THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 17, Issue of April 23, pp. 12049–12054, 1999

Printed in U.S.A.

Association of Sterol- and Glycosylphosphatidylinositol-linked Proteins with *Drosophila* Raft Lipid Microdomains*

(Received for publication, December 8, 1998, and in revised form, January 27, 1999)

Anton Rietveld, Stephanie Neutz, Kai Simons, and Suzanne Eaton‡

From the Cell Biology Programme, European Molecular Biology Laboratory, Meyerhofstrasse-1, 69117 Heidelberg, Germany

In vertebrates, the formation of raft lipid microdomains plays an important part in both polarized protein sorting and signal transduction. To establish a system in which raft-dependent processes could be studied genetically, we have analyzed the protein and lipid composition of these microdomains in *Drosophila melanogaster*. Using mass spectrometry, we identified the phospholipids, sphingolipids, and sterols present in *Drosophila* membranes. Despite chemical differences between *Drosophila* and mammalian lipids, their structure suggests that the biophysical properties that allow raft formation have been preserved. Consistent with this, we have identified a detergent-insoluble fraction of *Drosophila* membranes that, like mammalian rafts, is rich in sterol, sphingolipids, and glycosylphosphatidylinositol-linked proteins. We show that the sterol-linked Hedgehog N-terminal fragment associates specifically with this detergent-insoluble membrane fraction. Our findings demonstrate that raft formation is preserved across widely separated phyla in organisms with different lipid structures. They further suggest sterol modification as a novel mechanism for targeting proteins to raft membranes and raise the possibility that signaling and polarized intracellular transport of Hedgehog are based on raft association.

Recent evidence suggests that lipids in mammalian membranes are not uniformly miscible, but that lateral separation of specific lipid species leads to the formation of specialized phase domains called rafts. In mammals, the association of membrane proteins with raft lipid microdomains has emerged as an important regulator of polarized intracellular sorting and signal transduction (1, 2).

Raft formation is based on the tendency of cholesterol to organize the bilayer into cholesterol-rich liquid ordered and cholesterol-poor liquid disordered domains (3), a process that is enhanced by the preferential interaction of cholesterol with sphingolipids and the fact that sphingolipids have higher melting temperatures than phospholipids (Ref. 4; reviewed in Ref. 5). Rafts form when the sphingolipid/cholesterol-rich phase separates from the phospholipid-rich phase that constitutes the rest of the membrane. In model membranes, the formation of the liquid ordered phase correlates with the acquisition of insolubility in the nonionic detergent Triton X-100 (6). Insolubility in Triton X-100 or in Triton X-114, a related detergent, has been used as a criterion for isolation of rafts from cellular membranes (7, 8).

We decided to establish a system to study rafts in a genetic model organism with well characterized development: *Drosophila melanogaster*. Genetics would provide a powerful tool with which to identify molecules involved in raft formation, trafficking, and function. Furthermore, since raft formation is thought to play important roles in cell polarization and signal transduction, examining their functions in *Drosophila* may provide insights into the control of important developmental processes.

We began by asking whether *Drosophila* membranes contained membrane domains similar to mammalian rafts. *Drosophila* cannot synthesize sterols and require a dietary source. Furthermore, their membranes must remain fluid at lower temperatures than those of mammals; thus, the biophysical properties of their lipids might be expected to differ. We wondered whether raft formation would occur under these conditions and, if so, to what extent *Drosophila* rafts would resemble their mammalian counterparts. Our data show that, despite differences in the chemical structure of their lipids, *Drosophila* membranes contain rafts with a similar protein and lipid composition.

We then examined membrane-associated proteins of well characterized developmental pathways to establish which of them might constitute a suitable system in which to study raft function. In particular, we wished to examine whether the N-terminal fragment of the Hedgehog protein might be raft-associated. Hedgehog protein undergoes autocatalytic cleavage that results in covalent linkage of its N-terminal fragment to cholesterol (9). Since cholesterol is enriched in raft membranes, it seemed possible that cholesterol linkage might be a raft-targeting signal.

EXPERIMENTAL PROCEDURES

Preparation of Drosophila Embryonic Membranes—Drosophila were reared on yeast-based medium, and adults in population cages (10) were fed fresh yeast on apple juice/molasses agar plates twice/day. Flies were allowed to lay eggs for 14 h overnight, and embryos from eight population cages were collected onto stacked coarse, medium, and fine wire mesh screens with a paint brush. The embryos were washed in water and with 0.9% NaCl + 0.1% Triton X-100 (embryo wash) to remove debris and yeast and then dechorionated in 250 ml of 20% bleach and 80% embryo wash for 3 min. After decanting onto the fine wire mesh, the embryos were sprayed vigorously with water to remove residual chorion and collected into 50-ml Falcon tubes (~25 ml of packed embryos/tube). The embryos were then washed twice in embryo wash, three times in 0.9% NaCl, twice in TNE buffer (100 mM Tris (pH 7.5), 150 mM NaCl, and 0.2 mM EGTA) + 0.3 M sucrose, and resuspended in 40 ml/tube TNE buffer + 0.3 M sucrose + 0.001 volume of CLAP (10 mg/ml each chymostatin, leupeptin, antipain, and pepstatin in Me_2SO). Embryos were broken in a Potter-Elvehjam and then in a Dounce homogenizer with a loose pestle followed by a tight pestle until the pestle moved smoothly (approximately five times). The embryo homogenate was spun at 5000 rpm for 10 min at 4 °C to pellet nuclei, and the post-nuclear supernatant was removed, adjusted to 1.4 M sucrose, and distributed into SW 27 centrifuge tubes. Post-nuclear supernatants were overlaid with 10 ml of 1.22 M sucrose in TNE buffer and

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 49-6221-387-285; Fax: 49-6221-387-512; E-mail: eaton@embl-heidelberg.de.

10 ml of 0.1 M sucrose in TNE buffer and then spun for 18 h at 25,000 rpm at 4 °C. Membranes with a white, flocculent appearance were observed floating above the 1.22/0.1 M interface, and membranes with a more yellowish homogeneous appearance were observed at the 1.4/1.22 M interface and occasionally at the 1.22/0.1 M interface. The heavier membranes at the 1.4/1.22 M interface were enriched in endoplasmic reticular and mitochondrial NADH-cytochrome *c* reductase, whereas the lighter membranes above the 1.22/0.1 M interface were enriched in plasma membrane proteins. The Golgi enzymes galactosyltransferase and *N*-acetylglucosaminyltransferase were found in both fractions (data not shown). Membranes floating above the 1.22/0.1 M interface were collected, diluted three times in TNE buffer, and spun onto a 62% sucrose cushion in an SW 27 rotor for 20 min at 25,000 rpm. The membranes were then resuspended in TNE buffer, aliquoted, and stored at −80 °C. These membranes contained ~33% of the total membrane protein.

Purification of Detergent-insoluble Embryonic Membranes—Membranes were thawed and washed in 3 volumes of TNE buffer to remove residual sucrose. They were then resuspended in 350 μl of ice-cold TNE buffer to a phospholipid concentration of 880 nmol/ml. An equal volume of prechilled 2% Triton X-114 or Triton X-100 was added, and the membranes were solubilized at 0 °C for 30 min in SW 60 tubes. After solubilization, the membranes were brought to a final concentration of 24% Optiprep™ (Nycomed Pharma AS); overlaid with 1 ml of 21, 15, and 6% Optiprep in TNE buffer; and then spun for 5 h at 36,000 rpm at 2 °C. Insoluble membranes could be observed between the 6% and the 15% layers.

Western Blotting—After polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose at 4 °C in 40 mM Tris/glycine (pH 8.8) + 20% methanol for 2 h at 0.7 mA. Nitrocellulose blots were blocked with 5% powdered milk in Tris-buffered saline (10 mM Tris-HCl (pH 7.2), 150 mM NaCl, and 0.1% Tween 20) for 20 min and then incubated with primary antibody overnight at 4 °C. The dilutions of antibodies used were as follows: anti-Hedgehog, 1:2000; anti-Notch, 1:1000; anti-fasciclin-1, 1:2000; and anti-Drac1, 1:2000. Blots were washed three times in Tris-buffered saline for 15 min each and then bound to horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit, goat anti-rat, and goat anti-mouse; Bio-Rad) at a 1:1000 dilution for 2 h at room temperature. After washing three times in Tris-buffered saline for 15 min each, the signal was detected with an ECL™ kit (Amersham Pharmacia Biotech).

Analysis of Lipids by TLC—Phospholipids and sterols were prepared from membranes by Bligh and Dyer extraction (11). Phospholipids were quantified according to Rouser *et al.* (12). Sphingolipids were prepared by a Folch total lipid extraction (13), followed by mild alkaline hydrolysis of the glycerophospholipids for 2 h at 30 °C in methanolic 0.1 M NaOH. The base-resistant sphingolipids were desalted on an RP18 reversed-phase column. Lipid samples in chloroform (phospholipids) or chloroform/methanol (1:2; glycolipids) were applied to activated high performance TLC plates (Merck) and developed in chloroform/methanol/water (60:35:8; phospholipids) or chloroform/methanol/water (60:40:10; glycolipids) in a Whatman lined tank. The plates were dried at room temperature and stained with iodine vapor for detection of phospholipids and sterol or with ninhydrin spray reagent (Sigma) to detect amino phospholipids. Glycolipids were stained with orcinol/sulfuric acid reagent (14).

Lipid Analysis with Electrospray Ionization Tandem Mass Spectrometry—Sphingolipids were extracted and purified as described above and stored as a dried lipid film. Just prior to analysis, the lipids were dissolved in methanol containing 5 mM ammonium acetate and centrifuged for 15 min in a tabletop centrifuge at full speed. Sterols were identified and quantified after sulfation in the presence of [3,4-$^{13}C_2$]cholesterol as an internal standard. After addition of standard to membranes and subsequent extraction in 1,4-dioxane, sulfation was performed by adding sulfur trioxide-pyridine complex in absolute pyridine to the dried lipid samples. The reaction was quenched with barium acetate, and the lipids were diluted with methanol prior to measurement.[1] Mass spectrometric analysis was performed with a triple quadrupole instrument equipped with a nanoelectrospray ionization interface and a Dynolyte TM detector system (Micromass Quattro II). The first and third quadrupoles are used as independent analyzers, whereas the second serves as a hexapole collision cell. Ceramides and glycosphingolipids were detected in the positive ion mode, and phospholipid-derived fatty acids as well as the sulfated sterols were detected in the negative ion mode. The spray was induced with a capillary voltage of ±0.8–1.2 kV. The tandem mass spectrometric precursor scans were performed with argon as a collision gas at a nominal pressure of 2 millitorrs. Spectra were obtained by averaging 30–100 repetitive scans of 4 s.

Assaying Membrane Proteins for GPI[2] Linkage—Floating, detergent-insoluble membranes were washed once in PIPES (pH 6.8), 0.5 mM $CaCl_2$, and 150 mM NaCl and resuspended in 300 μl of 25 mM Tris-HCl (pH 7.5) + 0.5 mM $CaCl_2$ + 0.001 volume of CLAP. Half of each sample was treated with 0.75 units of phosphatidylinositol-specific phospholipase C (Sigma) for 3 h at 30 °C. The other half was mock-treated for the same length of time under the same conditions. Samples were then subjected to Triton X-114 phase separation (16), and proteins in the pellet, detergent, and aqueous fractions were precipitated by adding 4 volumes of methanol + 1 volume of chloroform, mixing, adding 3 volumes of H_2O, spinning for 2 min at 14,000 rpm, removing the upper phase, adding 3 of volumes methanol, spinning as before, and removing the supernatant from the protein pellet. Proteins were then analyzed by two-dimensional gel electrophoresis (8), and gels were stained with a SilverXpress™ kit (Novex).

RESULTS

Drosophila Lipids Are Shorter than Mammalian Lipids, but Retain the Structural Properties Required for Raft Formation—To identify the lipids present in *Drosophila* embryonic membranes, we used TLC analysis with standards (data not shown) and mass spectrometry (Fig. 1*A*). These data are summarized in Fig. 1 (*B–D*). We found that both the sphingolipids and phospholipids were shorter than those found in mammals. The sphingolipids were based on a tetradeca-4-sphingenine backbone (C_{14}) rather than the C_{18} backbone found in mammals. The amide-linked fatty acids present in the sphingolipids were also shorter; the most abundant fatty acid was arachidic acid ($C_{20:0}$) (Fig. 1, *A* and *B*), shorter than the lignoceric acid ($C_{24:0}$) often found in mammalian sphingolipids. Consistent with this, the free ceramide precursors of the sphingolipids contained the same fatty acids (Fig. 1, *A*, *B*, and *D*). The fatty acids in *Drosophila* sphingolipids were completely saturated (Fig. 1, *B* and *D*). The most abundant glycosphingolipids were glucosylceramide and mannosylglucosylceramide. The only phosphosphingolipid in mammalian membranes, sphingomyelin, was not present in *Drosophila*; instead, like other insects (17), *Drosophila* membranes contained phosphoethanolamine ceramide (PECer).

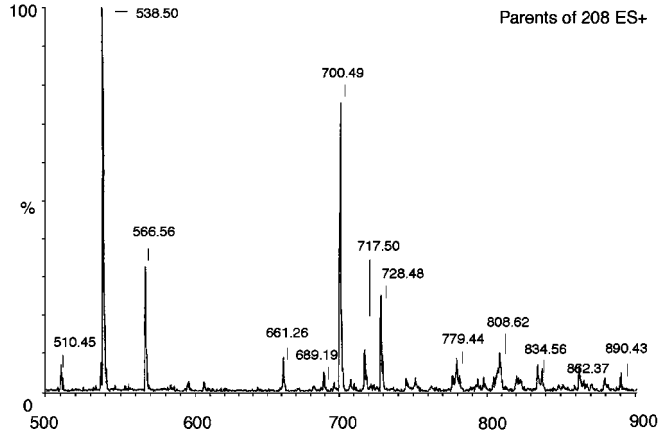
Drosophila membranes contained phosphoglycerolipids with the same head groups as those of mammalian cells (phosphocholine, phosphoethanolamine, phosphoserine, and phosphoinositol); however, they differed in having shorter fatty acyl chains. Whereas the longest fatty acid found in *Drosophila* phosphoglycerolipids was C_{18} (Fig. 1*C*), mammalian phosphoglycerolipids contain up to C_{24} (18, 19). Approximately 60% of the fatty acids were unsaturated (Fig. 1*C*), similar to ratios found in several mammalian cells (19).

In summary, both the sphingolipids and phosphoglycerolipids are shorter than those found in mammals and would therefore be predicted to have lower melting temperatures, consistent with the requirement that *Drosophila* membranes remain fluid at lower temperatures. Despite these differences, sphingolipids are still longer and more saturated than phosphoglycerolipids, as they are in mammals, and would therefore be predicted to have higher melting temperatures than phosphoglycerolipids. Thus, the structural properties of *Drosophila* sphingolipids and phosphoglycerolipids are consistent with the ability to separate into liquid ordered and disordered phases.

[1] Sandhoff, R., Brügger, B., Jeckel, D., Lehman, W. D., and Wieland, F. T. (1999) *J. Lipid Res.* **40,** 126–132.

[2] The abbreviations used are: GPI, glycosylphosphatidylinositol; PIPES, 1,4-piperazinediethanesulfonic acid; PECer, phosphoethanolamine ceramide; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

A

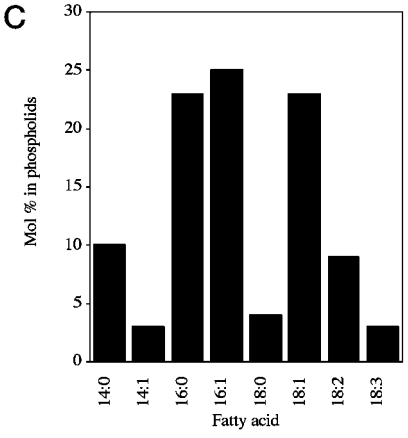


B

Major sphingolipid species in *Drosophila* (m/z).

	N-linked fatty acid carbon no.: no. double bonds		
	18:0	20:0	22:0
Ceramide	510	538	566
PE Cer	-	661	689
Gluc Cer	-	700	728
Man Gluc Cer	834	862	890

C



D

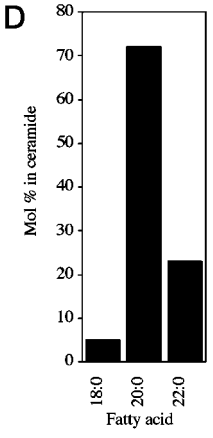


FIG. 1. **Structure of *Drosophila* sphingolipids and phospholipids.** *A*, sphingolipid classes in a total sphingolipid extract from *Drosophila* embryos were detected by positive mode electrospray ionization tandem mass spectrometry. $[M + H]^+$ ions were detected by precursor scanning for *m/z* 208 with a collision offset of −40 eV. Fragment 208 is a typical derivative of the hexadeca-4-sphingenine backbone. The peaks at 779 and 808 *m/z* are unidentified non-ceramide-related compounds, based on daughter ion analysis (data not shown). *B*, shown is the assignment of peaks shown in *A*. *C*, fatty acid length and degree of saturation of the major phospholipids were analyzed qualitatively by identification of intact molecular ions and collision-induced daughter ions (data not shown). *D*, quantitative analysis of fatty acid composition of the free ceramides was based on relative peak intensities in the mass spectra of intact ceramide molecular ions.

Ergosterol Is the Predominant Membrane Sterol in Drosophila Fed on Yeast—In model membranes, cholesterol must be present at between 7 and 30 mol % to induce the formation of the liquid ordered phase (3, 4). Consistent with this, cholesterol is essential for raft formation in vertebrate cells (20, 21). To determine the levels and types of sterols in *Drosophila* membranes, we performed mass spectrometry on lipid extracts and quantified sterols after sulfation in the presence of an internal ^{13}C-labeled standard.[1] The relative percentages of the different types of sterols observed are shown in Table I. Ergosterol is the most abundant sterol (69% of the total). Ergosterol, along with campesterol and sitosterol, could be absorbed directly from the diet in population cages (wet yeast on apple juice/beet syrup/agar plates). 24-Methylenecholesterol and fucosterol are intermediates in the conversion of campesterol and sitosterol, respectively, to cholesterol. Although the position of the double bond in dehydrocholesterol cannot be determined from this analysis, this sterol may be Δ7-dehydrocholesterol, an intermediate in the conversion of ergosterol to cholesterol (22). Cholesterol itself represents only 3% of the total membrane sterol. Taken together, sterols represent 18 mol % relative to phospholipids, similar to levels of cholesterol found in vertebrates. Because ergosterol, campesterol, and sitosterol are even more efficient than cholesterol at ordering acyl chains (23, 24), these levels should be sufficient to induce formation of the liquid ordered phase.

TABLE I

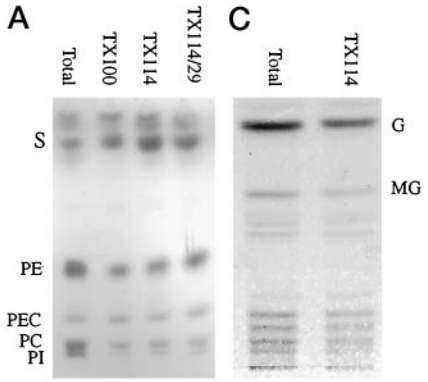
Sterol composition of Drosophila embryonic membranes

The sterols in *Drosophila* embryonic membranes were identified and quantified after sulfation in the presence of 200 pmol of $[^{13}C_2]$-cholesterol. Sulfated sterols were detected by negative mode electrospray ionization tandem mass spectrometry precursor scanning for *m/z* 97 with a collision offset of +60 eV. The values were corrected for differences in sulfation and ionization efficiencies with respect to the standard by measuring known amounts of pure sterol in the presence of $[^{13}C_2]$cholesterol standard (data not shown).

Sterol	*m/z*	mol %
Dehydrocholesterol	463	11
Cholesterol	465	3
$[^{13}C_2]$Cholesterol standard	467	
Brassicasterol	473	<1
Ergosterol	475	69
24-Methylene cholesterol	477	6
Campesterol	479	7
Fucosterol	491	<1
Sitosterol	493	2

The Lipid Composition of Drosophila Detergent-insoluble Membranes Resembles That of Mammalian Rafts—Mammalian raft membranes, rich in cholesterol and sphingolipids, can be isolated on the basis of their insolubility in the detergents Triton X-100 and Triton X-114 at low temperature (7, 8). To determine whether similar membrane domains form in *Drosophila*, we isolated detergent-insoluble membranes and analyzed their lipid composition. *Drosophila* embryonic membranes were treated with 1% Triton X-114 or Triton X-100 for 30 min at 0 °C, and insoluble membranes were purified from solubilized material by their ability to float through a density gradient. We separated lipids derived from the insoluble fraction or from membranes that had not been solubilized by TLC and stained them with iodine vapor (Fig. 2*A*). This showed that sterols were clearly enriched relative to the phospholipids (PI, PC, and PE) in detergent-insoluble membranes. The mole percent sterol relative to phospholipids in the Triton X-114-insoluble membranes (30.5%) is similar to what has been reported for cholesterol in mammalian rafts (31%) (7). The relative enrichment over the amount in unsolubilized membranes was less than that observed when solubilization is performed on whole Madin-Darby canine kidney cells, however. We began with a membrane fraction that was already enriched in plasma membrane with respect to the endoplasmic reticulum and mitochondria (see "Experimental Procedures"). TLC analysis showed that this fraction already contains proportionally more sterol than the total membrane fraction (data not shown) and would be expected to contain more sphingolipid as well (25, 26). Therefore, the final levels of enrichment of both sterol and sphingolipid should be lower than if rafts were isolated from whole cells.

PECer, like its mammalian counterpart, sphingomyelin, also appeared to be enriched in insoluble membranes (Fig. 2*A*). To



B

	Embr. membr.	TX114 insoluble	Fold enrichm.
Mole% PEC	5.3	14.7	2.8
Mole% Sterols	18	30.5	1.7

FIG. 2. **Sterol, phosphoethanolamine ceramide, and glycolipids are enriched in insoluble membranes.** *A*, membranes were solubilized with the detergents indicated at either 0 or 29 °C, and insoluble membranes were collected by flotation through a density gradient. Lipid extracts from insoluble membranes or from half of the corresponding amount of starting material were separated on TLC plates and stained with iodine vapor. The positions of PE, PECer (*PEC*), PC, PI, and sterols (*S*) are indicated. The identities of the lipids were determined by running cholesterol, sitosterol, campesterol, ergosterol, PE, PI, and PC standards. All sterols migrated indistinguishably from each other. PECer was identified by its reactivity with ninhydrin and resistance to mild base cleavage (data not shown). *B*, the mole percent of PECer was calculated by determining the moles of phosphate in the PECer, PC, and PE spots in *A* and expressing PECer as a percent of the total. The mole percent sterol relative to phospholipid in unsolubilized membranes was determined by quantifying the amount of each sterol by mass spectrometry, as shown in Fig. 2, and comparing the sum to the total moles of phospholipid. The mole percent sterol in Triton X (*TX*)-114-insoluble membranes was determined enzymatically using lipids from unsolubilized membranes as a standard. *C*, lipids from Triton X-114-insoluble membranes or unsolubilized membranes were analyzed by TLC and stained with orcinol. Triton X-114-insoluble membranes had 69 and 66% of the glucosylceramide (*G*) and mannosylglucosylceramide (*MG*), respectively (determined by scanning band intensities), but 24% of the phospholipid (determined by quantifying moles of phosphate) present in unsolubilized membranes. *Embr. membr.*, embryonic membranes; *enrichm.*, enrichment.

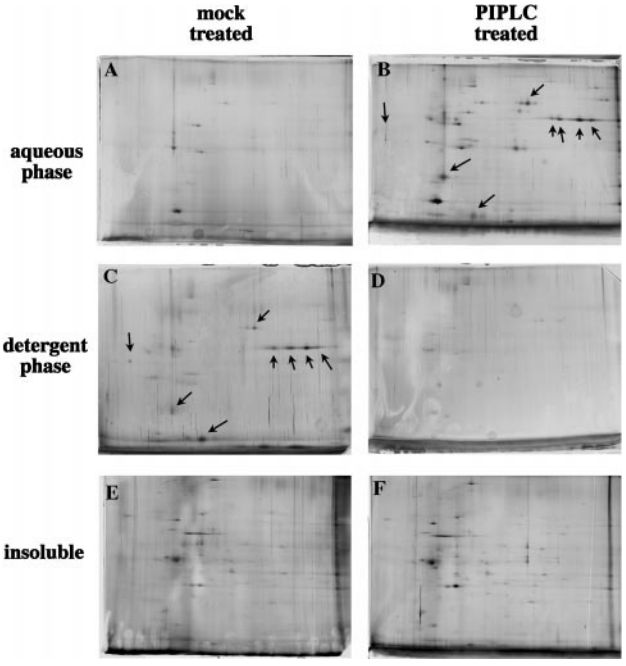


FIG. 3. **GPI-linked proteins are abundant in detergent-insoluble membranes.** Triton X-100-insoluble membranes were either treated with PI-specific phospholipase C (*PIPLC*; *A*, *C*, and *E*) or mock-treated (*B*, *D*, and *F*) and then subjected to phase separation in Triton X-114 and two-dimensional gel analysis. *A* and *B*, proteins in the aqueous phase; *C* and *D*, proteins in the detergent phase; *E* and *F*, the insoluble pellet. *Arrows* indicate proteins that shift from the detergent to aqueous phase after PI-specific phospholipase C treatment.

determine the mole percent of PECer relative to other phospholipids, we determined the number of moles of PECer, PE, and PC present in the TLC spots shown in Fig. 2*A*. PECer was present at 14.7 mol % in insoluble membranes, comparable to the level of sphingomyelin found in mammalian rafts (14.2%) (7). PECer was 2.8-fold more abundant in the Triton X-114-insoluble membranes than in unsolubilized material (Fig. 2*B*).

Next, we investigated whether glycosphingolipids were enriched in the insoluble fraction. We performed TLC on mild base-resistant lipids prepared from either total membranes or Triton X-114-insoluble membranes and stained them with orcinol (Fig. 2*C*). The intensity of the glucosylceramide and mannosylglucosylceramide bands before and after solubilization was quantified, and the percent that remained insoluble was compared with the percent of phospholipid that remained insoluble. By this estimate, these glycosphingolipids are enriched by ~3-fold relative to phospholipids in detergent-insoluble membranes. The orcinol-stained bands migrating closer to the origin represent more complex glycolipids (27), which have not been further identified by mass spectrometry. These glycolipids also appear to be enriched in the detergent-insoluble fraction. These experiments show that detergent solubilization can be used to isolate a *Drosophila* membrane fraction that is rich in sterol and sphingolipids, like mammalian rafts.

Drosophila Detergent-insoluble Membranes Are Rich in GPI-linked Proteins—In mammals, linkage to GPI targets proteins to raft membranes, and GPI-linked proteins are an abundant component of mammalian rafts (7, 28). To determine whether the *Drosophila* detergent-insoluble membrane fraction was similar to mammalian raft membranes in this regard, we analyzed its content of GPI-linked proteins. The Triton X-100 detergent-insoluble membrane fraction was isolated by solubilization in 1% Triton X-100 at 0 °C and flotation through a density gradient. These membranes were treated with PI-specific phospholipase C to release the lipid moiety from GPI-linked proteins. As a control, Triton X-100-insoluble membranes were mock-treated under the same conditions in the absence of PI-specific phospholipase C. Membranes were then dissolved in 1% Triton X-114 first at 4 °C and then at 29 °C. At 29 °C, solutions of Triton X-114 separate into detergent and aqueous phases; lipid-linked proteins and transmembrane proteins partition into the detergent phase, and other proteins partition into the aqueous phase (16). We analyzed the proteins present in the detergent and aqueous phases, as well as those that remained insoluble in Triton X-114 at 29 °C, by two-dimensional gel electrophoresis. Treatment of Triton X-100-insoluble membranes with PI-specific phospholipase C caused most major protein constituents to shift from the detergent to the aqueous phase of Triton X-114, indicating that a lipid anchor had been removed (Fig. 3, compare *A* and *B* with *C* and *D*). Because GPI-linked proteins are so abundant in *Drosophila* rafts, we conclude that, as in mammals, GPI linkage constitutes a specific targeting signal for these membrane domains in *Drosophila*.

We were surprised at how few raft proteins were left in the Triton X-114 detergent phase (and by implication, associated with raft membranes) after PI-specific phospholipase C treatment. Taken at face value, this would indicate that almost all of the non-peripherally associated membrane proteins in *Drosophila* rafts were either GPI-linked or linked to insoluble

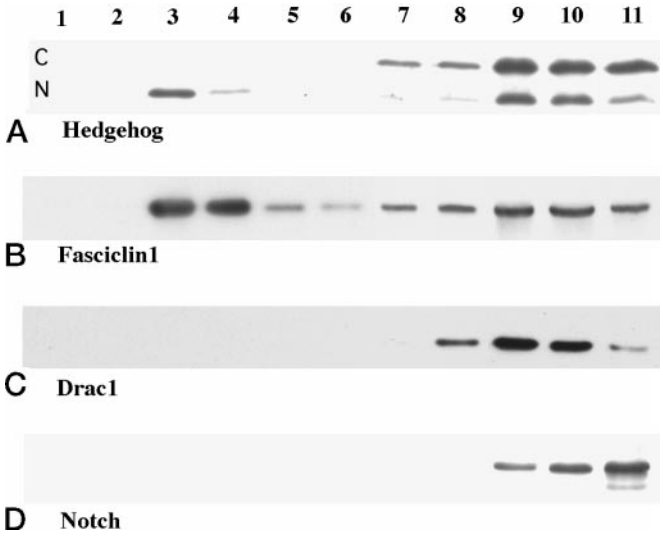


FIG. 4. **Hedgehog and fasciclin-1 associate specifically with detergent-insoluble membranes.** *Drosophila* embryonic membranes were solubilized with 1% Triton X-114 and then floated through a density gradient. Western blots of identical gradient fractions are shown probed with antibodies to detect Hedgehog N- and C-terminal fragments (*A*); fasciclin-1, a GPI-linked protein (*B*); Drac1, an isoprenylated protein (*C*); or Notch, a transmembrane protein (*D*). We detected very low levels of Notch in insoluble membranes after longer exposures, but not comparable to the level of Hedgehog N-terminal fragment.

cortex. It has been reported that a fraction of raft membrane lipid remains insoluble in Triton X-100 at temperatures between 13 and 37 °C (28). This led us to wonder whether some raft membranes might have remained insoluble in Triton X-114 even at the higher temperature required for phase separation of the detergent and thus constitute part of the "pellet" fractions shown in Fig. 3 (*E* and *F*). To address this possibility, we subjected membranes solubilized in Triton X-114 at the higher temperature to flotation through a density gradient. Floating insoluble membranes were observed just as when solubilization was performed at 0 °C. When we examined their lipid composition by TLC, we found that it was similar to that of raft membranes isolated at 0 °C (Fig. 2). This shows that a subfraction of raft membranes are resistant to Triton X-114 solubilization even at 29 °C. The protein and lipid composition of Triton X-100- and Triton X-114-insoluble membranes indicates that these detergents can be used to isolate *Drosophila* membranes that resemble mammalian rafts.

The Hedgehog N-terminal Fragment Associates Specifically with Detergent-insoluble Membranes—To determine whether the Hedgehog N-terminal fragment associated with *Drosophila* rafts, we solubilized embryonic membranes in 1% Triton X-114 at 0 °C for 30 min, floated the material though a density gradient, and investigated whether Hedgehog was found in the floating insoluble membranes. Western blotting of gradient fractions showed that a significant amount of the N-terminal fragment of Hedgehog remained associated with insoluble membranes (Fig. 4*A*). Although no lipid modification has yet been found on the Hedgehog C-terminal fragment, it copurifies with embryonic membranes, and it partitions almost exclusively into the detergent phase of Triton X-114 when the temperature is raised to 29 °C, suggesting that it is significantly hydrophobic (data not shown). Nevertheless, unlike the N-terminal fragment, it is completely solubilized by Triton X-114 at 0 °C (Fig. 4*A*).

To show that insoluble Hedgehog was found in the same fractions as raft-associated GPI-linked proteins, we probed the gradient fractions with an antibody to fasciclin-1 (41). We found this GPI-linked protein in the same low density gradient fractions as Hedgehog (Fig. 4*B*). This indicates that Hedgehog associates with raft membranes.

To demonstrate that the conditions we used were sufficiently stringent to solubilize other membrane proteins, the same gradient fractions were probed with antibodies to Drac1 (42) and Notch (43). Drac1 attaches to membranes via an isoprenyl group. Drac1 did not float out of the loaded volume of the gradient, indicating that it is completely solubilized by this treatment (Fig. 4*C*). Furthermore, the transmembrane protein Notch also remained predominantly in the fractions containing solubilized material (Fig. 4*D*). These results show that the association of Hedgehog with insoluble membranes is specific and takes place under conditions that solubilize other membrane proteins.

The proportion of Hedgehog present in detergent-insoluble membranes, although significant compared with that of Drac1 and Notch, does not appear to be as great as that of fasciclin-1. To determine whether this might be due to the existence of a non-sterol-linked population of Hedgehog molecules, we examined the sterol linkage of Hedgehog in embryonic membranes. Removing covalently linked cholesterol by base treatment causes Hedgehog to migrate more slowly on polyacrylamide gels (29). Base treatment of embryonic membranes caused all of the Hedgehog N-terminal fragment to shift to a lower mobility, indicating that all the Hedgehog in embryonic membranes is linked to sterol (data not shown). Failure of a fraction of Hedgehog molecules to associate with rafts cannot therefore be due to lack of sterol linkage (see "Discussion").

DISCUSSION

Our data show that *Drosophila* membranes contain raft lipid microdomains that resemble their mammalian counterparts in both lipid and protein composition. They further demonstrate that the cholesterol-linked Hedgehog N-terminal fragment and GPI-linked fasciclin-1 associate specifically with *Drosophila* rafts.

Analysis of the lipids present in *Drosophila* membranes shows that, despite differences in chemical structure between mammalian and *Drosophila* lipids, the properties of sterols, sphingolipids, and phosphoglycerolipids that allow formation of the liquid ordered (raft) phase have been preserved. One factor that allows the formation of laterally separated liquid ordered and disordered phases is the different melting temperatures of sphingolipids *versus* phosphoglycerolipids. Mammalian sphingolipids have a higher melting temperature than phosphoglycerolipids in part because their fatty acyl chains are longer and more saturated. We find that the fatty acids present in *Drosophila* sphingolipids are longer and more saturated than those in their phosphoglycerolipids, even though both sphingolipids and phosphoglycerolipids are shorter in *Drosophila* than in mammals (consistent with *Drosophila* membranes remaining fluid at lower temperatures). Thus, as in mammals, these lipids would be predicted to have different melting temperatures.

Despite being unable to synthesize sterols, *Drosophila* accumulate 18 mol % sterol in their membranes, relative to phospholipids, a level comparable to that of cholesterol in mammalian membranes. Dietary sterol appears to be incorporated largely without modification (ergosterol, campesterol, and sitosterol together account for 79% of the total membrane sterol). Nevertheless, small amounts of cholesterol (along with intermediates in the conversion of the dietary sterols to cholesterol) are observed. It will be interesting to determine whether these small amounts of cholesterol are necessary for modification of Hedgehog, or whether Hedgehog is modified by other sterols *in vivo*, as can apparently occur *in vitro* (30).

The concept of raft association as an important mediator of

protein localization has been developed based on experiments in mammalian tissue culture cells. We have shown that similar membrane domains rich in sterol and sphingolipid exist in *D. melanogaster*, an organism at some evolutionary distance from mammals, with different lipid classes and species. Detergent-insoluble membranes enriched in proteins similar to those found in mammalian rafts have also been recovered from *Dictyostelium* (31) and *Saccharomyces cerevisiae* (32). The preservation of the biophysical lipid properties allowing raft formation in such widely separate phyla suggests that these phase domains perform important cellular functions.

The functional importance of sterol in fly membranes has been questioned because fly tissue culture cells can be maintained in media depleted of sterols (33) and because adults survive when fed a sterol-free diet (although the efficiency of sterol depletion in adults is unknown) (34). Nevertheless, when *Drosophila* feed on an ergosterol-deficient strain of yeast, they produce embryos that fail to develop (34). This indicates that sterols are essential for the more complex cellular functions involved in embryonic development.

Our data show that the sterol-linked N-terminal fragment of the Hedgehog protein associates with raft membranes, suggesting that sterol linkage may be a raft-targeting signal. Cholesterol prefers to associate with sphingolipids rather than phospholipids due in part to the higher degree of saturation of the fatty acids in sphingolipids, which allows for a more favorable packing of the planar rings of cholesterol (35, 36). Cholesterol that is covalently linked to Hedgehog should retain its ability to associate with sphingolipids in this manner; this property would be predicted to confer affinity for the sphingolipid-rich raft membranes.

Although a significant amount of Hedgehog is present in detergent-insoluble membranes, it does not resist solubilization as efficiently as GPI-linked fasciclin-1 (Fig. 4). This indicates that a subpopulation of Hedgehog may not associate with rafts, although all Hedgehog in our membrane preparation is sterol-linked. Recent evidence indicated that a variable proportion of the sonic Hedgehog N-terminal fragment is also covalently linked to palmitic acid (37). Double acylation is known to be a raft-targeting signal for Src-related kinases, whereas modification by a single palmitoyl group is insufficient (38). If *Drosophila* Hedgehog is also palmitoylated, it will be interesting to determine whether sterol or palmitate or both are required to direct Hedgehog to raft membranes.

A question of converging interest for cell and developmental biologists is how proteins that pattern developing tissues move within and between cells, and how the regulation of this traffic contributes to the activity and distribution of morphogens. The limited spread of Hedgehog protein from a spatially restricted subset of producing cells is critical for patterning a variety of tissues. Covalent modification of Hedgehog by cholesterol has been postulated to influence the activity of the protein by conferring a general membrane affinity and thereby limiting its diffusion (29). Our data raise the possibility that sterol modification may play a more specific role by targeting Hedgehog to raft membranes. Rafts have been shown to play important roles in axonal trafficking of proteins in neurons and apical trafficking in epithelia (1). The Hedgehog protein moves axonally in photoreceptor neurons (39), and recent evidence suggests that epithelial cells actively transport Hedgehog protein through the plane of the epithelium (40); it will be interesting to determine whether proper cellular trafficking of Hedgehog depends on its association with raft membranes.

Rafts are also thought to play roles in signal transduction. Many G protein-coupled receptors, upon binding to their ligands, acquire affinity for raft membranes where mediators of signal transduction such as G proteins and adenylate cyclase are concentrated (15). Hedgehog signals through Smoothened, a putative G protein-coupled receptor. Although Hedgehog that is not modified by cholesterol is capable of signaling (29), modification of Hedgehog by cholesterol increases its potency in signal transduction by 30-fold (37). This raises the possibility that the efficiency of Hedgehog signaling might depend on its localization to raft membranes. The identification of rafts in a genetic model organism and the finding that Hedgehog associates with them will foster new approaches to understanding raft membrane microdomains and the role of polarized protein trafficking in development.

Acknowledgments—We are grateful to Roger Sandhoff for help with mass spectrometry and to Peter Becker for use of *Drosophila* population cages. We thank Steve Cohen, Tony Hyman, Stan Leibler, Andrew Murray, and Marino Zerial for helpful comments on the manuscript.

REFERENCES

1. Simons, K., and Ikonen, E. (1997) *Nature* **387,** 569–572
2. Brown, D. A., and London, E. (1997) *Biochem. Biophys. Res. Commun.* **240,** 1–7
3. Sankaram, M. B., and Thompson, T. E. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 8686–8690
4. Sankaram, M. B., and Thompson, T. E. (1990) *Biochemistry* **29,** 10670–10675
5. Rietveld, A. G., and Simons, K. (1998) *Biochim. Biophys. Acta*. **1376,** 467–479
6. Ahmed, S. N., Brown, D., and London, E. (1997) *Biochemistry* **36,** 10944–10953
7. Brown, D. A., and Rose, J. K. (1992) *Cell* **68,** 533–544
8. Fiedler, K., Kobayashi, T., Kurzchalia, T. V., and Simons, K. (1993) *Biochemistry* **32,** 6365–6373
9. Porter, J. A., Young, K. E., and Beachy, P. A. (1996) *Science* **274,** 255–259
10. Shaffer, C. D., Wuller, J. M., and Elgin, S. C. (1994) *Methods Cell Biol.* **44,** 99–108
11. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917
12. Rouser, G., Fleischer, S., and Yamamoto, A. (1970) *Lipids* **5,** 494–496
13. Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226,** 497–509
14. Schnaar, R. L., and Needham, L. K. (1994) *Methods Enzymol.* **230,** 371–389
15. Huang, C., Hepler, J. R., Chen, L. T., Gilman, A. G., Anderson, R. G. W., and Mumby, S. M. (1997) *Mol. Biol. Cell* **8,** 2365–2378
16. Bordier, C. (1981) *J. Biol. Chem.* **256,** 1604–1607
17. Wiegandt, H. (1992) *Biochim. Biophys. Acta* **1123,** 117–126
18. Klenk, H. D., and Choppin, P. W. (1969) *Virology* **38,** 255–268
19. Klenk, H. D., and Choppin, P. W. (1970) *Virology* **40,** 939–947
20. Keller, P., and Simons, K. (1998) *J. Cell Biol.* **140,** 1357–1367
21. Harder, T., Scheiffele, P., Verkade, P., and Simons, K. (1998) *J. Cell Biol.* **141,** 929–942
22. Svoboda, J. A., Ross, S. A., and Nes, W. D. (1995) *Lipids* **30,** 91–94
23. Schuler, I., Duportail, G., Glasser, N., Benveniste, P., and Hartmann, M. A. (1990) *Biochim. Biophys. Acta* **1028,** 82–88
24. Urbina, J. A., Pekerar, S., Le, H. B., Patterson, J., Montez, B., and Oldfield, E. (1995) *Biochim. Biophys. Acta* **1238,** 163–176
25. Van Helvoort, A., and Van Meer, G. (1995) *FEBS Lett.* **369,** 18–21
26. Simons, K., and Van Meer, G. (1988) *Biochemistry* **27,** 6197–6202
27. Sugita, M., Itonori, S., Inagaki, F., and Hori, T. (1989) *J. Biol. Chem.* **264,** 15028–15033
28. Melkonian, K. A., Chu, T., Tortorella, L. B., and Brown, D. A. (1995) *Biochemistry* **34,** 16161–16170
29. Porter, J. A., Ekker, S. C., Park, W. J., von Kessler, D. P., Young, K. E., Chen, C. H., Ma, Y., Woods, A. S., Cotter, R. J., Koonin, E. V., and Beachy, P. A. (1996) *Cell* **86,** 21–34
30. Cooper, M. K., Porter, J. A., Young, K. E., and Beachy, P. A. (1998) *Science* **280,** 1603–1607
31. Xiao, Z., and Devreotes, P. N. (1997) *Mol. Biol. Cell* **8,** 855–869
32. Kubler, E., Dohlman, H. G., and Lisanti, M. P. (1996) *J. Biol. Chem.* **271,** 32975–32980
33. Silberkang, M., Havel, C. M., Friend, D. S., McCarthy, B. J., and Watson, J. A. (1983) *J. Biol. Chem.* **258,** 8503–8511
34. Bondarenko, L. V., Luchnikova, E. M., and Inge-Vechtomov, S. G. (1989) *Ontogenez* **20,** 141–148
35. Slotte, J. P. (1992) *Biochemistry* **31,** 5472–5477
36. Bittman, R., Kasireddy, C. R., Mattjus, P., and Slotte, J. P. (1994) *Biochemistry* **33,** 11776–11781
37. Pepinsky, R. B., Zeng, C., Wen, D., Rayhorn, P., Baker, D. P., Williams, K. P., Bixler, S. A., Ambrose, C. M., Garber, E. A., Miatkowski, K., Taylor, F. R., Wang, E. A., and Galdes, A. (1998) *J. Biol. Chem.* **273,** 14037–14045
38. Shenoy-Scaria, A. M., Dietzen, D. J., Kwong, J., Link, D. C., and Lublin, D. M. (1994) *J. Cell Biol.* **126,** 353–363
39. Huang, Z., and Kunes, S. (1996) *Cell* **86,** 411–422
40. Bellaiche, Y., The, I., and Perrimon, N. (1998) *Nature*. **394,** 85–88
41. Hortsch, M., and Goodman, C. S. (1990) *J. Biol. Chem.* **265,** 15104–15109
42. Luo, L, Liao, Y. J., Jan, L. Y., and Jan, Y. N. (1994) *Genes Dev.* **8,** 1787–1802
43. Fehon, R. G., Johansen, K., Rebay, I., and Artavanis-Tsakonas, S. (1991) *J. Cell Biol.* **113,** 657–659