolae/lipid rafts. This would suggest that cholesterol moving through the caveolae/lipid raft domain rapidly moves to extracellular or intracellular acceptors while only slowly equilibrating with the nonlipid raft sterol domain. These data are supported by intact cell studies indicating that caveolar cholesterol poorly equilibrates with bulk plasma membrane cholesterol unless the caveolae are disrupted (reviewed in refs *8* and *54*).

Fifth, the data for the first time show that the intracellular cholesterol binding protein SCP-2 dramatically and selectively enhances sterol transfer from caveolae/lipid rafts, but not nonlipid rafts. SCP-2 is known to bind cholesterol (*36*, *69*−*71*), enhance sterol transfer from plasma membranes in vitro (*43*, *44*) and from plasma membrane to endoplasmic reticulum of sphingomyelinase-treated (i.e., disrupts caveolae) SCP-2 overexpressing cells (*34*, *72*), and redistribute cholesterol from the plasma membrane to lipid droplets in living cells (*33*, *65*). Consistent with this selective action of SCP-2 via caveolae/lipid rafts, recent immunofluorescence studies showed that SCP-2 colocalizes with caveolin-1 at the plasma membrane of intact cells (*73*). Since direct binding of SCP-2 to membranes is essential for SCP-2's ability to facilitate intermembrane sterol transfer (*74*−*77*), these data suggest that SCP-2 may target sterol transfer to caveolae/lipid rafts possibly by direct interaction of SCP-2 with caveolin-1, a possibility beyond the scope of the present investigation. The importance of nonvesicular pathways is illustrated by a recent real-time fluorescence imaging study of sterol transport between plasma membrane domains of polarized hepatic cells (*78*), which together with earlier studies with liver (*79*−*82*), indicates that the majority of liver cholesterol trafficks from the basolateral membranes by nonvesicular pathways to the bile canalicular region. It is of note that SCP-2 is highly expressed in liver hepatic cells (reviewed in ref *35*).

Sixth, overexpression of SCP-2 in intact transfected cells significantly altered the lipid and protein constituents of caveolae/lipid rafts such that spontaneous sterol transfer was reduced and these caveolae/lipid rafts became more resistant to direct actions of SCP-2. In contrast, in vitro studies from this and other laboratories show that SCP-2 added to purified membranes enhances sterol transfer (reviewed in ref *35*) and, in addition, SCP-2 directly interacts with caveolin (*73*). The apparent discrepancy in the intact cell SCP-2 overexpression vs in vitro studies with SCP-2 may be reconciled as follows: Kinetic analysis of HDL-mediated cholesterol efflux from L-cells revealed two kinetic pools, one rapid (half-life of 1min) and the other slow (half-life of 15 min), consistent with protein-mediated and vesicular transfer of sterol, respectively (*65*). Furthermore, while SCP-2 expression did not alter sterol pool sizes, the half-life of the rapid pool was decreased 36% (i.e., faster protein mediated sterol transfer), while the half-life of the slower pool increased 113% (i.e., slower vesicular sterol transfer) in SCP-2 overexpressing L-cells (*65*). Since vesicular transfer accounted for 80−90% of sterol exchange, the overall net effect was reduction of cholesterol efflux in cells overexpressing SCP-2. Thus, SCP-2 enhances molecular sterol transfer in both in intact cells and in vitro. While the mechanism whereby SCP-2 influences vesicular cholesterol trafficking remains to be resolved, SCP-2 is known to bind and transfer phosphatidylinositol (PI) and polyphosphoinositides (reviewed in ref *73*), enhance phospholipase mediated IP_3 production (*73*), and bind fatty acyl CoA (*83*, *84*). Both polyphosphoinositides (reviewed in ref *85*) and fatty acylCoAs (*86*, *87*) influence

vesicle formation at the Golgi. These data suggest that SCP-2 may affect the intracellular transfer of PI, poly-PIs, and fatty acyl CoAs to influence vesicle budding from the Golgi and vesicle trafficking from the Golgi through interactions with these ligands.

In summary, the present findings demonstrate the utility of con-A affinity chromatography for simultaneous isolation of caveolae/lipid raft and nonlipid raft domains from plasma membranes. These domains differed markedly in both protein and lipid constituents. The purity of the caveolae/lipid raft fraction as well as the nonlipid raft fraction was defined both by selective protein and lipid markers which included not only the presence and/or absence of specific proteins such as caveolin-1, flotillin, SR-B1, and Na, K-ATPase, but also total lipid content, cholesterol, total phospholipid, sphingomyelin, and gangliosides such as GM1. The enrichment and/or depletion of each of these markers are widely accepted in the literature as defining caveolae/lipid raft and nonlipid raft domains. The adherent fraction isolated from the plasma membrane by con-A sepharose was designated as enriched in caveolae/lipid rafts because this fraction was enriched in (i) protein markers (4-, 5-, and 10-fold in caveolin-1, flotillin, and SR−B1, respectively); (ii) total lipid content (3-fold); (iii) cholesterol (2.5-fold); (iv) specific phospholipids (15.2- and 3.1-fold in PC and PI); (v) specific sphingolipids (spingomyelin, 5.2-fold; ganglioside GM1, 5-fold). Concomitantly, the nonadherent fraction isolated from the plasma membrane by con-A sepharose was designated as enriched in nonlipid rafts because this fraction was enriched in Na^+,K^+-ATPase and lipid poor. The nonadherent fraction was also relatively deficient in SM, GM, PC, and cholesterol as compared to the adherent caveolae/lipid raft enriched fraction. It was also shown for the first time that spontaneous sterol transfer was 7−12-fold faster from caveolae/lipid rafts than nonlipid raft domains. This was largely due to the near absence of exchangeable sterol in the nonlipid rafts. SCP-2 dramatically and selectively enhanced sterol transfer from caveolae/lipid rafts, but not from nonlipid rafts. Finally, overexpression of SCP-2 significantly altered the sterol dynamics of caveolae/lipid rafts to facilitate retention of cholesterol within the cell. These data may help contribute to our understanding of abnormalities in the uptake, intracellular trafficking, or efflux of cholesterol which occurs in human diseases such as macrophage foam cell formation (*88*), cardiovascular (reviewed in refs *8* and *64*), Niemann-Pick C (*89*), and Alzheimer's (*28*, *57*). The present work allowed resolution of caveolae/lipid rafts from nonlipid raft domains and established for the first time that (i) caveolae/lipid rafts, rather than the nonlipid raft domains, contain significant levels of rapidly transferable sterol, consistent with their role in spontaneous sterol transfer from and through the plasma membrane, and (ii) SCP-2 participates in regulating how caveolae/lipid rafts, but not nonlipid raft domains, mediate cholesterol trafficking through the plasma membrane.

REFERENCES

1. Lavie, Y., and Liscovitch, M. (2000) *Glycoconjugate J. 17*, 253−259.
2. Garrigues, A., Escargueil, A. E., and Orlowski, S. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 10347−10352.
3. Frank, P. A., Pedraza, A., Cohen, D. E., and Lisanti, M. P. (2001) *Biochemistry 40*, 10892−10900.
4. Wood, W. G., Schroeder, F., Avdulov, N. A., Chochina, S. V., and Igbavboa, U. (1999) *Lipids 34*, 225−234.
5. Schroeder, F., Jefferson, J. R., Kier, A. B., Knittell, J., Scallen, T. J., Wood, W. G., and Hapala, I. (1991) *Proc. Soc. Exp. Biol. Med. 196*, 235−252.
6. Schroeder, F., Frolov, A. A., Murphy, E. J., Atshaves, B. P., Jefferson, J. R., Pu, L., Wood, W. G., Foxworth, W. B., and Kier, A. B. (1996) *Proc. Soc. Exp. Biol. Med. 213*, 150−177.
7. Anderson, R. (1998) *Annu. Rev. Biochem. 67*, 199−225.
8. Smart, E. J., and van der Westhuyzen, D. R. (1998) in *Intracellular Cholesterol Trafficking* (Chang, T. Y., and Freeman, D. A., Eds.) pp 253−272, Kluwer Academic Publishers, Boston.
9. Bretscher, M. S., and Munro, S. (1993) *Science 261*, 1280−1281.
10. Schroeder, F., and Nemecz, G. (1990) in *Advances in Cholesterol Research* (Esfahami, M., and Swaney, J., Eds.) pp 47−87, Telford Press, Caldwell, NJ.
11. Abrami, L., Liu, S., Cosson, P., Leppla, S. H., and van der Groot, F. G. (2003) *J. Cell Biol. 160*, 321−328.
12. Sandvig, K., and van Deurs, B. (1999) *FEBS Lett. 452*, 67−70.
13. Bavari, S., Bosio, C. M., Wiegand, E., Ruthel, G., Will, A. B., Geisbert, T. W., Hevey, M., Schmaljohn, C., and Aman, M. J. (2002) *J. Exp. Med. 195*, 593−602.
14. Empig, C. J., and Goldsmith, M. A. (2002) *J. Virol. 76*, 5266−5270.
15. Marjomaki, V., Pietiainen, V., Upla, P., Ivaska, J., Nissinen, L., Reunanen, H., Huttunen, P., Hyypia, T., and Heino, J. (2002) *J. Virol. 76*, 1856−1865.
16. Scheiffele, P., Roth, M. G., and Simons, K. (1997) *EMBO J. 16*, 5501−5508.
17. Marsh, M., and Pelchen-Matthews, A. (2000) *Traffic 1*, 525−532.
18. Sieczkarski, S. B., and Whittaker, G. R. (2002) *J. Gen. Virol. 83*, 1535−1545.
19. Shin, J.-S., and Abraham, S. N. (2002) *Microbes Infection 3*, 755−761.
20. Norkin, L. C. (2001) *Adv. Drug Delivery Rev. 49*, 301−315.
21. Marx, J. (2001) *Science 294*, 1862−1865.
22. Masserini, M., and Ravasi, D. (2001) *Biochim. Biophys. Acta 1532*, 149−161.
23. Brown, D. A., and London, E. (1998) *Annu. Rev. Cell Dev. Biol. 14*, 111−136.
24. London, E., and Brown, D. A. (2000) *Biochim. Biophys. Acta 1508*, 182−195.
25. Hooper, N. M. (1999) *Mol. Membr. Biol. 16*, 145−156.
26. Ahmed, S. N., Brown, D. A., and London, E. (1997) *Biochemistry 36*, 10944−10953.
27. Smart, E. J., Ying, Y., Mineo, C., and Anderson, R. G. W. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10404−10408.
28. Eckert, G. P., Igbavboa, U., Muller, W., and Wood, W. G. (2003) *Brain Res. 962*, 144−150.
29. Pike, L. J., Han, X., Chung, K.-N., and Gross, R. W. (2002) *Biochemistry 41*, 2075−2088.
30. Atshaves, B. P., Petrescu, A., Starodub, O., Roths, J., Kier, A. B., and Schroeder, F. (1999) *J. Lipid Res. 40*, 610−622.
31. McIntosh, A., Gallegos, A., Atshaves, B. P., Storey, S., Kannoju, D., and Schroeder, F. (2003) *J. Biol. Chem. 278*, 6384−6403.
32. Higuchi, K. (1970) *J. Cell Physiol. 75*, 65−72.
33. Gallegos, A. M., Atshaves, B. P., Storey, S., McIntosh, A., Petrescu, A. D., and Schroeder, F. (2001) *Biochemistry 40*, 6493−6506.
34. Moncecchi, D. M., Murphy, E. J., Prows, D. R., and Schroeder, F. (1996) *Biochim. Biophys. Acta 1302*, 110−116.
35. Gallegos, A. M., Atshaves, B. P., Storey, S. M., Starodub, O., Petrescu, A. D., Huang, H., McIntosh, A., Martin, G., Chao, H., Kier, A. B., and Schroeder, F. (2001) *Prog. Lipid Res 40*, 498−563.
36. Schroeder, F., Frolov, A., Schoer, J., Gallegos, A., Atshaves, B. P., Stolowich, N. J., Scott, A. I., and Kier, A. B. (1998) in *Intracellular Cholesterol Trafficking* (Chang, T. Y., and Freeman, D. A., Eds.) pp 213−234, Kluwer Academic Publishers, Boston.
37. Schroeder, F., Fontaine, R. N., and Kinden, D. A. (1982) *Biochim. Biophys. Acta 690*, 231−242.
38. van der Luit, A. H., Budde, M., Ruurs, P., Verheij, M., and van Blitterswijk, W. J. (2002) *J. Biol. Chem. 277*, 39541−39547.
39. Hara, A., and Radin, N. S. (1978) *Anal. Biochem. 90*, 420−426.
40. Jefferson, J. R., Slotte, J. P., Nemecz, G., Pastuszyn, A., Scallen, T. J., and Schroeder, F. (1991) *J. Biol. Chem. 266*, 5486-5496.
41. Marzo, A., Ghirardi, P., Sardini, D., and Meroni, G. (1971) *Clin. Chem. 17*, 145−147.
42. Bradford, M. (1976) *Anal. Biochemistry 72*, 248−254.

43. Frolov, A., Woodford, J. K., Murphy, E. J., Billheimer, J. T., and Schroeder, F. (1996) *J. Biol. Chem. 271*, 16075−16083.
44. Frolov, A. A., Woodford, J. K., Murphy, E. J., Billheimer, J. T., and Schroeder, F. (1996) *J. Lipid Res. 37*, 1862−1874.
45. Kavecansky, J., Joiner, C. H., and Schroeder, F. (1994) *Biochemistry 33*, 2880−2890.
46. Gallegos, A. M., Schoer, J., Starodub, O., Kier, A. B., Billheimer, J. T., and Schroeder, F. (2000) *Chem. Phys. Lipids 105*, 9−29.
47. Schoer, J., Gallegos, A., Starodub, O., Petrescu, A., Roths, J. B., Kier, A. B., and Schroeder, F. (2000) *Biochemistry 39*, 7662−7677.
48. Harder, T., Schieffele, P., Verkade, P., and Simons, K. (1998) *J. Cell. Biol. 141*, 929−942.
49. Johannes, L., and Lamaze, C. (2002) *Traffic 3*, 443−451.
50. Goldstein, J. L., and Brown, M. S. (1992) *Eur. J. Biochem. 13*, 34−36.
51. Fielding, C. J., and Fielding, P. E. (1997) *J. Lipid Res. 38*, 1503−1521.
52. Brown, D. A., and London, E. (1998) *J. Membr. Biol. 164*, 103−114.
53. Anderson, R. G. W., and Jacobson, K. (2002) *Science 296*, 1821−1825.
54. Schroeder, F., Gallegos, A. M., Atshaves, B. P., Storey, S. M., McIntosh, A., Petrescu, A. D., Huang, H., Starodub, O., Chao, H., Yang, H., Frolov, A., and Kier, A. B. (2001) *Exp. Biol. Med. 226*, 873−890.
55. Liu, P., Rudick, M., and Anderson, R. G. W. (2002) *J. Biol. Chem. 277*, 41295−41298.
56. Brown, D. A., and London, E. (2000) *J. Biol. Chem. 275*, 17221−17224.
57. Wood, G. W., Schroeder, F., Igbavboa, U., Avdulov, N. A., and Chochina, S. V. (2002) *Neurobiol. Aging 23*, 685−694.
58. Baenziger, J. U., and Fiete, D. (1979) *J. Biol. Chem. 254*, 2400−2407.
59. Frolov, A., Petrescu, A., Atshaves, B. P., So, P. T. C., Gratton, E., Serrero, G., and Schroeder, F. (2000) *J. Biol. Chem. 275*, 12769−12780.
60. Wood, W. G., Schroeder, F., Hogy, L., Rao, A. M., and Nemecz, G. (1990) *Biochim. Biophys. Acta 1025*, 243−246.
61. Murata, M., Peranen, J., Schreiner, R., Wieland, F., Kurzchalia, T. V., and Simons, K. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10339−10343.
62. Reaven, E., Tsai, L., and Azhar, S. (1996) *J. Biol. Chem. 271*, 16208−16217.
63. Azhar, S., Nomoto, A., Leers-Sucheta, S., and Reaven, E. (1998) *J. Lipid Res. 39*, 1616−1628.
64. Fielding, C. J., Bist, A., and Fielding, P. E. (1998) in *Intracellular Cholesterol Trafficking* (Chang, T. Y., and Freeman, D. A., Eds.) pp 273−288, Kluwer Academic Publishers, Boston.
65. Atshaves, B. P., Starodub, O., McIntosh, A. L., Roths, J. B., Kier, A. B., and Schroeder, F. (2000) *J. Biol. Chem. 275*, 36852−36861.
66. Schroeder, F., and Nemecz, G. (1989) *Biochemistry 28*, 5992−6000.
67. Schroeder, F., Nemecz, G., Wood, W. G., Joiner, C., Morrot, G., Ayraut-Jarrier, M., and Devaux, P. F. (1991) *Biochim. Biophys. Acta 1066*, 183−192.
68. Nemecz, G., and Schroeder, F. (1988) *Biochemistry 27*, 7740−7749.
69. Colles, S. M., Woodford, J. K., Moncecchi, D., Myers-Payne, S. C., McLean, L. R., Billheimer, J. T., and Schroeder, F. (1995) *Lipids 30*, 795−804.
70. Stolowich, N. J., Frolov, A., Petrescu, A. D., Scott, A. I., Billheimer, J. T., and Schroeder, F. (1999) *J. Biol. Chem. 274*, 35425−35433.
71. Schroeder, F., Frolov, A., Starodub, O., Russell, W., Atshaves, B. P., Petrescu, A. D., Huang, H., Gallegos, A., McIntosh, A., Tahotna, D., Russell, D., Billheimer, J. T., Baum, C. L., and Kier, A. B. (2000) *J. Biol. Chem. 275*, 25547−25555.
72. Murphy, E. J., and Schroeder, F. (1997) *Biochim. Biophys. Acta 1345*, 283−292.
73. Schroeder, F., Zhou, M., Swaggerty, C. L., Atshaves, B. P., Petrescu, A. D., Storey, S., Martin, G. G., Huang, H., Helmkamp, G. M., and Ball, J. M. (2003) *Biochemistry 42*, 3189−3202.
74. Woodford, J. K., Colles, S. M., Myers-Payne, S., Billheimer, J. T., and Schroeder, F. (1995) *Chem. Phys. Lipids 76*, 73−84.
75. Huang, H., Ball, J. A., Billheimer, J. T., and Schroeder, F. (1999) *Biochemistry 38*, 13231−13243.
76. Huang, H., Ball, J. A., Billheimer, J. T., and Schroeder, F. (1999) *Biochem. J. 344*, 593−603.
77. Huang, H., Gallegos, A., Zhou, M., Ball, J. M., and Schroeder, F. (2002) *Biochemistry 41*, 12149−12162.
78. Wustner, D., Herrmann, A., Hao, M., and Maxfield, F. R. (2002) *J. Biol. Chem. 277*, 30325−30336.
79. Fuchs, M., Lammert, F., Wang, D. Q. H., Paigen, B., Carey, M. C., and Cohen, D. E. (1998) *Biochem. J. 336*, 33−37.
80. Fuchs, M., Lammert, F., Wang, D. Q. H., Paigen, B., Carey, M. C., and Cohen, D. E. (1997) *FASEB J. 11*, A1060.
81. Hafer, A., Katzberg, N., Muench, C., Scheibner, J., Stange, E. F., Seedorf, U., and Fuchs, M. (2000) *Gastroenterology 118* (Part 1 Suppl 2), 135.
82. Fuchs, M., Hafer, A., Muench, C., Kannenberg, F., Teichmann, S., Scheibner, J., Stange, E. F., and Seedorf, U. (2001) *J. Biol. Chem. 276*, 48058−48065.
83. Frolov, A., Cho, T. H., Billheimer, J. T., and Schroeder, F. (1996) *J. Biol. Chem. 271*, 31878−31884.
84. Seedorf, U., Raabe, M., Ellinghaus, P., Kannenberg, F., Fobker, M., Engel, T., Denis, S., Wouters, F., Wirtz, K. W. A., Wanders, R. J. A., Maeda, N., and Assmann, G. (1998) *Genes Dev. 12*, 1189−1201.
85. Cockcroft, S. (1998) *BioEssays 20*, 423−432.
86. Pfanner, N., Orci, L., Glick, B. S., Amherdt, M., Arden, S. R., Malhotra, V., and Rothman, J. E. (1989) *Cell 59*, 95−102.
87. Pfanner, N., Glick, B. S., Arden, S. R., and Rothman, J. E. (1990) *J. Cell Biol. 110*, 955−961.
88. Hirai, A., Kino, T., Tokinaga, K., Tahara, K., Tamura, Y., and Yoshida, S. (1994) *J. Clin. Invest. 94*, 2215−2223.
89. Neufeld, E. B. (1998) in *Intracellular Cholesterol Trafficking* (Chang, T. Y., and Freeman, D. A., Eds.) pp 93−121, Kluwer Academic Publishers, Boston.

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