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Epidermal Growth Factor Receptors Are Localized to Lipid Rafts That Contain a Balance of Inner and Outer Leaflet Lipids

A SHOTGUN LIPIDOMICS STUDY* [S]

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The epidermal growth factor (EGF) receptor partitions into lipid rafts made using a detergent-free method, but is extracted from low density fractions by Triton X-100. By screening several detergents, we identified Brij 98 as a detergent in which the EGF receptor is retained in detergent-resistant membrane fractions. To identify the difference in lipid composition between those rafts that harbored the EGF receptor (detergent-free and Brij 98-resistant) and those that did not (Triton X-100-resistant), we used multidimensional electrospray ionization mass spectrometry to perform a lipidomics study on these three raft preparations. Although all three raft preparations were similarly enriched in cholesterol, the EGF receptor-containing rafts contained more ethanolamine glycerophospholipids and less sphingomyelin than did the non-EGF receptor-containing Triton X-100 rafts. As a result, the detergent-free and Brij 98-resistant rafts exhibited a balance of inner and outer leaflet lipids, whereas the Triton X-100 rafts contained a preponderance of outer leaflet lipids. Furthermore, in all raft preparations, the outer leaflet phospholipid species were significantly different from those in the bulk membrane, whereas the inner leaflet lipids were quite similar to those found in the bulk membrane. These findings indicate that the EGF receptor is retained only in rafts that exhibit a lipid distribution compatible with a bilayer structure and that the selection of phospholipids for inclusion into rafts occurs mainly on the outer leaflet lipids.

Lipid rafts are small, low density plasma membrane domains that contain high levels of cholesterol and sphingolipids (1–3). Tight interactions between the sterol and the sphingolipids result in the formation of a domain that is resistant to solubilization in detergents (4–6). This property is often used to separate lipid rafts from bulk plasma membrane fractions (1).

GPI[1]-anchored proteins (1, 7–9) and dually acylated proteins (10–12) selectively partition into lipid rafts by virtue of the interaction of their hydrophobic anchors with raft domains. Transmembrane proteins such as flotillin have also been shown to be enriched in lipid rafts compared with the bulk plasma membrane (13). Of special interest has been the finding that many molecules involved in cell signaling are enriched in lipid rafts. This includes proteins such as receptor and nonreceptor tyrosine kinases, serpentine receptors, and heterotrimeric and low molecular weight G proteins (for review, see Refs. 14 and 15). As a result of the selective localization of signaling molecules in lipid rafts, these domains are thought to serve as organizational platforms for the process of signal transduction.

Recent studies have suggested that lipid rafts represent a heterogeneous collection of domains showing differences in both protein and lipid composition. For example, Madore *et al.* (16) showed that, in lipid raft preparations, the GPI-anchored prion protein can be selectively immunoprecipitated away from a second GPI-anchored protein, Thy-1, suggesting that the two GPI-anchored proteins exist in physically separate domains. Gomez-Mouton *et al.* (17) used immunofluorescence to demonstrate that the raft proteins urokinase plasminogen activator receptor and CD44 and the raft lipids GM1 and GM3 distribute asymmetrically in cells. The urokinase plasminogen activator receptor and GM3 localized to the leading edge of migrating T cells, whereas CD44 and GM1 were found at the trailing edge of the cells. Because all four components were isolated in the same lipid raft fraction, these findings suggest that rafts with distinct protein and lipid compositions coexist within cells and show differences in spatial localization.

Differential sensitivity of proteins to extraction by various detergents has provided additional evidence for heterogeneity among lipid rafts (18–20). The classical method for the preparation of lipid rafts involves the extraction of cells in 1% Triton X-100, followed by separation of the low density raft membranes in a sucrose gradient (1). The use of other detergents to extract membranes has demonstrated that, even among a single class of raft proteins, there is variability in their resistance to detergent extraction. For example, GPI-anchored Thy-1 was shown to be associated with low density membrane domains when cells were extracted with 0.5% Triton X-100 or 0.5% Brij 96. However, another GPI-anchored protein, NCAM-120, was completely solubilized by both detergents (16). Thus, these two similarly anchored proteins must exist in domains of different composition that are differentially sensitive to detergent ex-

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[S] The on-line version of this article (available at http://www.jbc.org) contains Supplemental Tables 1–3.

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[1] The abbreviations used are: GPI, glycosylphosphatidylinositol; EGF, epidermal growth factor; ESI/MS, electrospray ionization mass spectrometry; CHO, Chinese hamster ovary; GM1, ganglioside GM1; GM3, ganglioside GM3; MES, 4-morpholineethanesulfonic acid; PS, serine glycerophospholipids; PE, ethanolamine glycerophospholipids; PC, choline glycerophospholipids; PNS, post-nuclear supernatant; SPM, sphingomyelin.

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traction. Schuck *et al.* (21) reported that rafts made using different detergents do indeed contain different complements of proteins and are variably enriched in cholesterol and sphingolipids compared with total cell membranes.

The EGF receptor, a type I transmembrane protein with tyrosine kinase activity (22), has been shown to be enriched in lipid rafts (23, 24). Localization of the EGF receptor to rafts appears to modulate both its ligand binding and tyrosine kinase activities because the disruption of lipid rafts by acute cholesterol depletion leads to an enhancement of both these activities (24–27). Unlike traditional raft proteins, the EGF receptor is solubilized by treatment with 1% Triton X-100 (28), but is enriched in lipid rafts prepared using a detergent-free protocol (23, 24). This suggests that the rafts into which the EGF receptor partitions may be different from classical Triton X-100-resistant rafts.

In this work, we screened a variety of detergents to determine which supported the retention of the EGF receptor in a low density, detergent-resistant fraction. Among the detergents tested, only Brij 98 produced a distinct EGF receptor-containing raft fraction. Subsequently, multidimensional ESI/MS was used to quantitate the differences in lipid composition of rafts that contained the EGF receptor (Brij 98-resistant membranes and detergent-free raft preparations (29)) and those that did not retain the EGF receptor (Triton X-100-resistant membranes). The results of this lipidomics analysis demonstrate that, although all rafts are similarly enriched in cholesterol, the EGF receptor-containing rafts possess a balance of inner and outer leaflet lipids, whereas non-EGF receptor-containing rafts contain principally outer leaflet lipids. In addition, the data demonstrate that phospholipids in the outer leaflet of rafts undergo a significant selection for inclusion into rafts, whereas inner leaflet lipids show relatively little selection compared with the bulk membrane.

EXPERIMENTAL PROCEDURES

Materials—Triton X-100, Tween 20, Brij 98, and Percoll were obtained from Sigma. Octyl glucoside was purchased from Calbiochem. Brij 96 was from Fluka. The anti-EGF receptor and anti-G_q polyclonal antibodies were from Santa Cruz Biotechnology, Inc. The anti-transferrin receptor monoclonal antibody was obtained from Zymed Laboratories Inc.. The monoclonal antibodies against flotillin-1 and annexin II and the polyclonal antibody against caveolin-1 were purchased from Transduction Laboratories. The anti-β-COP polyclonal antibody was from Sigma, and the anti-calnexin polyclonal antibody was from Stressgen Biotechnologies Corp. The monoclonal antibody against the Na^+/K^+-ATPase β-subunit was from BIOMOL Research Labs Inc. The anti-prohibitin monoclonal antibody was from Neomarkers. Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG and chemiluminescent reagents were from Amersham Biosciences. Effectene transfection reagent was obtained from Qiagen Inc. OptiPrep was purchased from Grainer Bio-One. All of the lipid internal standards were purchased from Avanti Polar Lipids. All of the solvents used for sample preparation and for MS analyses were obtained from Burdick & Jackson.

Cells and Tissue Culture—CHO cells were maintained in Ham's F-12 medium containing 10% fetal calf serum in 5% CO_2. Cells were transfected with human wild-type EGF receptor in pcDNA3.1(−) (Invitrogen) using Effectene according to the manufacturer's instructions. Transfected cells were passaged in Ham's F-12 medium containing 10% fetal calf serum, and colonies stably expressing the EGF receptor were selected upon addition of 400 μg/ml G418 to the growth medium. Isolated clones were maintained in Ham's F-12 medium containing 10% fetal calf serum plus 200 μg/ml G418.

Preparation of Detergent-resistant Lipid Rafts—One D150 mm plate of confluent cells was washed three times with phosphate-buffered saline and drained well. To the plate was added 1 ml of MES-buffered saline (50 mM MES (pH 6.5) and 150 mM NaCl) containing Triton X-100, Brij 98, Brij 96, Tween 20, or octyl glucoside. All detergents were used at a concentration of 1%, except octyl glucoside, which was used at a concentration of 2%. The detergent/protein ratio was 10:1 for the four detergents used at 1% and 20:1 for octyl glucoside. Cells were scraped into the detergent-containing buffer and mechanically disrupted by passage 20 times through a 3-inch 22-gauge needle. The lysate was mixed with an equal volume of 80% sucrose in MES-buffered saline. The material was placed in the bottom of a 12-ml ultracentrifuge tube, and a 10-ml 5–30% linear sucrose gradient in MES-buffered saline was poured on top. The gradients were centrifuged for 3 h at 175,000 × *g* and then fractionated into 12 fractions (1 ml each). Fractions 3–5 of the sucrose gradients were used for MS analysis of lipids.

Preparation of Non-detergent Lipid Rafts—The method of Macdonald and Pike (30) was used for the preparation of non-detergent lipid rafts. Briefly, four D150 mm plates of confluent cells were scraped into base buffer (250 mM sucrose and 20 mM Tris-HCl (pH 7.8)) to which 1 mM $CaCl_2$ and 1 mM $MgCl_2$ had been added. Cells were pelleted and then lysed in 1 ml of base buffer with calcium and magnesium by passage 20 times through a 3-inch 22-gauge needle, and a post-nuclear supernatant was obtained by low speed centrifugation. The post-nuclear supernatant was made 25% in OptiPrep by addition of an equal volume of 50% OptiPrep in base buffer. Rafts were isolated by centrifugation in a 0–20% OptiPrep gradient in base buffer. Fractions 1 and 2 were pooled for MS lipid analysis.

Western Blotting—For analysis, 100 μl of each fraction from a gradient was separated by SDS-PAGE. Gels were transferred electrophoretically to nitrocellulose, which was blocked by incubation with 10% nonfat powdered milk. The nitrocellulose strips were incubated for 2 h at room temperature with primary antibody, washed, and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. After washing, antibodies were detected by chemiluminescence.

Preparation of Lipid Extracts and MS Analysis of Lipids—Lipids were extracted by the Bligh and Dyer procedure with modifications as described previously (31–33). Briefly, to each lipid raft sample (~100 μg of protein) were added internal standards, including 14:0-14:0 PS (40 nmol/mg of protein), 15:0-15:0 phosphatidylglycerol (9 nmol/mg of protein), 15:0-15:0 PE (57 nmol/mg of protein), and 14:1-14:1 PC (45.0 nmol/mg of protein). Lipids from each sample were extracted twice against 2 ml of 50 mM LiCl, back-extracted twice against 2 ml of 10 mM LiCl, filtered with a 0.2-μm PFTE syringe filter, and finally stored in 200 μl of 1:1 (v/v) chloroform/methanol. Each lipid solution was further diluted ~20-fold just prior to infusion and lipid analysis.

Multidimensional ESI/MS analyses were performed utilizing a ThermoFinnigan TSQ Quantum Ultra triple-quadrupole mass spectrometer equipped with an electrospray ion source as described previously (31, 32). Typically, a 1-min period of signal averaging in the profile mode was employed for each mass spectrum, and a 1–2-min period of signal averaging was used for each tandem mass spectrum. Identification and quantitation of each individual molecular species were performed in a multidimensional MS array format as described previously (31–34)

Protein and Cholesterol Assays—Proteins were determined using the precipitation Lowry method described by Peterson (35). Cholesterol was determined using a Wako CII cholesterol assay kit.

RESULTS

EGF Receptors in Detergent-resistant Membranes—Five different detergents were screened for their ability to generate EGF receptor-containing lipid rafts. These included Triton X-100, Tween 20, Brij 98, Brij 96, and octyl glucoside. The first four detergents were used at 1%, whereas the latter detergent was used at 2%. Solubilization was aided by passage of the detergent lysates through a 22-gauge needle. After cell solubilization and centrifugation through a 5–30% sucrose gradient as described under "Experimental Procedures," the gradients were fractionated and analyzed by Western blotting for the distribution of a variety of plasma membrane proteins. The distribution of marker proteins in a detergent-free raft preparation was included for comparison. The results are shown in Fig. 1.

In the detergent-free raft preparation, the EGF receptor was recovered in the three lightest fractions of the gradient along with other raft proteins such as flotillin and the dually acylated G_q protein. These fractions were distinct from those that contained a plasma membrane marker protein, the transferrin receptor, indicating that rafts had been separated from the bulk plasma membrane. Caveolin was broadly distributed in this gradient possibly because of the interaction of caveolae with cytoskeletal elements.

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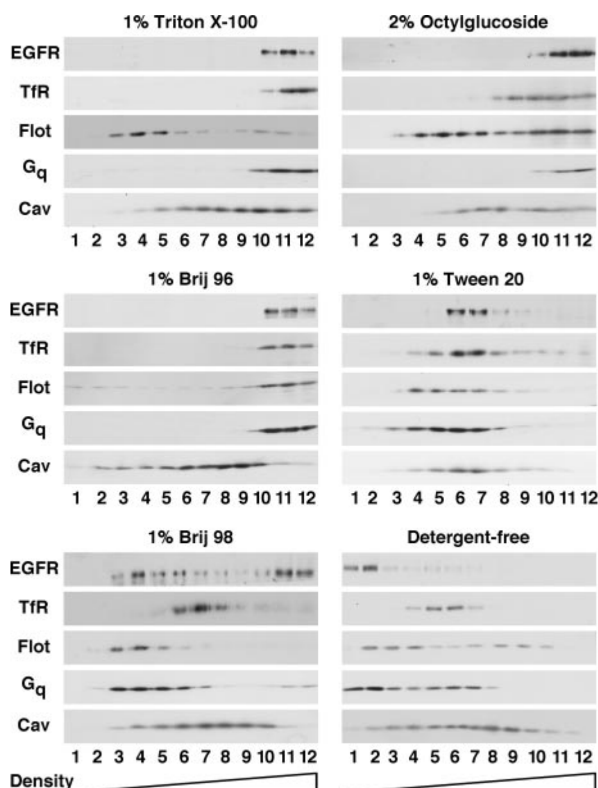

FIG. 1. **Density gradient analysis of lipid rafts prepared using different detergents.** Detergent-resistant membranes or detergent-free lipid rafts were prepared from CHO cells as described under "Experimental Procedures." Extracts were separated by density gradient centrifugation, and the gradients were fractionated into 12 fractions. An equal volume of each fraction was analyzed by SDS-PAGE, followed by Western blotting with the indicated antibody. *EGFR*, EGF receptor; *TfR*, transferrin receptor; *Flot*, flotillin; *Cav*, caveolin.

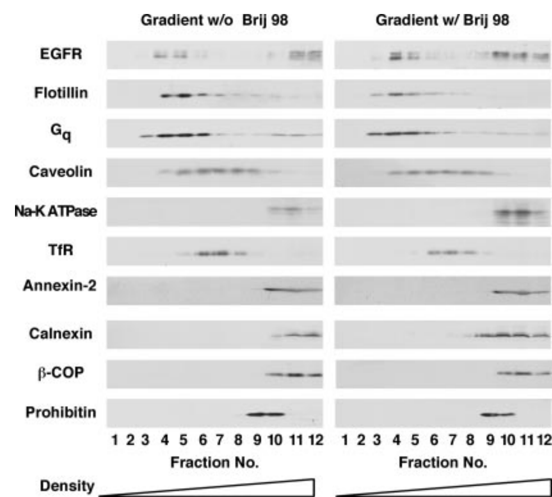

FIG. 2. **Characterization of detergent-resistant membranes prepared using 1% Brij 98.** CHO cells were solubilized with 1% Brij 98, and the extracts were analyzed by sucrose density gradient centrifugation as described under "Experimental Procedures." Gradients were fractionated, and equal volumes of each fraction were separated by SDS-PAGE. Gels were transferred to Immobilon and subjected to Western blotting using the indicated antibody. *Left*, sucrose solutions contained no Brij 98. *Right*, sucrose solutions contained 0.5% Brij 98. *EGFR*, EGF receptor; *TfR*, transferrin receptor.

Extraction of cells with 1% Triton X-100 resulted in the complete solubilization of the EGF receptor, the transferrin receptor, and the heterotrimeric G_q protein as evidenced by the recovery of these proteins in the high density portion of the gradient. By contrast, the raft protein flotillin floated into the low density region of the gradient, identifying the location of the lipid raft fraction in this gradient. As in the detergent-free preparation, caveolin was recovered throughout the gradient. Membranes solubilized with 2% octyl glucoside showed a pattern of marker protein distribution similar to that observed for Triton X-100-solubilized membranes in which the EGF receptor was excluded from the low density fraction marked by flotillin.

Unexpectedly, solubilization of CHO cells with 1% Brij 96 led to the recovery of almost all proteins, including the raft marker flotillin, in the high density non-raft fractions of the gradient. The lone exception to this rule was caveolin, which was partially recovered in the upper fractions of the gradient. At the other end of the spectrum, treatment of cells with 1% Tween 20 resulted in the recovery of all marker proteins in the middle third of the gradient, with little distinction in the distribution of the different proteins. This indicates that, even at a high concentration and when used with mechanical agitation, Tween 20 does not differentially solubilize raft and non-raft membranes and thus does not permit isolation of a distinct low density raft fraction.

Among the detergents tested, only Brij 98 appeared to generate a distinct low density, detergent-resistant fraction that contained the EGF receptor as well as known raft proteins. Approximately one-third to one-half of the EGF receptor was recovered in the low density region of the gradient (fractions 3–5) that also contained flotillin and G_q. The transferrin receptor, a plasma membrane marker, was found in the middle of the gradient at a position distinct from that of the lipid raft proteins. Caveolin again distributed broadly throughout the gradient.

Characterization of Brij 98-resistant Membranes—Additional studies were undertaken to determine whether the low density membrane fraction obtained by solubilization of cells in Brij 98 effectively separated plasma membrane raft proteins from proteins present on intracellular membranes. In addition to the distribution of the EGF receptor, flotillin, G_q, the transferrin receptor, and caveolin, Fig. 2 (*left*) shows the distribution of Na^+/K^+-ATPase, an intrinsic plasma membrane protein; annexin-2, an extrinsic plasma membrane protein; calnexin, a marker for the endoplasmic reticulum; β-COP, a marker for the Golgi; and prohibitin, a mitochondrial membrane protein. All of the intracellular membrane marker proteins were recovered in the high density portion of the gradient, well separated from the raft fractions that contained the EGF receptor, flotillin, and G_q. Thus, solubilization of membranes with Brij 98 resulted in the production of a low density fraction that was devoid of markers for intracellular membranes and non-raft plasma membrane proteins, but that contained the EGF receptor and other known raft proteins.

The data shown in Fig. 2 (*left*) were generated under conditions in which there was no detergent present in the sucrose gradient. However, as also shown in Fig. 2 (*right*), inclusion of 0.5% Brij 98 in the gradient fractions did not alter the distribution of any of the proteins. These data indicate that the distribution of proteins observed using this procedure was not the result of a "reconstitution" of membrane domains associated with removal of detergent during gradient centrifugation.

MS Analysis of Raft Lipid Composition—To begin to identify the differences between raft preparations that retained the EGF receptor (detergent-free preparations and Brij 98-resistant membranes) and those that did not (Triton X-100-resistant membranes), lipid rafts of each type were prepared and subjected to multidimensional ESI/MS analysis. Table I compares the lipid composition by class of each of the three raft preparations as well as membranes from the PNS fraction. The values for the abundance of each individual species are presented in Supplemental Tables 1–3.

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TABLE I
Lipid content of rafts and PNS membranes

PNS membranes and lipid rafts were isolated from CHO cells as described under "Experimental Procedures." Lipids were extracted from samples containing 100 μg of membrane protein and analyzed for phospholipid content by multidimensional ESI/MS. Results represent the mean ± S.D. of three separate experiments.

	PNS			Detergent-free rafts			Brij 98 rafts			Triton X-100 rafts		
	Average	S.D.	Phospholipid	Average	S.D.	Phospholipid	Average	S.D.	Phospholipid	Average	S.D.	Phospholipid
	nmol/mg protein		*mol %*	*nmol/mg protein*		*mol %*	*nmol/mg protein*		*mol %*	*nmol/mg protein*		*mol %*
PE	127.17	45.11	49.95	384.24	36.33	41.25	1394.70	99.38	41.46	700.30	59.28	28.20
PC	62.24	14.59	24.44	158.64	17.52	17.03	332.73	87.35	9.89	285.30	64.65	11.50
SPM	35.17	4.96	13.81	257.99	44.56	27.70	1057.54	143.69	31.44	1142.18	202.56	46.04
Phosphatidylserine	14.37	3.75	5.65	84.35	10.53	9.06	428.90	56.09	12.75	272.70	6.36	10.99
Phosphatidylinositol	11.02	2.14	4.33	20.05	2.67	2.15	48.06	7.53	1.43	42.74	4.33	1.72
Phosphatidic acid	2.41	1.17	0.95	5.73	1.10	0.62	72.99	7.17	2.17	28.84	5.40	1.16
Phosphatidylglycerol	2.22	1.23	0.87	20.52	3.11	2.20	29.10	2.89	0.87	8.55	1.06	0.34
Total phospholipid	254.60	25.30	100.00	931.52	53.60	100.00	3364.02	399.80	100.00	2480.61	331.90	100.00
Cholesterol	67.80	2.35		884	79		2688	320		2215	103	
Mole % cholesterol	21.03			48.69			44.41			47.17		

All three raft fractions had a substantially higher mole percent of cholesterol and SPM than did the PNS membranes. By contrast, the mole percent PC and phosphatidylinositol in the raft preparations was ~2-fold lower than that seen in the PNS. As observed previously (36), PS was enriched ~2-fold in the raft preparations compared with the PNS. These findings demonstrate that all three lipid raft preparations had a composition that was distinct from that of the starting membranes and exhibited the enrichment in cholesterol and SPM that is characteristic of these domains.

Although many of the general characteristics of the lipid raft fractions were shared among all three preparations, the phospholipid compositions of the two raft preparations that retained EGF receptors were similar to each other, but differed significantly from the lipid composition of the Triton X-100-resistant rafts that excluded EGF receptors (Table I). For example, the detergent-free and Brij 98 raft preparations contained ~40 mol % PE, whereas the Triton X-100 rafts contained about one-third less of this lipid. Conversely, the detergent-free and Brij 98-resistant membranes contained ~30 mol % SPM, whereas the Triton X-100-resistant rafts contained ~46 mol % SPM, a 50% increase compared with the receptor-containing rafts. Thus, the EGF receptor-containing rafts had a phospholipid composition that was distinct from that of the non-EGF receptor-containing Triton X-100 rafts.

PE and the acidic phospholipids tend to be found mainly in the inner leaflet of the membrane, whereas PC and SPM are most often found in the outer leaflet of the membrane. The data in Table I indicate that the PNS and EGF receptor-containing lipid raft fractions had slightly more of these inner leaflet-preferring lipids than of the outer leaflet-preferring lipids, whereas the situation was reversed in the non-EGF receptor-containing Triton X-100 rafts. To determine whether there were any additional general differences between inner and outer leaflet lipids in these membrane preparations, the chain length and saturation of the fatty acyl groups were compared in these subsets of lipids.

Fig. 3 shows the distribution of inner and outer leaflet phospholipids with respect to the chain length of the fatty acyl groups. For purposes of this analysis, PC and SPM were considered to be outer leaflet lipids, whereas PE and the anionic phospholipids were considered to be inner leaflet lipids. Fig. 3 shows that there appeared to be relatively little selection of inner leaflet lipids based on chain length. The fraction of inner leaflet lipids containing C_{16}, C_{18}, C_{22}, and C_{24} fatty acyl side chains was similar in the PNS and all three lipid raft preparations. Only for the lipids containing C_{20} fatty acyl groups were there any differences, with the EGF receptor-containing rafts containing modestly fewer such lipids and the non-EGF receptor-containing Triton X-100 rafts showing significantly fewer such lipids compared with the PNS. By contrast, there were significant differences in the chain length of the outer leaflet lipids in the PNS compared with the three raft preparations. In particular, the rafts appeared to select for phospholipids containing C_{16} and C_{24} fatty acyl groups and to select against phospholipids containing C_{18}, C_{20}, and C_{22} fatty acyl groups.

Fig. 4 compares the saturation of fatty acyl groups in inner and outer leaflet lipids. For purposes of this analysis, a phospholipid was deemed saturated if it contained no more than one double bond between the two fatty acyl chains. Inner leaflet lipids (PE and acidic phospholipids) were significantly less saturated than outer leaflet lipids (PC and SPM). This is largely because of the highly saturated nature of SPM. For both inner and outer leaflet lipids, however, raft lipids showed a higher degree of saturation than those in the PNS. Among the raft preparations, the non-EGF receptor-containing Triton X-100 rafts were more saturated than either of the EGF receptor-containing rafts. Overall, the EGF receptor-containing detergent-free and Brij 98 rafts exhibited greater similarity to each other in terms of lipid saturation than they did to the non-EGF receptor-containing Triton X-100 rafts.

The individual classes of phospholipids were then examined for differences between the PNS and the various raft preparations. Fig. 5 presents a scatter plot of the mole fraction (relative abundance) of the individual molecular species of PC obtained by multidimensional ESI/MS analysis in positive-ion mode in the presence of LiOH. The data are calculated as the nanomoles of a particular species/mg of protein divided by the total amount of PC present in the preparation. This allows a determination of whether a particular species is selectively enriched or depleted in a membrane preparation, regardless of the overall mole percent of that class of phospholipid in a given preparation. Species are organized into groups that show enrichment in two or more of the raft preparations relative to the PNS, depletion in the rafts relative to the PNS, or no consistent change relative to the PNS.

Among the PC species, the most prominent was 16:0-18:1 phosphatidylcholine (Fig. 5, *inset*), which accounted for ~40% of the total PC in all three raft preparations. The lipid rafts are enriched in this species relative to the PNS in which 16:0-18:1 PC represented only ~30% of the total PC. Other PC species that were enriched relative to the PNS included 16:0-16:0, 16:1-16:0, and 18:0-18:1. In general, the PC species that were depleted in the raft preparations were those that contained polyunsaturated chains such as 20:4 and 22:6.

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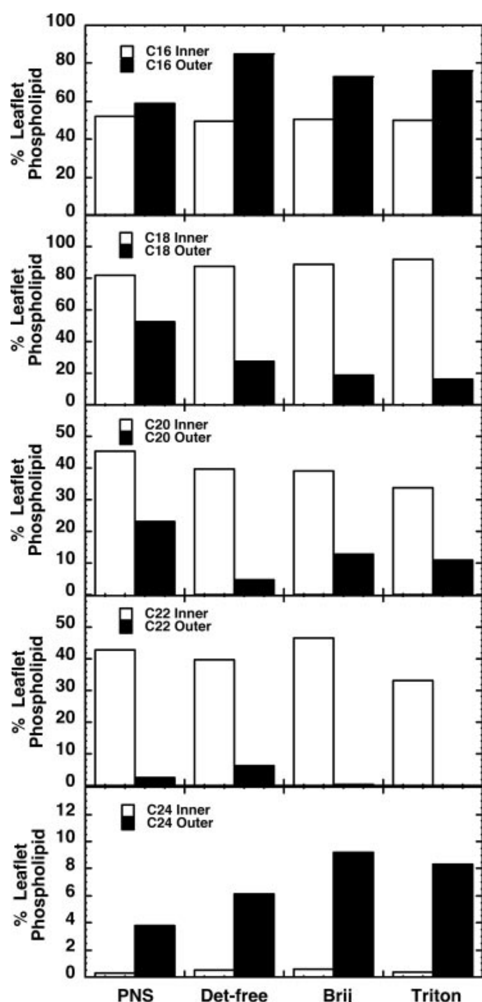

FIG. 3. **Comparison of fatty acyl chain length in inner and outer leaflet lipids.** Inner leaflet lipids were defined as PE, PS, phosphatidylinositol, phosphatidic acid, and phosphatidylglycerol. Outer leaflet lipids included PC and SPM. The values were calculated as the nanomoles/mg of protein for all species of inner or outer leaflet phospholipid containing at least one chain of a given length divided by the total nanomoles/mg of protein for inner or outer leaflet lipids. The value was multiplied by 100 to obtain the percent of the total. Because each phospholipid species contains two fatty acyl groups, the inner leaflet lipids total ~200%. SPM has only one fatty acid group (in addition to the C_{18} backbone of sphingosine), and thus, the outer leaflet lipids show variable totals depending on the mole percent of SPM in the membrane. *Det-free*, detergent-free.

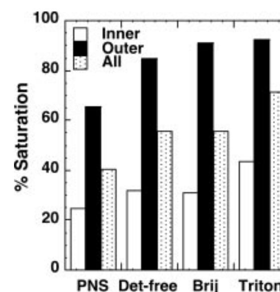

FIG. 4. **Fractional saturation of fatty acyl chains in inner and outer leaflet lipids.** A saturated phospholipid was defined as one in which there was no more than one double bond between the two fatty acyl chains in the lipid. The total nanomoles of a saturated species/mg of protein was divided by the total nanomoles of that class of phospholipid/mg of protein and multiplied by 100 to obtain the percent saturation. The results are from the averaged data sets for each class of phospholipids. *Det-free*, detergent-free.

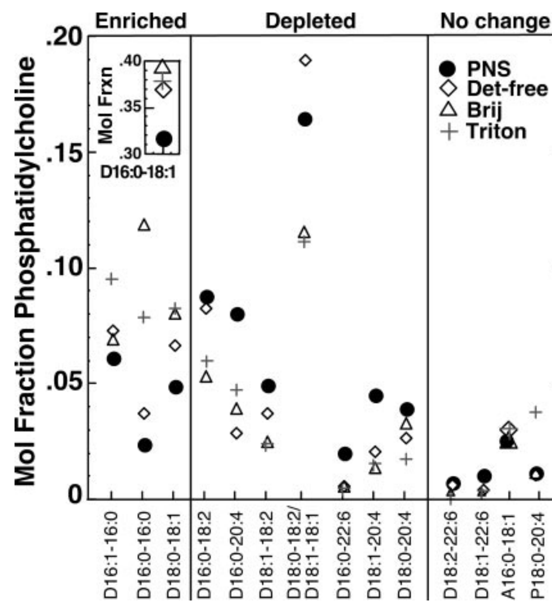

FIG. 5. **Mole fraction of PC species in the PNS and the lipid raft preparations.** The mole fraction (*Mol Frxn*; relative abundance) of each species was calculated by dividing the actual abundance of that species by the total amount of PC present in that particular membrane preparation. Each *symbol* represents the mole fraction of the species indicated on the *x axis* in the indicated membrane preparation. The first number in each pair on the *x axis* refers to the number of carbon atoms in the fatty acyl chain. The number after the colon refers to the number of double bonds. The two fatty acyl chain designations are separated by a hyphen. The prefix *D* indicates a diacyl compound. The prefix *A* indicates a plasmanyl compound. The prefix *P* indicates a plasmenyl compound. A species was designated as "enriched" if at least two of the three raft preparations showed a greater mole fraction of that species compared with the PNS. Data represent the average of three experiments. The absolute abundance data are given in Supplemental Table 1. *Det-free*, detergent-free.

These data indicate that PC species containing more saturated fatty acyl groups were enriched in all three raft preparations, whereas species containing polyunsaturated fatty acyl groups were relatively depleted.

For all three raft preparations, the major SPM species was N16:0 (Table II). However, this species accounted for ~50% of the total SPM in the PNS, but ~70–80% of the total SPM in the three raft preparations. Interestingly, the N20:0 species of SPM was nearly as abundant (32%) as the N16:0 species in the PNS membranes, but was significantly less represented in the raft preparations. In the detergent-free rafts, it accounted for only ~2% of the total SPM, whereas this species represented ~10% of the total SPM in the Brij 98- and Triton X-100-resistant rafts. These findings suggest that there is some selectivity with respect to which SPM species partition into what type of lipid raft. Detergent-free rafts had a clear preference for SPM species with shorter fatty acyl groups. The fact that this preference was not as sharp in Brij 98 and Triton X-100 rafts suggests that these detergents may extract out the shorter chain SPM species, giving a somewhat skewed composition relative to the original membranes.

Fig. 6 shows the mole fraction (relative abundance) of phosphatidylethanolamine species present in each of the membrane preparations. These data were obtained using negative-ion multidimensional ESI/MS. Unlike PC and SPM, there was not a single major species of phosphatidylethanolamine, but rather a collection of many species that represented 6–12% of the total. As noted previously, the Triton X-100 rafts contained substantially less PE than did the EGF receptor-containing rafts. Nonetheless, all three raft preparations showed similar patterns of enrichment or depletion of specific species compared with the PNS membranes. For example, all raft prepa-

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TABLE II
Relative abundance of SPM species in lipid rafts and the PNS

The fractional abundance of each species was calculated by dividing the nanomoles of a particular species/mg of protein by the total nanomoles of SPM/mg of protein present in that particular membrane preparation and multiplying by 100. Data represent the average of three experiments. The absolute abundance data are given in Supplemental Table 1.

m/z	Assignment	PNS		Detergent-free		Brij 98		Triton X-100	
		Average	Total SPM	Average	Total SPM	Average	Total SPM	Average	Total SPM
		nmol/mg protein	*%*	*nmol/mg protein*	*%*	*nmol/mg protein*	*%*	*nmol/mg protein*	*%*
707.5	N16:1	1.27	3.61	12.88	4.99	71.26	6.74	47.60	4.17
709.5	N16:0	17.87	50.82	209.07	81.04	703.74	66.54	840.36	73.58
735.5	N18:1	0.26	0.75	0.99	0.38	2.51	0.24	2.51	0.22
737.5	N18:0	0.69	1.95	3.50	1.36	7.16	0.68	10.76	0.94
765.5	N20:0	11.36	32.31	6.09	2.36	145.45	13.75	121.80	10.66
817.5	N24:2	0.96	2.73	3.69	1.43	22.38	2.12	18.02	1.58
819.5	N24:1	2.24	6.36	17.15	6.65	84.18	7.96	80.10	7.01
821.5	N24:0	0.51	1.46	4.63	1.79	20.87	1.97	21.04	1.84
Total		35.17	100.00	257.99	100.00	1057.54	100.00	1142.18	100.00

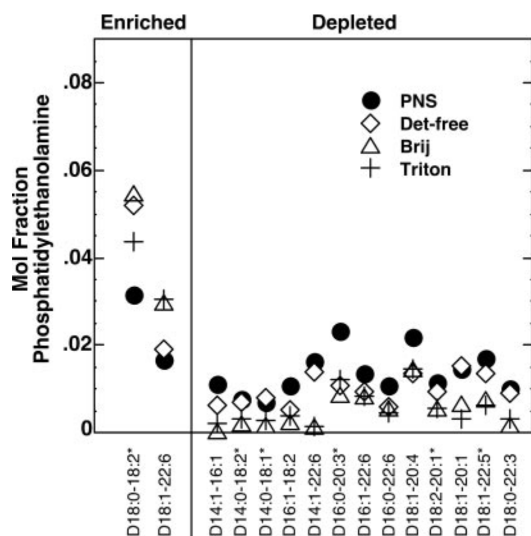

FIG. 6. **Mole fraction of phosphatidylethanolamine species in the PNS and the lipid raft preparations.** The mole fraction (relative abundance) of each species was calculated by dividing the actual abundance of that species by the total amount of PE present in that particular membrane preparation. Each *symbol* represents the mole fraction of the species indicated on the *x axis* in the indicated membrane preparation. All species are diacyl (*D*) compounds. Species marked with an *asterisk* are those for which there are other isobaric species. A species was designated as enriched if at least two of the three raft preparations showed a greater relative abundance of that species compared with the PNS. Data represent the average of three experiments. The absolute abundance data are given in Supplemental Table 2. *Det-free*, detergent-free.

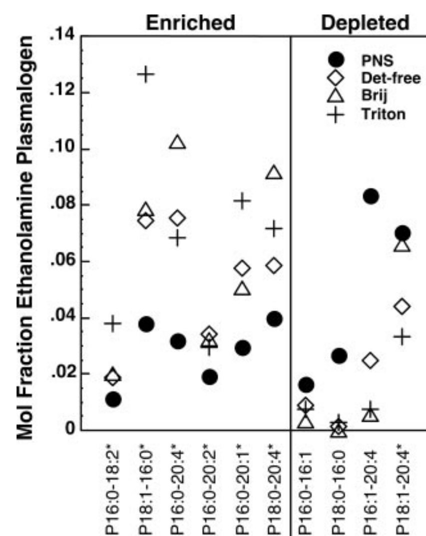

FIG. 7. **Mole fraction of ethanolamine plasmalogens in the PNS and the lipid raft preparations.** The mole fraction (relative abundance) of each species was calculated by dividing the actual abundance of that species by the total amount of PE present in that particular membrane preparation. Each *symbol* represents the fractional abundance of the species indicated on the *x axis* in the indicated membrane preparation. The prefix *P* indicates a plasmenyl compound. Species marked with an *asterisk* are those for which there are other isobaric species. A species was designated as enriched if at least two of the three raft preparations showed a greater relative abundance of that species compared with the PNS. Data represent the average of three experiments. The absolute abundance data are given in Supplemental Table 2. *Det-free*, detergent-free.

rations tended to be depleted of phosphatidylethanolamine species that contained a C_{14} fatty acyl group. Such PE species were not highly represented within this class of phospholipids, and they were not present in any other phospholipids in these cells. Nonetheless, as a group, they appeared to be excluded from lipid rafts. All raft preparations were also relatively depleted of species that contained polyunsaturated fatty acyl groups compared with the PNS, consistent with a preference for more saturated acyl groups. By contrast, the raft preparations were enriched in ethanolamine plasmalogens relative to the PNS (Fig. 7), and this enrichment was apparent in the overall composition of these preparations as well (Supplemental Table 3).

Anionic phospholipids were quantitated by multidimensional ESI/MS in negative-ion mode without addition of LiOH. Fig. 8 shows the mole fraction (relative abundance) of the various species of PS. There was a single major species of PS (18:0-18:1) in all membrane preparations, and this species was more abundant in rafts compared with the PNS. Similarly, the 16:0-18:1 species was markedly enriched in all raft preparations. Together, the increased absolute abundance of these two species accounted for the majority of the increase in PS observed in the three raft preparations. Overall, the lipid rafts tended to be enriched in saturated PS species and depleted of species containing polyunsaturated fatty acyl groups.

Although, as a class, phosphatidylinositol was depleted in the lipid raft preparations, no species were significantly enriched or depleted in this class (Supplemental Table 3). This suggests that, outside of the selection based on head group, there was no selective partitioning of specific phosphatidylinositol species into or out of lipid rafts.

DISCUSSION

The traditional method for preparation of lipid rafts involves solubilization of cells in Triton X-100, followed by isolation of a low buoyant density fraction by density gradient centrifugation. Many different variations of this method have been used to isolate lipid rafts, in particular, changes in the concentration and type of detergent used for extraction. These changes lead to

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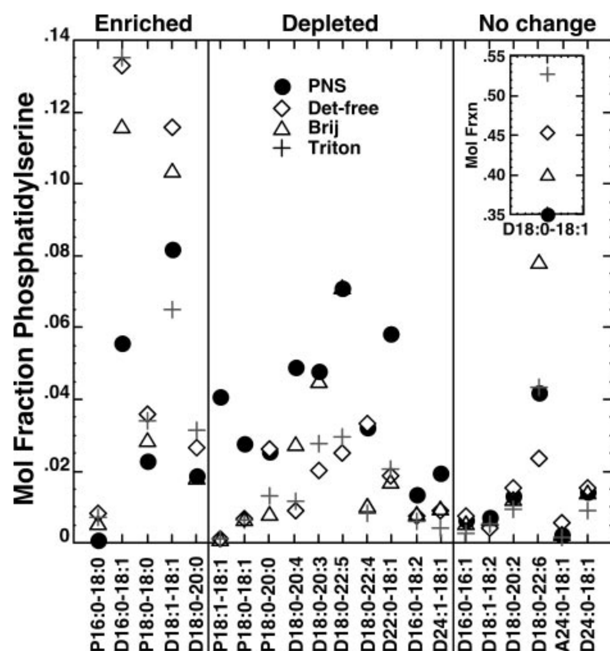

FIG. 8. **Mole fraction of PS species in the PNS and the lipid raft preparations.** The mole fraction (*Mol Frxn*; relative abundance) of each species was calculated by dividing the actual abundance of that species by the total amount of PS present in that particular membrane preparation. Each *symbol* represents the fractional abundance of the species indicated on the *x axis* in the indicated membrane preparation. The prefix *P* indicates a plasmenyl compound. The prefix *D* indicates a diacyl compound. The prefix *A* indicates a plasmanyl compound. A species was designated as enriched if at least two of the three raft preparations showed a greater relative abundance of that species compared with the PNS. Data represent the average of three experiments. The absolute abundance data are given in Supplemental Table 3. *Det-free*, detergent-free.

the inclusion or exclusion of a variety of different proteins and lipids into the resulting rafts (16, 17, 21). Although it is clear that there are differences between the rafts prepared by these various procedures, little is known about how such rafts differ from each other and why some proteins are retained in the detergent-resistant domains, whereas others are not.

In this study, we focused on the behavior of the EGF receptor, a transmembrane protein known to be present in detergent-free preparations of lipid rafts (23, 37). A screen of five different detergents demonstrated that, under most conditions, the EGF receptor was not isolated in the detergent-resistant fraction. Triton X-100 and octyl glucoside both produced rafts that contained the raft marker flotillin and some caveolin, but lacked EGF receptors and G_q. Brij 96 appears to have a greater tendency than either Triton X-100 or octyl glucoside to disrupt lipid rafts because even flotillin was excluded from the low density fractions prepared using this detergent. In addition, only a small portion of caveolin was found in the low density region of the gradient. This differs from previous reports suggesting that Brij 96 is a less stringent solubilizer of cell membranes compared with Triton X-100 (21). The difference may be due to the fact that, in the previous experiments, 0.5% Brij 96 and 1% Triton X-100 were compared, whereas in our experiments, both detergents were used at a final concentration of 1%. In addition, the protocols for solubilization were different in the two studies. These results make it clear that methodology plays a key role in the outcome of any detergent solubilization experiment.

Although the EGF receptor was not retained in most detergent-resistant membrane fractions, our studies indicate that solubilization of cells in 1% Brij 98 resulted in the generation of a distinct low density membrane fraction that contained the EGF receptor and other raft markers, but was devoid of non-raft plasma membrane or intracellular membrane proteins. Of interest is the observation that the transferrin receptor was recovered in a portion of the gradient that was of intermediate density, at a position distinct from that occupied by other plasma membrane proteins such as Na^+/K^+-ATPase. The transferrin receptor (a non-raft protein) is known to be palmitoylated (38) and may therefore be solubilized in a more lipid-rich, lower density complex compared with non-acylated proteins. That Brij 98 solubilization can distinguish this class of proteins from others in the membrane may be useful in studies of acylated proteins.

We next addressed the question of why the EGF receptor was included in some detergent-resistant membrane fractions, but not in others. Analyses of the lipid composition of the two raft preparations that retained the EGF receptor and one that did not (Triton X-100-resistant rafts) were used to determine whether there was a correlation between lipid content and retention of the EGF receptor.

The lipid analyses indicated many general similarities among the three raft preparations. For example, all three preparations were enriched in cholesterol, SPM, and saturated acyl side chains compared with the PNS membranes. In addition, all were enriched in PS and ethanolamine plasmalogens relative to the PNS. Thus, all three preparations exhibited characteristics consistent with the known properties of lipid rafts.

Although many general characteristics were similar among the lipid rafts examined, our findings indicate that there were clear-cut differences in the lipid composition of rafts that retained the EGF receptor and those that did not. The major difference observed was in the relative abundance of the major phospholipids, PE and SPM. In both the EGF receptor-containing detergent-free and Brij 98 rafts, PE accounted for ~40 mol % of the total phospholipid species, whereas in the non-EGF receptor-containing Triton X-100 rafts, PE represented only ~28% of the total phospholipid species. Conversely, SPM represented ~30 mol % in the EGF receptor-containing rafts, but 47 mol % in the Triton X-100 rafts.

Because of these differences in the abundance of these major phospholipid species, there was a difference in the relative levels of inner and outer leaflet lipids in the three raft preparations. PE is an inner leaflet-preferring lipid, whereas PC and SPM are outer leaflet-preferring lipids. Typically, the PE/(PC + SPM) ratio is near unity in any given membrane, and this was the case for both EGF receptor-containing raft preparations (0.92 and 1.0 for detergent-free and Brij 98 rafts, respectively). However, this ratio was only 0.47 in the non-receptor-containing Triton X-100 rafts. These data indicate that the Triton X-100-resistant rafts were relatively depleted of inner leaflet lipids.

Looking at the inner and outer leaflet lipids as groups, the outer leaflet lipids appear to undergo a more stringent selection for inclusion into lipid rafts than do the inner leaflet lipids. In terms of head group and fatty acyl chain length and saturation, the outer leaflet lipids in rafts were distinctly different from those in the PNS, whereas the inner leaflet lipids differed only marginally from those found in the PNS membranes. An exception to this rule was PS, which was selected for inclusion into lipid rafts, representing 9% of the inner leaflet lipids in the PNS, but 16, 22, and 26% of the inner leaflet lipids in the detergent-free, Brij 98, and Triton X-100 rafts, respectively. Similarly, phosphatidylinositol appeared to be specifically excluded from the rafts, representing 7% of the inner leaflet lipids in the PNS, but only 4, 2.5, and 4% in the detergent-free, Brij 98, and Triton X-100 rafts, respectively. The observation that all lipid rafts, no matter how they were made, exhibited this

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leaflet-dependent difference in lipid selectivity suggests that it is an intrinsic feature of lipid rafts, not one that is introduced by methodological differences in preparation. These findings imply that lipid rafts are largely outer leaflet structures with substantially less rigorously selected inner leaflet lipids.

These data also provide insight into the compositional differences of rafts made by extraction with different detergents. The data in Fig. 3 show that extraction with Brij 98 or Triton X-100 resulted in a similar degree of selection for phospholipids containing fatty acyl groups of particular lengths. In this regard, the two detergent raft preparations were more similar to each other than either was to the detergent-free raft preparation. In addition, both detergents appear to preferentially exclude the N16:0 SPM species from rafts because they contained 5-fold less of this lipid than did the detergent-free raft preparation. However, Triton X-100 selectively extracted inner leaflet lipids, but Brij 98-resistant rafts had a normal balance of inner and outer leaflet lipids. Thus, the ability to deplete inner leaflet lipids is not a general feature of all detergents, but rather depends on the properties of the individual detergents.

Several studies have suggested that Triton X-100 induces the formation of lipid domains in ternary mixtures of SPM, PC, and cholesterol (39, 40). Triton X-100 is membrane-disordering, but through unfavorable interactions with SPM, it drives the separation of SPM and cholesterol into a liquid ordered phase, distinct from the liquid disordered phase that contains most of the PC. When applied to our data, these findings suggest that outer leaflet phospholipids are likely to be positively selected for retention in lipid rafts rather than selectively extracted from these domains. Although this suggests that the detergent-resistant domains isolated here may not accurately reflect the domains that exist within the cell, it should be noted that all raft preparations showed a similar pattern of selection of outer leaflet lipids. Thus, although detergent extraction may enhance or promote the formation of domains of specific lipid content, it builds on a foundation that is already apparent in rafts made using detergent-free methods.

Rafts appear to exist on both the outer and inner leaflets of the membrane, with outer leaflet rafts harboring GPI-anchored proteins and inner leaflet rafts containing acylated proteins. Outer leaflet rafts are stabilized by the interaction of SPM and cholesterol. By comparison, inner leaflet rafts are significantly less stable because of the lack of SPM in these leaflets (41) and hence the absence of its stabilizing interaction with cholesterol. Our data suggest that inner leaflet rafts are preferentially disrupted by treatment with Triton X-100, giving rise to membrane preparations with a preponderance of outer leaflet lipids. By contrast, inner leaflet rafts were retained in both the detergent-free and Brij 98-resistant raft preparations. The hypothesis that Brij 98 (but not Triton X-100) solubilization results in the maintenance of inner leaflet rafts is supported by the observation that G_q, which is targeted to the cytoplasmic face of the membrane via protein acylation, was retained in Brij 98-resistant membranes, but was lost from Triton X-100-resistant membrane fractions.

Together, these data provide a picture of the type of raft into which the EGF receptor partitions. The observation that the EGF receptor was present only in rafts (detergent-resistant or detergent-free) that contained significant levels of both inner and outer leaflet lipids suggests that, for this transmembrane protein, the presence of a bilayer structure reminiscent of the original membrane is required for the retention of the receptor in the lipid raft. Furthermore, the lower degree of saturation suggests that these bilayer rafts are likely to be less ordered and hence more fluid than other types of rafts. This may be important for enabling the types of conformational changes than must occur in the receptor when it dimerizes and transduces its signal through the membrane. Indeed, Evans and Needham (42) showed that incorporation of a transbilayer peptide into PC/cholesterol mixtures reduces the compressibility modulus of the resulting bilayers, enhancing their elasticity. Thus, the transmembrane EGF receptor may play a role in defining the properties of the rafts into which it partitions.

A recent study has suggested that outer and inner leaflet rafts are only loosely associated under steady-state conditions (43). However, co-localization of inner leaflet rafts containing H-Ras with outer leaflet rafts is observed when the outer leaflet rafts are aggregated with antibodies directed against a GPI-anchored protein (43). Transmembrane domain proteins such as the EGF receptor that appear to interact with both outer and inner leaflet rafts may enhance the coupling of rafts in the two leaflets. This could promote the co-localization of signaling molecules present in the different leaflets, thereby enhancing the efficiency of downstream signaling upon receptor activation.

In summary, our studies demonstrate that rafts made using different methodologies exhibit significant differences in lipid composition. Despite these differences, all raft preparations show a more stringent selection for specific characteristics in outer leaflet compared with inner leaflet lipid species. These findings suggest that raft biogenesis may be driven by the formation of an outer leaflet structure, with inner leaflet rafts forming in response to an additional organizing element. The fact that the EGF receptor is able to partition only into rafts that exhibit a bilayer-like composition raises the possibility that this receptor, as well as other transmembrane raft proteins, may participate in the organization of rafts on the inner leaflet of the membrane.

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