Fluorimetric Evaluation of the Affinities of Isoprenylated Peptides for Lipid Bilayers†

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ABSTRACT: A fluorescence-enhancement assay has been used to monitor the association of a series of fluorescent isoprenylated di- to tetrapeptides, whose sequences represent the carboxyl termini of several isoprenylated proteins (Ki-*ras*, *ral* 1, *rac* 2, and *rho* C), with phospholipid vesicles. These lipopeptides, containing mainly hydrophilic amino acid residues, all rapidly equilibrate (in seconds or faster) between the aqueous phase and the outer surfaces of lipid vesicles, in a manner that is well-modeled as a simple two-phase partitioning equilibrium. Farnesylated or geranylgeranylated peptides with methylated C-terminal cysteine residues exhibit half-maximal binding to 9:1 phosphatidylcholine (PC)/phosphatidylethanolamine (PE) vesicles at lipid concentrations on the order of 5–40 μM or 200–800 nM, respectively. Removal of the methyl group from the carboxyl-terminal cysteine residue decreases the affinity of a given lipopeptide for neutral (PC/PE) vesicles by 10- to 20-fold and the affinity for vesicles with a physiological surface charge by 40-fold or more. Cysteine-linked farnesyl and geranylgeranyl residues are found to be equivalent to cysteine-linked *n*-alkyl chains of roughly 11 and 14 carbons, respectively, in the strength of their interactions with lipid bilayers. Variations in vesicle lipid composition (cholesterol or aminophospholipid content) only modestly alter the affinity of isoprenylated peptides for the lipid bilayer. Our data suggest that a C-terminal geranylgeranylcysteine or O-methylated farnesylcysteine residue can by itself confer efficient (but rapidly reversible) membrane binding to proteins bearing these modifications, while an unmethylated C-terminal farnesylcysteine residue by itself would be only marginally efficient as a membrane 'anchor' under physiological conditions.

A small but important group of intracellular proteins, including many with key regulatory functions such as the members of the *ras* superfamily, is now known to be post-translationally modified through the S-isoprenylation (with a farnesyl [C_{15}] or a geranylgeranyl [C_{20}] group), and often additionally by the O-methylation, of a cysteine residue at the carboxyl terminus of the mature protein. These modifications have been shown to be critically important to the normal functioning of such proteins *in situ* (and to the transforming and other activities of certain mutant forms as well), as well as to the ability of such proteins to associate with cellular membranes (for reviews see: Takai *et al.*, 1992; Clarke, 1992; Schafer and Rine, 1992; Cox & Der, 1992).

Many isoprenylated proteins appear to shuttle between membrane and cytoplasmic compartments as an essential aspect of their function, and the isoprenylated cysteine residue has been suggested as an important potential mediator of protein–protein as well as protein–lipid interactions during such processes (Araki *et al.*, 1990; Hori *et al.*, 1991; Shirataki *et al.*, 1991; Kurzchalia *et al.*, 1992; Backlund, 1992; Kawamura *et al.*, 1992; Regazzi *et al.*, 1992; Hancock & Hall, 1993; Soldati *et al.*, 1993; Marshall, 1993). An important element in understanding such phenomena is an assessment of the interaction of the isoprenylated protein with lipid bilayers (Epand *et al.*, 1993). This interaction may contribute to or even dominate the interaction of the protein with cellular membranes; as well, it will have to be masked or supplanted by a still more favorable interaction if the isoprenylated protein is to be transferred to a different environment. In this study we have examined the intrinsic strength of this interaction by using a fluorescence-enhancement assay to quantitate the affinities of isoprenylated peptides, which replicate the carboxyl-terminal sequences of several naturally occurring isoprenylated proteins, for binding to phospholipid vesicles. Our results indicate that the carboxyl-terminal structures of these proteins possess a relatively strong intrinsic affinity for the membrane bilayer, which at least equals and in most cases considerably exceeds the bilayer-partitioning affinity characteristic of an N-terminal myristoylglycyl residue.

MATERIALS AND METHODS

Materials

Protected amino acids were obtained from Sigma (St. Louis, MO); other peptide-synthetic reagents were obtained from Aldrich (Milwaukee, WI). Dimethylformamide (DMF)[1] was redistilled under vacuum from ninhydrin and stored over

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[1] Abbreviations: BimTA-, *S*-bimanylthioacetyl; Boc, *tert*-butyloxycarbonyl-; DABS-PC, 1-palmitoyl-2-((12-[4-[[4-(dimethylamino)phenyl]azo]phenyl]sulfonyl)methylamino)stearoyl PC; DCCD, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DMF, dimethylformamide; DOPS, dioleoyl phosphatidylserine; DTPA, diethylenetriaminepentaacetic acid; ePC, egg phosphatidylcholine; egg PG, phosphatidylglycerol prepared by transphosphatidylation from egg PC; Fmoc, 9-fluorenylmethoxycarbonyl; HOBT, 1-hydroxybenzotriazole hydrate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; POPE, 1-palmitoyl-2-oleoyl PE; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; tPE, PE prepared by transphosphatidylation from egg PC; Tris, tris(hydroxymethyl)-aminomethane. Peptide and lipopeptide structures are abbreviated using the one-letter amino acid code and the following additional abbreviations: Farn, *trans,trans*-farnesyl; GerGer, *all-trans*-geranylgeranyl; -OFm, 9-fluorenylmethoxy; StBu, *tert*-butylmercapto; tBu, *tert*-butyl.

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Molecular Sieve 4A; trifluoroacetic acid (TFA) was freshly redistilled before use. Diethyl ether for precipitations was freshly redistilled from $LiAlH_4$; all other solvents were of reagent grade or better.

The fluorenylmethyl ester of *S-tert*-butylcysteine was synthesized (as the trifluoroacetate salt) using the procedure described by Xue *et al.* (1990) for the corresponding ester of *S*-acetamidomethylcysteine; the methyl ester was prepared in the same way, using methanol in place of fluorenylmethanol. Di-*trans*-farnesol (Aldrich) and *all-trans*-geranylgeraniol (initially a generous gift from Dr. Robert Coates, University of Illinois, Champaign-Urbana, and later obtained from ARC [St. Louis, MO], giving essentially identical results) were converted to the corresponding bromides as described previously (Corey *et al.*, 1972). *N*-Myristoylglycylcysteine was prepared by acylating glycine methyl ester in DMF with succinimidyl myristate, saponifying, and coupling the resulting acylglycine, as the succinimidyl ester, to cysteine (Lapidot *et al.*, 1967). The product dipeptide was labeled with monobromobimane (Molecular Probes, Eugene, OR) and purified by TLC in 75:25:0.5 (v/v/v) $CHCl_3/CH_3OH/CH_3COOH$.

Methods

Peptide Synthesis. Peptides terminating in *O*-methylcysteine were synthesized by standard solution methods, employing Fmoc chemistry (Fields & Noble, 1990) with O-*tert*-butyl protection for the side chains of Glu and Thr and S-(*tert*-butylmercapto) protection for the side chain of cysteine. Peptides terminating in unmethylated cysteine were similarly prepared using fluorenylmethyl ester protection of the cysteine carboxyl group (Xue *et al.*, 1990) and Boc protection of α-amino groups until the final stage of coupling, when Fmoc-substituted amino acids were again used. Peptide structures were confirmed by ^{1}H-NMR. Representative synthesis are described below.

FmocERC(StBu)–OMe and -RERC(StBu)–OMe. Fmoc–arginine (396 mg, 1 mmol) and the TFA salt of *O*-methyl-*S-tert*-butylcysteine (273 mg, 1.05 mmol) were stirred in 1.5 of dry DMF for 3 h at 0 °C with 203 mg (1.5 mmol) of HOBT, 227 mg (1.1 mmol) of DCCD, and 8.7 μL (50 μmol) of DIEA. After filtration, the products were twice precipitated with cold ether and deprotected with 9:1 (v/v) DMF/piperidine (2 mL, 0 °C, 1 h). The mixture was concentrated *in vacuo* and then mixed with 1 M HCl in ether (2 equiv) and three times precipitated with cold ether. After drying under vacuum, half the product from the above reaction was coupled to Fmoc–glutamic acid γ-*tert*-butyl ester (1.1 equiv, in 1.2 mL of DMF with 1.5 equiv of HOBT and 1.1 equiv of DCCD, stirring at 0 °C for 1 h before and 3 h after addition of 1 equiv each of dipeptide plus DIEA), filtered, and ether-precipitated as above. The crude products were purified by 'flash' chromatography on silica gel 60 (Still *et al.*, 1978), eluting first with 2% methanol in chloroform (discarded) and then with 6% methanol in chloroform to recover 386 mg of Fmoc–E(tBu)-RC(StBu)–OMe (95% yield based on Fmoc–arginine).

Half of the above material was deprotected with DMF/piperidine as above and then coupled to Fmoc–arginine (1.2 equiv) in the presence of 1.3 equiv of DCCD, 2 equiv each of HOBT and DIEA, and 1 equiv of TFA in DMF (0.5 mL). After filtration and two precipitations from cold ether, the products were purified by 'flash' chromatography on silica gel 60, eluting first with 10% (discarded) and then with 15% methanol in chloroform to yield 127 mg of Fmoc–RE(tBu)-RC(StBu)–OMe (53% yield based on tripeptide).

The products from the above syntheses were incubated with 1:1 TFA/CH_2Cl_2 (1 h, 25 °C) to remove O-*tert*-butyl groups and then recovered by evaporation of solvent and cold ether precipitation. The above procedures were also used to synthesize Fmoc–TRC(StBu)–OMe, Fmoc–RTRC(StBu)–OMe, and Fmoc–ERC(StBu)–OFm, in the latter case using Boc chemistry to synthesize the dipeptide RC(StBu)–OFm (Xue *et al.*, 1990).

FmocRGC(StBu)–OFm. Boc–GC(StBu)–OFm was prepared from *N-tert*-Boc–glycine and the TFA salt of *O*-(fluorenylmethyl)-*S-tert*-butylcysteine as described by Xue *et al.* (1990) for the corresponding *S*-(acetamidomethyl)-protected species. The dipeptide (120 mg = 210 μmol) was deprotected (25:75 TFA/CH_2Cl_2, 15 °C, 2 h), and the resulting TFA salt was coupled to Fmoc–arginine (250 μmol, plus 250 μmol of DCCD, 350 μmol of HOBT, and 35 μmol of TFA in 0.3 mL of DMF for 2 h at 0 °C. The products were precipitated three times with cold diethyl ether (10 mL) and purified by 'flash' chromatography on silica gel 60, eluting first with 2.5% methanol in chloroform (discarded) and then with 8% methanol in chloroform to yield 170.5 mg (185 μmol) of Fmoc–RGC(StBu)–OFm.

Peptide Alkylation and (S-Bimanyl)thioacetyl Labeling. *N*-Fmoc–*S*-(*tert*-butylmercapto)-protected peptides were S-alkylated and fluorescent-labeled by the following reactions, all carried out under nitrogen and in the dark. The cysteine sulfhydryl group was first deprotected by incubation (4 h, 25 °C) with 1:1:1 (v/v/v) DMF/β-mercaptoethanol/0.5 M aqueous Tris-Cl, pH 8.0 (1 mL/200 mg of peptide), containing 10 mg/mL of dithiothreitol. The incubation mixture was concentrated under N_2 with gentle warming and the residue three times precipitated with cold ethyl ether. The washed precipitates, redissolved in DMF (5 μL/mg of peptide), were stirred with a twofold molar excess of farnesyl or geranylgeranyl bromide (1 h, 0 °C) or saturated *n*-alkyl iodide (5 h, 25 °C) in acetonitrile (30 μL/mg of peptide) containing excess solid KF·2H_2O (Xue *et al.*, 1990, 1991). The products were partitioned in the two-phase system chloroform/methanol/1 M aqueous KCl (2:1:1, v/v/v), and the chloroform phase was concentrated under nitrogen. The residue was applied in chloroform to a small column of silica gel 60 (1 g/20 mg of peptide), which was successively eluted with 20 volumes each of chloroform (discarded) and 50:50:5:0.1:0.03 $CHCl_3/CH_3OH/H_2O$/triethylamine/TFA. The latter eluate was shaken with half the volume of 1 M aqueous KCl, and the recovered chloroform layer was dried under nitrogen.

After cleavage of Fmoc protecting groups (80:20 DMF/piperidine, 0 °C, 1 h, followed by removal of solvents under high vacuum), the S-alkylated peptides were reacted for 1 h at 0 °C with succinimidyl *S*-acetylthioacetate (1.3 equiv based on starting protected peptide) in DMF (20 μL/mg of peptide) containing DIEA and TFA (2 and 1.2 equiv, respectively). Methanol and cysteine-saturated 0.5 M aqueous hydroxylamine, pH 7.0 (200 and 50 μL/mg of peptide, respectively) were then added, and the mixture was incubated for 45 min at 25 °C. After partitioning of the products in 2:1:1 $CHCl_3/CH_3OH$/1 M aqueous KCl, the chloroform layer was dried down under nitrogen and the residue immediately reacted with monobromobimane (roughly 50% excess) in 5:1:1 $CH_3OH/CHCl_3$/0.2 M Tes, pH 7.6 (15 min, 25 °C). After the reaction was quenched with excess aqueous cysteine, the labeled S-alkylated peptides were extracted into chloroform as above and purified by preparative thin-layer chromatography on silica gel 60 plates, developing with $CHCl_3$/acetone/$CH_3OH/CH_3COOH/H_2O$ (50:20:10:10:5 for dibasic peptides and 50:18:7.5:7.5:3 for other species). For the syntheses of isoprenylated peptides, di-*tert*-butylphenol (50 μg/mg of

peptide) was included at all stages except the final TLC purification.

S-Methylated peptides were synthesized using the above reaction sequence with the following modifications: solvent-partitioning steps were replaced by ether precipitation from methanolic solution (after evaporation of the original solvents); the chromatographic step after alkylation was omitted; and dithiothreitol (5 mM) was used in place of cysteine in the hydroxylamine deprotection step.

Except where otherwise indicated, lipid vesicles were prepared by hand-extruding 10–40 mM lipid dispersions, initially vortexed in 150 mM KCl, 5 mM Tes, 20 μM DTPA, pH 7.0, and five times freeze/thawed, through 0.1-μm pore-size polycarbonate filters (MacDonald *et al.*, 1991). For PE-containing vesicles the fraction of exposed lipid was estimated using the TNBS-accessibility assay of Nordlund *et al.* (1981). The concentration of total surface-exposed lipid (L_{eff}) was estimated from this value and the total lipid concentration (assessed by phosphorus assay [Lowry and Tinsley, 1974]) on the assumption that PE was randomly distributed across the bilayers of these relatively large vesicles, as concluded by Nordlund *et al.* (1980).

The affinity of lipopeptide association with lipid vesicles was determined by 'titration' of peptide fluorescence with increasing concentrations of vesicles, using two protocols. In the 'methanol-injection' protocol, peptides (50–150 pmol) were injected from 6 μL of methanol into a stirred cuvette containing varying concentrations of vesicles in 3 mL of buffer, and the fluorescence was measured at excitation and emission wavelengths of 390 and 468 nm, respectively. In the 'vesicle-injection' protocol, mixtures containing lipid vesicles and lipopeptides (0.5 mM and 5 μM, respectively) were first preincubated for 30 min at 20 °C. Small aliquots of these mixtures were then injected into a stirred cuvette containing additional (peptide-free) vesicles, and the fluorescence was measured as above. While the methanol-injection method was preferred for technical reasons, for species with very high bilayer-partitioning affiniites ($K_d^{eff} < 1$–2 μM), fluorescence data acquired by this method showed small but significant deviations from the theoretical partitioning equation; the partition coefficients for these species were therefore measured using the vesicle-injection method exclusively. For species with lower bilayer-partitioning affinities, the two methods gave essentially indistiguishable partition coefficients.

RESULTS

The lipopeptides prepared in this study,[2] labeled at their amino termini with a fluorescent *S*-bimanylthioacetyl (Bim-TA) residue, correspond to the C-terminal sequences of isoprenylated proteins from several different subgroups of the *ras* superfamily: human *rac* 2 (-RAC[GerGer]), *rho* C (-RRGC[GerGer]), and *ral* 1 (-RERC[GerGer]) and murine Ki-*ras* B (-RTRC[Farn]) (Maltese, 1990; Casey *et al.*, 1989; Hancock *et al.*, 1989; Adamson *et al.*, 1992; Kinsella *et al.*, 1991). The C-terminal cysteines of *rho* C and Ki-*ras* have been found to be methylated in cultured cell lines (Adamson *et al.*, 1992a; Hancock *et al.*, 1989) while that of *rac* 2 has been reported to undergo methylation in response to activation of specific cell surface receptors in human neutrophils (Philips *et al.*, 1993). The methylation status of the C-terminal cysteine of *ral* A has not been reported to date. While both lysine and arginine residues are common in the C-terminal sequences of isoprenylated proteins (Maltese, 1990; Cox & Der, 1992), arginine-containing peptides were preferred here both for synthetic reasons and to minimize the presence of alternate charged forms for each peptide under our experimental conditions.

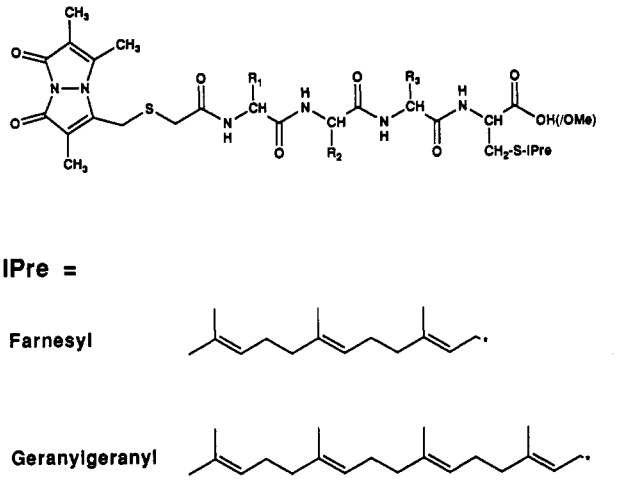

FIGURE 1: Representative structure of an N-(*S*-bimanylthioacetyl)-labeled lipopeptide (here, a tetrapeptide) of the type examined in this study.

When injected from methanol into buffer, the bimanyl-labeled lipopeptides gave a relatively low fluorescence, which slowly declined with time due to adsorption of probe molecules to the cuvette. At the low concentrations employed here (15–50 nM), most of the lipopeotides examined gave a very similar ratio of fluorescence intensities in buffer *vs* methanol, regardless of the nature of the S-alkyl (C_{10}- to C_{15}-alkyl) or -isoprenyl group attached. However, some C_{16}- and a few C_{15}-alkylated peptides gave a substantially lower value for this ratio, which in these cases alone varied significantly with the lipopeptide concentration. We suggest that in the absence of lipids these latter species exist partly as micelles or other aggregates, while the other lipopeptides exist in monomeric form under our normal experimental conditions.

As shown in Figure 2A, the addition of extruded PC/PE vesicles to lipopeptides initially dispersed in buffer led to a rapid (≤1 s) increase in fluorescence. Addition of similar vesicles containing 2 mol% of DABS-PC, an energy-transfer acceptor capable of quenching bimanyl fluorescence (Silvius *et al.*, 1987), led to rapid quenching rather than enhancement of the lipopeptide fluorescence (Figure 2A, lower curve). These fluorescence changes appear to reflect the rapid binding of the lipopeptides to the vesicles upon mixing. In both types of experiments the amplitude of the fluorescence changes varied in a hyperbolic manner with the concentration of added lipid.

Two lines of evidence indicated that the isoprenylated peptides examined here can rapidly dissociate from, as well as bind to, lipid vesicles. First, when peptide/vesicle dissociation was triggered by diluting concentrated vesicle/lipopeptide suspensions into buffer, the fluorescence signal relaxed within the mixing time (ca. 3 s) to the level characteristic of the diluted mixture. Second, when lipopeptides were first incubated in the fluorimeter with DABS-PC-containing vesicles, at concentrations such that virtually all of the lipopeptide was vesicle-bound, a subsequent addition of quencher-free vesicles produced a rapid rise in fluorescence (Figure 2B). As the intermixing of lipid components between

[2] In this paper the designation 'lipopeptide' will refer only to peptides substituted with long alk(en)yl or acyl chains and not to the S-methylated peptides used for certain control experiments.

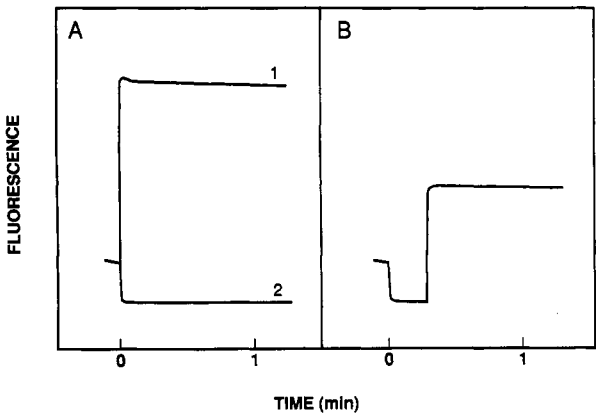

FIGURE 2: (A) Time courses of fluorescence change at 37 °C when lipid vesicles (200 μM) where added at time $t = 0$ to buffer containing lipopeptide (BimTA–RGC[GerGer]–OH, 50 nM) injected into the cuvette from methanolic solution at time $t = -10$ s: curve 1, 90:10 ePC/POPE vesicles added; curve 2, 88:10:2 ePC/POPE/DABS–PC vesicles added. Similarly rapid kinetics of fluorescence change were observed in these experiments for all the lipopeptides examined in this study. (B) Time course of fluorescence change at 37 °C when 88:10:2 ePC/POPE/DABS–PC vesicles (200 μM) were added at time $t = 0$, followed by 90:10 ePC/POPE vesicles (200 μM) at time $t = 20$ s, to a cuvette into which BimTA–RGC[GerGer]–OH was injected (to 50 nM) at time $t = 10$ s. Very similar results were obtained (with appropriate adjustment of vesicle concentrations for peptides with low bilayer-partitioning affinities) for all of the lipopeptides examined in this study.

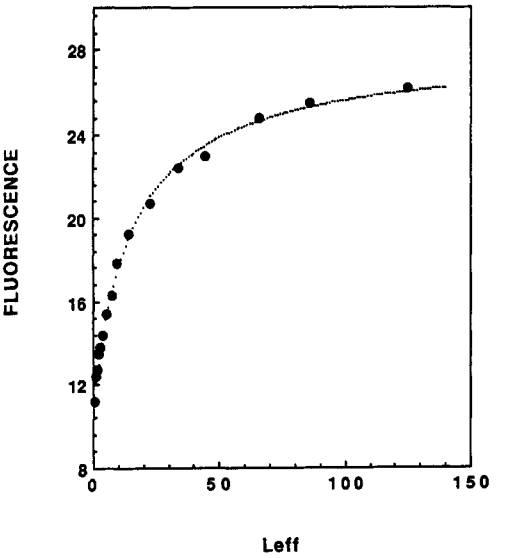

FIGURE 3: Representative titration of the fluorescence of a bimane-labeled lipopeptide (here, BimTA–ERC[GerGer]–OH) with 90:10 ePC/POPE vesicles at 37 °C. The parameter L_{eff}, given in units of μM, represents the concentration of *surface-exposed* vesicle lipids as described in the text. Nonlinear least-squares fitting of the data points (large filled circles) to eq 1 (small points) yields estimates of $K_d^{eff} = 15.87 \pm 1.21$ μM, $F_0 = 11.12 \pm 0.19$ fluorescence units, and $F_{max} = 27.91 \pm 0.27$ fluorescence units.

such vesicles is negligible on this time scale (Brown, 1992), this rapid fluorescence increase presumably reflects a rapid dissociation of the lipopeptides from DABS-PC-containing vesicles and subsequent binding to quencher-free vesicles. Significantly, geranylgeranylated as well as farnesylated peptides showed rapid dissociation kinetics in the above experiments, indicating that neither type of isoprenyl group can mediate long-lived anchorage to a particular membrane surface through hydrophobic interactions with the lipid bilayer alone.

Several types of experiments suggested that the lipopeptides examined here rapidly equilibrate only between the aqueous phase and the outer surfaces of lipid vesicles. First, we prepared 'prelabeled' 90:10:2:0.5 (molar proportions) ePC/POPE/DABS-PC/geranylgeranyl–peptide vesicles by either sonication (to near-clarity) or simple vortexing, at lipid concentrations such that >99% of each peptide was vesicle-bound. 'Postlabeled' vesicles were also prepared by dispersing lipids alone by the above methods and then adding lipopeptides to the same final concentration. Small samples of each preparation of vesicles were then mixed with a 20-fold excess of sonicated egg PG vesicles, and the resultant rapid fluorescence increase was measured to estimate the fraction of total lipopeptide that was rapidly exchangeable between vesicles. In these experiments, for each lipopeptide, <20%, 40–60%, and >90% of the total peptide was rapidly exchangeable from prelabeled/vortexed, prelabeled/sonicated, and postlabeled vesicles (sonicated or vortexed), respectively. As sonicated PC/PE vesicles expose a much larger fraction of their total PE than do multilamellar vortexed vesicles (ca. 55% *vs* 15–20%, assayed by the procedure of Nordlund *et al.* [1981]), these results suggest that only lipopeptides present at the outer surfaces of PC/PE vesicles can undergo rapid intervesicle exchange. In a second set of experiments, we found that the binding of various mono- and dibasic farnesylated peptides to extruded PC/PE vesicles was not detectably enhanced by the presence of an internally acidic transmembrane pH gradient (internal pH 4.2 *vs* 7.0, external pH 7.0). This result strongly suggests that these basic lipopeptides do not undergo significant translocation across the membrane bilayer on the time scale of the binding measurements (Chakrabarti *et al.*, 1992). Therefore, the bilayer/aqueous partitioning of the lipopeptides examined here appears to depend on the concentration of surface-exposed rather than total vesicle lipids.

When a fixed quantity of a given labeled lipopeptide is mixed with varying concentrations of (quencher-free) lipid vesicles, the amplitude of the lipopeptide fluorescence shows a hyperbolic dependence on the lipid concentration, as illustrated in Figure 3. This variation is well-described by the equation

$$F = F_0 + (F_{max} - F_0)(K_p^{eff}[L]_{eff}/(1 + K_p^{eff}[L]_{eff})) \quad (1)$$

where L_{eff} is the concentration of *surface-exposed* lipids in a given lipopeptide/vesicle sample, F is the fluorescence measured for that sample, and F_0 and F_{max} represent the fluorescence values expected at $L_{eff} = 0$ and L_{eff} = 'infinity', respectively, for the same amount of peptide. The effective partition coefficient K_p^{eff} determined from eq 1 can be converted to various alternate forms, two of which will be used in this paper. The first, K_p, is a dimensionless, mole fraction-based form defined by the expression

$$K_p = \frac{X(\text{peptide})_{ves}}{X(\text{peptide})_{aq}} \quad (2)$$

where the numerator and denominator terms represent the mole fraction of the peptide in the outer-surface monolayer of the vesicles and in the aqueous phase, respectively. The second, which we term the effective dissociation constant K_d^{eff}, is the reciprocal of K_p^{eff} in eq 1 above and represents the concentration of exposed (vesicle outer-monolayer) lipids at which the peptide is 50% partitioned into the lipid bilayer.[3]

In Table 1 are summarized the values of K_d^{eff} and K_p, estimated as just described for the binding of various farnesylated peptides to extruded ePC/POPE (9:1) vesicles at 37 °C and pH 7.0. For a representative series of

Table 1: Effective Dissociation Constants K_d^{eff} for Binding of Isoprenylated Peptides to Large Unilamellar PC/PE (9:1) Vesicles

peptide	K_d^{eff}, μM[a]	K_p[b]
Bim–TA		($\times 10^{-4}$)
–RGC(Farn)–OMe	8.32 ± 0.63	667 ± 51
–RGC(Farn)–OH	114 ± 6	48.7 ± 2.6
–RRGC(Farn)–OMe	13.2 ± 0.6	421 ± 19
–RAC(Farn)–OMe	10.3 ± 0.3	539 ± 16
–RAC(Farn)–OH	131 ± 5	42.4 ± 1.6
–RC(Farn)–OMe	4.92 ± 0.63	1128 ± 147
–TRC(Farn)–OMe	8.58 ± 0.88	647 ± 58
–TRC(Farn)–OH	213 ● 48	26.0 ± 6.2
–RTRC(Farn)–OMe	15.2 ± 1.6	365 ± 39
–ERC(Farn)–OMe	27.4 ± 1.0	203 ± 7
–RERC(Farn)–OMe	38.7 ± 2.5	143 ± 9
		($\times 10^{-6}$)
–RGC(GerGer)–OMe	0.231 ± 0.015	240 ± 16
–RGC(GerGer)–OH	2.28 ± 0.06	24.3 ± 0.6
–RRGC(GerGer)–OMe	0.349 ± 0.027	159 ± 12
–RAC(GerGer)–OMe	0.266 ± 0.004	209 ± 3
–RAC(GerGer)–OH	2.55 ± 0.22	21.8 ± 1.9
–TRC(GerGer)–OMe	0.197 ± 0.004	282 ± 6
–RTRC(GerGer)–OMe	0.353 ± 0.007	157 ± 3
–ERC(GerGer)–OMe	0.634 ± 0.017	87.5 ± 2.3
–ERC(GerGer)–OH	16.36 ± 0.33	3.39 ± 0.28
–RERC(GerGer)–OMe	0.711 ± 0.027	78.1 ± 3.0

[a] Determined by the methanol-injection and vesicle-injection methods (two experiments each) for farnesylated peptides and by the vesicle-injection method (three experiments) for geranylgeranylated peptides, as described in Materials and Methods. [b] Calculated from the relation $K_p = ((55.5\ M)/K_d^{eff})$ and defined as the equilibrium ratio of the lipopeptide mole fraction in the bilayer over than in the aqueous phase, as discussed in the text.

lipopeptides, very similar values of K_d^{eff} were estimated using vortexed or sonicated/freeze-thawed vesicles (Silvius and Leventis, 1993) in place of extruded lipid vesicles, *if* the value L_{eff} was taken in all cases as the concentration of *surface-exposed* lipids presented by each type of vesicle. This result suggests that bilayer-curvature effects have little effect on lipopeptide binding to vesicles as large as the extruded vesicles used here (mean diameter ca. 80 nm [MacDonald *et al.*, 1991]). The K_d^{eff} values for the different cysteine-methylated/farnesylated peptides vary over a roughly eightfold range, generally increasing with increasing peptide chain length. Striking differences in K_d^{eff} values are observed comparing cysteine-methylated to -unmethylated lipopeptides with the same sequence; the value of K_d^{eff} increases by roughly 12-fold upon demethylation of the species BimTA–RGC(Farn)–OMe and -RAC(Farn)–OMe and by over 20-fold upon demethylation of BimTA–TRC(Farn)–OH.

Also shown in Table 1 are the values of K_d^{eff} (and K_p) measured for a series of geranylgeranylated peptides related to the farnesylated species just discussed. The pattern of variation of K_p with the structure of the peptide moiety is quantitatively very similar to that observed for the farnesylated peptides discussed above; the K_p values for C_{20}-isoprenylated species averaged 45-fold higher (range = 36- to 54-fold) than those for the corresponding C_{15}-isoprenylated species. Again, methylation of the cysteinyl carboxyl group produces a very substantial increase (from 10- to over 20-fold) in the strength of partitioning of the geranylgeranylated species into the lipid bilayer. Expressing these differences in terms of free energies of lipopeptide partitioning, we calculate that carboxyl methylation decreases (i.e., enhances) the free energy of partitioning ΔG_p (defined for transfer from the aqueous to the bilayer phase) by ca. 1.4–2.0 kcal mol^{-1}, while replacement of a farnesyl by a geranylgeranyl group decreases this quantity by 2.3–2.5 kcal mol^{-1}.

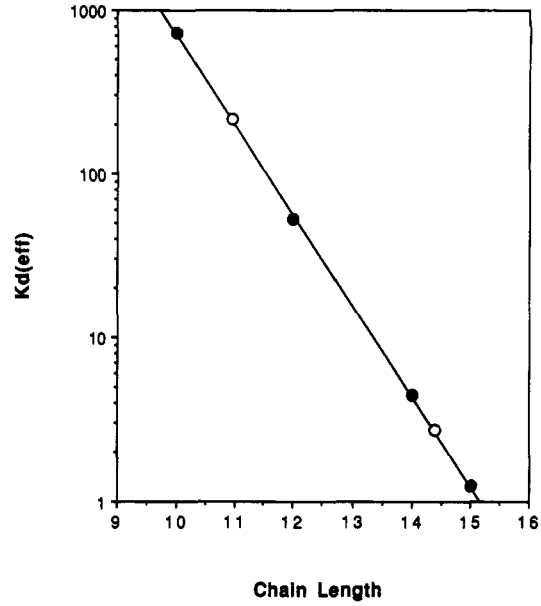

FIGURE 4: Closed circles, variation of K_d^{eff} with alkyl chain length for a series of lipopeptides BimTA–RGC(alkyl)–OH; open circles, log(K_d^{eff}) values for the corresponding farnesylated (upper point) and geranylgeranylated species (lower point).

Farnesyl and geranylgeranyl groups, by virtue of their branched and multiply unsaturated structures, may differ significantly from simple alkyl groups of equal carbon number in their affinity for lipid bilayers. To examine this point, we compared the bilayer partitioning of two sets of lipopeptides, BimTA–RGC(alkyl)–OH and BimTA–RGC(alkyl)–OMe, whose cysteine side chains were alkylated either with simple *n*-alkyl groups or with polyisoprenyl residues. In Figure 4 the logarithm of the effective dissociation coefficient for the series of lipopeptides BimTA–RGC(*n*-alkyl)–OH is plotted as a function of the *n*-alkyl chain length. For these species the value of log(K_d^{eff}) decreases linearly with *n*-alkyl chain length in the range C_{10}–C_{15}, with a slope that indicates a 781 ± 6 cal mol^{-1} decrease in the free energy of partitioning, ΔG_p, per added methylene unit. The value of this decrement is similar to that measured for other series of charged and uncharged amphiphiles at physiological ionic strength (Tanford, 1980; Peitzsch and McLaughlin, 1993). Extrapolating the line indicated in Figure 4, we predict that ΔG_p (=–RTln[55.5 M/K_d^{eff}]) will be very nearly zero (0.1 ± 0.2 kcal mol^{-1}) for an alkyl chain length of 1, *i.e.*, for the homologous S-methylated peptide.[4] This point will be considered further in the Discussion.

Also plotted in Figure 4 are the values of log(K_d^{eff}) measured for the farnesyl- and geranylgeranyl-substituted forms of the peptide BimTA–RGC(isoprenyl)–OH. By interpolation, we

[3] K_d^{eff} is used here as a strictly empirical (though intuitively useful) parameter, as denoted by the superscript ^{eff}, and should not be confused with the classical dissociation constant for a saturable binding isotherm with a defined number of binding sites. Likewise, our use in this paper of the term 'binding' to describe lipopeptide assocition with lipid bilayers should be understood to imply a two-phase partitioning process rather than binding to a finite number of 'sites' in the bilayer.

[4] The linear extrapolation made from Figure 4 of course assumes that the incremental change in partitioning free energy per added methylene unit remains fairly constant even for very short alkyl chain lengths. Evidence to support this assumption can be found in the data summarized by Cevc and Marsh (1987) for the partitioning of shorter chain *n*-alkanes and -alkanols into lipid bilayers. The data of Peitzsch and McLaughlin (1993), in their study of the partitioning of saturated fatty acids and *N*-acylglycines into lipid bilayers, are also consistent with this conclusion.

Table 2: Effective Dissociation Constants K_d^{eff} for Binding of Isoprenylated Peptides to Lipid Vesicles of Varying Compositions

peptide	vesicle composition	K_d^{eff}, μM[a]
BimTA		
–RGC(Farn)–OMe	ePC/ePG/tPE (7:2:1)	2.36 ± 0.24
–RGC(Farn)–OH		118 ± 12
–RRGC(Farn)–OMe		1.95 ± 0.36
–RAC(Farn)–OMe		2.73 ± 0.39
–RAC(Farn)–OH		108 ± 33
–TRC(Farn)–OMe		1.83 ± 0.18
–RTRC(Farn)–OMe		1.14 ± 0.26
–ERC(Farn)–OMe		21.9 ± 1.9
–RERC(Farn)–OMe		7.07 ± 0.6
–RGC(GerGer)–OH	ePC/tPE/chol (9:1:6.7)	3.93 ± 0.65
–RGC(Farn)–OH		319 ± 18
–RGC(Farn)–OMe		15.9 ● 1.8
–RGC(GerGer)–OH	tPE/ePC/DOPS (6:2:2)	2.11 ± 0.43
–RGC(Farn)–OH		67.4 ± 11.2
–RGC(Farn)–OMe		2.84 ± 0.40

[a] Values were determined by the vesicle-injection method (duplicate experiments), as described in Materials and Methods.

estimate that the farnesyl and geranylgeranyl groups are equivalent to *n*-alkyl chains of 11.0 and 14.3 carbons, respectively, in terms of their abilities to promote bilayer association of this peptide. Parallel experiments using a series of lipopeptides BimTA–RGC(*n*-alkyl/isoprenyl)–OMe (not shown) gave a comparable increment in ΔG_p per added methylene residue (706 ± 56 cal mol^{-1} $(CH_2)^{-1}$) and similar effective chain lengths for the farnesyl and geranylgeranyl groups (10.9 and 14.1 carbons, respectively). The extrapolated estimate of ΔG_p for BimTA–RGC(methyl)–OMe, obtained as described above, is –2.3 ± 0.6 kcal mol^{-1}.

The methods used above were also applied to measure the bilayer-partitioning affinity of the peptide *N*-(myristoylglycyl)(*S*-bimanyl)cysteine. The measured value of K_d^{eff} = 126 ± 22 μM for this species is several-fold greater than those predicted for S-tridecyl derivatives of peptides such as BimTA–RGC–OH and -RGC–OMe (e.g., ca. 16 μM predicted for BimTA–RGC(tridecyl)–OH from the data of Figure 4), suggesting that a C-terminal S-alkylcysteinyl residue, methylated or unmethylated, allows greater penetration of the coupled alkyl chain into the bilayer and/or provides additional types of interactions with the lipid bilayer, compared to an N-acylated glycinyl residue. The calculated value of K_p for *N*-(myristoylglycyl)(*S*-bimanyl)cysteine ((4.4 ± 0.8) × 10^5) is in excellent agreement with the equivalent value (ca. 1 × 10^4 in volume fraction units, equivalent to 4.4 × 10^5 in mole fraction units) reported by Peitzsch and McLaughlin (1993) for binding of the peptide *N*-(myristoylglycyl)alanine to PC vesicles. As the two peptides differ only in the presence or absence of the bimanylmercapto group, we conclude that this fluorescent group does not itself contribute significantly to the free energy of peptide–bilayer association.

In experiments summarized in Table 2, we examined how variations in vesicle lipid composition affected the partitioning of isoprenylated peptides. The affinity of binding of representative lipopeptides to 54:6:40 PC/PE/cholesterol vesicles was two- to three-fold weaker than that to similar vesicles without cholesterol. Inclusion of 20 mol% of the anionic lipid phosphatidylglycerol in PC/PE vesicles had little effect on vesicle binding for farnesylated peptides with no net charge but enhanced the binding of peptides with net charge +1 by 3.5–5.5-fold and that of peptides with net charge +2 by roughly twice this factor. The affinity of lipopeptide binding to vesicles composed of 60:20:20 tPE/ePC/DOPS, a composition chosen to model that of the cytoplasmic faces of cellular membranes, was similar to that for binding to PC-rich vesicles. It thus appears that the carboxyl terminus of an isoprenylated protein would interact with similar affinity with the lipid bilayers of various cellular membranes, regardless of modest differences in their lipid compositions.

DISCUSSION

The isoprenylated peptides examined here exhibit very substantial affinities for lipid bilayers. Lipopeptides terminating in an unmethylated *S*-farnesylcysteine residue exhibit bilayer/aqueous partition coefficients similar to those reported by Peitzsch and McLaughlin (1993), and also observed here, for N-myristoylated peptides. Analogous lipopeptides bearing an *S*-farnesyl-*O*-methylcysteine residue show roughly 10- to 20-fold greater affinities for neutral lipid vesicles, and this difference can increase to 40-fold or more for lipopeptide binding to vesicles of physiological surface charge (Table 2). Replacement of a farnesyl by a geranylgeranyl residue enhances peptide lipid-partitioning affinity still further (by roughtly 45-fold). These values are somewhat smaller than those estimated by Black (1992) on the basis of theoretical modeling but nonetheless indicate very substantial free energies of interaction between an isoprenylated carboxyl-terminal cysteinyl residue (even if unmethylated) and the lipid bilayer: from roughly –8 kcal mol^{-1} for an unmethylated farnesylcysteine residue to as much as –12 kcal mol^{-1} for a methylated carboxylterminal geranylgeranylcysteine residue.

Several observations suggest that the peptide portions of the lipopeptides examined here (beyond the modified terminal cysteine itself) make little if any favorable contribution to the free energy of the lipopeptides' interactions with uncharged lipid bilayers. First, S-methylated analogues of a representative sample of these lipopeptides (BimTA–RC[Me]–OMe, -RGC[Me]–OMe, -RGC[Me]–OH, -RTRC[Me]–OMe, and -RRGC[Me]–OMe) showed no detectable interaction with either PC/PE (9:1) or PC/PG/PE (7:2:1) vesicles up to at least L_{eff} = 5 mM, the practical upper limit of our fluorescence assay (data not shown). If we assume that K_d^{eff} must then be at least 10-fold greater than this value, the free energy of water-to-bilayer partitioning (ΔG_p) for all these species must be more positive than –RT ln(55.5 M/50 mM) = –4.3 kcal mol^{-1}. This lower limit is consistent with the values of ΔG_p predicted for the S-methyl derivatives of BimTA–RGC–OH and -OMe (0.1 ± 0.2 and –2.3 ± 0.6 kcal mol^{-1}, respectively) by extrapolation of data for peptides with longer S-alkyl chains as shown in Figure 4. Given that an S-methyl substituent should itself contribute some –1 to –2 kcal mol^{-1} to the value of ΔG_p (Tanford, 1980; Engelman *et al.*, 1986), we conclude that the peptide moieties of these species show little if any net affinity for association with neutral lipid bilayers. We also note that among the isoprenylated peptides examined here, bilayer-partitioning affinity generally *decreases* somewhat with increasing peptide chain length (Table 1). For the lipopeptides studied here, all bearing relatively hydrophilic peptide moieties (as occur at the carboxyltermini of most known isoprenylated proteins [Cox & Der, 1992]), the affinity for neutral lipid bilayers thus appears to be dominated by the properties of the alkylated (*O*-methyl-) cysteine residue and may even be slightly antagonized by nearby amino acid residues.[5]

[5] Portions of the polypeptide chain of an isoprenylated protein, either at the extreme carboxyl terminus or elsewhere, may of course exhibit additional, distinct interactions (electrostatic, hydrophobic, etc.) with the membrane lipid bilayer and/or with putative membrane 'targeting receptors'. Possible implications of such interactions are discussed later in this section.

To what degree can the isoprenylated peptides examined here model the bilayer associations of isoprenylated proteins? The membrane binding of a given isoprenylated protein may obviously be modulated by factors not represented in the current experiments, including proteins that can bind the isoprenyl group in the aqueous phase (Araki *et al.*, 1990; Hori *et al.*, 1991; Shirataki *et al.*, 1991; Kawamura *et al.*, 1992; Kurzchalia *et al.*, 1992; Backlund, 1992; Regazzi *et al.*, 1992; Hancock & Hall, 1993) and/or the presence of membrane-binding sequences in the protein distinct from the isoprenylated carboxyl terminus (Hancock *et al.*, 1990, 1991; Chavrier *et al.*, 1991; Adamson *et al.*, 1992b). Nonetheless, as discussed below, the present results provide some guidance and useful constraints for formulating realistic models of the interactions that govern the distributions of isoprenylated proteins between the membrane and cytoplasmic environments.

The effective concentration of membrane lipids exposed to the cytoplasm in a typical eukaryotic cell is on the order of several millimolar. If isoprenylated proteins bound to cellular membranes with affinities comparable to those of the lipopeptides examined here, we would thus predict that geranylgeranyl-modified proteins should be overwhelmingly (albeit reversibly), and farnesylated proteins preponderantly, membrane-bound. In principle, several factors could perturb the membrane/aqueous partitioning of isoprenylated proteins (relative to that of the peptides examined here) sufficiently to alter this conclusion. The association of any molecule with a membrane (or other large structure) may entail some loss of translational and rotational degrees of freedom (Janin & Chothia, 1978; Finkelstein & Janin, 1989) and/or of conformational entropy (Whittington, 1975; Silvius & Zuckermann, 1993). Theoretical considerations suggest that the resulting entropy losses from these sources will increase with molecular size, although the actual magnitude of these effects remains controversial (Finkelstein & Janin, 1989; Peitzsch & McLaughlin, 1993). As well, steric interactions between 'permanent' membrane proteins and isoprenylated (or acylated) proteins may render a significant fraction of the total membrane lipid surface inaccessible for binding of the isoprenylated (/acylated) species. The magnitude of this effect will depend on the size, geometry, and flexibility of the lipid-modified protein and on the amount and the distribution (lateral and transverse) of proteins in the membrane. We estimate that the above effects may collectively reduce the net strength of membrane association for lipid-anchored proteins by roughly 1 order of magnitude relative to that observed for small molecules carrying the same lipophilic group.

Combining the above considerations with our present results, we predict that cysteine-farnesylated/methylated proteins that are anchored to a membrane merely by their lipophilic carboxyl termini will exhibit apparent bilayer dissociation constants (K_d^{eff}) on the order of hundreds of micromolar. This range of values appears consistent with published data describing the binding of farnesylated/methylated rhodopsin kinase and β-adrenergic receptor kinase (Inglese *et al.*, 1992) and transducin γ-subunit (Ohguro *et al.*, 1991) to rod outer segment membranes in the absence of rhodopsin activation.[6] Demethylation of the carboxyl-terminal cysteine of such proteins would be expected to decrease the bilayer-partitioning affinity by more than 1 order of magnitude, to the extent that a substantial fraction of the protein could be present in the aqueous compartment even at physiological concentrations of membrane lipids and in the absence of any cytoplasmic factors to shield the isoprenyl group from the aqueous phase. (De)methylation of the terminal isoprenylcysteine residue could thus serve a significant role in modulating the membrane/cytoplasmic distributions of farnesylated proteins, purely by altering the affinity of the modified carboxyl terminus for the lipid bilayer.

In contrast to the above predictions for farnesylated proteins, our results suggest that geranylgeranylated proteins should partition strongly in favor of cellular membranes, regardless of their methylation status, unless the isoprenyl group is to some degree shielded from the aqueous phase by protein–protein interactions. This conclusion agrees well both with some previous conjectures and with most available experimental data, which indicate that geranylgeranylated proteins exist in the cytoplasmic phase only when complexed to other proteins which appear to sequester the isoprenyl group (Araki *et al.*, 1990; Hori *et al.*, 1991; Shirataki *et al.*, 1991; Kawamura *et al.*, 1992; Kurzchalia *et al.*, 1992; Backlund, 1992; Regazzi *et al.*, 1992; Hancock & Hall, 1993; Marshall, 1993), otherwise binding avidly to cellular membranes even in the absence of specific targeting information (Hancock *et al.*, 1991; Inglese *et al.*, 1992). However, from data like those shown in Figure 4, we can calculate that even a partial sequestration or comparatively weak binding of the polyisoprenyl group upon complexation of a geranylgeranylated protein to a cytoplasmic binding protein (energetically equivalent to protection of less than half the C_{20} chain from the aqueous phase) could suffice to shift the geranylgeranylated protein predominantly into the cytoplasmic compartment. This may explain why at least one such cytoplasmic binding protein appears to show a rather weak specificity, and hence presumably a suboptimal affinity, for binding of the geranylgeranyl group (Shirataki *et al.*, 1991).

Specific delivery or binding of isoprenylated proteins to particular membranes presumably requires determinants within the protein structure other than the isoprenylated carboxyl-terminal cysteine residue. Such determinants have in fact been localized somewhat upstream from the carboxyl terminal in proteins such as Ki-*ras* (Hancock *et al.*, 1990, 1991), *rho* A/*rho* B (Adamson *et al.*, 1992b), and several members of the *rab* family (Chavrier *et al.*, 1991), and in a few cases putative membrane receptors for specific isoprenylated proteins or peptides have been identified (Shirataki *et al.*, 1992, 1993; Thissen & Casey, 1993). However, the strength (free energy) of binding between such 'targeting' determinants and their putative membrane receptors may be relatively weak; while the isoprenylated carboxyl terminus of a protein can contribute 8–12 kcal mol^{-1} to the free energy of binding of the protein to a membrane, an additional, membrane-specific interaction with a free energy of only 3–4 kcal mol^{-1} would be needed to favor the selective binding of the protein to a particular target membrane by factors of 100- to 1000-fold. Weak interactions of the latter type would be wholly undetectable in the absence of the isoprenylated cysteine moiety (or another group with comparable affinity for the lipid bilayer), regardless of whether (or not) the polyisoprenylated residue itself interacts with the putative targeting receptor. To address the latter issue it will be necessary to examine whether normally isoprenylated proteins, when modified with quite different lipophilic groups (e.g., an N-terminal acyl chain), retain normal selectivity in their binding to different cellular membranes.

[6] Estimates were derived using data provided in the papers cited, where membrane concentrations were specified as the concentration of rhodopsin, and a value of 35 mol of surface-exposed lipids per mol of rhodopsin in a typical rod outer segment vesicle preparation (Daemen, 1973).

An interesting corollary of the present results concerns the kinetics of dissociation of an isoprenylated protein from a membrane, an event that appears to be important in the functional cycles of many such proteins. The rate of spontaneous desorption of such a protein from the membrane will depend on the free energy of activation for desorption, ΔG_d^*, according to the equation

$$k_d = (\kappa kT/h) \exp(-\Delta G_d^*/RT) \quad (3)$$

In general, ΔG_d^* must exceed the equilibrium free energy of transfer of the desorbing species from the bilayer to the aqueous phase, $-\Delta G_p$, by at least a small margin. Using absolute reaction-rate theory (Glasstone *et al.*, 1941), we then calculate that the rate constant for 'spontaneous' desorption of a bound protein from a bilayer cannot exceed the value

$$k_d(\max) = (\kappa kT/h) \exp(\Delta G_p/RT) \quad (4a)$$

$$= (\kappa kT/h)(K_p)^{-1} \quad (4b)$$

which equates to 230 s^{-1} for $K_d^{eff} = 2$ μM, the latter value being a reasonable estimate for a geranylgeranylated protein that interacts with the membrane only through its modified carboxyl terminal (see above). Taking this rather liberal upper-bound estimate for k_d[7] together with our kinetic data, it seems reasonable to conclude that for geranylgeranylated proteins in particular, spontaneous membrane/cytoplasmic exchange can occur on a marginally subsecond time scale. Under this condition, significant additional interactions of the isoprenylated protein with the membrane (e.g., through favorable electrostatic interactions with the bilayer, or through binding of another portion of the protein to a membrane receptor) could easily retard the rate of spontaneous desorption to a point where some form of catalysis would be required for the protein to fulfill its normal regulatory functions at a reasonable rate.

The above kinetic arguments lead us to two conclusions. First, if rapid spontaneous desorption of an isoprenylated protein from a bilayer is important to the protein's physiological function, we may expect that the interactions between this protein and its membrane targeting receptors must be relatively weak (energies of a few kcal mol^{-1}, which would decrease the rate of spontaneous desorption by at most 2–3 orders of magnitude) or must substantially antagonize the association of the isoprenyl group with the lipid bilayer. Secondly, if these conditions are not fulfilled, some mechanism must exist to enhance the rate of desorption of the isoprenylated species from the membrane, even for proteins that bear a single isoprenylated residue and no other lipid modifications. It will be interesting to determine to what degree regulation of the kinetics, as well as the affinity, of partitioning of such proteins between the membrane and aqueous phases may play a role in regulating their overall function.

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[7] The alternative theory of Aniansson *et al.* (1976) would predict still slower kinetics of dissociation of lipopeptides from bilayers given equilibrium partition coefficients of the magnitudes indicated. Moreover, in general the free energy for desorption of an amphiphile from a membrane or other aggregate is expected to be significantly higher than the equilibrium free energy of transfer of the amphiphile from the membrane to water (compare for example the estimates of $[-\Delta G_p]$ *vs* ΔG_d^* for another class of amphiphiles in Nichols, 1985). The actual desorption rate constant is therefore likely to be considerably lower (plausibly, by orders of magnitude) than the maximum values given in the text.

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