Different Sphingolipids Show Differential Partitioning into Sphingolipid/Cholesterol-Rich Domains in Lipid Bilayers

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ABSTRACT Two fluorescence-based approaches have been applied to examine the differential partitioning of fluorescent phospho- and sphingolipid molecules into sphingolipid-enriched domains modeling membrane "lipid rafts." Fluorescence-quenching measurements reveal that N-(diphenylhexatrienyl)propionyl- (DPH3:0-)-labeled gluco- and galactocerebroside partition into sphingolipid-enriched domains in sphingolipid/phosphatidylcholine/cholesterol bilayers with substantially higher affinity than do analogous sphingomyelin, ceramide, or phosphatidylcholine molecules. By contrast, the affinity of sphingomyelin and ceramide for such domains is only marginally greater than that of a phosphatidylcholine with similar hydrocarbon chains. By using direct measurements of molecular partitioning between vesicles of different compositions, we show that the relative affinities of different C_6-NBD- and C_5-Bodipy-labeled sphingolipids for sphingolipid-enriched domains are quantitatively, and in most circumstances even qualitatively, quite different from those found for species whose N-acyl chains more closely resemble the long saturated chains of cellular sphingolipids. These findings lend support in principle to previous suggestions that differential partitioning of different sphingolipids into "raft" domains could contribute to the differential trafficking of these species in eukaryotic cells. However, our findings also indicate that short-chain sphingolipid probes previously used to examine this phenomenon are in general ill-suited for such applications.

INTRODUCTION

Recent evidence has pointed to the existence of sphingolipid- and cholesterol-enriched microdomains known as "lipid rafts" in the plasma and possibly other membranes of animal cells (reviewed in Harder and Simons, 1997; Simons and Ikonen, 1997; Brown and London, 1998a,b) as well as yeast (Kübler et al., 1996). Interest in the nature and properties of these structures has been stimulated by findings that rafts, while depleted in many integral membrane proteins, appear to be enriched in certain classes of lipidated proteins, including glycosylphosphatidylinositol-anchored proteins and doubly acylated intracellular proteins, and in a variety of other proteins implicated in signaling at the level of the plasma membrane (Brown and Rose, 1992; Cinek and Horejsí, 1992; Janes et al., 1999; Sargiacomo et al., 1993; Sheets et al., 1999; Shenoy-Scaria et al., 1992, 1993). It has also been suggested that partitioning into "raft" structures may be an important element in the intracellular trafficking of certain classes of cellular membrane lipids (Simons and van Meer, 1988; van Ijzendoorn and Hoekstra, 1999; van Meer, 1993; van Meer et al., 1987; van't Hof and van Meer, 1990; Zegers and Hoekstra, 1998) and possibly certain membrane proteins (Bretscher and Munro, 1993; Munro, 1995; Pelham and Munro, 1993; Scheiffele et al., 1997).

Studies of model membranes composed of lipids or lipids and reconstituted proteins strongly suggest that "lipid rafts" in biological membranes exist in an l_o state distinct from the l_d phase preferred by most natural membrane glycerophospholipids (Ahmed et al., 1997; Brown, 1998; Rietveld and Simons, 1998; Schroeder et al., 1994, 1998). Formation of l_o-phase domains in mixed-lipid bilayers has been shown to be favored by the presence of long-chain saturated phospho- or sphingolipids and physiological proportions of cholesterol (Brown, 1998; Reitveld and Simons, 1998). It has also been suggested that sphingolipids may exhibit a further specific affinity for cholesterol and/or for self-association by virtue of the hydrogen-bonding capacities of their sphingosine backbone and polar headgroups (Boggs, 1987; Thompson and Tillack, 1985), which in turn may enhance their abilities to associate with "raft" structures (Simons and Ikonen, 1997). Although different sphingolipids indeed exhibit distinctive physical properties in isolation or at high concentrations in model membranes, it is less clear to what extent such properties are manifested in complex lipid mixtures such as those found in biological membranes.

In this study we have used two approaches to monitor the association of different fluorescent phospho- and sphingolipids with sphingolipid-enriched liquid-ordered domains with compositions similar to those of the extracytoplasmic

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Abbreviations used: l_d, l_o, liquid-disordered, liquid-ordered; C_5-Bodipy, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl-; C_6-NBD-, 6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-aminohexanoyl-; C_6-NBD-PC, 1-palmitoyl-2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-aminohexanoyl)-PC; DPH3:0-, 3-(4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl)propanoyl-; EDTA, ethylenediaminetetraacetic acid, trisodium salt; LUV, large unilamellar vesicles; NBD-, 7-nitrobenz-2-oxa-1,3-diazol-4-yl-; PC, phosphatidylcholine; PE, phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoyl PC; 12SLPC, 1-palmitoyl-2-(12-doxylstearoyl)-phosphatidylcholine; Rho-PE, N-(lissamine rhodaminesulfony)-PE; Tes, (-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid. Fatty acyl chains of fluorescent lipids are designated using standard shorthand nomenclature as N:x or N:xc, where N = carbon number, x = number of double bonds, and *c* denotes a *cis* double bond configuration.

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faces of lipid raft domains. Using a novel method based on fluorescence quenching measurements (Wang et al., 2000), we have measured the relative tendencies of different diphenylhexatrienylpropionyl- (DPH3:0-) phospho and sphingolipids to partition into sphingolipid-enriched l_o-phase domains in sphingolipid/phosphatidylcholine/cholesterol bilayers. Second, by measurements of the intervesicle partitioning of exchangeable fluorescent sphingolipid probes commonly used to study lipid trafficking in mammalian cells, we have determined the partition coefficients governing the distribution of these species between sphingolipid-enriched l_o and unsaturated phospholipid-enriched l_d phases. Our results demonstrate that DPH3:0-monoglycosylsphingolipids, whose N-acyl chains approximate the long saturated N-acyl chains of natural membrane glycosphingolipids, indeed exhibit a significantly greater affinity for "raft"-like domains than do analogous phospho- or sphingolipids with different polar headgroups. Fluorescent C_6-NBD- and C_5-Bodipy-labeled sphingolipids, by contrast, exhibit generally very weak affinities for sphingolipid/cholesterol-rich liquid-ordered phases, which moreover vary in an unpredictable manner with changes in the sphingolipid composition of such phases. Our findings thus lend support to previous suggestions that differential intracellular trafficking of sphingolipids may be influenced by differential partitioning of these species into liquid-ordered, sphingolipid/cholesterol-enriched domains within cellular membranes. However, our results also underscore the need for caution in drawing such conclusions from the results of studies examining the cellular trafficking of short-chain fluorescent sphingolipids.

MATERIALS AND METHODS

Materials

Glucocerebroside was obtained from Sigma (St. Louis, MO) and sphingosinephosphorylcholine from Calbiochem (La Jolla, CA); other natural and synthetic lipids, lysolipids, and sphingosine were obtained from Avanti Polar Lipids (Alabaster, AL). C_5-Bodipy-lipids, C_6-NBD-sphingomyelin, and C_6-NBD-PC were obtained from Molecular Probes (Junction City, OR). 1-Acyl-2-DPH3:O-PCs were synthesized by reacting the appropriate 1-acyl-lysophosphatidylcholine with the anhydride of diphenylhexatrienylpropionic acid (Molecular Probes) as described previously (Mason et al., 1981). The succinimidyl esters of N-NBD-aminocaproic acid (Monti et al., 1977) or diphenylhexatrienylpropionic acid were generated by reacting 1, 1.2, and 2 eq, respectively, of fluorescent fatty acid, dicyclohexylcarbodiimide, and N-hydroxysuccinimide for 4 h at 25°C in 1:1 dry methylene chloride/dimethylformamide. Gluco- and galactosylpsychosines, prepared by alkaline hydrolysis of the corresponding cerebrosides (Koshy and Boggs, 1983), sphingosine, and sphingosinephosphorylcholine were re-N-acylated with the fluorescent succinimidyl esters by overnight reaction at 25°C in dry chloroform/methanol 4:1 containing 2 eq diisopropylethylamine. The resulting fluorescent-labeled cerebrosides and sphingomyelin were purified by thin-layer chromatography in 50:15:5:5:5 or 50:20:10:10:5 (v/v/v/v/v) CH_2Cl_2/acetone/methanol/CH_3COOH/H_2O, respectively, and the corresponding ceramide was similarly purified using 98:2:0.2 CH_2Cl_2/methanol/CH_3COOH. Fluorescent phosphatidylethanolamines were synthesized from the corresponding phosphatidylcholines by phospholipase D-mediated transphosphatidylation (Comfurius and Zwaal, 1977).

Fluorescence-quenching assays

Lipid mixtures (normally 75 nmol total lipid, including 0.1–0.6 mol % fluorescent lipid) were dried down from 3:1 (v/v) CH_2Cl_2/methanol under nitrogen, redissolved in 0.4 ml 90:10 cyclohexane/ethanol with brief warming to 80°C, then rapidly frozen in dry ice/ethanol and lyophilized. To the dried lipid samples was added 1 ml buffer (100 mM NaCl, 10 mM Tes, 0.1 mM EDTA, pH 7.2), and the samples were successively incubated at 45°C for 15 min at 75°C (or 85°C for cerebroside-containing samples) for 1 min and again at 45°C for 15 min, then cooled to 37°C and incubated at this temperature for 3–16 h. All data presented in this paper were obtained for samples incubated for 12–16 h at 37°C, although samples incubated for 3 h gave essentially identical results. Sample fluorescence was then determined at 37°C using a Perkin-Elmer LS-5 spectrofluorimeter, both before and after adding Triton X-100 (to 1% [v/v] final concentration) and heating to 75°C for 1 min, bath-sonicating for 10 sec, and finally incubating at 45°C for 15 min. The fluorescence of the DPH3:0-lipids was monitored at 37°C using excitation and emission settings of 362/435 nm (slitwidths 3 nm/20 nm). From these values was calculated the normalized fluorescence, F_N, defined as the ratio of the fluorescence measured for each sample (corrected using a suitable blank sample without probe) to the (corrected) fluorescence measured for the same sample after Triton solubilization.

Fluorescence-quenching data obtained as just described were analyzed in two ways to estimate the relative affinities of partitioning of different DPH3:0-lipids into the (quencher-depleted) l_o phase (Wang et al., 2000). First, data obtained for fluorescent lipids at each sample composition from 20% to 70% 12SLPC in the nonsterol fraction were analyzed according to Eq. 3 in the text and the mean determined for each fluorescent species, providing an average index of the relative extents of partitioning of different probes into the l_o phase over the range of compositions just noted. Second, quenching data were analyzed using the curve-fitting ("slope") method described in Wang et al. (2000). Results obtained using the two approaches were not substantially or systematically different; the values presented in Table 1 were obtained by the latter method.

Intervesicle-partitioning measurements

Dried lipid mixtures (6–15 μmol lipid), prepared as described above by lyophilization from 9:1 cyclohexane/ethanol, were rehydrated as indicated above, then extruded through 0.1 μm pore size polycarbonate filters (MacDonald et al., 1991) to prepare LUV. The relative affinities of NBD- and C_5-Bodipy-labeled lipids for lipid vesicles of different compositions were measured as described previously (Gardam et al., 1989; Shin et al., 1991) with minor modifications. Briefly, aliquots of "donor" LUV (99:1:50 [molar proportions] POPC/Rho-PE/cholesterol) were first labeled with a C_6-NBD- or C_5-Bodipy-labeled lipid (7 nmol/μmol vesicle lipid) by injecting the labeled lipid into the donor vesicle suspension in a small volume ($\leq$1% v/v) of methanol. After incubating at 37°C for 15 min (for NBD-labeled probes) or 1 h (for C_5-Bodipy-labeled species), replicate aliquots (75 nmol total lipid) of the probe-labeled donor vesicles were combined with varying amounts of acceptor vesicles (95:5:50 sphingolipid/POPC/cholesterol) in a volume of 1 ml, and the donor/acceptor mixtures were incubated for an additional 30 min (for C_6-NBD probes) or 1 h (for C_5-Bodipy probes) at 37°C. Control experiments showed that for all probe species the incubation conditions just noted allowed both complete initial insertion into the donor vesicles and subsequent complete equilibration between the outer leaflets of donor and acceptor vesicles. After equilibration as just described, the fluorescence of the co-incubated donor/acceptor vesicle mixtures was measured at 37°C before and after solubilization with Triton X-100 as described above, using emission and excitation settings of 470 nm/538 nm (slitwidths 15 nm/20 nm) and 480 nm/526 nm (slitwidths

5 nm/10 nm) for C_6-NBD- and C_5-Bodipy-lipids, respectively. Appropriate blank measurements were obtained using donor and acceptor vesicles without NBD- or Bodipy-labeled lipids.

Dithionite reduction of NBD-labeled probes in lipid vesicles was assayed as described previously (McIntyre and Sleight, 1991; Balch et al., 1994), adding sodium dithionite (final concentration 10 mM) to the vesicles at 37°C while continuously monitoring the fluorescence. Total concentrations of vesicle phospholipids were determined by organic phosphorus assay as described previously (Lowry and Tinsley, 1974).

RESULTS

Fluorescence-quenching measurements of fluorescent lipid partitioning in sphingolipid/PC/cholesterol bilayers

In the first series of experiments we used fluorescence-quenching measurements to compare the distributions of different DPH3:0-phospho- and -sphingolipids (structures shown in Fig. 1) between the l_o and l_d phases in mixtures of sphingolipids, the spin-labeled phosphatidylcholine 12SLPC, and cholesterol. The spin-labeled phosphatidylcholine 12SLPC has been shown to behave similarly to unsaturated phosphatidylcholines in ternary lipid systems like those studied here (Ahmed et al., 1997).

In phase-separated systems comprising only two major lipid components, one of which is a fluorescence quencher, the interphase partition coefficient for a fluorescent probe can be determined directly from such measurements (London and Feigenson, 1981; Huang et al., 1988). For ternary and higher-order systems such as the sphingolipid/phospholipid/cholesterol bilayers of interest here, such direct analysis is generally not feasible. However, as we have discussed previously (Wang et al., 2000), in these latter systems it is still possible to use fluorescence-quenching measurements to compare the *relative* affinities of different but structurally related fluorescent probes for one coexisting phase over the other.

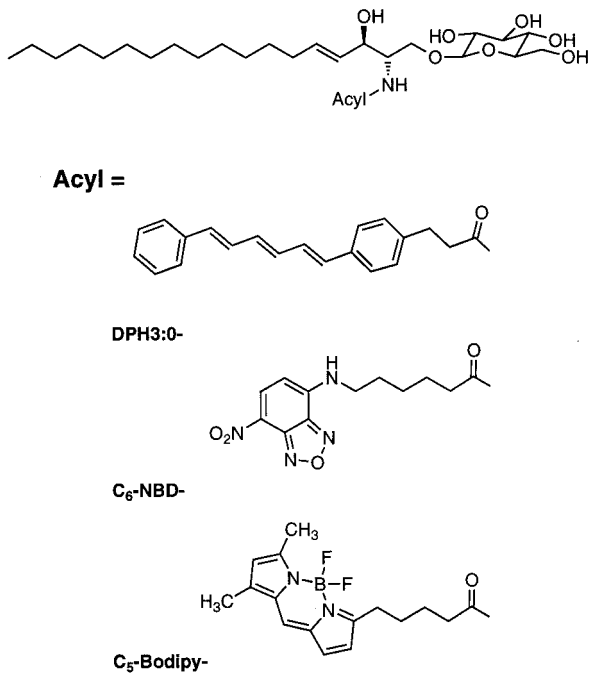

FIGURE 1 Representative structures of fluorescent lipids used in this study, illustrated for fluorescent glucocerebroside.

DPH3:0-labeled lipids were chosen for the measurements described below to approximate the structures of natural sphingolipids (which carry mainly saturated N-acyl chains), as the DPH3:0-acyl chain is entirely hydrocarbon, has an overall length similar to that of a long saturated acyl chain, and has an extended rod-like structure that is compatible with ordered and disordered lipid phases, as has been demonstrated for diphenylhexatriene itself (London and Feigenson, 1981). Previous studies have also concluded that within lipid bilayers the fluorescent acyl chain of 16:0/DPH3:0-PC adopts a disposition (orientation and depth of penetration into the bilayer interior) that is similar to that for unlabeled lipid acyl chains (Kaiser and London, 1998a and references therein). It should be noted, however, that the analysis described below is concerned only with the *relative* affinities of different DPH3:0-lipids for sphingolipid-enriched domains and neither implies nor requires that the *absolute* affinity of any given DPH3:0-labeled sphingolipid for such domains must be identical to that of the corresponding natural sphingolipid. The analysis rather assumes only that a lipid-coupled DPH3:0-chain approximates a long saturated acyl chain to the extent that it does not distort the contributions of *other* portions of the molecule (e.g., the "backbone" and headgroup regions) to the net affinity of the labeled species for sphingolipid-enriched domains.

In Fig. 2 are shown quenching curves determined at 37°C for various DPH3:0-phospho and sphingolipids incorporated into sphingomyelin/12SLPC/(33 mol % cholesterol) bilayers containing varying proportions of sphingomyelin versus spin-labeled PC. In the inset to Fig. 2 *A* representative quenching curves are shown as plots of the normalized fluorescence F_N versus mol % 12SLPC in the nonsterol fraction. In the main panel of this and subsequent figures fluorescence is plotted in the alternative scaled form $(F/F_o)_{cor} = (F_N - F_{100\%Q})/(F_{0\%Q} - F_{100\%Q})$ as suggested by London and Feigenson (1981), where F_N is the normalized fluorescence measured for the probe in a mixture containing a given molar percentage of quencher lipid, and $F_{100\%Q}$ and $F_{0\%Q}$ are the normalized fluorescence values measured in mixtures containing, respectively, 100% or 0% quencher lipid in the nonsterol fraction. As illustrated in Fig. 2 *A* (*inset*), all of the DPH3:0-lipids examined show very similar normalized fluorescence values in samples containing 0 mol % or 100 mol % SLPC in the nonsterol fraction, whereas the quenching curves diverge significantly at intermediate compositions. This observation can be explained by noting that at 37°C this system exhibits an l_o/l_d phase separation, with sphingomyelin enriched in the l_o phase and 12SLPC in the l_d phase, over a range of compositions estimated to extend from

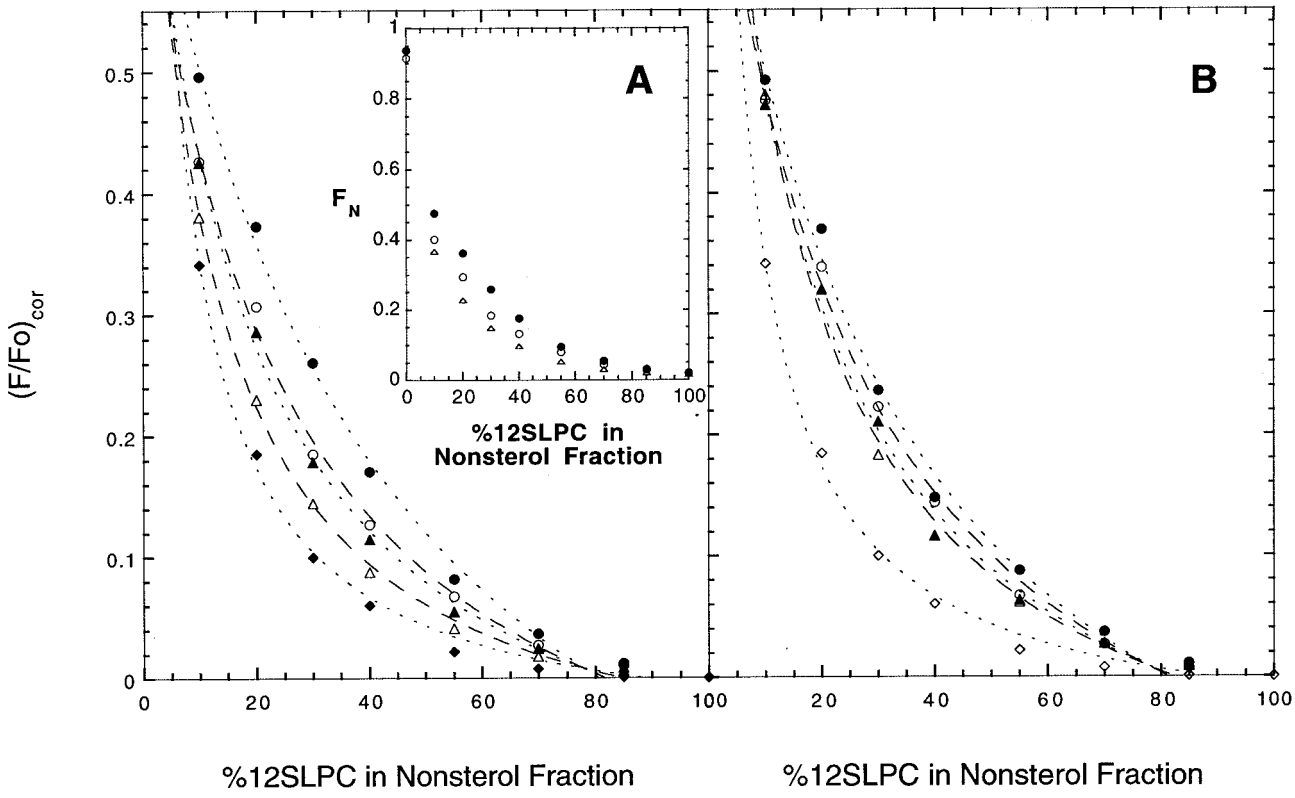

FIGURE 2 Quenching curves measured at 37°C for DPH3:O-phospho and sphingolipids in sphingomyelin/12SLPC/(33 mol % cholesterol) bilayers containing the indicated percentages of 12SLPC in the nonsterol fraction. Data points within the region of l_o/l_d phase separation (estimated as spanning the range of compositions from ~5 to 80 mol % sphingomyelin in the nonsterol fraction) were fitted empirically to a hyperbolic function of the form described in Ahmed et al. (1997) and Wang et al. (2000) to give the dashed curves shown. *(A)* Quenching data for (●) DPH3:O-glucocerebroside, (○) DPH3:O-sphingomyelin, (▲) 14:O/DPH3:O-PC, (△) 18:1c/DPH3:O-PC, and (◆) N-linoleoyl-18:1c/DPH3:O-PE. *Inset:* Quenching data for (●) DPH3:O-glucocerebroside, (○) DPH3:O-sphingomyelin, and (△) 18:1c/DPH3:O-PC, plotting the normalized fluorescence F_N rather than the scaled fluorescence $(F/F_o)_{cor}$. *(B)* Quenching curves for (●) DPH3:O-galactocerebroside, (○) 18:O/DPH3:O-PC, (▲) DPH3:0-ceramide, (△) 18:0/DPH3:O-PE, and (◇) N-linoleoyl-18:1c/DPH3:O-PE. Quenching curves were determined as described in Materials and Methods.

<20 mol % to 80–85 mol % 12SLPC in the nonsterol fraction (Ahmed et al., 1997) (see Note 1 at end of text). Within the region of phase separation, the different DPH3:0-labeled species partition differently between the quencher-depleted l_o and quencher-enriched l_d domains, and hence give different normalized fluorescence values, while for compositions outside this region all probes exist in a single phase and give comparable normalized fluorescence values.

As we have discussed previously (Wang et al., 2000), data like those shown in Fig. 2 can be analyzed quantitatively to determine the relative extents of partitioning of the different fluorescent probes into l_o-phase domains. For a given probe **X** in (l_o/l_d) phase-separated bilayers of a particular composition, the scaled fluorescence $(F/F_o)_{cor}(\mathbf{X})$ is given by the expression

$$(F/F_o)_{cor}(\mathbf{X}) = f_{ld}(\mathbf{X}) \cdot (F/F_o)^{ld}_{cor}(\mathbf{X}) + f_{lo}(\mathbf{X}) \cdot (F/F_o)^{lo}_{cor}(\mathbf{X}) \quad (1)$$

where $(F/F_o)^{ld}_{cor}(\mathbf{X})$ and $(F/F_o)^{lo}_{cor}(\mathbf{X})$ represent the normalized fluorescence values, and $f_{lo}(\mathbf{X})$ and $f_{ld}(\mathbf{X})$ the fractions, of the probe in the l_d and the l_o phases coexisting at that bilayer composition. For a series of fluorescent probes that all bear the same fluorophore and are closely structurally related, we assume that in phase-separated bilayers of a given composition the fluorescence of all the probes is quenched with similar efficiency in the l_o phase, and likewise for all probes in the coexisting l_d phase. This assumption is supported by the observation noted above that the normalized fluorescence values F_N are essentially identical for the different probes in (l_o-phase) bilayers composed purely of sphingomyelin and cholesterol, and similarly in (l_d-phase) bilayers composed of 12SLPC and cholesterol (Fig. 2 *A*, *inset*); similar results were obtained for the different DPH3:0-lipids in single-phase mixtures of dioleoyl PC, 12SLPC, and cholesterol (not shown). In this case we can rearrange Eq. 1 to the more immediately useful equation

$$f_{lo}(X) = \frac{((F/F_o)_{cor}(X) - (F/F_o)^{ld}_{cor})}{((F/F_o)^{lo}_{cor} - (F/F_o)^{ld}_{cor})} \quad (2)$$

where $f_{lo}(\mathbf{X})$ and $(F/F_o)_{cor}(\mathbf{X})$ are as defined above and the parameters $(F/F_o)^{ld}_{cor}$ and $(F/F_o)^{lo}_{cor}$ are now assumed to have

the same values for all the related fluorescence species examined.

Calculation from Eq. 2 of the *absolute* extent of partitioning of a given probe **X** into the l_o phase requires knowledge of the value of $(F/F_o)^{lo}_{cor}$, which is difficult to estimate experimentally (Wang et al., 2000). It is by contrast feasible to estimate the value of $(F/F_o)^{ld}_{cor}$ as a function of composition if one of the related fluorescent species examined exhibits negligible partitioning into the l_o phase, in which case the value of $(F/F_o)_{cor}$ measured for that species provides a direct estimate of the value of $(F/F_o)^{ld}_{cor}$ at each composition (see Note 2). It is then possible to calculate the *relative* extents of partitioning of any two probes $\mathbf{X_1}$ and $\mathbf{X_2}$ into the l_o phase in bilayers of a given composition, using the following modification of Eq. 2:

$$\frac{f_{lo}(\mathbf{X_1})}{f_{lo}(\mathbf{X_2})} = \frac{((F/F_o)_{cor}(\mathbf{X_1}) - (F/F_o)^{ld}_{cor})}{((F/F_o)_{cor}(\mathbf{X_2}) - (F/F_o)^{ld}_{cor})} \quad (3)$$

where the various terms in this equation are as described above.

N-linoleoyl-(18:1c/DPH3:0)-PE was designed to show minimal l_o-phase partitioning, and hence to provide estimates of the value of $(F/F_o)^{ld}_{cor}$ for each composition examined, based on our previous finding that multiply *cis*-unsaturated lipids show negligible affinities for the l_o phase (Wang et al., 2000). As shown in Fig. 2 *A*, consistent with this expectation the quenching curve for this species in the sphingomyelin/12SLPC/(33 mol % cholesterol) system lies below that for all other species examined, including 18:1c/DPH3:0-PC. A second multiply unsaturated species, a conjugate joining 18:1c/DPH3:0-PE to di18:1c-PE via a succinyl linker, gave a quenching curve very similar to that determined for N-linoleoyl-(18:1c/DPH3:0)-PE (not shown).

Using the quenching curve for N-linoleoyl-(18:1c/DPH3:0)-PE to estimate the value of $(F/F_o)^{ld}_{cor}$ in Eq. 3 as a function of composition, we calculated the relative affinities of partitioning of the different fluorescent species into l_o domains (expressed relative to those of the reference species 14:O/DPH3:0-PC) in (l_o/l_d) phase-separated sphingomyelin/12SLPC/(33 mol % cholesterol) bilayers, giving the results listed in Table 1. The values presented in this table were determined using a curve-fitting approach described previously (Wang et al., 2000) and provide an estimate of the value of the (l_o/l_d) phase partition coefficient for each DPH3:0-labeled lipid species divided by the corresponding value for 14:O/DPH3:0-PC.

It can be seen from the data of Table 1 that in the sphingomyelin/12SLPC/cholesterol system the gluco- and galactocerebroside probes show the greatest relative affinity for the l_o phase, while the corresponding labeled sphingomyelin and ceramide probes show a significantly lesser tendency to partition into l_o-phase domains. Among the 1-acyl-2-DPH3:0-phosphatidylcholines examined, as expected, affinity for the l_o phase decreases substantially both with decreasing chain length (compare 18:O/DPH3:0- and 14:O/DPH3:0-PC) and with unsaturation of the 1-position acyl chain (compare 18:O/DPH3:0- and 18:1c/DPH3:0-PC). Interestingly, 14:O/DPH3:0-PC shows an only slightly lower affinity for the l_o phase than does the labeled sphingomyelin, which it closely matches in hydrocarbon chain lengths. This result suggests that the sphingosine backbone and the amide linkage of sphingomyelin make only a small contribution to the affinity of this species for sphingolipid-enriched l_o-phase domains. The observation that the two DPH3:0-monoglucocerebrosides partition into the l_o phase with substantially greater affinity than does the corresponding ceramide (Table 1) indicates that the sugar residues of the former species contribute significantly to the overall free energy of partitioning into this phase.

TABLE 1 Relative affinities of diphenylhexatrienylpropionyl phospho- and sphingolipids for the liquid-ordered phase in sphingolipid/12SLPC/cholesterol bilayers

	$f_{lo}(\mathbf{X})/f_{lo}$(14:0/DPH3:0-PC)* in	
DPH3:0-Lipid	SM†/PC/Ch.	SM/GalCer/PC/Ch.
18:0/DPH3:0-PC	1.64 ± 0.16	2.06 ± 0.17
18:0/DPH3:0-PE	1.08 ± 0.14	2.07 ± 0.14
18:1c/DPH3:0-PC	0.48 ± 0.04	0.40 ± 0.04
14/DPH3:0-PC	(1)	(1)
DPH3:0-SM	1.29 ± 0.16	1.21 ± 0.08
DPH3:0-ceramide	1.28 ± 0.17	1.19 ± 0.11
DPH3:0-GalCer	1.97 ± 0.26	1.79 ± 0.12
DPH3:0-GlcCer	2.31 ± 0.22	1.77 ± 0.11

*Values listed were determined from the data shown in Figs. 2 and 3 as described in Materials and Methods.
†Additional abbreviations used: SM, sphingomyelin; GalCer/GlcCer, galactocerebroside/glucocerebroside; Ch., cholesterol.

Mammalian cells typically express glycosphingolipids and sphingomyelin in their plasma membranes, and glycosphingolipids are generally found to be highly enriched in the detergent-resistant membrane fractions isolated from cell membranes at low temperatures (Brown and London, 1998a,b). We accordingly also compared the l_o-phase partitioning of different DPH3:0-phospho- and -sphingolipids in sphingolipid/12SLPC/(33 mol % cholesterol) bilayers in which the sphingolipid component was a 1:1 mixture of sphingomyelin and galactocerebroside. This system has been shown previously to exhibit l_o/l_d phase separation over a wide range of proportions of sphingolipid relative to 12SLPC (Ahmed et al., 1997). The quenching curves obtained for various DPH3:0-lipids in this system are shown in Fig. 3, and the calculated relative affinities of these species for the sphingolipid-enriched l_o phase are listed in Table 1. In general, the partial replacement of sphingomyelin by galactocerebroside in these bilayers has little effect on the relative affinities of the different fluorescent phosphatidylcholines and sphingolipids for the l_o phase. However, the relative affinity of 18:0/DPH-3:0 PE for the l_o phase was

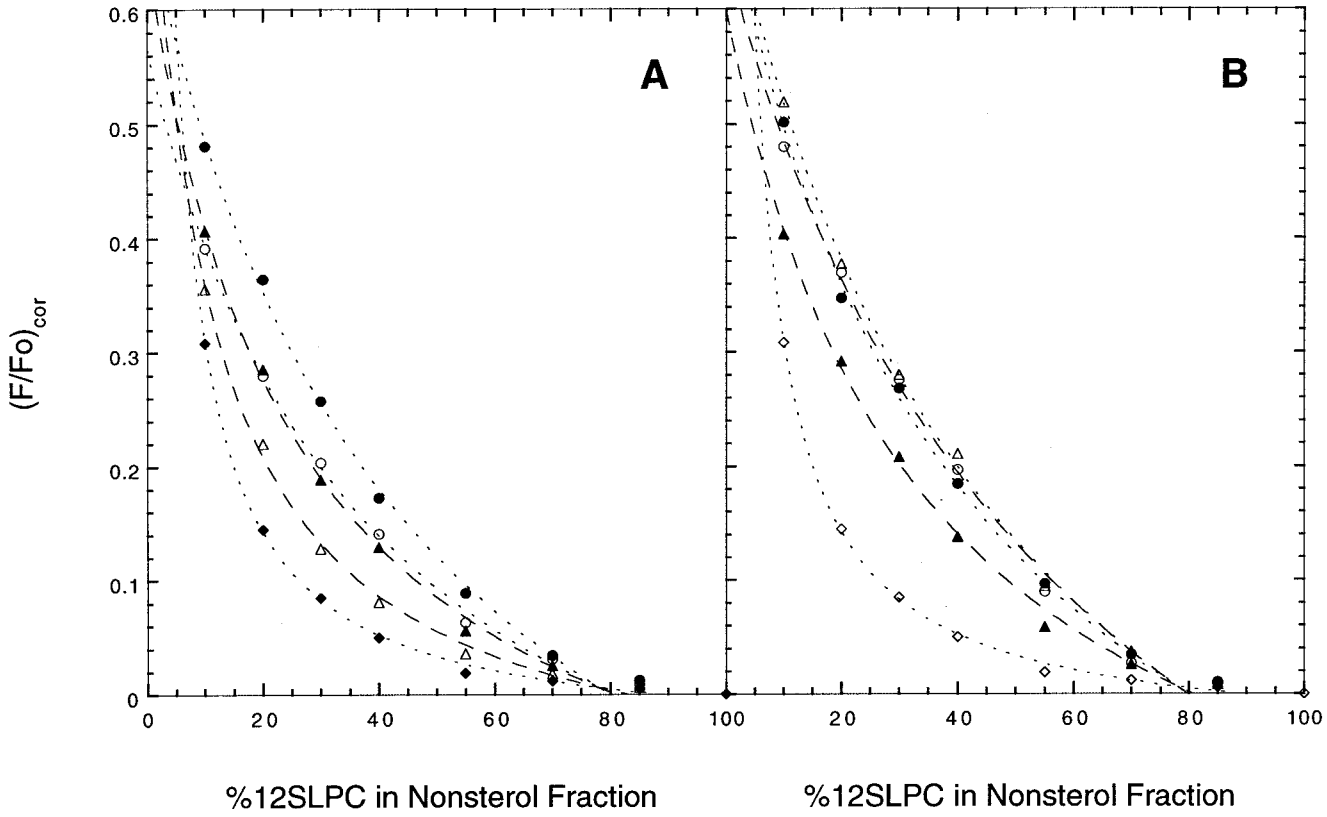

FIGURE 3 Quenching curves measured at 37°C for DPH3:0-phospho and sphingolipids in sphingolipid/12SLPC/(33 mol % cholesterol) bilayers containing equimolar sphingomyelin and galactocerebroside as the sphingolipid component and the indicated percentages of 12SLPC in the total nonsterol fraction. The hyperbolic curves shown were fitted empirically to data points within the region of l_o/l_d phase separation as indicated in the legend to Fig. 2. *(A)* Quenching data for (●) DPH3:0-glucocerebroside, (○) DPH3:0-sphingomyelin, (▲) 14:O/DPH3:0-PC, (△) 18:1c/DPH3:0-PC, and (◆) N-linoleoyl-18:1c/DPH3:0-PE. *(B)* Quenching curves for (▲) 18:O/DPH3:0-PE, (△) 18:O/DPH3:0-PE, (●) DPH3:0-galactocerebroside, (○) DPH3:0-ceramide, and (◆) N-linoleoyl-18:1c/DPH3:0-PE.

significantly greater in cerebroside-containing bilayers than in bilayers in which sphingomyelin comprised the only sphingolipid component (Table 1). By contrast, the affinities of the two fluorescent glycosphingolipids for l_o-phase domains are slightly *decreased,* relative to that of the corresponding fluorescent sphingomyelin or ceramide, when the sphingolipid fraction includes cerebroside. This latter result indicates that partitioning of at least simple (monoglycosyl) glycosphingolipids into l_o-phase domains is not enhanced by putative preferential interactions between sugar headgroups, contrary to some previous suggestions. Nonetheless, the presence of the sugar headgroup clearly enhances the affinity of a sphingolipid for l_o-phase domains in sphingolipid/PC/cholesterol bilayers whether the l_o phase-forming sphingolipid component is sphingomyelin alone or, more typical of mammalian cell plasma membranes, a mixture of sphingomyelin and glycosphingolipids.

Intervesicle-partitioning measurements

Fluorescence measurements can be used to monitor the distribution of exchangeable fluorescent molecules between two populations of lipid vesicles when one of the vesicle populations incorporates a nonexchangeable fluorescence quencher in its lipid bilayer (Gardam et al., 1989; Nichols and Pagano, 1982; Shin et al., 1991; Leventis and Silvius, 1998). We used this principle to monitor the partitioning of C_6-NBD- and C_5-Bodipy-labeled lipids between 100:50 (molar proportions) POPC/cholesterol "donor" LUV, incorporating 0.7 mol % of the nonexchangeable energy-transfer quencher Rho-PE, and 95:5:50 sphingolipid/POPC/cholesterol "acceptor" LUV without quencher. The sphingolipid component in the latter vesicles was either sphingomyelin alone or equimolar sphingomyelin and galactocerebroside. A low proportion of POPC was also incorporated in the acceptor vesicles to approximate the composition of the l_o phase at the left-hand (sphingolipid-rich) limit of the region of (l_o/l_d) phase separation (estimated from fluorescence-quenching data in this study and Ahmed et al., 1997; Wang et al., 2000).

To determine the relative affinity of the fluorescent probes for acceptor versus donor vesicles, a fixed amount of probe-labeled donor vesicles was incubated with varying amounts of unlabeled acceptor vesicles to allow equilibra-

tion of the exchangeable lipid probe between the two vesicle populations, and the normalized fluorescence F_N (determined as described in Materials and Methods) was then measured as a function of the ratio of donor to acceptor vesicle concentrations. In Fig. 4 *A* we present results from a representative experiment in which a fixed concentration of POPC/Rho-PE/cholesterol donor vesicles, labeled with different C_6-NBD lipids, was incubated with varying concentrations of sphingomyelin/POPC/cholesterol (95:5:50 molar proportions) acceptor vesicles as just described. As discussed previously (Gardam and Silvius, 1987; Shin et al., 1991; Leventis and Silvius, 1998), such data can be described by the equation

$$F_N = F_N^D + f_{exch} \cdot (F_N^A - F_N^D) \cdot \left(\frac{K_p \cdot [L_{eff}]_A}{K_p \cdot [L_{eff}]_A + [L_{eff}]_D} \right) \quad (4)$$

where F_N^D and F_N^A are the normalized fluorescence values for the probe in donor and in acceptor vesicles, respectively; f_{exch} is the fraction of the probes that is exchangeable between vesicles on the time scale of the experiment; K_p is the partition coefficient describing the relative affinity of the fluorescent lipid for the acceptor over the donor vesicles; and $[L_{eff}]_D$ and $[L_{eff}]_A$ represent respectively the (constant) concentration of donor vesicle lipids and the (variable) concentration of acceptor vesicle lipids to which the exchangeable fraction of the probe molecules has access on the time scale of the experiment.

To determine the pertinent values of the quantities L_{eff} in Eq. 4, it is necessary to determine whether transbilayer diffusion of the exchangeable fluorescent species is rapid or slow on the experimental time scale, a question that has not previously been addressed for some of the NBD-labeled species studied here. To this end we examined the kinetics of reaction of vesicle-bound C_6-NBD-labeled lipid probes with sodium dithionite, which rapidly reduces NBD-labeled molecules in the outer monolayers of vesicles to a nonfluorescent form (McIntyre and Sleight, 1991; Balch et al., 1994). As illustrated in Fig. 5 (*curve 1*), addition of dithio-

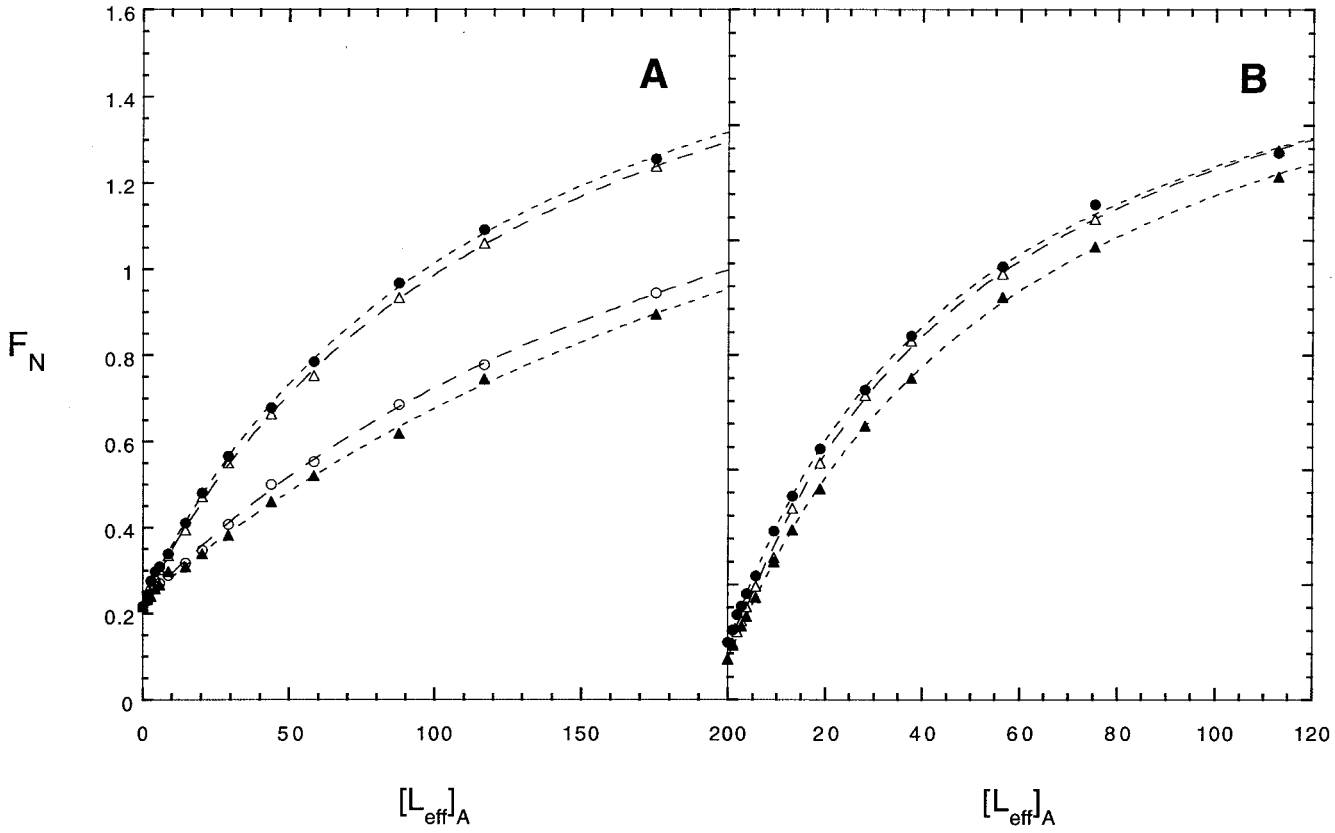

FIGURE 4 Partitioning of short-chain fluorescent sphingolipids and phosphatidylcholines between POPC/Rho-PE/cholesterol (99:1:50 molar proportions) donor LUV and sphingomyelin/POPC/cholesterol (95:5:50 molar proportions) acceptor vesicles. Data shown in each panel are from a representative individual experiment carried out as described in the text. The effective lipid concentrations $(L_{eff})_D$ and $(L_{eff})_A$ used to plot and to fit the data according to Eq. 4 were determined from the measured total concentrations of donor/acceptor vesicle lipids and using values of 0.47 and 0.56, respectively (determined as described in the text), for the fractions of total donor and acceptor vesicle lipids exposed to the extravesicular medium. *(A)* (●) C_6-NBD-galactocerebroside, (△) C_6-NBD-glucocerebroside, (○) C_6-NBD-sphingomyelin, and (▲) C_6-NBD-PC; $(L_{eff})_D$ in the experiment shown was determined to be 6.4 μM. *(B)* (●) C_5-Bodipy-GM_1, (△) C_5-Bodipy-glucocerebroside and (▲) C_5-Bodipy-PC; $(L_{eff})_D$ in the experiment shown was 7.7 μM.

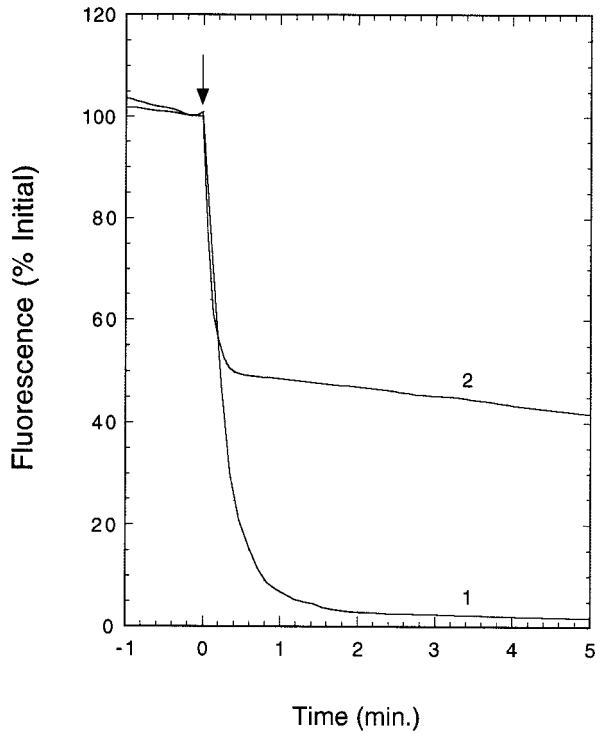

FIGURE 5 Time courses of dithionite reduction of C_6-NBD-galactocerebroside in POPC/cholesterol (100:50 molar proportions) LUV. *Curve 1:* sodium dithionite (10 mM final concentration) was added at time zero to vesicles that had been asymmetrically labeled with the fluorescent lipid by injecting a methanolic solution of the latter into a vesicle suspension (see Materials and Methods). The curve shown was obtained using vesicles incubated for 15 min at 37°C after addition of the fluorescent lipid, but essentially identical results were obtained when the vesicles were incubated for 1 h before dithionite addition. *Curve 2:* dithionite was added to a suspension of vesicles symmetrically labeled with the C_6-NBD-lipid by including the labeled species in the lipid mixture dried down for subsequent preparation of vesicles. The second, slow phase of fluorescence decrease in *curve 2* apparently reflected dithionite permeation into the vesicles rather than transbilayer diffusion of the NBD-labeled lipid, as the slope of this phase was proportional to the dithionite concentration (not shown).

nite to donor vesicles labeled with C_6-NBD-galactocerebroside as described in Materials and Methods caused a rapid reduction in fluorescence by >95%. The same result was observed using similarly labeled donor vesicles that were incubated for 1 h at 37°C after probe incorporation (not shown). By contrast, when donor vesicles are symmetrically labeled with the C_6-NBD-lipid (by including it in the original lipid mixture used to prepare the vesicles), addition of dithionite causes only a partial (≈50%) rapid reduction fluorescence. Taken together, these results indicate that transbilayer diffusion of the C_6-NBD-labeled species is very slow on the time scale of our experiments. Similar results were obtained for all C_6-NBD-labeled lipids studied using POPC/cholesterol and sphingolipid/cholesterol vesicles (data not shown).

The results just noted indicate that for the intervesicle-partitioning experiments described above the relevant effective lipid concentrations L_{eff} in Eq. 4 are the concentrations of donor and acceptor vesicle lipids that are exposed at the outer surface of these vesicles. To determine this quantity, we first prepared donor and acceptor vesicles without C_6-NBD-lipids or Rho-PE, but incorporating 0.5 mol % NBD-di18:0-PE. Because in large vesicles the transbilayer distribution of the different lipid components is expected to be symmetrical (Nordlund et al., 1981), we determined the fraction of the NBD-di18:0-PE that was rapidly reduced by extravesicular dithionite as a measure of the fraction of the total vesicle lipids that was exposed at the vesicles' outer surface (listed in the legend to Fig. 4). From these values and the measured total concentrations of donor and acceptor vesicle lipids, we determined the values of $[L_{eff}]_D$ and $[L_{eff}]_A$ used to analyze our intervesicle-partitioning data according to Eq. 4.

In Table 2 are summarized the measured values of the partition coefficients K_p, describing the relative affinity for acceptor (sphingolipid/cholesterol-rich) over donor (POPC/cholesterol) vesicles, for each of the C_6-NBD-labeled species examined here. Three noteworthy points emerge from these results. First, all of the NBD-labeled species show

TABLE 2 Partitioning of short-chain fluorescent lipids between large unilamellar POPC/cholesterol and sphingolipid/cholesterol vesicles

Acceptor Vesicle	Probe*	$(K_p^{-1})^{\dagger}$	$K_p/K_p(PC)^{\ddagger}$
SM/PC/Ch			
(95:5:50)	C_6-NBD-PC	15.9 ± 1.7	(1)
	C_6-NBD-SM	12.6 ± 1.8	1.27 ± 0.05
	C_6-NBD-GalCer	6.1 ± 0.5	2.62 ± 0.05
	C_6-NBD-GlcCer	7.1 ± 1.1	2.26 ± 0.11
	C_5-Bdpy-PC	7.2 ± 0.3	(1)
	C_5-Bdpy-GlcCer	5.8 ± 0.2	1.25 ± 0.04
	C_5-Bdpy-GM_1	6.9 ± 0.2	1.21 ± 0.03
SM/GalCer/PC/Ch			
(95:5:50)	C_6-NBD-PC	7.9 ± 0.9	(1)
	C_6-NBD-SM	6.1 ± 0.6	1.29 ± 0.03
	C_6-NBD-GalCer	7.8 ± 0.7	1.01 ± 0.02
	C_6-NBD-GlcCer	7.4 ± 1.0	1.08 ± 0.03
	C_5-Bdpy-PC	7.9 ± 0.1	(1)
	C_5-Bdpy-GlcCer	10.6 ± 0.7	0.75 ± 0.03
	C_5-Bdpy-GM_1	11.7 ± 0.4	0.68 ± 0.01

*Additional abbreviations used: SM, sphingomyelin; GalCer, galactocerebroside; GlcCer, glucocerebroside; GM_1, ganglioside GM_1.

$^{\dagger}(K_p^{-1})$ represents the affinity of each probe for donor over acceptor vesicles; increasing values of this parameter thus indicate an increasing preference for the POPC/Rho-PE/cholesterol donor vesicles.

‡Values of K_p (relative affinity for acceptor over donor vesicles) determined for the indicated fluorescent species in each experiment were divided by the value of K_p determined for the analogous fluorescent PC in the same experiment; values of this ratio greater (/lesser) than unity thus indicate that the fluorescent species shows a greater (/lesser) affinity for the acceptor vesicles than does the corresponding fluorescent PC. Table entries represent the mean (± half-range) of values determined in two independent experiments.

only a weak affinity for the l_o-phase (sphingolipid-containing) vesicles in competition with the l_d-phase (POPC/cholesterol) vesicles. Second, using acceptor vesicles in which the sphingolipid fraction is entirely sphingomyelin, the C_6-NBD-gluco- and galactocerebrosides show significantly greater affinity for the acceptor vesicles than do the sphingomyelin and phosphatidylcholine probes. In this system the *relative* affinities of the different C_6-NBD-lipid probes for the (sphingomyelin-containing) l_o-phase vesicles thus resemble those measured for partitioning of the corresponding DPH3:0-lipids into (sphingolipid-rich) l_o-phase domains in sphingomyelin/12SLPC/cholesterol bilayers (see previous section). However, this pattern of relative affinities is not maintained when the acceptor vesicles contain galactocerebroside and sphingomyelin, in which case the NBD-labeled cerebroside probes show affinities for the sphingolipid-containing vesicles that are very similar to those found for the labeled phosphatidylcholine.

The partitioning of C_5-Bodipy-labeled phosphatidylcholine, glucocerebroside, and ganglioside GM_1 between POPC/cholesterol and sphingolipid/cholesterol-rich vesicles was also analyzed as described above. As for the C_6-NBD-lipids discussed above, the C_5-Bodipy-labeled species were assumed to undergo very slow transbilayer diffusion on the time scale of our experiments, based on the previous findings of Bai and Pagano (1997) and our observation that the fraction of C_5-Bodipy-labeled lipids available for rapid exchange from asymmetrically labeled donor to acceptor vesicles did not change significantly when the donor vesicles were first incubated for up to 1 h at 37°C (results not shown). The values of the intervesicle partition coefficients determined by applying Eq. 4 (with the assumption just noted) to data like those shown in Fig. 4 *B* are summarized in Table 2. The two Bodipy-labeled glycosphingolipid probes partition with slightly greater affinity into sphingomyelin/cholesterol-rich acceptor vesicles, in competition with POPC/cholesterol vesicles, than does the phosphatidylcholine probe. However, the magnitude of this difference is relatively small. Moreover, the Bodipy-labeled glycolipid probes show a significantly *weaker* affinity than the corresponding phosphatidylcholine for the sphingolipid-containing acceptor vesicles when these vesicles contain equimolar sphingomyelin and galactocerebroside in place of sphingomyelin alone. In all cases, as for the NBD-labeled species examined above, the absolute values of the partition coefficients determined indicate that all the C_5-Bodipy-labeled species strongly prefer the POPC/cholesterol donor vesicles over the sphingolipid/cholesterol-rich acceptor vesicles.

DISCUSSION

Previous work has established clearly that among the major classes of cellular lipids, sphingolipids exhibit a particular tendency to support the formation of segregated liquid-ordered domains in mixed-lipid bilayers in the presence of cholesterol (Brown, 1998; Brown and London, 1998a,b; Rietveld and Simons, 1998). However, it has been shown as well that saturated long-chain phospholipids also can form l_o-phase domains in model membranes combining these species with unsaturated or shorter-chain phospholipids and cholesterol (Ahmed et al., 1997; Silvius et al., 1996). These findings leave open the question whether the unique headgroup and sphingosine backbone structures of sphingolipids contribute in any unique way to their proclivities to associate with raft structures in biological membranes, or whether these properties are attributable purely to the fact that most membrane sphingolipids are effectively disaturated lipids. The results of our fluorescence-quenching assays using DPH3:0-phospho- and sphingolipids suggest that the sphingosine backbone per se makes only a small contribution to the affinity of sphingolipids for raft domains. However, we also find that the corresponding monogluco- and monogalactocerebrosides show a significantly greater affinity for such l_o-phase domains than do other sphingolipids, indicating that the glycosyl residues of these species contribute significantly to their affinity for raft-like domains. Significantly, this effect is even stronger in sphingomyelin/12SLPC/cholesterol bilayers than in sphingomyelin/cerebroside/12SLPC/cholesterol bilayers, arguing that the effect does not reflect specific interactions between sugar headgroups.

It is interesting to note that DPH3:0-ceramide shows an affinity for l_o-phase domains that is almost identical to that determined for the corresponding sphingomyelin. This finding suggests that ceramide produced by hydrolysis of (or phosphorylcholine exchange from) sphingomyelin in membranes containing sphingolipid-enriched domains could itself become significantly enriched in these domains. The possible relevance of this behavior to ceramide-mediated signaling in the plasma or other membranes is difficult to assess, given our imprecise current understanding of the nature and localization of the pool(s) of sphingomyelin molecules that are converted to ceramide in such signaling events (Levade and Jaffrezou, 1999). However, evidence has been reported for compartmentalized production of ceramide within the plasma membrane in fibroblasts treated with interleukin-1β (Liu and Anderson, 1995).

Our results provide support for the principle that differential partitioning of different sphingolipids into l_o-phase "raft" domains could play a role in the differential cellular trafficking of sphingolipids within animal cells (Simons and van Meer, 1988; van Ijzendoorn and Hoekstra, 1999; Zegers and Hoekstra, 1998). Such sorting has been suggested based on the distinct sphingolipid compositions of the basolateral and apical plasma membrane domains of epithelial cells (Simons and van Meer, 1988), and on observations that glucocerebroside and sphingomyelin derived from exogenously added short-chain ceramides are differentially trafficked to the apical versus the basolateral membrane of MDCK and other epithelial cells in culture (van Ijzendoorn

et al., 1997; van Meer et al., 1987; van't Hof and van Meer, 1990; van't Hof et al., 1992). In various intracellular membranes, sphingolipid-enriched liquid-ordered domains, where present at all, are likely to comprise at most a modest fraction of the total membrane lipid bilayer. Given that both sphingomyelin and cerebroside exhibit a finite solubility in the liquid-disordered (phospholipid-rich) l_d phase in sphingolipid/phospholipid/cholesterol mixtures (Ahmed et al., 1997; Silvius, 1992), in such membranes under physiological conditions a large proportion of such sphingolipids are likely to exist outside of "raft" domains. It is under these conditions that differences in the intrinsic affinities of different sphingolipids for such domains may most strongly affect the intramembrane distributions of these lipids and, potentially, their intracellular trafficking.

As noted above, to date the experimental evidence for lipid sorting based on differential partitioning into sphingolipid/cholesterol-rich membrane domains has largely been derived from studies examining the intracellular trafficking of short-chain fluorescent sphingolipids, and here the implications of our present results are more equivocal. First, all of the short-chain fluorescent lipids examined here exhibit relatively low abilities to partition into a sphingolipid-enriched l_o phase, preferring (l_d-phase) POPC/cholesterol to (l_o-phase) sphingolipid/cholesterol-rich vesicles by factors of 6- to over 15-fold. By contrast, it is clear that in systems like those examined here naturally occurring membrane sphingomyelin and cerebrosides exhibit (l_o/l_d) partition coefficients that are substantially *greater* than unity (favoring association with the liquid-ordered phase by a significant margin) and that therefore exceed by a very large factor those determined for the corresponding short-chain fluorescent species. This finding may reflect the fact that C_6-NBD-, and to a lesser extent C_5-Bodipy-lipids exhibit a looping back of their labeled acyl chain to position the fluorescent group at the membrane interface (Chattopadhyay and London, 1987; Kaiser and London, 1998b). Sphingolipids carrying these acyl chains would therefore occupy a substantially larger molecular area within the membrane than do endogenous membrane sphingolipids, restricting their ability to associate with membrane domains exhibiting the close molecular packing and comparatively high degree of acyl chain ordering that are characteristic of the liquid-ordered phase (Brown, 1998; Rietveld and Simons, 1998).

In view of the conclusion just noted it is perhaps surprising to find that the *relative* affinities of partitioning of various C_6-NBD-labeled sphingolipids and phosphatidylcholine into sphingomyelin/cholesterol-rich bilayers (vesicles), in competition with POPC/cholesterol bilayers (Table 2), are quantitatively similar to those measured for the analogous DPH3:0-labeled species using the fluorescence-quenching assay (Table 1). However, for C_6-NBD-sphingolipids this pattern of partitioning behavior is abolished when cerebroside is included in the sphingolipid-rich l_o phase, in contrast to our findings with DPH3:0-sphingolipids. Taken together, these results suggest that although in some systems differences in partitioning into sphingolipid/cholesterol-rich l_o-phase domains could contribute to differential intracellular trafficking of different short-chain fluorescent sphingolipids (van Meer et al., 1987; van't Hof et al., 1990, 1992; van Ijzendoorn and Hoekstra, 1999), in general such effects cannot be taken to reliably reflect the nature or the extent of sorting of endogenous sphingolipids through the same cellular pathway. Recent work has in fact suggested that differential trafficking of short-chain sphingolipids to the apical and basolateral plasma membrane domains of MDCK cells may rest on factors unrelated to their differential partitioning into sphingolipid-rich domains, and unlikely to be relevant to the differential trafficking of endogenous cellular sphingolipids (van Helvoort et al., 1996).

Exchangeable fluorescent lipids have played a very useful role in elucidating the nature and subcellular localization of various lipid-metabolic and lipid-trafficking processes in animal cells, largely because such lipids appear to be recognized by many of the cellular proteins that mediate biochemical conversions or transmembrane translocation of the corresponding endogenous lipids (Pagano, 1990). It has long been recognized that the ability of short-chain fluorescent lipids to diffuse spontaneously between different membranes is not generally shared by their endogenous lipid counterparts. Our present findings demonstrate the need for an additional caution in interpreting the results of studies of intracellular trafficking of short-chain fluorescent sphingolipids, namely that the abilities of these species to partition into sphingolipid-rich "raft" domains may be quantitatively and even qualitatively different from those of long-chain cellular sphingolipids. Nonetheless, our results also support previous suggestions that the latter species can partition differentially into "raft" domains based on differences in their headgroup structure, and that such effects could thus contribute to the differential trafficking of sphingolipids within eukaryotic cells.

NOTES

1. The limits for the l_o/l_d phase separation noted in the text were deduced by Ahmed et al. (1997) by comparing quenching curves (plots of $(F/F_o)_{cor}$ versus mole fraction of quencher lipid) obtained for diphenylhexatriene in the system sphingomyelin/12SLPC/(33 mol % cholesterol) and in the homogeneous reference system dioleoyl PC/12SLPC/(33 mol % cholesterol). The results of detergent-solubilization assays reported by these authors, and our own measurements of quenching curves for other families of fluorescent probes (Wang et al., 2000), support these assignments of phase boundaries for the sphingomyelin/12SLPC/(33 mol % cholesterol) system.
2. For mixtures containing very high proportions of sphingolipid (and hence of l_o-phase domains), even fluorescent species with very low affinities for the l_o phase may begin to partition to a significant extent into such domains. The quantitative analyses described here were accordingly restricted to data obtained for mixtures containing $\leq$70 mol % sphingolipid in the nonsterol fraction.

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