Partitioning of Lipidated Peptide Sequences into Liquid-Ordered Lipid Domains in Model and Biological Membranes†

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ABSTRACT: We have used a fluorescence assay and detergent fractionation to examine the partitioning of different fluorescent lipidated peptides, with sequences and lipid substituents matching those found in various classes of lipidated cellular proteins, into liquid-ordered (raft-like) domains in lipid bilayers. Peptides incorporating isoprenyl groups, or multiple unsaturated acyl chains, show negligible affinity for liquid-ordered domains in mixed-phase liquid-ordered/liquid-disordered (l_o/l_d) bilayers composed of dipalmitoylphosphatidylcholine, a spin-labeled unsaturated phosphatidylcholine, and cholesterol. By contrast, peptides incorporating multiple *S*- and/or *N*-acyl chains, or a cholesterol residue plus an N-terminal palmitoyl chain, show significant partitioning into liquid-ordered domains under the same conditions. Interestingly, the affinity of a lipidated peptide for l_o domains can be strongly influenced, not only by the structures of the lipid substituents but also by the nature and the positions of their attachment to the peptide chain. These results are well correlated with those obtained from parallel assays based on low-temperature detergent fractionation. Using the latter approach, we further demonstrate that a truly minimal l_o domain partitioning motif [myristoylGlyCys(palmitoyl)-] can mediate efficient incorporation into the "raft" fraction of COS-7 cell membranes.

A number of proteins associated with the plasma membrane are modified with one or more lipid groups directly linked to the polypeptide backbone, including *N*-acyl, *S*-acyl, *S*-isoprenyl, and C-terminally linked cholesteryl residues (*1*−*6*). Recent evidence has suggested that the lipid-modified sequences of such proteins may serve not only to promote membrane anchorage but also to influence the partitioning of the proteins into particular submembrane domains, such as lipid rafts or related structures such as caveolae. Diverse proteins found at the cytoplasmic face of the plasma membrane, and modified with multiple saturated acyl chains, have been shown to be enriched in biochemically isolated membrane "lipid raft" fractions (*7*−*10*) and/or to colocalize with other raft-associated species when the latter are clustered in the plasma membrane (*11*−*15*). The cholesterol-modified Hedgehog protein, which may also be N-terminally palmitoylated (*5*), has similarly been found to be enriched in raft fractions isolated from embryonic *Drosophila* cells (*16*). For some proteins, including GAP-43, the T-cell adaptor protein LAT, and the *src*-homologous tyrosine kinase *fyn*, the membrane-binding and raft-directing properties of such lipid modifications have been shown to be at least partially dissociable (*15*, *17*−*19*).

Much current evidence suggests that the basic structural element of membrane lipid rafts is a cluster of membrane lipids which adopts a liquid-ordered (l_o) organization distinct from that of adjacent liquid-disordered (l_d) regions in the membrane bilayer (*20*−*27*). Lipids bearing saturated acyl chains associate preferentially with l_o domains in model systems, and this property has been suggested to account for the observed enrichment of species such as sphingolipids and glycosylphosphatidylinositol- (GPI-)[1] anchored proteins in raft fractions isolated from animal cells. It has further been suggested that saturated acyl chains or sterol residues coupled to proteins may promote association with membrane rafts by a similar mechanism.

In the present study, we have used a novel fluorescence-based assay (*28*, *29*) to examine directly the affinities of a variety of lipidated peptides, incorporating lipid-modified sequence motifs found in different classes of cellular proteins, for liquid-ordered domains in lipid bilayers that exhibit coexisting l_d and l_o domains. Our results indicate that both the nature of the peptide-coupled lipid groups and their positions of attachment to the peptide can exert important influences on affinity for l_o domains. We further show that, for these lipidated peptides (in contrast to some previously studied lipid species), our direct fluorescence assay and the

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[1] Abbreviations: DMF, *N*,*N*-dimethylformamide; DOPC, 1,2-dioleoylphosphatidylcholine; 12-doxyl-PC, 1-palmitoyl-2-(12-doxylstearoyl)-phosphatidylcholine; DPPC, 1,2-dipalmitoylphosphatidylcholine; (E)-GFP, (enhanced) green fluorescent protein; EDTA, ethylenediaminetetraacetic acid trisodium salt; GPI, glycosylphosphatidylinositol; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TEMPO-DOPC, 1,2-dioleoylphosphatidyl-*N*,*N*-dimethyl-*N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)ethanolamine; TLC, thin-layer chromatography. Peptides are designated using standard three-letter amino acid designations plus the following additional abbreviations: Bimta, *S*-(bimanylthio)acetyl; Boc, *N*-*tert*-butyloxycarbonyl; caBim, 2-(*S*-bimanylthio)ethylamide; Fmoc, 9-fluorenylmethoxycarbonyl; StBu, *S*-(*tert*-butylmercapto).

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commonly used low-temperature detergent solubilization approach provide very similar pictures of the relative affinities of different lipidated sequences for l_o domains. Finally, we demonstrate that a lipidated sequence motif as minimal as myristoylGlyCys(palmitoyl)- can associate efficiently with raft domains in mammalian cells.

MATERIALS AND METHODS

Materials. DPPC, DOPC, and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). TEMPO-DOPC was synthesized as described previously (*28*). [9,10-^{3}H(n)]-Palmitic acid (specific activity 60 mCi/μmol) was purchased from Amersham Pharmacia (Baie d'Urfé, Québec). Cell culture media were obtained from Life Technologies (Burlington, Ontario). All solvents and common chemicals used were of reagent grade or better. A sequence encoding the EGFP-derived chimeric protein lck-EGFP was prepared by standard polymerase chain reaction methods, using as template the plasmid pEGFP-C1 (Clontech) and the primers GGGGGGTACCATGGGCTGTGTCTGCAGCTCAAACCC-TGAAGGATCCGTGAGCAAGGGC and GGGGTCTA-GATTACTTGTACAGCTCGTCCATG, and cloned between the *Kpn*I and *Xba*I sites of the plasmid pcDNA3 (Invitrogen). A sequence encoding the construct lckSS-EGFP, in which the sequence underlined in the first primer above was altered to -TCTGTCTCC- to convert two cysteine residues to serines, was prepared by the polymerase chain reaction from the lck-EGFP-encoding plasmid and similarly cloned into pcDNA3.

N/S-diacylated and prenylated/S-acylated peptides were synthesized as described previously (*29*−*31*). Species with the general structure acylCys(acyl)Gly-caBim were prepared using similar methods, first coupling *S*-bimanylcysteamine to Fmoc-glycine and then coupling the product (after TLC purification and N-deprotection with DMF/piperidine) to *N*-Boc-*S*-tritylcysteine. The purified BocCys(trityl)Gly-caBim intermediate was N/S-deprotected in 63/35/5/2 CH_2Cl_2/CF_3-COOH/dimethyl sulfide/triethylsilane (25 °C, 1 h) and then reacted with 5 equiv of the appropriate acyl chloride in dry 9:1 (v/v) CH_2Cl_2/pyridine (*30*) to give the species acylCys-(acyl)Gly-caBim.

AcetylGlyCys(StBu)Cys(StBu)Gly-OH was prepared by solid-phase peptide synthesis using standard Fmoc chemistry, coupled to *S*-bimanylcysteamine (*30*), and then S-deprotected by incubation for 6 h at 37 °C, in the dark and under argon, with 10 equiv each of 1,4-butanedithiol and sodium 2-mercaptoethanesulfonate in 70/15/15 DMF/CH_2Cl_2/0.5 M aqueous HEPES, pH 7.6. The crude deprotected peptide was three times precipitated with diethyl ether from a minimum volume of methanol, then incubated overnight under high vacuum, and S-acylated by reacting for 2 h at 25 °C with 6 equiv each of the appropriate acyl chloride and dry pyridine in 9:1 (v/v) dry CH_2Cl_2/dry DMF (*30*). The recovered acyl peptides were purified by preparative TLC on silica gel 60 plates, developing with 90:10 (v/v) CH_2Cl_2/CH_3OH.

The cholesterol-anchored peptides Bimta-Gly-cholesterol and palmitoylXaaCys(bimanyl)Gly-cholesterol (Xaa = Ala or Cys) were prepared as follows. FmocGly-cholesterol was synthesized by stirring 1 equiv each of Fmoc-glycine and cholesterol (previously dried under high vacuum) overnight at 25 °C and under argon with 1.1 equiv of dicyclohexylcarbodiimide in 5:5:1:0.1 (v/v/v/v) CH_2Cl_2/acetonitrile/dimethylformamide/pyridine. The reaction mixture was partitioned between CH_2Cl_2 and 1:1 methanol/0.1 M HCOONa, pH 3.0, and the products were recovered by concentrating the CH_2Cl_2 phase and then purified by preparative TLC (solvent 199:1 CH_2Cl_2/methanol, R_f of FmocGly-cholesterol ≈ 0.6). The product was N-deprotected with 80:20 (v/v) DMF/piperidine (25 °C, 30 min) and dried thoroughly. Bimta-Gly-cholesterol was synthesized by coupling a portion of the deprotected glycylcholesterol to the *N*-hydroxysuccinimidyl ester of *S*-bimanylmercaptoacetic acid (*32*). Other cholesterol-conjugated peptides were synthesized by coupling glycylcholesterol to the appropriate protected amino acids in successive solution reactions, using methods described previously (*30*).

Fluorescence Assay of Peptide l_o/l_d Domain Partitioning. Lipid samples (75 nmol total lipid, containing 0.6 mol % fluorescent peptide) were prepared by combining lipids, cholesterol, and lipidated peptides from stock solutions in 2:1 (v/v) CH_2Cl_2/CH_3OH and then drying under nitrogen with warming to 45−50 °C. The mixtures were incubated under high vacuum for a minimum of 6 h and then redissolved at 37 °C in 30 μL of ethanol. Buffer (2.97 mL of 100 mM KCl, 10 mM potassium phosphate, 0.1 M EDTA, pH 5.0) preheated to 37 °C was added to the ethanolic lipid/peptide solutions while vortexing, and the resulting samples were incubated successively at 45 °C for 2 min and at 37 °C for 15 min before cooling at <0.3 °C/min to 25 °C. After overnight incubation at the latter temperature, the fluorescence of the samples was measured at 25 °C using a Perkin-Elmer LS-5 spectrofluorometer (390/468 nm, slit widths 15/20 nm). The samples were then mixed with 75 μL of 20% Triton X-100, heated to 45 °C for 2 min and to 65 °C for 15 s, and then bath-sonicated for 10 s and reincubated at 45 °C for 5 min before cooling to 25 °C and remeasuring the fluorescence. From these data normalized fluorescence values F_N were calculated by dividing the (blank-corrected) fluorescence of each sample by the (blank-corrected) fluorescence measured for the same sample after Triton solubilization. Most of the data described in this study are presented in the alternative scaled form $(F/F_o)_{cor}$ originally suggested by London and Feigenson (*33*):

$$(F/F_o)_{cor} = (F_N - F_N(100\%\ Q))/(F_N(0\%\ Q) - F_N(100\%\ Q)) \quad (1)$$

where $F_N(0\%\ Q)$ and $F_N(100\%\ Q)$ are the normalized fluorescence values obtained for a given fluorescent species in bilayers where the quencher lipid comprises 0 mol % or 100 mol % of the phospholipid fraction, respectively.

Quenching curves [plots of $(F/F_o)_{cor}$ vs content of quencher lipid in the bilayer], determined as just described for different lipidated peptides, were analyzed quantitatively by fitting to a function of the form

$$(F/F_o)_{cor} = (F/F_o)_{cor}^{ld}\left(\frac{\%\ Q - \%\ Q_{lo}}{K_p(\%\ Q_{ld} - \%\ Q) + (\%\ Q - \%\ Q_{lo})}\right) + (F/F_o)_{cor}^{lo} K_p\left(\frac{\%\ Q_{ld} - \%\ Q}{K_p(\%\ Q_{ld} - \%\ Q) + (\%\ Q - \%\ Q_{lo})}\right) \quad (2)$$

Table 1: Relative Affinities of Partitioning of Different Lipidated Peptides between l_o and l_d Domains in DPPC/TEMPO-DOPC/33 mol % Cholesterol Bilayers at 25 °C[a]

peptide	relative affinity for l_o domains [myrGC(palm)G-caBim = 1.00]
myr**GC**(palm)**GC**(palm)**G**-caBim	1.20 ± 0.34
palm**GC**(palm)**G**-caBim	2.03 ± 0.51
myr**GC**(palm)**G**-caBim	(1.00)
14:1c**GC**(palm)**G**-caBim	0.20 ± 0.03
14:1c**GC**(14:1c)**G**-caBim	0.012 ± 0.005
Ac**GC**(palm)**C**(palm)**G**-caBim	0.18 ± 0.03
Ac**GC**(14:1c)**C**(14:1c)**G**-caBim	0.016 ± 0.009
palm**C**(palm)-caBim	0.125 ± 0.027
14:1c**C**(14:1c)**G**-caBim	(0.00)
palm**CC**(bimanyl)**G**-cholesterol	0.136 ± 0.025
palm**AC**(bimanyl)**G**-cholesterol	0.125 ± 0.027
Bimta-**G**-cholesterol	0.039 ± 0.013
Bimta-**GC**(palm)**GC**(farnesyl)-OMe	0.002 ± 0.003
Bimta-**GC**(palm)**GC**(geranylgeranyl)-OMe	0.009 ± 0.003
Bimta-**GC**(14:1c)**GC**(farnesyl)-OMe	0.006 ± 0.006
Bimta-**GC**(18:0)**GC**(hexadecyl)-OMe	0.140 ± 0.012

[a] Tabulated values were determined from experimental quenching curves as described in Materials and Methods using data obtained in two to four independent experiments. Error limits were calculated using the estimated standard errors from curve fitting and standard propagation of error methods. For brevity the following special abbreviations are used: Ac = acetyl, myr = myristoyl, palm = palmitoyl, 14:1c = myristoleoyl, and 18:0 = stearoyl.

where % Q is the molar percentage of TEMPO-DOPC in the phospholipid fraction, % Q_{lo} and % Q_{ld} are the values of % Q defining the l_o-only and the l_d-only limits of the region of l_o/l_d domain segregation (estimated as 5 mol % and 90 mol % TEMPO-DOPC in the phospholipid fraction for DPPC/TEMPO-DOPC/33 mol % cholesterol mixtures at 25 °C), and K_p, $(F/F_o)_{cor}^{ld}$, and $(F/F_o)_{cor}^{lo}$ here serve as empirical fitting parameters (*29*). The relative affinities of different lipidated peptides for l_o domains (summarized in Table 1) were estimated from the calculated slopes of the fitted curves, evaluated for % Q = % Q_{ld}, as described previously (*29*). The relative amounts of different lipidated peptides (X and Y) present in l_o domains (f_{lo}) in bilayers composed of equimolar DPPC, TEMPO-DOPC, and cholesterol (Figure 5) were calculated using the equation

$$\frac{f_{lo}(X)}{f_{lo}(Y)} = \frac{(F/F_o)_{cor}(X) - (F/F_o)_{cor}(ref)}{(F/F_o)_{cor}(Y) - (F/F_o)_{cor}(ref)} \quad (3)$$

where the indicated values of $(F/F_o)_{cor}$ at this composition were calculated from the fitted quenching curves for each species and where the species (ref) is a lipidated peptide that shows negligible partitioning into l_o domains (*29*). Several peptides modified with two unsaturated chains gave nearly identical quenching curves that suggested negligible affinity for l_o domains (see Figures 2B and 3); of these, the peptide myristoleoylCys(myristoleoyl)Gly-caBim was chosen as the species (ref) for the above calculations.

Detergent Fractionation of Lipopeptide-Incorporating Liposomes. Lipid/lipopeptide mixtures (375 nmol total lipid, incorporating 1 mol % lipopeptide), prepared and dried from solvent as described above, were redissolved in 50 μL of ethanol at 37 °C and rapidly diluted into 4.95 mL of buffer at 37 °C as described for the above fluorescence experiments. The samples were incubated successively at 45 °C, at 37 °C, and at 25 °C as for the above fluorescence experiments, then mixed at 0 °C with 250 μL of 20% Triton, incubated at this temperature for 20 min, and finally centrifuged for 4 h at 250000*g* in a Beckmann SW55.1 ultracentrifuge rotor at 4 °C. Peptide fluorescence in the resulting pellet and supernatant fractions was determined as described previously (*29*).

Analysis of EGFP Chimeras and S-Acylated Peptides in "Raft" Fractions. Conditions used for culture and transfection of COS-7 cells were as described previously (*34*, *35*). Cells expressing EGFP-derived chimeric proteins were fractionated with Triton X-100 according to the procedure of Zlatkine et al. (*36*) with minor modifications. Briefly, cells harvested from one 100 mm dish of transfected cells were resuspended in 1 mL of 150 mM NaCl, 50 mM Tris-HCl, 2 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 5 mM iodoacetamide, and 10 μg/mL each soybean trypsin inhibitor, leupeptin, and aprotinin, then chilled to 0 °C, mixed with Triton to 1% (w/v), and incubated at this temperature for 20 min. The samples were centrifuged at 250000*g* for 45 min at 4 °C. After the supernatants were recovered, the pellets were resuspended in 1 mL of buffer at 37 °C, mixed with Triton X-100 to 1%, bath-sonicated to ensure complete dispersal (10−15 s), incubated for 15 min at 37 °C, and then recentrifuged as above but at 30 °C. The Triton-soluble supernatants from the two incubations were analyzed by SDS−PAGE and Western blotting with anti-EGFP antibody (a generous gift of Dr. Luc Berthiaume, University of Alberta) as described previously (*35*).

To examine the potential raft association of S-acylated peptides, COS-7 cells grown to confluency in a 100 mm culture dish were incubated for 4 h at 37 °C in serum-free medium with the peptides myristoylGlyCysGly-caBim or Bimta-GlyCysGlyCys(farnesyl)-OMe (50 μM) and [^{3}H]-palmitic acid (120 μCi); the cells were then fractionated by successive incubations with Triton X-100 at 0 and 37 °C as described above. The fractions solubilized at the two temperatures were mixed with 1 volume of methanol and 2 volumes of methylene chloride and mixed thoroughly. After phase separation the lower phase was recovered and the extraction twice repeated. The pooled methylene chloride extracts were concentrated under nitrogen, mixed with 10 nmol of S-acylated peptide standard and separated by two-dimensional TLC, and the S-acylated peptide radioactivity was quantitated as described previously (*34*). Autoradiography of TLC chromatograms of extracts from cells incubated with the myristoyl or farnesyl peptide and [^{3}H]palmitate showed well-resolved radioactive spots which in each case comigrated exactly with the fluorescent internal standard, was lost upon treatment with neutral hydroxylamine, and was absent in extracts prepared from cells incubated with radiolabeled palmitate alone.

RESULTS

Fluorescence Quenching Assays. As we and others have shown previously, quenching measurements can be used to examine the partitioning of a fluorescent lipid or peptide between distinct domains in lipid bilayers when the bilayers incorporate a fluorescence quencher that is unequally distributed between the coexisting domains (*28*, *29*, *33*, *37*). In the present study we have examined the distributions of

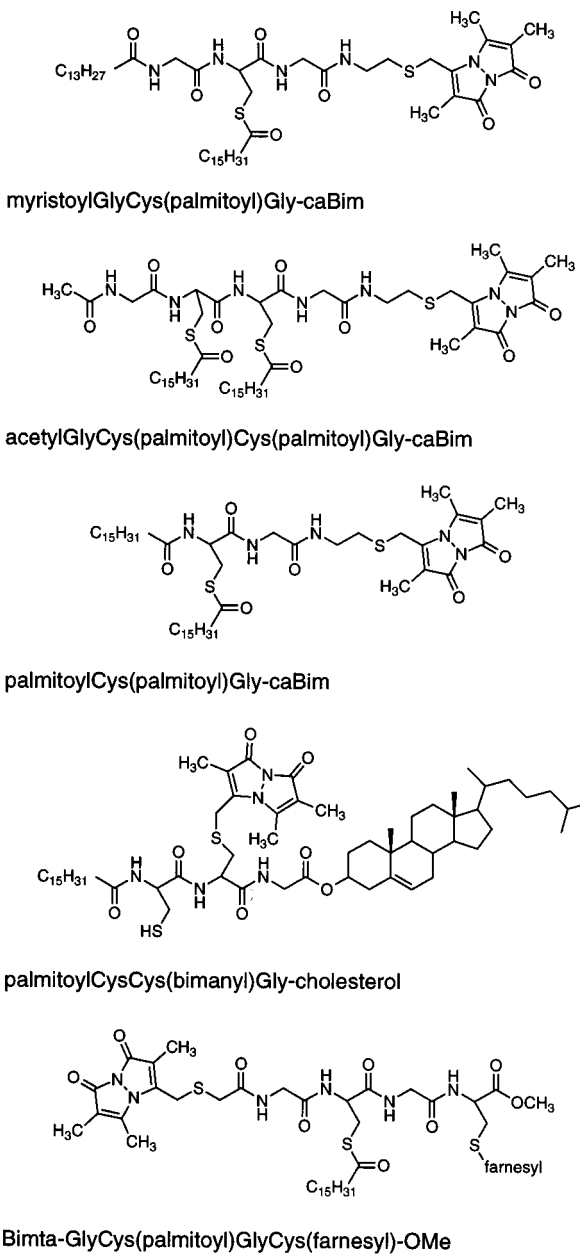

FIGURE 1: Representative structures of fluorescent lipidated peptides used in this study.

various bimane-labeled lipidated peptides (representative structures shown in Figure 1) between coexisting liquid-ordered (l_o) and liquid-disordered (l_d) domains in bilayers combining cholesterol (33 mol %) with DPPC and the spin-labeled quencher species TEMPO-DOPC. At 25 °C, mixed lipid bilayers incorporating these lipid components exhibit coexistence of l_o and l_d domains over a wide range of compositions ranging from ca. 5 mol % to 90 mol % TEMPO-DOPC in the phospholipid fraction, with the spin-labeled lipid and DPPC enriched in the l_d and in the l_o domains, respectively (*29*).

When the fluorescence of a labeled lipidated peptide in systems such as the above is measured as a function of the proportion of the quencher lipid (here, TEMPO-DOPC) in the bilayer, a quenching curve can be plotted as illustrated in Figure 2A. The form of the quenching curve for a given fluorescent species in the region of l_o/l_d domain segregation reflects the relative affinity of that species for l_o vs l_d domains (*21*). For systems such as the DPPC/TEMPO-DOPC/cholesterol bilayers examined here, if the fluorescent probe partitions exclusively into l_d domains, the quenching curve will exhibit a strong upward concavity within the region of domain coexistence, as illustrated by the lowest curve in Figure 2A. However, for fluorescent species that show increasing affinity for l_o domains, the quenching curve gradually changes in form as illustrated by the progression from the lowest to the uppermost curve in the figure.

Two significant modifications of our previous experimental methodology (*29*) were required to examine the l_o/l_d domain partitioning of lipidated peptides using the approach just outlined. First, TEMPO-DOPC was used in place of the 12-doxyl-PC quencher used previously, since the TEMPO-labeled PC quenched the fluorescence of the fluorescent peptides much more efficiently. The two spin-labeled phosphatidylcholines show very similar segregation of l_o and l_d domains when combined with DPPC and cholesterol (*29*). Second, an ethanol dilution method was used to generate lipid vesicles incorporating the lipidated peptide probes. This procedure allowed consistent and homogeneous incorporation of the lipidated peptides into the vesicles (as assessed by the low scatter and the reproducibility of the quenching curves obtained), which for many peptides was difficult to achieve by simply dispersing dried or lyophilized lipid/peptide mixtures in buffer.

To confirm the reliability of the modified experimental approach just described, we first determined the quenching curves for a set of previously studied prenylated peptides, using DPPC/TEMPO-DOPC/33 mol % cholesterol vesicles prepared by the ethanol dilution method. As shown in Figure 2B, the quenching curves obtained at 25 °C in this system for the peptides Bimta-GlyCys(palmitoyl)GlyCys(farnesyl)-OMe, Bimta-GlyCys(palmitoyl)GlyCys(geranylgeranyl)-OMe, and Bimta-GlyCys(myristoleoyl)GlyCys(farnesyl)-OMe are essentially superimposable and show very strong upward concavity. By contrast, the quenching curve obtained for the lipidated peptide Bimta-GlyCys(stearoyl)GlyCys-(hexadecyl)-OMe, which carries two long saturated hydrocarbon chains, is markedly different in form and indicates a considerably higher affinity for l_o domains. These results agree very well with those obtained previously for the same lipidated peptides in DPPC/12-doxyl-PC/33 mol % cholesterol vesicles prepared by a solvent evaporation/rehydration protocol (*29*).

As discussed later, quenching curves such as those shown in Figure 2B can be compared quantitatively when the different fluorescent species show comparable efficiencies of quenching as a function of the local concentration of spin-labeled lipid (*29*). To assess this latter point, we examined the quenching curves for the different fluorescent peptides studied here in homogeneous DOPC/TEMPO-DOPC/33 mol % cholesterol bilayers. As illustrated in Figure 3, the quenching curves obtained in this system for different lipidated peptides are virtually superimposable. [Plots of the unscaled data (F_N) for the same peptides are also quite similar though not identical, as shown in the figure inset.] Such results confirm that, for the various bimane-labeled lipidated peptides examined in this study, the efficiencies of fluorescence quenching vary in a nearly identical manner with the local concentration of the quencher lipid in the bilayer.

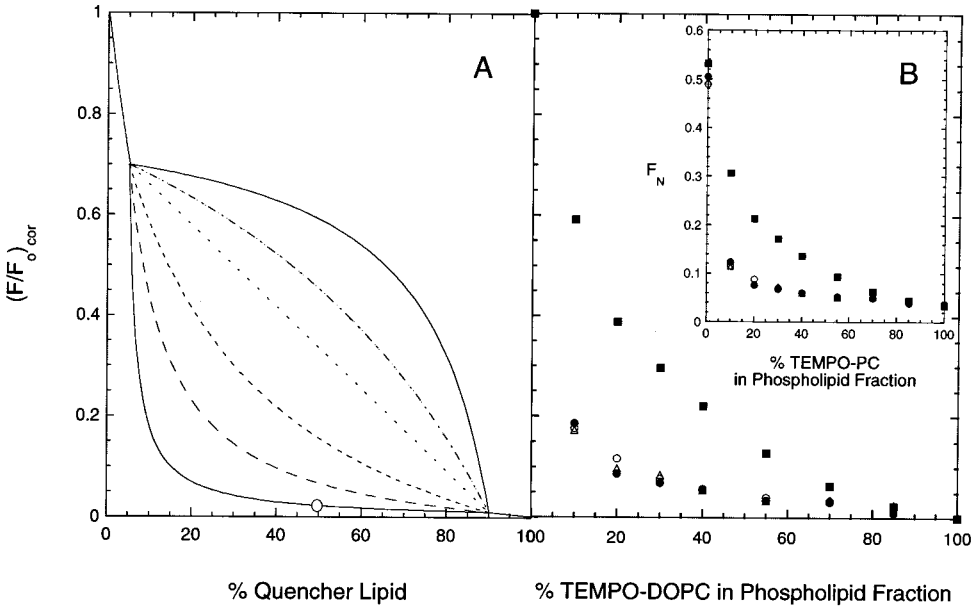

FIGURE 2: Panel A: Hypothetical quenching curves for related fluorescent species with varying affinities for l_o vs l_d domains in a ternary system exhibiting l_o/l_d domain segregation like the DPPC/TEMPO-DOPC/33% cholesterol system examined in this study. The lower and upper solid curves represent hypothetical quenching curves for fluorescent species that partition wholly into the l_d or the l_o domains, respectively. The dashed curves represent quenching curves that would be obtained in the same system for species showing progressively higher affinities for l_o over l_d domains. Panel B: Quenching curves determined at 25 °C for DPPC/TEMPO-DOPC/33 mol % cholesterol bilayers incorporating (●) BimtaGlyCys(palmitoyl)GlyCys(farnesyl)-OMe, (○) Bimta-GlyCys(palmitoyl)GlyCys(geranylgeranyl)-OMe, (△) Bimta-GlyCys(myristoleoyl)GlyCys(farnesyl)-OMe, or (■) Bimta-GlyCys(stearoyl)GlyCys(hexadecyl)-OMe. Quenching curves were determined as described in the text. Panel B insert: Quenching curves for the same fluorescent species as shown in panel B but plotting the normalized fluorescence F_N as the *y*-axis variable. The quenching curves shown are from a single experiment; very similar results were obtained in a replicate experiment.

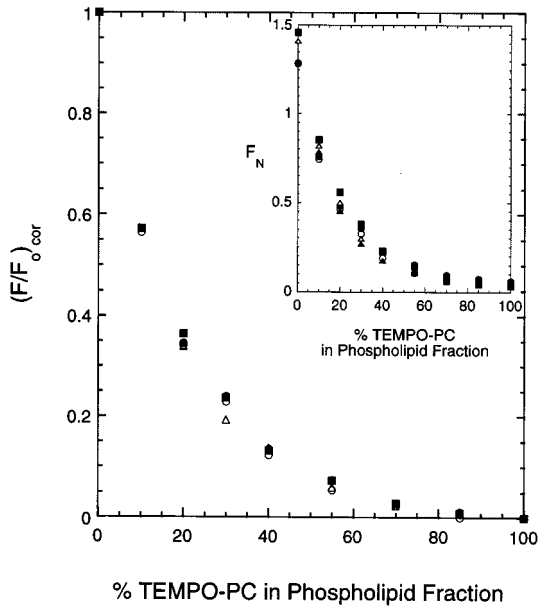

FIGURE 3: Quenching curves determined at 25 °C for DOPC/TEMPO-DOPC/33 mol % cholesterol bilayers incorporating (●) palmitoylAlaCys(bimanyl)Gly-cholesterol, (○) Bimta-Gly-cholesterol, (△) palmitoylCys(palmitoyl)Gly-caBim, (▲) myristoylGlyCys(palmitoyl)Cys-caBim, or (■) acetylGlyCys(palmitoyl)Cys-(palmitoyl)Gly-caBim. Inset: results obtained using the same species plotted as the normalized fluorescence F_N rather than the scaled fluorescence $(F/F_o)_{cor}$.

In Figure 4 are shown quenching curves obtained for a variety of bimane-labeled lipidated peptides in the DPPC/TEMPO-DOPC/33 mol % cholesterol system at 25 °C. As we have noted previously (*29*), the essentially identical quenching curves obtained for the peptides Bimta-GlyCys-(palmitoyl)GlyCys(farnesyl)-OMe and Bimta-GlyCys(myristoleoyl)GlyCys(farnesyl)-OMe indicate that both of these species show negligible affinity for l_o domains, since otherwise the replacement of a long saturated palmitoyl chain by an unsaturated myristoleoyl chain should markedly alter the l_o-domain partitioning of the peptide and hence its quenching curve. Very similar quenching curves are observed for other peptides bearing multiple unsaturated and/or prenyl chains, indicating that these species likewise show very low affinities for l_o domains. By contrast, the quenching curves determined for peptides modified with multiple saturated acyl chains (*N*/*S*-diacyl or di-*S*-acyl) indicate that these species all exhibit significant affinities for l_o domains.

As noted above, sets of quenching curves such as those presented in Figure 4 can be analyzed to determine quantitatively the relative affinities of the different fluorescent species for l_o vs l_d domains, using the curve fitting approach outlined in Materials and Methods (for further details see ref *29*). The results of this analysis are summarized in Table 1. Peptides bearing the N-terminal myristoyl-GlyCys(palmitoyl)- motif found in proteins including p56^{lck} and homologous kinases of the *src* family, as well as the α-subunits of the $G_{i/o}$ heterotrimeric G-proteins, show the highest affinities for l_o domains, as does a related peptide bearing the palmitoylGlyCys(palmitoyl)- motif recently found to occur at the N-terminus of $G_{\alpha s}$ (C. Kleuss, personal communication). A peptide bearing a -Cys(palmitoyl)Cys-(palmitoyl)- motif, as is found in proteins such as GAP-43 (*17*), shows a considerably lower, though still significant, affinity for l_o domains. The same is true for a peptide with a nonphysiological N/S-dipalmitoylated structure [palmitoylCys(palmitoyl)Gly-caBim] and for the peptide Bimta-

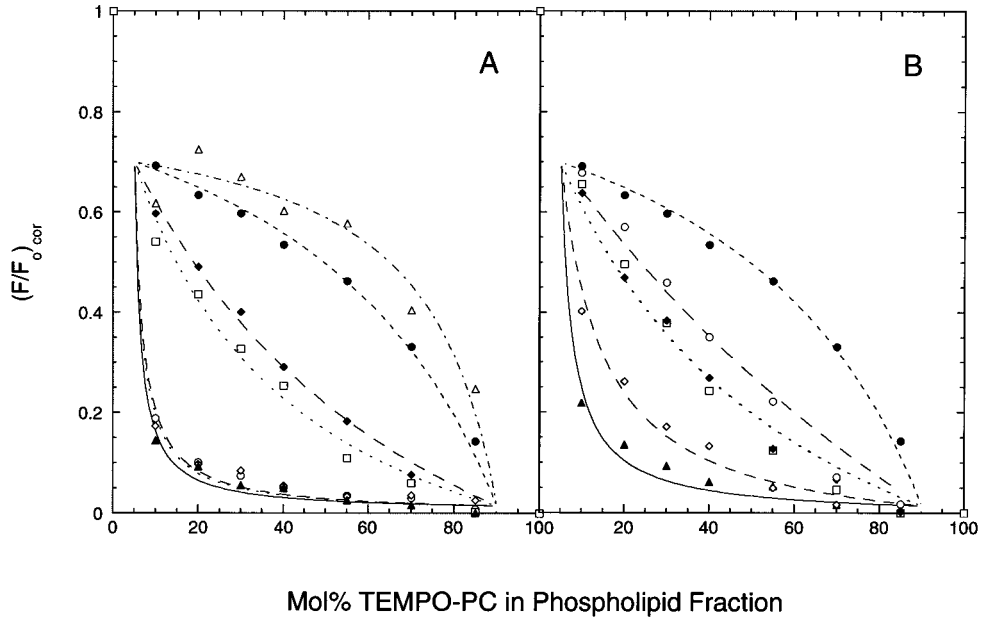

FIGURE 4: Quenching curves determined at 25 °C for DPPC/TEMPO-DOPC/33 mol % cholesterol bilayers incorporating various fluorescent peptides. Panel A: Quenching curves for (▲) myristoleoylCys(myristoleoyl)Gly-caBim, (◇) Bimta-GlyCys(myristoleoyl)GlyCys(farnesyl)-OMe, (○) Bimta-GlyCys(palmitoyl)GlyCys(farnesyl)-OMe, (□) palmitoylCys(palmitoyl)Gly-caBim, (◆) acetylGlyCys(palmitoyl)Cys-(palmitoyl)Gly-caBim, (●) myristoylGlyCys(palmitoyl)Gly-caBim, or (△) palmitoylGlyCys(palmitoyl)Gly-caBim. Panel B: Quenching curves for (▲) myristoleoylCys(myristoleoyl)Gly-caBim, (◇) Bimta-Gly-cholesterol, (□) palmitoylAlaCys(bimanyl)Gly-cholesterol, (◆) palmitoylCysCys(bimanyl)Gly-cholesterol, (○) myristoleoylGlyCys(palmitoyl)Gly-caBim, and (●) myristoylGlyCys(palmitoyl)Gly-caBim.

GlyCys(stearoyl)GlyCys(hexadecyl)-OMe, which carries two saturated chains coupled in the same manner (and at the same positions) as the *S*-palmitoyl and *S*-farnesyl residues coupled to the carboxy terminus of H-ras. It is clear from these results that affinity for l_o domains can be strongly influenced not only by the nature but also by the positions of attachment of the peptide-linked lipid substituents. Interestingly, the peptide myristoleoylGlyCys(palmitoyl)Gly-caBim also shows a significant affinity for l_o domains, consistent with our previous finding that lipids bearing one saturated and one monounsaturated acyl chain can partition to a measurable extent into l_o domains (*29*).

The processed forms of the morphogenetic signaling protein Hedgehog and its homologues are ester-linked to cholesterol via their carboxy-terminal glycine residue (*38*). At least one such species, Sonic Hedgehog, is further modified by an *N*-palmitoyl group amide-linked to the protein's N-terminal cysteine residue (*5*). To model the possible contributions of these lipid modifications to the raft affinity of such proteins, we also examined the behavior of cholesterol-modified peptides in DPPC/TEMPO-DOPC/33 mol % cholesterol bilayers. The quenching curve obtained for the model peptide palmitoylCysCys(bimanyl)Gly-cholesterol indicates a significant affinity for l_o domains (roughly two-thirds that observed for a di-S-palmitoylated peptide), as shown in Figure 4B and Table 1. To ensure that the behavior of this species was not affected by possible oxidation of the N-terminal cysteine residue, we also examined the partitioning behavior of the related species palmitoylAlaCys(bimanyl)Gly-cholesterol, which gave a very similar result. By contrast, the quenching curve determined for Bimta-Gly-cholesterol indicates a considerably lower affinity of this species for l_o domains.

Detergent Fractionation Assays. The methods most frequently used to isolate raft fractions from mammalian cells exploit the relative resistance of liquid-ordered membrane domains to low-temperature solubilization by detergents such as Triton X-100 (*39*). It was thus of interest to determine how faithfully low-temperature detergent insolubility would correlate with partitioning into l_o domains for the lipidated peptides examined here. To investigate this question, various lipidated peptides were incorporated into DPPC/DOPC/cholesterol (1:1:1 molar proportions) vesicles as described above, and the vesicles were subjected to low-temperature fractionation in the presence of Triton X-100 as described in Materials and Methods. In Table 2 we summarize the relative extents of recovery of different lipidated species in the detergent-insoluble fraction in these experiments. It is evident that lipidated peptides that show substantial affinities for liquid-ordered domains in the fluorescence quenching assay (e.g., species modified with multiple saturated acyl chains) also show substantial association with the low-temperature detergent-insoluble fraction. A similar pattern of results was obtained in a limited number of experiments using 1:1:1 DPPC/TEMPO-DOPC/cholesterol vesicles (not shown). By contrast, the different peptides were completely solubilized by cold Triton when incorporated into 2:1 DOPC/cholesterol vesicles, with the exception of N/S-diacylated peptides bearing two saturated acyl chains, a small proportion of which (<20%) could be pelleted under these conditions (not shown).

The correlation just noted was assessed more quantitatively by using our fluorescence quenching results to estimate the relative fractions of different fluorescent species that are partitioned into l_o domains in 1:1:1 DPPC/TEMPO-DOPC/cholesterol bilayers, as described in Materials and Methods. In Figure 5 we have plotted this quantity alongside the relative fractions of the same peptides that were recovered in the Triton-insoluble fraction in the detergent fractionation experiments described above. To facilitate comparison, the

Table 2: Distributions of Lipidated Peptides between Soluble and Insoluble Fractions after Incubation of 1:1:1 DPPC/DOPC/Cholesterol Vesicles with Triton X-100 at 0 °C[a]

peptide	% recovered in Triton-insoluble fraction[b]
palm**GC**(palm)**G**-caBim	57.2 ± 7.4
myr**GC**(palm)**G**-caBim	53.6 ± 2.9
myr**GC**(palm)**GC**(palm)**G**-caBim	52.6 ± 6.7
palm**C**(palm)**G**-caBim	20.7 ± 3.0
14:1c**C**(14:1c)**G**-caBim	2.5 ± 1.7
Ac**GC**(palm)**C**(palm)**G**-caBim	18.6 ± 3.8
Ac**GC**(14:1c)**C**(14:1c)**G**-caBim	3.2 ± 0.9
palm**CC**(bimanyl)**G**-cholesterol	23.1 ± 6.9
palm**AC**(bimanyl)**G**-cholesterol	13.3 ± 2.1
Bimta-**G**-cholesterol	3.6 ± 0.9
Bimta-**GC**(palm)**GC**(farnesyl)-OMe	3.0 ± 0.8
Bimta-**GC**(palm)**GC**(geranylgeranyl)-OMe	2.6 ± 1.7
Bimta-**GC**(14:1c)**GC**(farnesyl)-OMe	2.7 ± 0.6

[a] Vesicles composed of 1:1:1 (molar proportions) DPPC/DOPC/cholesterol and incorporating 0.6 mol % of different lipidated peptides were incubated with 1% Triton X-100 at 0 °C and then ultracentrifuged to separate the soluble and insoluble fractions. The proportions of the lipidated peptides in the two fractions were then determined by fluorescence. Further details of these experiments are described in Materials and Methods. Special abbreviations are as in Table 1. [b] Values listed as mean ± standard error of the mean, calculated from the results obtained in three to eight separate experiments.

two sets of results have been normalized to the value obtained in each type of experiment for the peptide acetylGlyCys-(palmitoyl)Cys(palmitoyl)Gly-caBim. An excellent correlation can be seen between the degree of l_o-domain partitioning and the degree of low-temperature detergent insolubility observed for the different peptides.

Raft Association of N-Myristoylated/S-Acylated GFP Chimeras and Peptides in COS-7 Cells. The above results suggest that a lipidated sequence as minimal as myristoyl-GlyCys(palmitoyl)- can direct association of proteins with liquid-ordered domains and, hence by extension, with membrane lipid rafts in mammalian cells. We tested this prediction in two ways using cultured COS-7 cells. First, using enhanced green fluorescent protein- (EGFP-) based chimeras, we confirmed previous reports that an amino acid sequence incorporating a short N/S-acylation motif can direct an otherwise soluble protein to associate with membrane lipid rafts. To this end we transfected COS-7 cells to express a chimeric protein (*40*), fusing the sequence myristoylG<u>C</u>V<u>C</u>-SSNPEG- from the tyrosine kinase *lck* to the N-terminus of EGFP (lck-EGFP) or a related construct in which the two underlined cysteine residues were mutated to serine residues (lckSS-EGFP) to abolish palmitoylation but not membrane association. Cells expressing the two constructs were incubated successively at 0 °C and at 37 °C with 1% Triton X-100 (*36*), and the proteins solubilized by each incubation were analyzed by SDS−PAGE and Western blotting with anti-GFP antibody. As illustrated in Figure 6A, a substantial fraction of the lck-EGFP chimera (61 ± 8% of total expressed chimera in four independent experiments) was raft-associated as assessed by Triton insolubility at 0 °C and solubilization by Triton at 37 °C.[2] By contrast, all of the control lckSS-EGFP construct was found in the low-temperature Triton-soluble fraction. These findings are consistent with those reported previously for chimeric

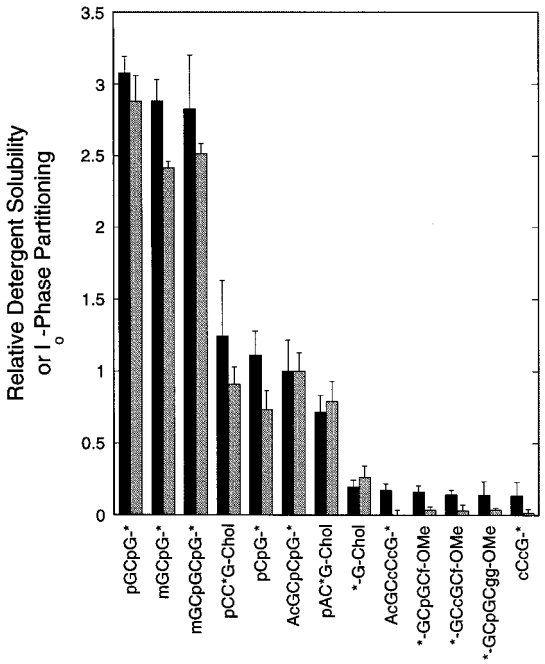

FIGURE 5: Comparison of the relative proportions of different lipidated peptides recovered in the 0 °C Triton-insoluble fraction isolated from 1:1:1 DPPC/DOPC/cholesterol vesicles (black bars) and the estimated relative fractions of the same species partitioning into l_o domains in 1:1:1 DPPC/TEMPO-DOPC/cholesterol vesicles at 25 °C (gray bars). The former values were determined from the results given in Table 2, and the latter were calculated from experimental quenching curves as described in the text (see eq 3 in Materials and Methods). The values calculated for the different peptides from either type of experiment have been normalized to the corresponding (averaged) value calculated for acetylGlyCys-(palmitoyl)Cys(palmitoyl)Gly-caBim. Error bars for the fluorescence-based results were calculated using the estimated errors of the quenching curve fits and standard propagation of error methods. Peptides are designated in the *x*-axis legend using the standard one-letter code for amino acids and the following additional abbreviations: Ac = acetyl, c = myristoleoyl, f = farnesyl, gg = geranylgeranyl, m = myristoyl, p = palmitoyl, and * = bimanyl group location.

proteins linking these *lck*-derived sequences to chloramphenicol acetyltransferase (*36*).

To assess whether an even more minimal sequence containing the myristoylGlyCys(palmitoyl)- motif would associate efficiently with lipid rafts, we examined whether peptides containing this motif or (as a control) a palmitoylated/farnesylated sequence would associate with the low-temperature detergent-insoluble membrane fraction in COS-7 cells. As we have described previously for related peptides (*32*, *34*), cells incubated with myristoylGlyCysGly-caBim or Bimta-GlyCysGlyCys(farnesyl)-OMe together with [^{3}H]-palmitate synthesized the S-[^{3}H]palmitoylated forms of these peptides, which were largely localized to the plasma membrane by fluorescence microscopy and which incorporated radiolabeled fatty acid chiefly as [^{3}H]palmitate (>90% as assessed by reverse-phase TLC), with the balance incorporated as [^{3}H]stearate (H. Schroeder and J. Silvius, unpub-

[2] The fraction of *palmitoylated* lck-EGFP chimera associated with the "raft" fraction may in fact be even higher than the figure given in the text, as Zlatkine et al. (*36*) demonstrated for an lck-chloramphenicol acyltransferase chimera, since a fraction of the expressed chimeric protein may be unpalmitoylated and, like the lckSS-EGFP chimera, partition entirely into the S1 fraction.

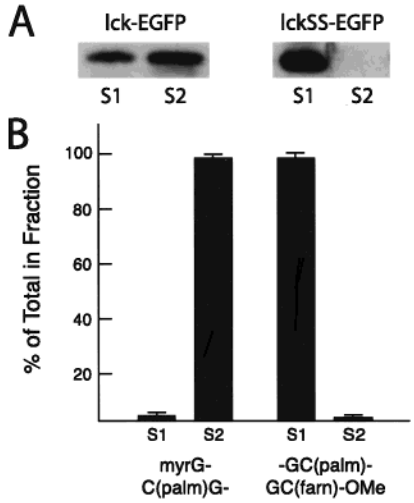

FIGURE 6: Panel A: Distribution of the chimeric proteins lck-EGFP (amino acids 1−11 of *lck* linked to the N-terminus of EGFP) or lckSS-EGFP (a palmitoylation-defective C3S,C5S mutant of lck-EGFP) between the cold Triton-insoluble and soluble fractions isolated from COS-7 cells. Transfected cells transiently expressing either construct were extracted sequentially with 1% Triton X-100 at 0 °C and then at 37 °C as described in Materials and Methods, and the soluble fraction obtained from each extraction (S1, 0 °C soluble; S2, 0 °C insoluble/37 °C soluble) was recovered and analyzed by SDS−PAGE and Western blotting with anti-GFP antibody. Other experimental details were as described in the text. Panel B: Distribution of myristoylGlyCys([^{3}H]palmitoyl)Gly-caBim or Bimta-GlyCys([^{3}H]palmitoyl)GlyCys(farnesyl)-OMe between the cold Triton-soluble and insoluble fractions isolated from COS-7 cells. Cells incubated with [^{3}H]palmitate and either myristoylGly-CysGly-caBim or Bimta-GlyCysGlyCys(farnesyl)-OMe, to synthesize the corresponding S-[^{3}H]palmitoylated peptides, were fractionated with Triton X-100 as outlined for panel A, and the content of radiolabeled *S*-palmitoyl peptide in the S1 and S2 fractions was determined as outlined in the text. The data shown represent the mean (± half-range) of results obtained in duplicate experiments.

lished observations). Cells synthesizing either S-[^{3}H]-palmitoylated peptide were extracted with Triton X-100 first at 0 °C and then at 37 °C as described above, and the relative amounts of the S-[^{3}H]palmitoylated peptide in the two solubilized fractions were determined as described in Materials and Methods. As shown in Figure 6B, the diacylated peptide myristoylGlyCys([^{3}H]palmitoyl)Gly-caBim was almost entirely recovered in the cold Triton-insoluble (S2) fraction. By contrast, under the same conditions the acylated/prenylated peptide Bimta-GC([^{3}H]palmitoyl)GC(farnesyl)-OMe was essentially completely associated with the cold Triton-soluble (S1) fraction. The Triton solubility properties of the prenylated peptide resemble those reported previously for prenylated proteins in model and biological membranes (*10*, *41*).

DISCUSSION

The results of our fluorescence quenching studies provide direct confirmation that certain lipidated motifs found in proteins, including the combination of a cholesteryl and an *N*-palmitoyl group as well as motifs combining multiple saturated acyl chains, confer significant affinities for liquid-ordered membrane domains. Interestingly, however, a given combination of lipidic residues can confer markedly different affinities for l_o domains, depending on the precise manner in which the lipid chains are attached to the peptide backbone. This is strikingly illustrated by comparing the affinities for l_o domains measured for peptides bearing the motifs palmitoylGlyCys(palmitoyl)-, -Cys(palmitoyl)Cys-(palmitoyl)-, and palmitoylCys(palmitoyl)-. While all of these peptides exhibit significant affinities for l_o domains, the first shows an affinity for l_o domains that exceeds by more than 10-fold those observed for the latter two species. The differing behavior of peptides bearing the palmitoylGlyCys-(palmitoyl)- and palmitoylCys(palmitoyl)- motifs, in which the acyl groups are attached by the same chemical linkages, suggests that, in addition to their structures, the relative spacing and/or orientation of the coupled acyl chains can be important determinants of affinity for l_o domains. This possibility has been previously suggested in a more general context by Mesquita et al. (*42*) and raises the interesting potential that the affinities of lipidated proteins for liquid-ordered domains could be modulated by factors that modify the local conformation of the lipidated sequence.

Our fluorescence quenching analysis can rigorously be used only to determine the *relative* extents of partitioning of different fluorescent species into l_o domains in ternary systems such as that examined here. However, using reasonable assumptions it is also possible (and instructive) to estimate from our data the *absolute* extents of partitioning of different peptides into l_o domains in bilayers of a specific composition, as we will here illustrate specifically for the case of 1:1:1 (molar proportions) DPPC/TEMPO-DOPC/33 mol % cholesterol bilayers.

The scaled fluorescence $(F/F_o)_{cor}$ observed for a given lipidated peptide in bilayers of any composition is simply the average of the scaled fluorescence values for peptide molecules present in l_o domains and for those present in l_d domains [which we will designate as $(F/F_o)_{lo}$ and as $(F/F_o)_{ld}$, respectively], weighted by the relative fractions of the peptide present in the two types of domains. If we can estimate the parameters $(F/F_o)_{ld}$ and $(F/F_o)_{lo}$ for 1:1:1 DPPC/TEMPO-DOPC/cholesterol bilayers, we can therefore estimate the proportion of the peptide molecules present in l_o vs l_d domains in bilayers of this composition. The quantity $(F/F_o)_{ld}$ can be estimated from the quenching curves obtained for species that partition entirely into l_d domains (e.g., species with multiple unsaturated acyl chains), as indicated schematically by the open circle in Figure 2A. An upper bound for $(F/F_o)_{lo}$ can be estimated by assuming (conservatively) that the value of this quantity will not exceed the value of $(F/F_o)_{cor}$ measured for quencher-free bilayers, i.e., 1.0. We can then calculate, for example, that a *minimum* of 50% of total myrGlyCys(palmitoyl)Gly-caBim will be partitioned into l_o domains in 1:1:1 DPPC/TEMPO-PC/cholesterol bilayers at 25 °C. It is clear that the absolute affinity of some of the peptides studied here for l_o domains is substantial. This conclusion is consistent with the results of our detergent fractionation experiments.

Our fluorescence quenching results indicate that peptides dually modified with an N-terminal palmitoyl and a C-terminal cholesteryl residue show significant affinity for l_o domains. Somewhat surprisingly, however, a C-terminal cholesteryl anchor alone appears to confer at best a very weak affinity for these domains. This latter result may reflect the fact that the free hydroxyl group of cholesterol (which becomes modified upon coupling to a protein or peptide) is important for the condensing effect of the sterol on phospholipid packing (*43*) and for its ability to partition into

liquid-ordered domains (*44*). The processed form of the protein Sonic Hedgehog has been shown to carry not only a carboxy-terminal cholesteryl residue but also a palmitoyl group amide-linked to its N-terminal cysteine residue (*5*). Other cholesterol-linked proteins of the Hedgehog family have highly homologous N-terminal sequences and are likely to be modified in the same manner. It is thus of interest that model peptides with the structure palmitoylXaaCys(bimanyl)-Gly-cholesterol (Xaa = Cys or Ala) show significant affinity for l_o domains, approaching that measured for a di-S-palmitoylated peptide. While the spatial relationship between the cholesteryl and *N*-palmitoyl residues in these model peptides may differ from those in the processed Hedgehog proteins themselves, our results nonetheless suggest that both cholesterol modification and palmitoylation may be important for the observed raft association of these proteins (*16*).

Our finding that the *S*-palmitoyl derivative of the simple peptide myristoylGlyCysGly-caBim becomes largely incorporated into the low-temperature Triton-insoluble fraction in COS-7 cells indicates that a truly minimal lipidated motif [myrGlyCys(palmitoyl)-] can mediate raft association. Direct quantitative comparison of these results to those obtained with proteins bearing the same motif, including the lck-EGFP chimera examined here, must be undertaken with some caution. Neutral lipidated peptides can in some cases undergo rapid transbilayer movement in lipid vesicles (*32*), and the N/S-diacylated form of the peptide may thus become partly incorporated into raft domains in the exofacial as well as the cytofacial leaflet of the plasma membrane. Nonetheless, our finding is consistent with previous suggestions that the raft localization of proteins bearing multiple saturated acyl chains can be attributed to the direct association of their lipidated sequences with raft lipids, without inherently requiring protein−protein interactions. By contrast, as we have noted previously (*29*), the reported association with rafts of proteins bearing an isoprenyl group (*45*−*48*) cannot be explained on the basis of lipid partitioning effects and must in fact reflect the influence of protein−protein associations that can overcome the intrinsically very low affinity of isoprenyl groups for liquid-ordered domains.

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