Doubly-Lipid-Modified Protein Sequence Motifs Exhibit Long-Lived Anchorage to Lipid Bilayer Membranes†

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ABSTRACT: To understand better the potential functional importance of the dual-lipid modifications found in a number of intracellular proteins of eukaryotes, we have examined how "tenaciously" various doubly-lipid-modified peptides, with sequences and lipid modifications reflecting those found in intracellular proteins, are anchored to lipid bilayer membranes. Fluorescent-labeled peptides bearing dual-lipid modifications were incorporated into large unilamellar egg phosphatidylcholine/phosphatidylglycerol vesicles, and the kinetics of spontaneous intervesicle transfer of the lipopeptides were monitored by a fluorescence-dequenching assay. Lipopeptides incorporating the stable "dual-anchor" motif -C(geranylgeranyl)XC(geranylgeranyl)-OMe found in several *rab* and homologous proteins exhibit very slow rates of interbilayer transfer ($t_{1/2}$ > 50 h), as do lipopeptides incorporating myristoyl-GC(palmitoyl)X- and -C(palmitoyl)XC(farnesyl)-OMe motifs found in various *src*-related intracellular tyrosine kinases and G-protein α-subunits and in $p21^{H\text{-}ras}$, respectively. Lipopeptides terminating in an ummethylated -C(geranylgeranyl)C(geranylgeranyl)-OH motif show somewhat greater but still very slow rates of spontaneous interbilayer transfer ($t_{1/2}$ = ca. 10 h). Extrapolating from these results, we estimate that the rate of spontaneous desorption of the corresponding doubly-anchored proteins from membranes should be much slower than that of regulated, protein-mediated release (effected by binding to an "escort" protein or by de-S-acylation). As a result the intracellular distributions of these species (and particularly their targeting to specific intracellular membranes) are likely to be governed (and regulated) primarily by kinetic rather than thermodynamic factors. In parallel vesicle-binding measurements, peptides modified with *S*-palmitoyl groups were found to associate with lipid bilayers even more avidly than comparable geranylgeranylated peptides, explaining the 'tenacious' membrane-binding properties of dual-anchor motifs incorporating an *S*-palmitoyl residue.

Among the various intracellular proteins of eukaryotes that are covalently modified with acyl or isoprenyl groups, a significant number carries more than one such hydrophobic substituent per protein molecule. Particularly interesting among the latter proteins, from a physical standpoint, are those species which possess no identifiable membrane-associating polypeptide sequences and which interact with the membrane lipid bilayer primarily if not exclusively by means of two (or, rarely, more) coupled acyl or isoprenyl substituents. Several different "dual-anchor" motifs of this type have now been identified in membrane-associating proteins. Various mammalian *rab* proteins, and homologous proteins such as yeast *ypt*5, terminate in a -CXC amino acid sequence which is geranylgeranylated on both cysteine residues and methylated at the carboxyl terminus (Farnsworth *et al.*, 1991; Horiuchi *et al.*, 1991; Cox & Der, 1992; Newman *et al.*, 1992). Other *rab* proteins, terminating in a -CC (or -CCXX) motif, have been shown to be doubly geranylgeranylated (Farnsworth *et al.*, 1994) but not methylated (Wei *et al.*, 1992; Li & Stahl, 1993a; Smeland *et al.*, 1994), and yeast *ypt* proteins terminating in a -CC sequence may be similarly modified in at least some cases (Moores *et al.*, 1991; Newman *et al.*, 1992; Giannakouros *et al.*, 1993). Distinct patterns of dual-lipid substitution, including palmitoyl/farnesyl and geranylgeranyl/farnesyl modifications, have been suggested for other types of proteins terminating in -CC sequences (Fujiyama *et al.*, 1991; Adamson *et al.*, 1992). Various nonreceptor tyrosine kinases of the *src* family, including $pp56^{lck}$, $pp59^{fyn}$, $pp55^{fgr}$, and $pp56^{hck}$ (Paige *et al.*, 1993; Shenoy-Scaria *et al.*, 1993, 1994; Rodgers *et al.*, 1994; Alland *et al.*, 1994), as well as the α-subunits of a variety of heterotrimeric G-proteins (Linder *et al.*, 1993; Wedegaertner *et al.*, 1993; Degtyarev *et al.*, 1993; Parenti *et al.*, 1993; Hallak *et al.*, 1994b; Wedegaertner & Bourne, 1994) are modified by N-terminal myristoylation and reversible S-acylation on a nearby cysteine residue. Finally, certain members of the *ras* superfamily of small monomeric G-proteins, including $p21^{H\text{-}ras}$ and $p21^{N\text{-}ras}$, are both farnesylated on their terminal cysteine residue and reversibly S-acylated on one or more nearby cysteines (Hancock *et al.*, 1989; Fujiyama *et al.*, 1991).

From a physical standpoint, the addition of two hydrophobic chains to a protein could fulfill two distinct (though not mutually exclusive) functions. Some types of protein-coupled lipophilic groups (notably an *N*-myristoyl residue) appear to exhibit rather marginal membrane-binding affinity in isolation (Kaplan *et al.*, 1990; Silverman & Resh, 1992; Taniguchi & Manenti, 1993; Peitszsch & McLaughlin, 1993; Kim *et al.*, 1994), and in some cases the pairing of such residues with a second hydrophobic moiety (usually an *S*-acyl residue) could serve simply to shift the membrane/cytoplas-

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mic equilibrium of the modified protein decisively in favor of the membrane phase.

An interesting alternative possibility is suggested by previous findings (Helms et al., 1993; Silvius & l'Heureux, 1994) that the association of monoacylated or monoprenylated peptides and proteins with the membrane lipid bilayer is very rapidly reversible, even when such binding is 'strong' in the thermodynamic sense. The presence of a second acyl or isoprenyl chain might serve biologically to suppress this rapid reversibility of membrane binding, allowing a doubly-lipid-modified protein to be efficiently targeted to a particular membrane compartment by a mechanism we will refer to here as "bilayer trapping". In a process of this type, a doubly-lipid-modified protein, after introduction to the correct membrane compartment by appropriate targeting factors, would remain for all practical purposes irreversibly associated with the lipid bilayer until the protein was released from the membrane by some controlled, protein-mediated process. Targeting mechanisms of this type, which could offer both high efficiency and high capacity (since they utilize targeting components in catalytic rather than stoichiometric amounts), have been suggested previously in various contexts (Hancock *et al.*, 1989; Pfeffer, 1992, 1994; Magee & Newman, 1992; Novick & Brennwald, 1993; Cadwallader *et al.*, 1994) but have not been tested experimentally.

To target a dual-anchored protein efficiently to a given membrane by a bilayer-trapping mechanism as just suggested, it is clear that the rate of *regulated* (protein-mediated) release of the protein from the membrane must considerably exceed that of *spontaneous* transfer of the protein to other membranes. To evaluate the tenacity with which different dual-anchor motifs could anchor proteins hydrophobically to a given membrane, we have here examined the rates at which various doubly-lipid-modified peptides, representing analogous sequences found in dual-anchored proteins, undergo spontaneous intermembrane transfer. Our results suggest that the lipid-modified sequences found in most dual-anchored proteins may indeed serve to retain these proteins in a given membrane over time scales long enough to permit efficient protein targeting by bilayer-trapping mechanisms as suggested above. As a corollary to this work, we have also shown that peptide-coupled long-chain *S*-acyl groups, which occur either singly or as components of dual-anchor motifs in a variety of intracellular proteins, partition with high affinity into the membrane lipid bilayer and in fact constitute the most hydrophobic individual lipid modification currently known to occur in intracellular proteins of eukaryotes.

MATERIALS AND METHODS

Materials. Protected amino acids and other reagents for peptide synthesis were obtained from NovaBiochem (La Jolla, CA) or Sigma (St. Louis, MO). *trans,trans*-Farnesol and *all-trans*-geranylgeraniol, purchased from Aldrich (Minneapolis, MN) and American Radiolabeled Chemicals (Bowling Green, IN), respectively, were converted to the corresponding bromides (Corey *et al.*, 1972) and stored at −80 °C under argon with 1% (w/v) BHT.[1] (*S*-Bimanylthio)acetic acid was synthesized by reacting monobromobimane with a 10% excess each of thioacetic acid and triethylamine in methanol for 30 min at room temperature and then drying exhaustively under high vacuum. FmocD(OFm)-OtBu was prepared from FmocD-OtBu, using the method described by Xue *et al.* (1990) for BocC(Acm)-OFm, and then deprotected with 1:1 CH_2Cl_2/trifluoroacetic acid and exhaustively dried *in vacuo* to give FmocD(OFm)-OH.

Synthetic Methods. The lipopeptides myrGCG-(BimCA) and myrGSG-(BimCA) were synthesized as described previously (Silvius & l'Heureux, 1994; Quesnel & Silvius, 1994). The peptides BimTA-ACR-OMe and -ACR-NH_2 were prepared essentially as described previously for acetyl-LCR-OMe (Quesnel & Silvius, 1994), using the succinimidyl ester of (*S*-bimanylthio)acetic acid (prepared using 2 and 1.2 equiv, respectively, of *N*-hydroxysuccinimide and DCCD in DMF at 25 °C) in place of acetic anhydride to acylate the N-terminal amino acid. *S*-Acyl and -isoprenyl derivatives of the latter peptides were prepared using conditions described previously (Silvius & l'Heureux, 1994; Quesnel & Silvius, 1994).

BimTA-GGGCC-OMe and -OH were synthesized by condensing Fmoc-GGG-OH, prepared from glycylglycylglycine (Sigma) using Fmoc-succinimidyl ester (Milton *et al.*, 1987), with C(StBu)C(StBu)-OMe or -OFm in the presence of 2 and 1.2 equiv of HOBT and DCCD, respectively, in dry DMF for 6 h at 0 °C. The product was purified by flash chromatography (Still *et al.*, 1978) on silica gel 60, using 2.5% methanol in chloroform as the eluant, and then deprotected with butane-1,4-dithiol (4 equiv) and 2-mercaptoethanesulfonic acid sodium salt (10 equiv) in 3:1 DMF/0.5 M aqueous HEPES, pH 7.6 (1 mL/50 μmol of peptide), for 5 h at 60 °C. The mixture was partitioned in 2:1:1 chloroform/methanol/0.1 M Mes, pH 5.0, and the chloroform phase was dried extensively under nitrogen, with gentle warming and with several additions of DMF, to evaporate most of the residual butanedithiol. The residue was reacted for 24 h at 0 °C with 3 mol equiv of geranylgeranyl or farnesyl bromide in 9:1 acetonitrile/DMF in the presence of KF·$2H_2O$ (Xue *et al.*, 1991); after phase partitioning as described above, the choroform extract was applied to a column of silica gel 60, which was successively eluted with 10 volumes of chloroform (discarded) and 20 volumes of 8:2 chloroform/methanol. The latter eluate was concentrated *in vacuo* and the residue deprotected with 4:1 DMF/piperidine for 45 min at 0 °C under argon. After removal of the solvents under high vacuum, the products were purified by preparative TLC in 80:20 chloroform/methanol (adding 100 μg of BHT/mL of solvent), recovering the major

[1] Abbreviations: BHT, 2,6-di-*tert*-butylphenol; DCCD, dicyclohexylcarbodiimide; DMF, dimethylformamide; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; ePC, phosphatidylcholine from egg yolk; ePG, phosphatidylglycerol prepared from ePC by transphosphatidylation; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid) sodium salt; HOBT, 1-hydroxybenzotriazole monohydrate; Mes, 2-(*N*-morpholino)ethanesulfonic acid sodium salt; MOPS, 3-(*N*-morpholino)propanesulfonic acid sodium salt; *N*-oleoyl-DABS-PE, *N*-oleoyl-1-palmitoyl-2-[12-[[[4-[[4-(dimethylamino)phenyl]azo]phenyl]sulfonyl]methylamino]stearoyl]-phosphatidylethanolamine; PEG-*x*, poly(ethylene glycol) of average molecular weight *x*; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; *rab*-GDI, *rab* guanine nucleotide dissociation inhibitor protein; REP, *rab* escort protein; SATA, (*S*-acetylthio)acetic acid *N*-hydroxysuccinimide ester; TLC, thin-layer chromatography; TNBS, trinitrobenzenesulfonic acid sodium salt. Peptide structures are abbreviated using the standard one-letter amino acid code plus the following additional abbreviations: -BimCA, -2-(*S*-bimanylthio)ethylamide; BimTA-, (*S*-bimanylthio)acetyl-; Boc, *N*-*tert*-butyloxycarbonyl-; Fmoc, *N*-fluorenylmethoxycarbonyl-; GerGer, geranylgeranyl; -MSBim-, -2-(*S*-bimanylthio)succinyl-; -OFm, *O*-fluorenylmethyl; StBu, *tert*-butylthio-.

ninhydrin-positive band (visualized on a thin strip of the developed plate) by elution of the scraped gel with 4:2:1 methanol/chloroform/water followed by solvent partitioning as above. The products were fluorescent-labeled by successive reactions with succinimidyl (*S*-acetylthio)acetate (SATA), neutral hydroxylamine, and monobromobimane as described previously (Silvius & l'Heureux, 1994) and then finally purified by preparative TLC as above. Purification and workup of the fluorescent products were carried out with exclusion of light and wherever possible under argon.

The lipopeptides BimTA-DC(GerGer)AC(GerGer)-OMe and MSBim-C(GerGer)AC(GerGer)-OMe were prepared as follows. FmocC(StBu)AC(StBu)-OMe was prepared starting from (*S-tert*-butylthio)cysteine methyl ester, using standard Fmoc chemistry with HOBT/DCCD-mediated coupling. The product tripeptide was S-deprotected, coupled to farnesyl or geranylgeranyl bromide, purified by flash chromatography on silica gel 60 (eluting with a gradient of 0.5−1% methanol in chloroform), and then deprotected with piperidine/DMF, all as described above for diprenylated GGGCC-OMe. One portion of the diprenylated tripeptide was condensed with FmocD(OFm)-OH, deprotected with DMF/piperidine, reacted successively with SATA, hydroxylamine, and monobromobimane as above, and then purified by preparative TLC in 90:10:0.5 chloroform/methanol/acetic acid to yield BimTA-DC(GerGer)AC(GerGer)-OMe. A second portion of the diprenylated tripeptide was reacted successively with (*S*-acetylthio)succinic anhydride, neutral hydroxylamine, and monobromobimane as described (Silvius & Zuckermann, 1993) to yield MSBim-C(GerGer)AC(GerGer)-OMe, which was coupled either to ethanolamine or to α-amino-ω-methoxy-PEG-350, -750, or -2000 as described previously (Silvius & Zuckermann, 1993).

The lipopeptide SC(StBu)RC(farnesyl)-OMe was prepared using Fmoc chemistry as above, introducing the farnesyl group at the stage of the Fmoc-protected dipeptide, and coupled to (bimanylthio)acetic acid and subsequently S-deprotected as described above. The product, BimTA-SCRC(farnesyl)-OMe, could be selectively S-acylated in excellent yield by incubation under argon for 2 h at 37 °C in an aqueous mixture containing 0.8 mM lipopeptide, 400 mM NaCl, 100 mM HEPES, 3 mM $MgCl_2$, 1 mM DTT, 4 mM long-chain acyl-CoA, pH 7.6; after incubation the mixture was acidified to pH 5 and extracted in the cold with chloroform (2 volumes) after adding 1 volume of methanol. The S-acylated lipopeptides were purified by preparative TLC in 50:15:5:5:2 chloroform/acetone/methanol/acetic acid/water and recovered from the scraped gel by elution and solvent partitioning (at 0 °C) as described above.

The structure and purity of the lipopeptides generated in this study were assessed by ^{1}H-NMR and TLC in two different solvent systems, in the latter case testing for potential impurities both by fluorescence and by charring with sulfuric acid.

Methods. Large unilamellar lipid vesicles were prepared by hand extrusion of lipid dispersions, prepared from well-dried lipid samples by vortexing and repeated freeze−thawing, through 0.1 μm pore size poly(carbonate) filters (MacDonald *et al.*, 1991). All operations were performed under argon and in dim light or darkness for vesicle samples containing fluorescent probes. Vesicles were prepared and incubations carried out in 150 mM NaCl, 10 mM MOPS, 0.5 mM EDTA, 0.05 mM DTPA, pH 6.5, except where otherwise indicated. The fraction of PE exposed at the outer surfaces of PE-containing vesicles was estimated using the procedure of Nordlund *et al.* (1981), shortening the time of vesicle reaction with TNBS to 10 min.

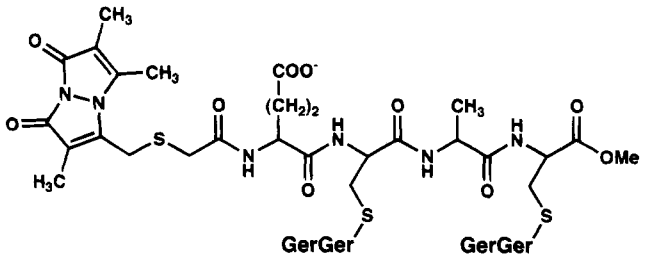
The affinity of binding of monoacylated or monoisoprenylated fluorescent peptides to 90:10 ePC/POPE vesicles was determined as described previously (Silvius & l'Heureux, 1994). Briefly, the fluorescence of a fixed amount of lipopeptide (0.1 nmol) in 3 mL of buffer was measured as a function of the concentration of added lipid vesicles (90:10 ePC/POPE). The resulting data were fit to the equation

$$F = F_0 + (F_{max} - F_0)([L]_{eff}/(K_d^{eff} + [L]_{eff}))$$

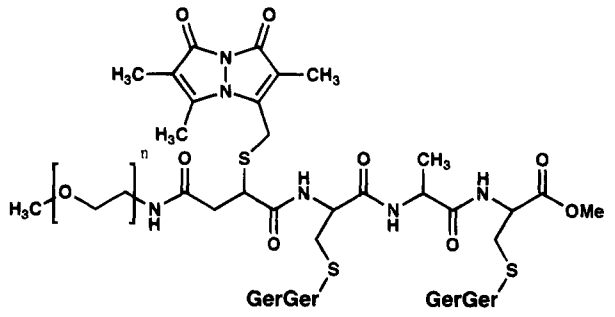
where F is the sample fluorescence at a given concentration of *surface-exposed* vesicle lipids ($[L]_{eff}$), F_0 and F_{max} represent the fluorescence values for the same amount of lipopeptide at zero and saturating lipid concentrations, respectively, and K_d^{eff} is the value of $[L]_{eff}$ at which 50% of the lipopeptide is vesicle-bound. The quantity $[L]_{eff}$ was determined by assaying the fraction of surface-exposed vesicle PE and the total lipid concentration in vesicle preparations as described previously (Silvius & l'Heureux, 1994).

The transfer of fluorescent lipopeptides between lipid vesicles was monitored essentially as described previously (Nichols & Pagano, 1982; Silvius & Zuckermann, 1993; Silvius & Leventis, 1993). Large unilamellar donor vesicles, containing 0.4 mol % fluorescent lipopeptide and 1.5 mol % *N*-oleoyl-DABS-PE, a nonexchangeable energy-transfer quencher of bimane fluorescence (Leventis & Silvius, 1993), were mixed at time zero with bath-sonicated acceptor vesicles of the same composition but lacking the probe and quencher, and the subsequent time-dependent enhancement of fluorescence was monitored to determine the rate of intervesicle probe transfer. Samples were incubated either in the fluorimeter cuvette, at donor and acceptor vesicle concentrations of 15 and 150 μM, respectively, or, for longer time courses, in glass tubes under argon at donor and acceptor concentrations of 0.2 and 2 mM, respectively.

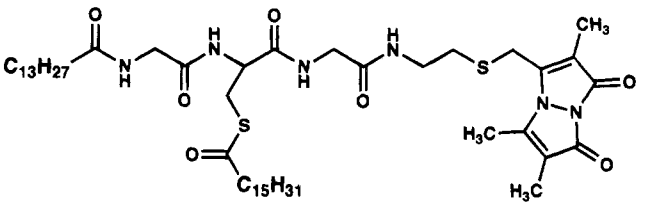
To analyze the complete time course of interbilayer transfer of amphipathic molecules like the lipopeptides examined here, it is necessary to know (or conjecture) whether the rate of transbilayer flip-flop for such species is slower or faster than that of their interbilayer transfer [see Homan and Pownall (1988), Wimley and Thompson (1990)]. However, in either circumstance the *initial slope* of the fluorescence time course (scaled to the maximal fluorescence change expected when the lipopeptide redistributes completely to equilibrium) is simply equal to the product of the "true" first-order rate constant for lipopeptide desorption from the bilayer surface, *multiplied by the proportion of total lipopeptide that is initially present at the outer surfaces of the donor vesicles.* To estimate the latter quantity we assumed that the lipopeptides at least initially adopt a symmetrical transbilayer distribution in the large unilamellar donor vesicles employed here. In this case the quantity just noted can be equated to the fraction of total donor vesicle lipids exposed at the outer surface, which for these large vesicles can be reliably estimated by determining the fraction of surface-exposed PE (Nordlund *et al.*, 1981). Due to spectral interference, this procedure could not be applied to vesicles containing *N*-oleoyl-DABS-PE, precluding



BimTA-DC(GerGer)AC(GerGer)-OMe



PEG-x-MSBim-C(GerGer)AC(GerGer)-OMe



myrGC(palmitoyl)G-(BimCA)

FIGURE 1: Structures of three of the lipid-modified peptides examined in this study, illustrating the general structures of the fluorescent-labeled lipopeptides used here.

its routine application in each kinetic experiment. However, in control experiments we found that the fraction of surface-exposed POPE in 70:20:10 ePC/ePG/POPE vesicles, prepared identically to the donor vesicles used for lipopeptide-transfer measurements, was 46 ± 2% in four independent preparations. We thus used this value in all our measurements of intervesicle lipopeptide transfer to estimate the percentage of fluorescent lipopeptide that was exposed at the donor−vesicle surface at time zero.

RESULTS

In Figure 1 are illustrated the general structures of the lipid-modified peptides examined in this study. The lipopeptides were labeled with a fluorescent bimane group, which in previous studies has been shown to make no detectable contribution to the interactions of labeled (lipo)peptides with lipid vesicles (Skerjanc *et al.*, 1987; Silvius & l'Heureux, 1994). The sequence BimTA-ACR-NH_2 was chosen as a simple model of the sequences surrounding many protein S-acylation sites [which are usually enriched in basic and/or aliphatic amino acid residues (Schlesinger *et al.*, 1993)], with a minimal expected intrinsic affinity for uncharged lipid bilayers. The labeled lipopeptide myrGCG-(BimCA), representing the N-terminus of the human nonreceptor tyrosine kinase $pp56^{lck}$, resembles the N-terminal sequences (myrGCX-, where X is an aliphatic, hydroxylic, or basic residue) of a variety of *lck* homologues and heterotrimeric G-protein α-subunits (Parenti *et al.*, 1993; Mumby *et al.*, 1994) which are dynamically S-acylated on a cysteine residue near the terminal myristoylglycyl residue. The lipopeptide BimTA-SCRC(farnesyl)-OMe represents the C-terminus of $p21^{H\text{-}ras}$, whose nonterminal cysteine can be S-acylated in mammalian cells (Hancock *et al.*, 1989); the lysine residue of the naturally occurring (murine) protein was replaced by an arginyl residue in the lipopeptide to facilitate chemical synthesis of authentic *S*-acylpeptide standards. The lipopeptide BimTA-DC(GerGer)AC(GerGer)-OMe represents the C-terminus of *rab* 3A, which physiologically is modified as indicated (Horiuchi *et al.*, 1991; Farnsworth *et al.*, 1991). The sequence -GGGC(GerGer)C(GerGer)-OH represents the C-termini of *rab1A* and *rab2* (Wei *et al.*, 1992; Farnsworth *et al.*, 1994); yeast *ypt1*, with the same C-terminal sequence, may be analogously modified (Moores *et al.*, 1991; Newman *et al.*, 1992).

Table 1: Effective Dissociation Constants for Association of Lipopeptides with 90:10 ePC/POPE Vesicles[a]

lipopeptide	K_d^{eff} (μM)
BimTA-AC(X)R-NH_2, X =	
palmitoyl	0.196 ± 0.013
geranylgeranyl	0.96 ± 0.10
farnesyl	18.5 ± 1.4
myristoyl	1.78 ± 0.06
BimTA-AS(X)R-NH_2, X =	
palmitoyl	0.60 ± 0.04
myristoyl	5.47 ± 0.39
BimTA-AC(X)R-OMe, X =	
palmitoyl	0.126 ± 0.003
geranylgeranyl	0.61 ± 0.04
farnesyl	12.5 ± 1.5

[a] Values of K_d^{eff}, determined at 37 °C, pH 6.5, as described in the text, were determined in duplicate experiments using different preparations of lipid vesicles.

Bilayer-Partitioning Affinities of S-Acylated Peptides. While the lipid-binding affinities of representative N-acylated and S-isoprenylated peptides have been estimated directly (Peitzsch & McLaughlin, 1993; Silvius & l'Heureux, 1994), no similar determination has been reported to date for species bearing a thioester-linked fatty acyl group. As *S*-acyl groups constitute the only lipid modification of certain membrane-associating proteins and serve as a reversible "secondary" lipid modification in many others, we examined the affinities with which representative S-acylated (and, for comparison, S-isoprenylated) peptides partition into lipid bilayers. As described previously (Silvius & l'Heureux, 1994), partitioning of a bimane-labeled lipopeptide from the aqueous phase into the surfaces of lipid vesicles leads to an enhancement of fluorescence, allowing ready determination of both the kinetics of partitioning and the water/bilayer partition coefficient. Kinetic measurements similar to those described previously (Silvius & l'Heureux, 1994) showed that the mono-S-acylated and -isoprenylated lipopeptides examined insert into and spontaneously transfer between lipid vesicles on a very rapid time scale (<seconds) but show very slow rates of transbilayer flip-flop (not shown).

In Table 1 are summarized the affinities measured for binding of several S-acylated and S-isoprenylated derivatives of the peptides BimTA-ACR-NH_2 and BimTA-ACR-OMe to large unilamellar 90:10 ePC/POPE vesicles. These

affinities are quantitated through the parameter K_d^{eff}, which represents the concentration of *surface-exposed* vesicle lipids (to which the added lipopeptide has ready access) at which 50% of a given lipopeptide is vesicle-bound. As discussed previously (Silvius & l'Heureux, 1994), this parameter is inversely related to the dimensionless mole fraction-based bilayer/water partition coefficient for the lipopeptides, K_p, by the relationship $K_d^{eff} = 55.5\ M/K_p$. It can be seen from Table 1 that an *S*-palmitoyl substituent confers on these peptides an affinity for lipid bilayers that is some 5-fold greater than that conferred by a geranylgeranyl group and almost 100-fold greater than that conferred by a farnesyl group, in the same sequence context. The values of K_d^{eff} measured for the farnesylated and geranylgeranylated peptides listed in Table 1 fall in the same range as those previously measured for a series of C-terminally-isoprenylated peptides (l'Heureux & Silvius, 1994). C-terminally-amidated and -carboxylmethylated peptides give very similar patterns of results, although the binding of the methylated species is somewhat stronger than that of the corresponding amidated lipopeptides.

Replacement of an *S*-palmitoyl by an *S*-myristoyl substituent in the peptide BimTA-ACR-NH_2 increases the value of K_d^{eff} by roughly 9-fold (Table 1), indicating that the free energy of bilayer/water partitioning decreases (becomes more negative) by ca. -680 cal mol^{-1} per methylene group added to the acyl chain. This incremental value is comparable to those measured previously for the bilayer/water partitioning of a homologous series of *S*-alkylcysteinyl peptides (Silvius & l'Heureux, 1994) but is somewhat lower than the value reported by Peitzsch and McLaughlin (1993) for a series of homologous *N*-acylglycines (-825 cal mol^{-1}). Replacement of an *S*-acyl by a (serine) *O*-acyl linkage in the above lipopeptides weakens the bilayer-partitioning affinity by roughly 3-fold (Table 1) but does not detectably alter the incremental change in the free energy of partitioning per added methylene residue.

Extrapolating from our above results, assuming a linear variation of the free energy of water/bilayer partitioning with acyl chain length (Tanford, 1980; Peitzsch & McLaughlin, 1993), we predict the value of K_d^{eff} for BimTA-AC(stearoyl)R-NH_2 to be of the order of 25 nM. The value of K_d^{eff} for the *S*-stearoyl peptide was found experimentally to be too low to measure accurately using our assay, implying that for this species $K_d^{eff} < 0.05\ \mu M$, consistent with the above prediction.

Kinetics of Intervesicle Transfer of Doubly-Anchored Lipopeptides. In Table 2 are summarized the first-order rate constants determined, as described in Materials and Methods, for the spontaneous interbilayer transfer of a series of fluorescent lipopeptides bearing various double-anchor motifs. Control experiments showed that under our experimental conditions the kinetics of probe transfer were in all cases independent of the total vesicle concentration, indicating that transfer is independent of vesicle−vesicle interactions and rate-limited by desorption from the donor vesicles (Roseman & Thompson, 1980; Nichols & Pagano, 1982; Jones & Thompson, 1989).

Table 2: Rate Constants (k_d)[a] and Half-Times ($t_{1/2}$) for Transfer of "Doubly-Anchored" Lipopeptides between Large Unilamellar 70:20:10 ePC/ePG/POPE Vesicles at 37 °C

lipopeptide	k_d (h^{-1})	$t_{1/2}$ (h)[b]
myrGC(palmitoyl)G-(BimCA)[d]	$(9.96 \pm 0.54) \times 10^{-3}$	70
myrGC(oleoyl)G-(BimCA)[d]	$(7.00 \pm 0.40) \times 10^{-3}$	99
myrGS(palmitoyl)G-(BimCA)[d]	$(2.88 \pm 0.06) \times 10^{-2}$	24
BimTA-SC(palmitoyl)RC(farnesyl)-OMe[d]	$(8.41 \pm 0.36) \times 10^{-3}$	82
BimTA-SC(myristoyl)RC(farnesyl)-OMe[d]	$(4.20 \pm 0.06) \times 10^{-3}$	16.5
BimTA-SC(GerGer)RC(farnesyl)-OMe[d]	$(1.34 \pm 0.04) \times 10^{-3}$	52
BimTA-DC(GerGer)AC(GerGer)-OMe[c]	$(1.12 \pm 0.06) \times 10^{-2}$	62
BimTA-GGGC(GerGer)C(GerGer)-OMe[c]	$(5.93 \pm 0.20) \times 10^{-3}$	117
BimTA-GGGC(GerGer)C(GerGer)-OH[c]	$(8.02 \pm 0.18) \times 10^{-2}$	8.6
BimTA-GGGC(farnesyl)C(farnesyl)-OMe[c]	$(3.82 \pm 0.10) \times 10^{0}$	0.19
BimTA-GGGC(farnesyl)C(farnesyl)-OH[c]	$(5.23 \pm 0.72) \times 10^{1}$	0.013
X-MSBim-C(GerGer)AC(GerGer)-OMe,[e] X =		
ethanolamine	$(6.07 \pm 0.47) \times 10^{-3}$	114
PEG-350	$(2.22 \pm 0.04) \times 10^{-2}$	31
PEG-750	$(3.01 \pm 0.10) \times 10^{-2}$	23
PEG-2000	$(4.82 \pm 0.24) \times 10^{-2}$	14.4

[a] Spontaneous transfer of lipopeptides between lipid vesicles was assayed and analyzed as described in the text. Values shown represent the mean (±half-range) determined in two separate experiments using different preparations of donor and acceptor vesicles. [b] Calculated as $-\ln(1/2)/k_d$. [c] Incubations of donor and acceptor vesicles were carried out at pH 7.4. [d] Incubations of donor and acceptor vesicles were carried out at pH 6.5. [e] Incubations of donor and acceptor vesicles were carried out at pH 6.5 and 7.4 with essentially identical results.

Lipopeptides incorporating the S-acylated dual-anchor sequences myrGC(palmitoyl)X- (found in certain nonreceptor tyrosine kinases and the α-subunits of several heterotrimeric G-proteins) and -C(palmitoyl)-XC(farnesyl)-OMe (found in $p21^{H\text{-}ras}$) show very slow spontaneous transfer between vesicles (halftimes of ca. 60−110 h). Replacement of the palmitoyl residue in the former species by an *S*-oleoyl group, which can also be found in S-acylated proteins (Bizzozero *et al.*, 1986; O'Brien *et al.*, 1987; Hallak *et al.*, 1994b), further decreases the rate of intervesicle transfer somewhat. By contrast, replacement of the palmitoyl residue in the lipopeptide BimTA-SC(palmitoyl)RC(farnesyl)-OMe by a nonphysiological myristoyl or geranylgeranyl group increases the rate of spontaneous intermembrane transfer by roughly 5- or 2-fold, respectively. The effects of these latter substitutions reflect (in slightly attenuated form) those expected given the relative affinities of the different *S*-acyl and -isoprenyl groups for the lipid bilayer as determined in the vesicle-partitioning assays discussed above. Intervesicle

[2] The binding of a hydrophilic macromolecule to a lipid bilayer via a coupled hydrophobic "anchor" leads to some loss of configurational, translational, and rotational entropy. The potential to regain this entropy upon transfer to the aqueous phase confers upon the "anchored" macromolecule a greater tendency to undergo desorption from the bilayer than would be observed for the "anchor" moiety alone [see Silvius and Zuckermann (1993) and references therein]. From a previous analysis of the kinetics of interbilayer transfer of various types of phospholipid-anchored macromolecules (Silvius & Zuckermann, 1993), we have estimated that a lipid-anchored conjugate of a species such as a globular protein, which exhibits only a modest degree of configurational freedom, may undergo spontaneous interbilayer transfer at a rate typically 5−10-fold faster than would the corresponding "anchor" structure alone.

[3] A few proteins have been reported to be modified to some extent with other *S*-acyl groups, including arachidonyl (Muszbek & Laposata, 1993; Hallak *et al.*, 1994a) or palmitoleoyl (Casey *et al.*, 1994), which may be somewhat less hydrophobic than palmitoyl chains (Tanford, 1980; Anel *et al.*, 1993; Peitzsch & McLaughlin, 1994). It is not known to what extent (if any) the introduction of such alternative *S*-acyl modifications may affect the function of these proteins *in situ*.

[4] On the basis of our present findings, we can estimate that a putative palmitoyl/geranylgeranyl dual modification of a terminal -CC-OH sequence (Molenaar *et al.*, 1988) would confer slightly more "tenacious" membrane anchorage than would a bis(geranylgeranyl) modification.

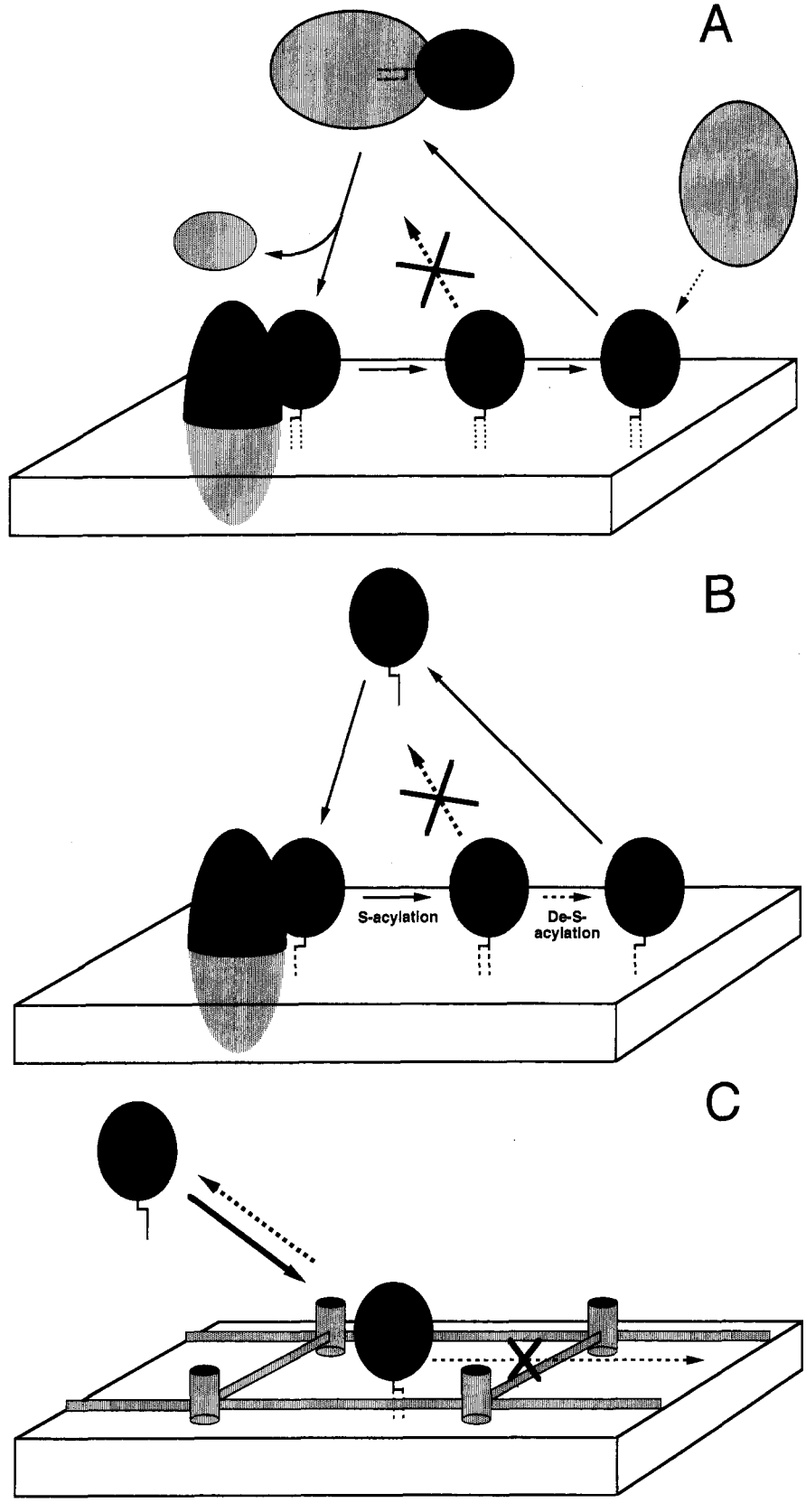


FIGURE 2: Schematic illustrations of potential mechanisms for specific membrane targeting of dual-anchored proteins via bilayer-trapping mechanisms. Panel A: A permanently-dual-modified species (e.g., a diprenylated *rab* or *rab*-like protein) is delivered to a specific membrane compartment by the interaction between a cytoplasmic carrier protein, which shields the lipid groups from the aqueous phase, and a putative membrane-targeting receptor (left). The dual-anchored protein, once released from association with both of these latter species, may subsequently associate with a variety of other membrane proteins (not shown) by free lateral diffusion but remains in essence irreversibly anchored to the lipid bilayer until abstracted from the membrane by a cytoplasmic carrier protein (e.g., a *rab*-GDI). Panel B: A singly-lipid-modified protein bearing an S-acylation site near the "primary" acylated or isoprenylated residue can diffuse to the membrane via the aqueous phase without the obligate participation of a cytoplasmic carrier protein. S-acylation at the membrane, in association with a putative membrane-targeting receptor, produces a dual-anchored species which again is "permanently" anchored to that membrane compartment

transfer of S-acylated peptides was measured at pH 6.5 to minimize the rate of thioester bond hydrolysis, which was estimated to be no more than 0.15%/h at this pH (not shown). These rates of thioester hydrolysis could make at most a small (<20%), and in most cases a negligible, contribution to the rates of intervesicle transfer measured for the S-acylated lipopeptides listed in Table 2.

Also shown in Table 2 are the rates of spontaneous interbilayer transfer measured for lipopeptides carrying several diprenyl "dual-anchor" motifs. The lipopeptides BimTA-DC(GerGer)AC(GerGer)-OMe (representing the C-terminus of *rab3A*) and BimTA-GGGC(GerGer)C(GerGer)-OMe (an unphysiologically methylated variation of the *rab1A/rab2/ypt1* C-terminal sequence) transfer between lipid vesicles at comparably low rates, suggesting that the separation of the prenylated cysteines in the former motif does not significantly affect its membrane-anchoring properties. By contrast the unmethylated lipopeptide BimTA-GGGC-(GerGer)C(GerGer)-OH, representing the physiologically-modified C-terminus of *rab1A* and *rab2*, transfers between lipid vesicles some 13-fold faster than does its carboxylmethylated counterpart. Intervesicle transfer of the lipopeptide BimTA-GGGC(farnesyl)C(farnesyl)-OH is likewise ca. 14-fold faster than that of the equivalent carboxylmethylated lipopeptide; both difarnesylated peptides transfer between lipid vesicles more than 600-fold faster than their bis-(geranylgeranyl)-modified counterparts.

We have shown previously (Silvius & Zuckermann, 1993) that conjugates linking a given phospholipid "anchor" to various hydrophilic macromolecules, including species as diverse as proteins and poly(ethylene glycols), undergo transfer between membranes at rates significantly faster (typically, by ca. 5−10-fold) than does the corresponding lipid alone. Such effects, which rest mainly on very general properties (size and configurational flexibility) of the "tethered" macromolecule, are important to consider in predicting the likely rates of spontaneous interbilayer transfer of lipid-anchored *proteins* based on the transfer rates measured here for analogous lipid-anchored peptides. To assess whether such effects in the present context are likely to be comparable to those observed previously for phospholipid-anchored macromolecules, we determined the rates of spontaneous interbilayer transfer for a series of conjugates linking the lipopeptide MSBim-C(GerGer)AC(GerGer)-OMe to poly-(ethylene glycols) (PEGs) of increasing molecular weight (see Figure 1). These conjugates, while artificial, can be readily prepared, characterized, and manipulated as homogeneous species, and as we have discussed previously, they well-replicate the essential physical factors that determine the rates of intermembrane transfer of various types of lipid-anchored hydrophilic macromolecules (Silvius & Zuckermann, 1993). As shown in Table 2, the rate of spontaneous interbilayer transfer for the different lipopeptide−PEG conjugates steadily increases with increasing molecular weight of the coupled PEG and is roughly 8-fold greater for the PEG-2000 conjugate than for the ethanolamine ("PEG-44") conjugate. The pattern of results observed for the different lipopeptide−PEG conjugates is very similar to that we have observed previously using phospholipid-anchored conjugates of the same PEGs (Silvius & Zuckermann, 1993), suggesting that coupled hydrophilic macromolecules exert quantitatively similar effects on the kinetics of bilayer desorption of both phospholipid and lipopeptide "anchor" moieties. We can then estimate that a lipid-modified globular protein, which apart from its lipid anchor behaves as a typical "soluble" protein molecule, may desorb from a lipid bilayer up to 5−10-fold faster than would a small peptide bearing the same lipid modifications.[2]

Applying the conclusion just noted to the data presented in Table 2, we predict that proteins anchored to the membrane lipid bilayer by any of the following naturally-occurring dual-anchor motifs will exhibit half-times for spontaneous intermembrane transfer of at least several hours: myrGC(palmitoyl)-, as is found in a number of nonreceptor tyrosine kinases and heterotrimeric G-protein α-subunits; -C(GerGer)XC(GerGer)-OMe, where X is a neutral amino acid residue, as is found in a variety of *rab* and *rab*-homologous proteins; and -C(palmitoyl)YC-(farnesyl)-OMe, where Y is a basic amino acid residue, as occurs in $p21^{H\text{-}ras}$. The dual-anchor motif -C(GerGer)C-(GerGer)-OH, which as already noted occurs in certain *rab* proteins, is predicted to allow somewhat more rapid intermembrane transfer of proteins so anchored (with a half-time potentially as short as 1−2 h).

DISCUSSION

Our present estimates of the bilayer-partitioning affinities of S-acylated peptides complement previous determinations of the same property for N-myristoylated and S-isoprenylated peptides (Peitzsch & McLaughlin, 1993; Silvius & l'Heureux, 1994). In contrast to the previous theoretical estimates of Black (1992), we find a cysteine-coupled *S*-palmitoyl chain to be a more lipophilic modification than is a cysteine-coupled geranylgeranyl (C_{20}-isoprenoid) chain; this is true whether we compare the two modifications in a common sequence context (-AC(X)R-) or, more "fairly", each in their respective physiological sequence contexts [for example, using 90:10 ePC/POPE large unilamellar vesicles K_d^{eff} = 0.196 ± 0.013 μM for BimTA-AC(palmitoyl)R-NH_2 (this study), 0.266 ± 0.004 μM for BimTA-RAC(GerGer)-OMe, and 2.55 ± 0.22 μM for BimTA-RAC(GerGer)-OH (Silvius & l'Heureux, 1994)].

Prior studies of isoprenylated proteins (Hancock *et al.*, 1991) and peptides (Silvius & l'Heureux, 1994) have indicated that the presence of an O-methylated/S-geranylgeranylated cysteine residue at the carboxyl terminus of a protein is sufficient to promote efficient membrane binding under intracellular conditions. We can thus conclude that the same should be true for even a single protein-coupled *S*-palmitoyl chain, at least when it is present in a surface-exposed terminal or loop region of the protein, as for example

until the protein is de-S-acylated, either hydrolytically or, potentially, by acyl transfer to another membrane protein. A very similar scheme can be applied to proteins that bear no permanent lipid modifications but can be S-acylated at multiple nearby sites: in this case dual S-acylation is predicted to be required to anchor the protein irreversibly to the membrane bilayer. Panel C: Barriers to free lateral diffusion on the cytoplasmic face of a membrane can confine a dual-anchored protein at least transiently to the lateral "domain" in which it is initially inserted. In principle this effect can create some degree of steady-state enrichment of the protein in particular membrane domains, if the sites of membrane insertion of the dual-anchored species are inhomogeneously distributed within the membrane.

is the case for the nonmyristoylated α-subunits of several heterotrimeric G-proteins (Linder *et al.*, 1993; Veit *et al.*, 1994). The *S*-acyl chains of some "S-palmitoylated" proteins appear to be somewhat heterogeneous, including notably oleoyl and/or stearoyl as well as palmitoyl residues (Bizzozero *et al.*, 1986; O'Brien *et al.*, 1987; Fujimoto *et al.*, 1993; Hallak *et al.*, 1994b). However, these alternative *S*-acyl groups should exhibit bilayer-partitioning affinities comparable to or greater than that observed for an *S*-palmitoyl residue [this work and Anel *et al.* (1993), Peitzsch and McLaughlin (1993)] and should thus anchor a protein to the membrane bilayer at least as strongly as a palmitoyl chain.[3] The high affinity of palmitoyl and related *S*-acyl groups for the lipid bilayer is reflected in the very long-lived bilayer association that we observe for dual-anchored peptides incorporating long-chain *S*-acyl groups.

Our measurements of the kinetics of interbilayer transfer for doubly-lipid-modified peptides suggest that a variety of physiological dual-anchor motifs, notably myrGC(palmitoyl)-, -C(GerGer)XC(GerGer)-OMe, and -C(palmitoyl)-YC(farnesyl)-OMe (X = a neutral amino acid, Y = a basic amino acid), could anchor an associated protein to the membrane lipid bilayer "tenaciously", with half-times for spontaneous interbilayer transfer in excess of several hours. These half-times are considerably longer than those reported for turnover of the *S*-acyl chains of membrane-associating proteins such as $p21^{H\text{-}ras}$, $G_{s\alpha}$, and $pp56^{lck}$, which are typically of the order of 1−2 h or less (Magee *et al.*, 1987; Paige *et al.*, 1993; Mumby *et al.*, 1994; Wedegaertner & Bourne, 1994), and longer than those expected for the carrier protein-mediated membrane/cytoplasmic cycling of *rab* and homologous proteins (Goud *et al.*, 1988; Ferro-Novick & Novick, 1993; Zerial & Stenmark, 1993; Pfeffer, 1994). Proteins bearing the *N*-myristoyl/*S*-acyl, isoprenyl/*S*-acyl, and diprenyl modifications noted above are thus good potential candidates for targeting to specific membrane compartments by mechanisms based on bilayer trapping, as illustrated in Figure 2. As shown in panel A, diprenylated *rab* proteins, once delivered to a particular cellular membrane by a carrier protein such as a *rab*-GDI or REP (Seabra *et al.*, 1992a,b; Andres *et al.*, 1993; Ullrich *et al.*, 1993, 1994; Soldati *et al.*, 1994; Dirac-Svejstrup *et al.*, 1994; Alexandrov *et al.*, 1994), would be predicted from our results to remain tenaciously associated with the lipid bilayer of that membrane, such that release of the protein from the "target" membrane will be wholly dependent upon the action of carrier proteins (e.g., *rab*-GDIs). As shown in panel B, monoacylated or monoprenylated proteins that additionally become S-acylated in a particular membrane compartment are likewise predicted to remain tenaciously bound to the lipid bilayer, such that release of the protein from the "target" membrane is in essence entirely dependent upon removal of the *S*-acyl chain, either by thioesterase-catalyzed hydrolysis or, potentially, by transfer to another protein (Quesnel & Silvius, 1994).

In both of the models shown in Figure 2A, B, elements of which have been discussed previously (Hancock *et al.*, 1989; Araki *et al.*, 1990; Pfeffer, 1992, 1994; Magee & Newman, 1992; Novick & Brennwald, 1993; Ullrich *et al.*, 1993, 1994; Elazar *et al.*, 1994; Cadwallader *et al.*, 1994), efficient targeting of dual-anchored proteins to particular membranes can be achieved using specific "targeting factors" in merely catalytic amounts. Dual-anchored species, once integrated into the target membrane, need not remain persistently bound to specific "targeting receptors" in order to maintain their proper membrane localization, as would be the case for a protein less tenaciously anchored to the membrane lipid bilayer. A dual-anchored protein may thus be able to associate reversibly and flexibly with a number of different membrane proteins during a single "residence cycle" on a target membrane yet remain strictly confined to that membrane until released in a controlled manner. This potential would be at least advantageous if not essential for species such as various *rab* proteins, which appear to interact successively with multiple membrane components during each functional cycle (Shirataki *et al.*, 1992; Moya *et al.*, 1993; Ullrich *et al.*, 1994; Soldati *et al.*, 1994; Li *et al.*, 1994; Søgaard *et al.*, 1994).

Our prediction of a comparatively short half-time (possibly as little as 1−2 h) for spontaneous intermembrane transfer of *rab* and homologous proteins bearing a -C(GerGer)-C(GerGer)-OH motif suggests that a membrane-targeting mechanism based on bilayer trapping could be marginally less "tight" for these species than for the other types of dual-anchored proteins discussed above.[4] The extent of spontaneous transfer of such proteins to inappropriate membrane compartments could still be very limited, if the proteins normally cycle rapidly (in tens of minutes) between membrane- and cytoplasmic carrier-bound states. Alternatively, spontaneous transfer of such proteins to heterologous membranes may occur to a limited extent but be functionally tolerable, a possibility suggested by experiments using "mistargeted" chimeras of *rab* and *rab*-like proteins (Brennwald & Novick, 1993; Dunn *et al.*, 1993; Stenmark *et al.*, 1994). A more interesting case is that of the monoprenylated *rab8* and *rab13* proteins, both of which appear to be targeted to specific regions of the plasma membrane in epithelial cells (Huber *et al.*, 1993; Jober *et al.*, 1993; Zahraoui *et al.*, 1994). As monoprenylated peptide sequences dissociate very rapidly from lipid bilayers (Silvius & l'Heureux, 1994), these latter *rab* proteins must presumably be targeted by mechanisms other than bilayer trapping (e.g., by persistent binding to particular proteins in the "target" membranes). It will clearly be of great interest to determine to what extent changes in the C-terminal lipid modifications of various rab proteins affect their function *in situ* (Li & Stahl, 1993b).

As illustrated in Figure 2C, dual-anchor motifs like those examined here could be exploited to target a modified protein not only to a particular membrane but even, in principle, to particular lateral domains within the membrane if significant barriers to free lateral diffusion are present. Such barriers appear to be present in at least the plasma membrane of mammalian cells, where subcortical cytoskeletal elements impede the long-range lateral diffusion of tightly-membrane-bound proteins (including, in principle, dual-anchored proteins) that project more than 1−2 nm from the cytoplasmic surface (Edidin *et al.*, 1994). If a given dual-anchored protein is preferentially incorporated (by S-acylation or "escort protein"-mediated delivery) into particular regions within the plasma membrane, the action of such diffusional barriers could produce a significant steady-state enrichment of the dual-anchored species within these membrane domains. While this possibility is at present entirely speculative, it has been suggested that the dual-anchored (*N*-myristoyl/*S*-acyl) forms of certain nonreceptor protein tyrosine kinases may be inhomogenously distributed within the

plasma membrane and that the S-acylation of these proteins may be critical to this behavior [Paige *et al.*, 1993; Shenoy-Scaria *et al.*, 1993, 1994; Alland *et al.*, 1994; for a contrary view, see Rodgers *et al.* (1994)]. Further studies of these and related systems will be useful to define more precisely both the intramembrane distribution of such proteins in their dual-anchored form and the importance of the dual-anchor motif in maintaining this distribution.

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