Binding of Prenylated and Polybasic Peptides to Membranes: Affinities and Intervesicle Exchange†

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ABSTRACT: The small GTP-binding protein G25K and the protein K-Ras 4B contain prenyl groups (geranylgeranyl and farnesyl, respectively) that are thioether linked to a C-terminal cysteine which is methylated on its α-carboxyl group. These proteins, like many other prenyl proteins, also have a string of basic residues near their C-termini. A series of prenylated peptides based on the C-terminal sequences of human brain G25K and human K-Ras 4B were synthesized and analyzed for their membrane binding affinities. G25K peptides containing an N-terminal *N*-acetyltryptophan group were studied because their binding to membranes containing a trace of dansylated phospholipid could be detected by fluorescence resonance energy transfer. The G25K peptide lacking a prenyl group and a C-terminal methyl ester did not detectably bind to vesicles, and binding was enhanced by more than 500-fold if the peptide was geranylgeranylated. For the farnesylated peptide, methylation of the C-terminus increased membrane affinity by at least 60-fold if the vesicles contained phosphatidylserine and by 3-fold if they lacked this acidic lipid. The geranylgeranylated and methylated G25K peptide remains irreversibly attached to vesicles over several minutes only if the vesicles contain phosphatidylserine, whereas the corresponding nonmethylated or farnesylated and methylated peptides dissociate rapidly (less than a few seconds) from neutral or anionic vesicles. Farnesylation of the nonmethylated K-Ras 4B peptide enhances its affinity to vesicles containing acidic phospholipids (phosphatidylglycerol or phosphatidylserine) by 70-fold, and methylation leads to an additional dramatic (150-fold) increase in membrane affinity. Replacement of the farnesyl group by the longer geranylgeranyl group enhances membrane binding by 8-fold. Methylation of the geranylgeranylated K-Ras peptide enhances its binding to vesicles with acidic lipids by 100-fold; the corresponding enhancement with pure phosphatidylcholine vesicles (no net charge) is only 3-fold. The presence of 20% acidic phospholipid in phosphatidylcholine vesicles enhances the binding of the farnesylated and geranylgeranylated K-Ras 4B methylated peptides by 300- and 700-fold, respectively, compared to pure phosphatidylcholine vesicles. A simple model is presented to rationalize the synergistic effects of a C-terminal prenylated cysteine methyl ester combined with a polybasic peptide sequence on the binding of prenylated peptides to vesicles containing acidic phospholipids.

A specific set of proteins in eukaryotic cells are posttranslationally modified by attachment of prenyl groups (Casey, 1992; Clarke, 1992; Glomset & Farnsworth, 1994; Glomset et al., 1990; Maltese, 1990; Sinensky & Lutz, 1992). These proteins contain a C-terminal cysteine residue that is modified by a thioether-linked farnesyl group (15 carbons) or a geranylgeranyl group (20 carbons), and some proteins contain a pair of C-terminal geranylgeranylated cysteines (Farnsworth et al., 1994). In addition, the α-carboxyl group of the prenylated cysteine is often methylated. Evidence suggests that the role of the prenyl group is either to anchor proteins to cellular membranes and/or to direct interactions with other proteins (Glomset & Farnsworth, 1994; Marshall, 1993). For example, the γ-subunits of heterotrimeric G proteins and the small GTP-binding proteins G25K and rap 1B are C-terminally geranylgeranylated and methylated, and proteins that lack these modifications do not associate with membranes (Kawata et al., 1990; Yamane et al., 1990, 1991). Some of the Rab GTP-binding proteins are doubly geranylgeranylated, and the prenyl groups are required for the binding of these proteins to membranes and to a water-soluble protein that modulates the function of Rab proteins (Araki et al., 1990; Beranger et al., 1994; Farnsworth et al., 1991; Musha et al., 1992).

The Ras family of GTP-binding proteins is universally prenylated (Buss et al., 1991; Casey et al., 1989; Hancock et al., 1989; Schafer et al., 1990), and their is general consensus that such lipidation is required for their binding to the plasma membrane and for transformation of cells by oncogenic forms of these proteins [for example, see Cos and Der (1992), Der and Cox (1991a,b), Gibbs (1991), James et al. (1993), and Kohl et al. (1993)]. Ras proteins contain additional elements at their C-terminus that modulate their function. Of the four Ras proteins found in human cells, H-Ras, N-Ras, and K-Ras 4A have 1−2 cysteines that are close to the C-terminal farnesylated cysteine, and these are palmitoylated (Casey et al., 1989a; Hancock et al., 1989). On the other hand K-Ras 4B lacks these cysteines but contains a run of eight lysine residues. It has been shown that palmitoylation or the polylysine sequence enhances the association of Ras with membranes (Hancock et al., 1989, 1991a; Jackson et al., 1994). The polylysine sequence may engage in electrostatic binding with acidic phospholipids in

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the plasma membrane since a K-Ras 4B mutant in which all of the lysines have been changed to arginines retains plasma membrane binding, but the variant with all lysines changed to glutamine does not (Hancock et al., 1991a).

The role of C-terminal methylation of prenylated proteins is poorly understood, but there are some data to indicate that this modification increases the affinity of proteins to membranes. For example, K-Ras 4B that is farnesylated *in vitro* (but not methylated) binds more weakly to membranes than the fully processed protein (Hancock et al., 1991), but accurate affinities have not been measured. Expression of yeast RAS2 in a mutant yeast strain that lacks the methyltransferase leads to a slight reduction in the amount of membrane-bound RAS2 protein (Hrycyna et al., 1991).

To formulate working hypotheses for the role of C-terminal modifications in supporting the function of prenylated proteins, it is necessary to accurately determine their membrane affinities. In this study, we describe the synthesis of a series of prenylated peptides and their affinities for membranes. The peptides are patterned after the C-terminus of K-Ras 4B or the small GTP-binding protein G25K. The role of the prenyl chain and its length, C-terminal methylation, polylysine residues, and membrane phospholipid composition on the affinities of these peptide for membranes is explored. In the case of the G25K peptides, fluorescence spectroscopy was used to measure the kinetics of exchange of these peptides between different phospholipid vesicles; such results bear on whether prenylated and methylated proteins are expected to exchange between different cellular membrane compartments. During the course of these experiments, a study of the affinity of short prenylated peptides for membranes has appeared (Silvius & l'Heureux, 1994), and the results of these two studies are compared.

EXPERIMENTAL PROCEDURES

Materials. *all-trans*-Farnesyl bromide was from Aldrich, and *all-trans*-geranylgeranyl chloride (and bromide) was prepared as described (Liu et al., 1994). Purified water is from a Milli-Q system (Millipore). ePC,[1] ePE, POPC, POPG, DOPS, and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine were obtained from Avanti Polar Lipids, and dansyl-DTPE was obtained as a generous gift from Prof. M. K. Jain (University of Delaware) (Jain & Vaz, 1987).

Peptide Synthesis. All peptides were prepared by machine synthesis on a typical scale of 0.25 mmol as described (Liu et al., 1994), and additional details are given here. The structure of the peptides were verified by either electrospray mass spectrometry as described (Liu et al., 1994) or by liquid secondary ion mass spectrometry (LSIMS) on a VG 70-SEQ machine. The G25K peptide Ac-WKKSRRC-COOMe (Ac designates an acetyl group at the N-terminus, and COOMe designates a methyl ester of the C-terminal α-carboxyl group) and the K-Ras 4B peptides Ac-C(S-S-*t*-Bu)GK_6SKTKC-COOMe and Ac-C(S-S-*t*-Bu)GK_6SKTKS-COOMe were prepared using the resin FMOC-C(Trityl)-Sasrin (Bachem), which permitted site-selective methylation of the C-terminal carboxyl group (Liu et al., 1994). The peptide Ac-WKKSRC-COOH was made using the resin FMOC-C(Trityl)-Wang. During the synthesis of the K-Ras 4B peptides, a small aliquot of resin was submitted to the Kaiser test (Kaiser et al., 1990) after each residue coupling to judge the completion of the coupling. The synthesis of these peptides was problematic and required the following number of repetitive coupling steps for each residue (given in brackets): Ac-C(S-S-*t*-Bu)[3]-G[2]-K[4]-K[4]-K[4]-K[3]-K[3]-K[2]-S[3]-K[2]-T[4]-K[1]-C-resin, and the same number of couplings were done with Ac-C(S-S-*t*-Bu)GK_6SKTKS-COOMe. N-terminal acetylation was carried out by treating the resin-bound peptide (after the last FMOC group was removed) in CH_2Cl_2 with acetic anhydride (3.0 equiv) and diisopropylethylamine (3.2 equiv, Aldrich) for 3 h at room temperature. The reaction was complete when a small aliquot of resin gave a negative Kaiser test. Cleavage from the resin, C-terminal methylation, and side chain deprotection were carried out as described (Liu et al., 1994).

Peptides were purified by HPLC (Liu et al., 1994) using a semipreparative reverse phase column (Vydac 218TP1010). In all cases peptide methyl esters eluted 1−2 min after peptide free acids. The column was developed with solvent A (0.06% trifluoroacetic acid in purified water) and solvent B [0.06% trifluoroacetic acid in acetonitrile (Resi-Analyzed, Baker)]. The flow rate was 1.5 mL/min, and the following solvent programs were used: program 1, 0% B to 70% B in 70 min and then to 100% B in 50 min; program 2, 0% B for 15 min, then to 50% B over 30 min, then to 80% B over 20 min, and then to 100% B over 20 min. The purified peptides are as follows: Ac-WKKSRRC-COOMe, retention time 32 min (program 1), calculated monoisotopic mass for the +1 ion 1019.5, observed 1019 (LSIMS); Ac-WKKSRRC-COOH, retention time 30 min (program 1), calculated monoisotropic mass for the +1 ion 1005.5, observed 1005 (LSIMS); Ac-C(S-S-*t*-Bu)GK_6SKTKC-COOMe, retention time 40 min (program 2), calculated average mass for the +2, +3, and +4 ions 820, 547, and 411, observed 820, 547, and 411 (electrospray); Ac-C(S-S-*t*-Bu)GK_6SKTKS-COOMe, retention time 37 min (program 2), calculated average mass for the +2, +3, and +4 ions 812, 542, and 407, observed 812, 542, and 407 (electrospray).

Peptides were prenylated essentially as described (Liu et al., 1994). Ac-WKKSRRC-COOMe or Ac-WKKSRRC-COOH (2 mg) was dissolved in 0.5 mL of DMF/100 μL of water/100 μL of 0.5 M $KHCO_3$ in a small glass vial with a teflon septum-lined screw cap containing a small magnetic stir bar. Prenyl halide (2 equiv in 20 μL of DMF) was added, the vial was purged with argon and wrapped with foil to exclude light, and the mixture was stirred overnight at room temperature. Aqueous HCl (1 N) was added to bring the pH to about 6−7, the mixture was injected directly onto the HPLC column (see above), and the column was developed with program 1: Ac-WKKSRRC(F)-COOMe [C(F) designates a farnesyl group thioether linked to cysteine], retention time 54 min, yield 1.2 mg, calculated monoisotopic for the +1 ion 1224.5, observed 1224 (LSIMS); Ac-WKKSRRC-(F)-COOH, retention time 53 min, yield 0.6 mg, calculated monoisotopic for the +1 ion 1210.5, observed 1211 (LSIMS); Ac-WKKSRRC(GG)-COOMe [C(GG) designates a geranylgeranyl group thioether linked to cysteine], retention time 71 min, yield 1.6 mg, calculated monoisotopic for the +1

[1] Abbreviations: dansyl-DTPE, *N*-dansyl-1,2-ditetradecyl-*sn*-glycero-3-phosphoethanolamine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; ePC, egg phosphatidylcholine; ePE, egg phosphatidylethanolamine; LUV, large unilamellar vesicles; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol.

ion 1291.5, observed 1292 (LSIMS); Ac-WKKSRRC(GG)-COOH, retention time 70 min, yield 0.25 mg, calculated monoisotopic for the +1 ion 1277.5, observed 1278 (LSIMS).

Ac-C(S-S-*t*-Bu)GK_6SKTKC-COOMe was farnesylated under acidic conditions (conditions provided by Prof. F. Naider, CUNY Staten Island), which gave much better results than under basic conditions. The peptide (9.9 mg) was dissolved in 242 μL of DMF/121 μL of acetonitrile/121 μL of 0.1% trifluoroacetic acid in water. Zinc acetate dihydrate (4 mg) was added, and the pH of the reaction mixture was adjusted to $\approx$4 with small portions of 10% trifluoroacetic acid in water (monitored with moist pH paper). A 19 μL portion of a freshly made stock solution of farnesyl bromide in DMF (93 mg/mL) was added, the reaction vessel was purged with argon and wrapped with foil to exclude light, and the mixture was stirred at room temperature. After 3 h a second portion of farnesyl bromide was added. After an additional 6 h of stirring at room temperature, the mixture was injected directly onto the HPLC column. The column was developed with program 2, and the nonfarnesylated peptide (6.5 mg) and the farnesylated peptide (retention time 53 min, 3.2 mg) were obtained after removal of the solvent in a Speed-Vac. In the same way, 10 mg of Ac-C(S-S-*t*-Bu)GK_6SKTKC-COOMe was geranylgeranylated with geranylgeranyl bromide. Purification on HPLC gave the geranylgeranylated peptide (retention time 59 min, 5 mg) and the nonprenylated peptide (6 mg). ^{1}H NMR spectra (500 MHz) of the prenylated peptides showed the characteristic resonances for the prenyl groups, and when the vinyl proton resonances were integrated relative to the resonance of the acetyl group, it was determined that there was one prenyl group per peptide. Since the peptides gave a negative test for SH with Ellman's reagent, it was concluded that the prenyl group is on cysteine.

The S-*t*-Bu protection group was removed form the K-Ras 4B peptides as follows. Peptide (3.2 mg) was dissolved in 0.32 mL of DMF/64 μL of 0.25 M $KHCO_3$, and 33 mg of dithiothreitol was added. The vial was purged with argon, and the reaction mixture was left overnight at room temperature. The mixture was loaded onto a small column (0.6 $\times$ 8 cm) of Sephadex G15 (Pharmacia) that was equilibrated with 5% acetic acid. Column fractions were screened with Ellman's reagent, peptide containing fractions were pooled, and the solvent was removed by lyophilization (typical yields of 60−80%). ^{1}H NMR analysis (500 MHz) of the peptides showed that the S-*t*-Bu was completely removed and that the C-terminal methyl ester was still intact.

After removing the S-*t*-Bu group, the K-Ras 4B peptides were labeled with *N*-[*ethyl*-2-^{3}H]ethylmaleimide as follows: Peptide (1−3 mg) was dissolved in 50−100 μL of 20 mM HEPES, pH 8.1. The concentration of SH groups was determined by submitting a small aliquot to analysis with Ellman's reagent. To the peptide in buffer was first added radiolabeled *N*-ethylmaleimide in pentane (as supplied by NEN, 56 Ci/mmol, 0.5 μCi per nmol of peptide). The mixture was vortexed, and the pentane was removed with a gentle stream of nitrogen. The vial was purged and left at room temperature for 0.5 h. Then, 3 equiv (based on peptide SH) of nonradiolabeled *N*-ethylmaleimide was added from a stock solution in ethanol. After an additional 1 h at room temperature (at which point analysis of a small aliquot with Ellman's reagent gave a negative test for SH), the mixture was submitted to gel filtration as described above. Product-containing fractions were pooled, and the solvent was removed by lyophilization. ^{1}H NMR analysis (500 MHz) of the peptides showed that the C-terminal methyl ester was still intact and that 1 equiv of *N*-ethylmaleimide was incorporated.

The K-Ras 4B peptide methyl esters were converted to the corresponding free acids as follows. Peptide methyl ester (typically 1 mg) was dissolved in 0.22 mL of methanol/0.18 mL of water, and 2.2 μL of 12 N NaOH was added. The mixture was placed in a 40 °C water bath for 7 h. The mixture was neutralized with small aliquots of acetic acid (pH paper), and the sample was lyophilized. The peptides were purified by HPLC as described above for the S-*t*-Bu-protected K-Ras 4B peptides. ^{1}H NMR spectra (500 MHz) of the peptides showed the loss of the methyl ester resonance.

The G25K and K-Ras 4B peptides were stored in DMF and water, respectively, in vials under argon at −80 °C. Argon was used to prevent oxidation at sulfur; dry films of prenylated peptides exposed to air for several hours undergo thioether oxidation to give sulfoxides and sulfones (Liu et al., 1994).

Preparation of Phospholipid Vesicles. Stock solutions of phospholipids in $CHCl_3$ were stored under argon at −80 °C. The appropriate volumes of each stock solution of phospholipids were mixed together in a glass tube, and the solvent was removed first with a stream of nitrogen and then *in vacuo* for 30 min. The following types of vesicles were prepared: ePC/ePE (24.6%/75.4%), ePC/ePE/DOPS (16.8%/51.6%/31.6%), POPC (100%), POPC/POPG (80%/20%, unless otherwise noted). Vesicles used in fluorescence studies also contained 5% dansyl-DTPE. Stock solutions of LUVs (typically 1−20 mM total lipid) were prepared by suspending the mixed lipid films in the desired buffer, briefly vortexing, and subjecting the sample to 10 freeze/thaw cycles. The suspension was passed 29 times through a LiposoFast syringe device (Avestin) containing two 100 nm filters (Nucleopore) as described by the manufacturer. By doping the vesicles with a small amount of radiolabeled phospholipid (1-palmitoyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine, 50 mCi/mmol, NEN), it was found that the yield of total lipid after extrusion was always >90%.

Peptide−Vesicle Binding Measured by Fluorescence Resonance Energy Transfer. Phospholipid vesicles with Dansyl-DTPE (4 mM total lipid) were prepared in 20 mM HEPES and 100 mM NaCl, pH 7.4, and then diluted into 1 mL of the same buffer to give a final total lipid concentration of 6 μM. The buffer was prefiltered through a 0.45 μm filter cartridge to remove dust. The samples were placed in a quartz cuvette in a Jasco 821-FP fluorimeter thermostated at 21 °C. Various amounts of peptide were added (see figures), and the emission at 510 nm was recorded with excitation at 280 nm. In some experiments, peptide was first added to 1 mL of buffer containing 50 μM dansyl-DTPE-loaded vesicles, and the fluorescence was recorded before and after the addition of 50 μM of the same vesicles but without dansyl-DTPE.

Peptide−Vesicle Binding Measured by Centrifugation. Essentially the procedure of Buser et al. (1994) was followed. LUVs used for centrifugation studies with the K-Ras 4B peptides were prepared as 4−20 mM stock solutions as follows. Lipids were mixed in chloroform, and the solvent was removed as described above. Buffer (1 mM MOPS, 176 mM sucrose, pH 7.0) was added, and LUVs were

Table 1: G25K Peptide−LUV Dissociation Constants

peptide	abbreviated name	K_D (ePC/ePE/DOPS LUVs) (μM)	K_D (ePC/ePE LUVs) (μM)
Ac-WKKSRRC-COOH	G25K-COOH	>500	>500
Ac-WKKSRRC-COOMe	G25K-COOMe	108, 108	423, 480[a]
Ac-WKKSRRC(F)-COOH	^{F}G25K-COOH	68, 57	31, 32
Ac-WKKSRRC(F)-COOMe	^{F}G25K-COOMe	<0.3−0.6	12, 9
Ac-WKKSRRC(GG)-COOH	^{GG}G25K-COOH	<0.3−0.6	<0.3−0.6
Ac-WKKSRRC(GG)-COOMe	^{GG}G25K-COOMe	<0.3−0.6	<0.3−0.6

[a] The two values correspond to the K_Ds determined via eq 1 with 0.5 or 1 nmol of added peptide.

prepared by extrusion as described above. LUV stock solutions were stored at room temperature under argon and were used the same day. A second sample of LUVs were prepared that also contained a small amount of 1-palmitoyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine. The concentration of total lipid in the radiolabeled LUV stock solution and the amount of radioactivity were such that these LUVs, when diluted into mixtures for centrifugation (see below), gave the lowest desired total lipid concentration and contained 500−1000 cpm of radiolabel.

Polyallomar microfuge tubes (1.5 mL, Beckman) were treated with Sigmacote (Sigma, as described by the manufacturer). Tritiated K-Ras 4B peptide (150 pmol) was added to the tube containing centrifugation buffer (1 mM MOPS, 0.1 M KCl, pH 7.0). The same amount of radiolabeled LUV stock solution was added to all of the tubes. The appropriate amounts of nonradiolabeled LUV stock solution were added to all of the tubes (except the tube with the lowest lipid concentration which contained only radiolabeled LUVs) to bring the total lipid concentrations to the desired final values. The total volume of each tube was 1 mL. Samples were centrifuged at 21 °C at 100000g_{av} for 1 h in a TLA45 rotor (Beckman). Most of the supernatants (0.8 mL) were removed by pipet and transferred to scintillation vials. The tubes containing the pellet and 0.2 mL of supernatant were vortexed for 1 min, and the liquid was transferred to scintillation vials. Samples were submitted to scintillation counting, and the amount of ^{3}H and ^{14}C was determined by standard double-channel analysis.

Equilibrium dissociation constants (K_Ds) for the peptide−LUV interaction are expressed as [peptide in the aqueous phase][total lipid]/[vesicle-bound peptide] and were calculated as follows. Very little ^{14}C (<10%) was found in the supernatant fraction, indicating that the LUVs were fully pelleted. Since the pellet fraction contains 20% of the supernatant, 25% of the ^{3}H cpm measured in the supernatant fraction was subtracted from the ^{3}H cpm in the pellet; the remaining ^{3}H cpm in the pellet fraction is due to vesicle-bound peptide. By knowing the amount of ^{14}C LUV added to the tube and the amount recovered in the pellet fraction, it was possible to correct the amount of peptide recovered in the pellet (^{3}H cpm) for the amount of LUV stuck to the wall of the tube (at the lowest lipid concentration, 0.05 μM, the yield of recovered lipid was typically 20−30%). The recoveries from the centrifuge tubes of all of the K-Ras peptides except the prenylated and methylated peptides were high (typically >80%). For the latter, the yields were lower (30−50%), but this does not effect the calculation of K_D since this constant depends only on the ratio of concentrations of peptide in the supernatant and pellet.

Studies with Ac-WKKSRRC(GG)-COOMe were carried out with sucrose-loaded LUVs. Lipids (ePC/ePE/DOPS and ePC/ePE, as above) were converted to LUVs (4 mM stock solution) by extrusion in 10 mM HEPES and 150 mM sucrose, pH 7.4 as described above. Vesicles also contained a small amount of 1-palmitoyl-2-[1-^{14}C]-arachidonyl-*sn*-glycero-3-phosphocholine (40 000 cpm per μmol of lipid). LUVs were added to 10 mM HEPES and 75 NaCl, pH 7.4, to give a lipid concentration of 1 μM and a total volume of 3 mL in a polyallomar tube. Peptide (150 pmol) was added, and the sample was centrifuged at 400000g_{av} in a TLA100.3 rotor (Beckman) for 1.5 h at 21 °C. Most of the supernatant (2.85 mL) was transferred to a new tube, and 200 μL was submitted to scintillation counting to verify that the LUVs were pelleted. The remaining supernatant was concentrated to dryness in a Speed-Vac (Savant Instruments). The residue was dissolved in 20 μL of DMF/140 μL of water, and the solution was injected onto the HPLC column. HPLC was carried out as described for the purification of the G25K peptides except that an analytical size column of the same packing was used (Vydac 218 TP52) and a fluorescence detector (Jasco 821-FP) was used to detect the tryptophan signal.

For all vesicle binding experiments, data were fit to the standard hyperbolic equation by nonlinear regression using Fortran programs provided by Professor Paul Cook (University of North Texas).

Circular Dichroism Spectroscopy. Spectra were recorded on a Jasco J-720 spectropolarimeter using a quartz cuvette (2 mm path length). Samples at room temperature contained ^{GG}K-Ras-COOMe (10 μM) in 50 mM potassium phosphate, pH 7.0, with or without 2 mM POPC/POPG (2/1) LUVs. The final spectra are the average of 100 spectral scans from 250 to 200 nM.

RESULTS

Preparation of Peptides. The sequence of the peptides prepared in this study are given in Tables 1 and 2. They will be referred to in the text using the shortened notation listed in the tables. The G25K peptides have the sequence corresponding to the C-terminal six residues of the G25K small GTP-binding protein from human brain (Munemitsu et al., 1990). An *N*-acetyltryptophan was placed at the N-terminus of these peptides so that peptide−vesicle interactions could be monitored with fluorescence resonance energy transfer. The K-Ras 4B peptides have the sequence corresponding to the C-terminal 12 residues of the human K-Ras 4B protein (McGrath et al., 1983). An *N*-acetylcysteine was placed at the N-terminus of these peptides so that they could be conveniently radiolabeled in the last synthetic step by alkylation with tritiated *N*-ethylmaleimide. The structures of representative peptides are shown in Figure 1.

All peptides were prepared by standard automated solid-phase synthesis using a protocol that permits the C-terminal α-carboxyl group to be selectively methylated (Liu et al.,

Table 2: K-Ras 4B Peptide-LUV Dissociation Constants

peptide	abbreviated name	K_D (POPC/POPG, 4:1) (μM)	K_D (POPC) (μM)
Ac-C(S-NEM)GK$_6$SKTKS-COOH	K-Ras-COOH	42% bound at 4 mM lipid	no binding at 4 mM lipid
Ac-C(S-NEM)GK$_6$SKTKS-COOMe	K-Ras-COOMe	450 ± 60[a]	14% bound at 2 mM lipid
Ac-C(S-NEM)GK$_6$SKTKC(F)-COOH	^{F}K-Ras-COOH	70 ± 4	≈10% bound at 2 mM lipid
Ac-C(S-NEM)GK$_6$SKTKC(F)-COOMe	^{F}K-Ras-COOMe	0.48 ± 0.2	150 ± 25
Ac-C(S-NEM)GK$_6$SKTKC(GG)-COOH	^{GG}K-Ras-COOH	6.2 ± 1.5	120 ± 20
Ac-C(S-NEM)GK$_6$SKTKC(GG)-COOMe	^{GG}K-Ras-COOMe	0.06 ± 0.02	40 ± 15
N-acetyl-*S*-farnesylcysteine		30% bound at 4 mM lipid	≈10% bound at 2 mM lipid

[a] The indicated standard errors come from nonlinear regression analysis of the vesicle binding data.

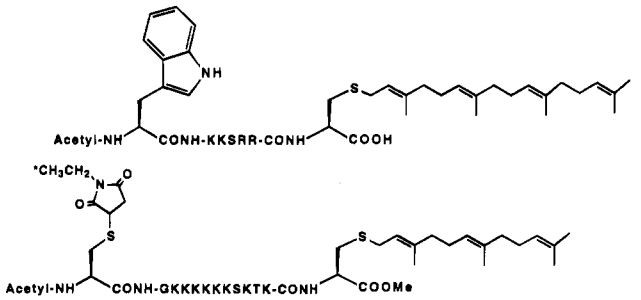

FIGURE 1: Peptide structures: (Top) Geranylgeranylated and nonmethylated G25K peptide containing an N-terminal *N*-acetyltryptophan; (bottom) farnesylated and methylated K-Ras 4B peptide containing an N-terminal *N*-acetylcysteine modified by alkylation at sulfur with tritiated (*) *N*-ethylmaleimide.

1994). In the case of the K-Ras 4B peptides, the cysteines were differentially protected so that they could be individually modified at different stages of the synthesis (Liu et al., 1994). Prenyl groups were introduced by treating the peptides in solution with prenyl halides (Liu et al., 1994). The K-Ras 4B peptides were conveniently radiolabeled in the last synthetic step by alkylation with tritiated *N*-ethylmaleimide.

Binding of G25K Peptides to Membranes. Binding of the G25K peptides to LUVs brings the N-terminal tryptophan in close proximity to the fluorescent probe dansyl-DTPE (present in small amounts in LUVs) such that fluorescence resonance energy transfer from tryptophan to dansyl occurs. Mixed-lipid LUVs were prepared that contained ePC, ePE, and DOPS in roughly the same proportion that is found on the cytoplasmic face of erthryrocyte plasma membranes [pure plasma membranes can be obtained from erthryrocytes, and the asymmetric distribution of phospholipids across the bilayer is well determined (Devaux, 1991; Zwaal et al., 1975)]. Vesicles lacking DOPS were also used in order to evaluate the importance of this negatively charged phospholipid in supporting peptide binding.

In order to obtain dissociation constants for the peptide–LUV complex, the following approach was taken. First, for the set of peptides, their relative intensities of energy transfer per mole of vesicle-bound peptide are needed. These were obtained by measuring the energy transfer of a fixed amount of peptide (2 nmol) in the presence of different LUV concentrations for each of the G25K peptides (except the nonprenylated peptides). With 10 and 20 μM amounts of LUV, there were some differences in the energy transfer intensities for each peptide, but at a higher lipid concentration, 100 μM, the energy transfer efficiencies of all of the peptides were the same within experimental error (data not shown). This was true with both ePC/ePE and ePC/ePE/DOPS LUVs, although the absolute intensities measured with the former were about 2-fold higher than those measured with latter. From these experiments, it can be concluded that all four prenylated G25K peptides are fully bound to LUVs when the lipid concentration is ≥100 μM. Furthermore, the energy transfer intensities per mole of lipid-bound peptide are virtually the same for all peptides. As a control, it was found that addition of prenylated peptides to LUVs lacking dansyl-DTPE gave rise to an increase in fluorescence that was only 1−2% of that measured in the presence of vesicles containing dansyl-DTPE, which shows that the fluorescence increase being monitored is due to peptide-to-vesicle resonance energy transfer.

For the second stage of the analysis, the fraction of ^{GG}G25K-COOMe bound to LUVs was determined be pelleting the vesicles in an ultracentrifuge and measuring the amount of peptide remaining in the supernatant by HPLC with fluorimetric detection (see Experimental Procedures). In the presence of 1 μM ePC/ePE/DOPS or 1 μM ePC/ePE LUVs, no aqueous phase ^{GG}G25K-COOMe was detected, whereas in the absence of LUVs, a strong HPLC peak (10-fold above the noise) was detected (data not shown). Thus the K_D values for the complexes of this peptide with ePC/ePE/DOPS and ePC/ePE LUVs are <0.3−0.6 μM.

The last stage of the analysis made use of the fluorescence resonance energy transfer data shown in Figure 2A,B. In these experiments, the concentration of LUVs is held contant (6 μM total phospholipid), and increasing amounts of peptides were added. This lipid concentration is well above the K_D for ^{GG}G25K-COOMe. Thus, these curves (Figure 2A,B) gives the maximum fluorescence energy transfer signal for fully bound peptide as a function of increasing amount of added peptide. The curvature in this plot above about 1−1.5 nmol of added peptide is almost certainly due to crowding of the peptides on the vesicle since the peptide/lipid ratio is approaching 1/2. For the other peptides, the fraction of vesicle-bound peptide at a given amount of added peptide is simply I_i/I_{GG}, where I_i and I_{GG} are the energy intensities observed for the i^{th} peptide and ^{GG}G25K-COOMe, respectively, at a given concentration of lipid. The K_Ds for the peptides can be calculated from eq 1

$$K_D^{\,i} = \frac{[\text{lipid}]\left\{1 - \left(\frac{I_i}{I_{GG}}\right)_L\right\}}{\left(\frac{I_i}{I_{GG}}\right)_L} \qquad (1)$$

using the I_i/I_{GG} data (at 0.5 and 1 nmol of added peptide) shown in Figure 2A,B.

For this calculation, only the data points obtained with 0.5 and 1 nmol of added peptides were used (Figure 2A,B) to avoid the effects of peptide crowding. The K_D^i values for the various G25K peptides are listed in Table 1. No

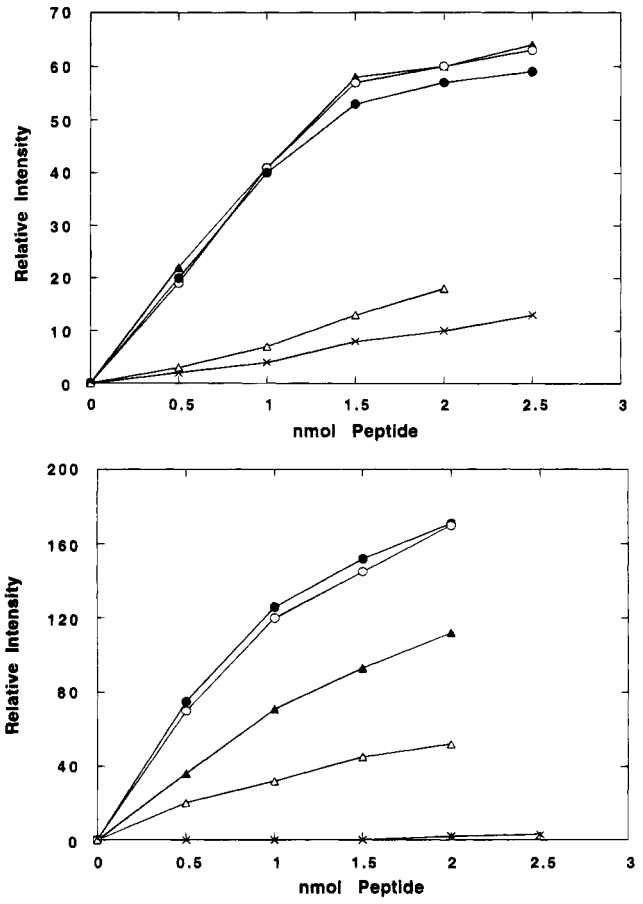

FIGURE 2: Binding of the G25K peptides to vesicles measured by fluorescence energy transfer. The indicated amount of the peptides [G25K-COOMe (crosses), ^{F}G25K-COOMe (filled triangles), ^{F}G25K-COOH (open triangles), ^{GG}G25K-COOMe (filled circles), ^{GG}G25K-COOH (open circles)] were added to (A, top) 6 μM ePC/ePE/DOPS LUVs or (B, bottom) 6 μM ePC/ePE LUVs. The curves have been drawn through the data points.

binding of the nonprenylated and nonmethylated peptide G25K-COOH to vesicles with or without DOPS could be detected. The binding of the nonprenylated and methylated peptide G25K-COOMe to vesicles without DOPS could barely be detected, and the binding is enhanced about 4-fold when DOPS is present. Farnesylation enhances the binding of these peptides to membranes. Compared to G25K-COOH, ^{F}G25K-COOH binds at least 8- and 16-fold tighter to vesicles with or without DOPS, respectively. It is interesting to note that this peptide binds about 2-fold tighter to vesicles without DOPS compared to those with DOPS. Methylation of ^{F}-G25K-COOH enhances its affinity for LUVs with DOPS by at least 6-fold and by 3-fold for LUVs without DOPS. Binding of the geranylgeranylated peptides to vesicles with and without DOPS was too tight (K_Ds < 6 μM) to be measured by this method. Lower concentrations of lipid could not be used because of sensitivity limitation intrinsic to this fluorimetric approach.

Intervesicle exchange of the G25K peptides was also analyzed. Peptides were prebound to 50 μM LUVs containing dansyl-DTPE, and then an equal amount of LUVs without fluorophore was added. Hopping of the peptide between vesicle would lead to a decrease in the intensity of fluorescence energy transfer as part of the peptide partitions into LUVs without fluorophore. As a control, it was found that addition of the G25K peptides to a mixture of equal

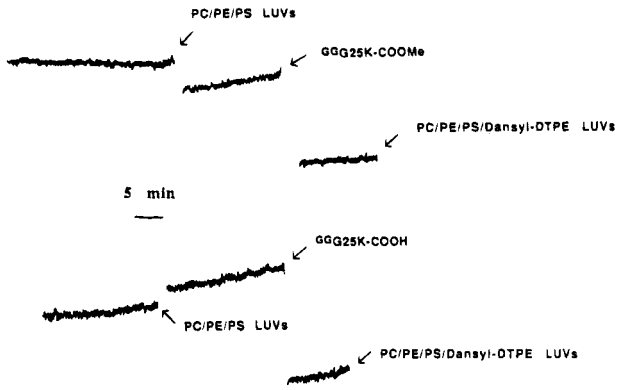

FIGURE 3: Intervesicle exchange of G25K peptides. The abscissa is time which progresses from right to left. (Top) LUVs containing fluorophore are first added to buffer at the first arrow. At the second arrow ^{GG}G25K-COOMe is added which results in an increase in fluorescence due to energy transfer. At the third arrow, LUVs without fluorophore are added, and the signal increases by the same amount that is seen when these vesicles are added in the absence of peptide. The gap in time between the arrows corresponds to the 5−10 s needed to make the addition to the sample cuvette. (Bottom) Same as top except that the nonmethylated peptide ^{GG}G25K-COOH is used.

amounts of LUVs with and without dansyl-DTPE gave half the fluorescence energy transfer signal that was seen for the addition of the peptides to the same amount of LUVs with dansyl-DTPE alone (not shown). As shown in Figure 3 (top), addition of ^{GG}G25K-COOMe to ePC/ePE/DOPS/dansyl-DTPE LUVs gives rise to an energy transfer signal. Subsequent addition of ePC/ePE/DOPS LUVs gives rise to a further slight increase in intensity, and this increase is identical to that seen in the absence of peptide when LUVs without fluorophore are added to those with fluorophore (not shown). This is presumably the result of scattering by the vesicles. From this experiment it is concluded that ^{GG}G25K-COOMe remains irreversibly bound to the vesicles over the duration of the experiment (several minutes). As shown in Figure 3 (bottom), when the intervesicle transfer experiment is carried out with ^{GG}G25K-COOH and ePC/ePE/DOPS LUVs, the fluorescence signal immediately drops after the second portion of LUVs are added (if the increase due to scattering is subtracted from the final signal, it is found that the signal drops to half of its original value as expected). Thus the geranylgeranylated, nonmethylated peptide undergoes rapid (less than several seconds) intervesicle exchange. The same behavior is seen with ^{GG}G25K-COOH and ePC/ePE LUVs, with ^{F}G25K-COOH and ^{F}G25K-COOMe bound to LUVs with or without DOPS, and with ^{GG}G25K-COOMe bound to vesicles without DOPS (not shown).

Binding of K-Ras 4B Peptides to Membranes. The binding of K-Ras 4B peptides to membranes was studied using a radiometric analysis since such as method is typically more sensitive than fluorimetric approaches. LUVs made of POPC with or without the negatively charged phospholipid POPG were used since these, when loaded with sucrose, are readily pelleted in an ultracentrifuge (Buser et al., 1994). A fixed amount of peptide was incubated with various concentrations of LUVs, and, after ultracentrifugation, the amount of radiolabeled peptide in the supernatant and membrane pellet was analyzed by scintillation counting (see Experimental Procedures).

Values of K_D for the interaction of the K-Ras 4B peptides with POPC and POPC/POPG (4:1) LUVs were determined

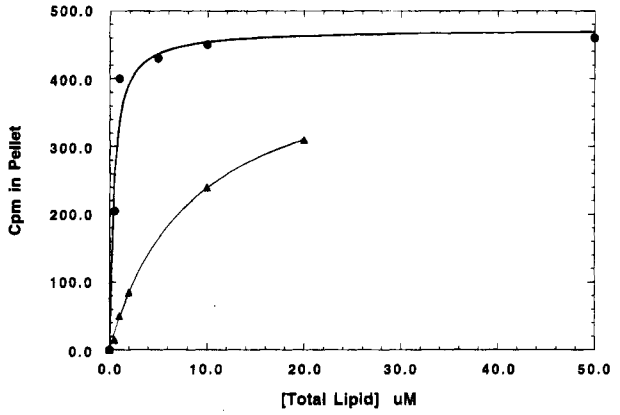

FIGURE 4: Binding of K-Ras 4B peptides to sucrose-loaded POPC/POPG (4:1) LUVs measured by ultracentrifugation: ^{GG}K-Ras-COOH (triangles); ^{F}K-Ras-COOMe (circles). The curves denote the regression fit of the data to the mass action equation.

by fitting the binding data to the standard mass action equation (Experimental Procedures). Binding data for the peptides ^{GG}K-Ras-COOH and ^{F}K-Ras-COOMe interacting with POPC/POPG LUVs are shown in Figure 4, and the K_D values for all of the K-Ras peptides are listed in Table 2. The nonprenylated, nonmethylated peptide K-Ras-COOH bound weakly to POPC/POPG LUVs, and binding was enhanced by about 70-fold if the peptide was farnesylated and by about 10-fold if the peptide was methylated. No binding of K-Ras-COOH was detected in the presence of 4 mM POPC LUVs, and a small amount of peptide binding was detected if this peptide was methylated or farnesylated. In the presence of POPC/POPG LUVs, methylation of the farnesylated peptide ^{F}K-Ras-COOH results in a dramatic (150-fold) increase in membrane affinity. The corresponding value for the geranylgeranylated peptide GGK-Ras-COOH is 100-fold. With the neutral POPC LUVs, the binding enhancement due to methylation is smaller, 3-fold for the geranylgeranylated peptides. ^{F}K-Ras-COOMe and ^{GG}K-Ras-COOMe bind 300- and 700-fold, respectively, tighter to vesicles that contain POPG compared to POPC LUVs which have no net charge. The peptide ^{GG}K-Ras-COOH binds 10-fold tighter to LUVs containing 33% POPG than to those containing 10% POPG, and there was no difference in affinities when the POPG was replaced with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine. Increasing the length of the prenyl chain of ^{F}K-Ras-COOMe from 15 to 20 carbons results in an 8-fold increase in affinity for POPC/POPG LUVs and a 4-fold increase for POPC LUVs; the corresponding change for the nonmethylated peptides binding to POPC/POPG is 11-fold. Interestingly, *N*-acetyl-*S*-farnesylcysteine binds to POPC vesicles with about the same affinity as ^{F}K-Ras-COOH; however, in the presence of POPC/POPG LUVs, the latter binds about 70-fold tighter.

Circular Dichroism Spectra. Figure 5 shows the circular dichroism spectra (200−250 nm) of ^{GG}K-Ras-COOMe in buffer alone or bound to POPC/POPG (2:1) vesicles. In the absence of lipid, the peptide exists mainly in a random coil conformation (negative elipticity below 210 nm), and a small amount of α-helix is also detected (negative elipticity at 215−235 nm). The only difference in the spectrum when LUVs are added is the loss of the signal due to α-helix.

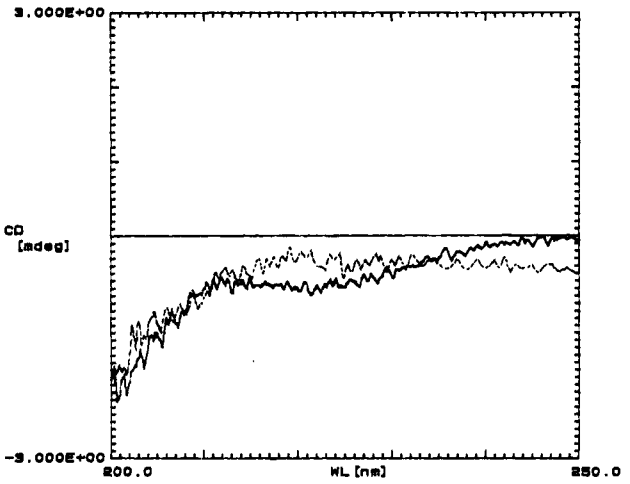

FIGURE 5: Circular dichroism spectra of ^{GG}K-Ras-COOMe in the absence of membranes (solid line) or in the presence of POPC/POPG (2:1) LUVs (dotted line).

DISCUSSION

What emerges from the present is study is that the prenyl group, the C-terminal methyl ester, and the string of polybasic amino acids all contribute to promote the binding of peptides to membranes containing acidic phospholipids. The results are consistent with the rough estimates of relative binding affinities that have been reported for prenylated proteins (Hancock et al., 1991a,b; Hrycyna et al., 1991). The results of this study are reminiscent of those recently reported for myristoylated peptides corresponding to the N-terminus of the src protein kinase (Buser et al., 1994). In that study, a myristoylated peptide with a net charge of +5 bound 4000-fold tighter to POPC/POPG (2/1) LUVs than to pure POPC LUVs. For the K-Ras 4B peptides the analogous enhancement is 300−700-fold for the methylated peptides containing a farnesyl group or a geranylgeranyl group, respectively, binding to POPC/POPG (4/1) LUVs. Interestingly, farnesylated and geranylgeranylated nonmethylated peptides bind only 20- and 50-fold, respectively, tighter to POPC/POPG LUVs than to POPC LUVs. Presumably the juxtaposition of the anionic C-terminus of the peptide to acidic phospholipids at the interface somewhat offsets the favorable interactions between the lysines and the POPG. However, this argument assumes that the C-termini of these prenylated peptides bound to membranes are ionized; this may not be the case if the pK_a of the carboxyl group for the membrane-bound peptide is elevated to near 7 as is the case, for example, with fatty acids present in bilayered vesicles.

What also emerges from this study is the fact that *N*-acetyl-*S*-farnesylcysteine and ^{F}K-Ras-COOH show about the same affinity to POPC LUVs. This suggests that only the farnesyl group contributes to the binding of ^{F}K-Ras-COOH to neutral LUVs, and that the peptide portion is presumably in the aqueous phase and not in contact with the interface. The same result was obtained with myristoylated src peptides (Buser et al., 1994).

Farnesylation of K-Ras-COOMe enhances its binding to POPC/POPG LUVs by 940-fold. Buser et al. (1994) have shown that myristoylation of the polybasic peptides patterned after the N-terminal portion of src protein kinase enhances their binding to negatively charge LUVs by about 30 000-fold. Thus it appears that the 15-carbon farnesyl group is

less hydrophobic than the 14-carbon myristoyl group. This is not unreasonable since the farnesyl group is branched and polyunsaturated. Indeed, Silvius and l'Heureux (1994) have estimated that farnesyl group is equivalent to a saturated linear-chain hydrocarbon of 11 carbons in terms of its ability to partition from the aqueous phase into the membrane phase.

The trends in membrane-binding affinities of a series of prenylated tri- and tetrapeptides reported recently (Silvius & l'Heureux, 1994) are similar to results described herein for the G25K peptides. The one difference is that the geranylgeranyl tripeptide RGC(GG)-COOMe bearing a fluorescent *N*-(*S*-bimanylthioacetyl) group on its N-terminus undergoes rapid intervesicle exchange (less than a few seconds) (Silvius & l'Heureux, 1994), whereas ^{GG}G25K-COOMe, but not ^{GG}G25K-COOH, remains bound at least for several minutes to ePC/ePE/DOPS (Figure 3). Rapid intervesicle exchange of the geranylgeranylated and methylated peptide is seen when the vesicles lack DOPS, which supports a role for the electrostatic interactions between basic residues of the peptide and anionic phospholipids in the LUVs in supporting tight membrane association. The results in this study suggest that methylation of some geranylgeranylated proteins such as G25K may trap the protein in the cellular membrane in which the methylation occurs.

Animal cells have a radius of 5−15 μm, and thus the concentration of plasma membrane phospholipid that a prenylated peptide inside the cell "sees" is 70−200 μM [assuming 50 Å^2 per phospholipid (Cevc & Marsh, 1987) and roughly 10% of the membrane lipid not bound to other cellular components (Yeagle, 1989) and thus available for binding peptide]. Thus, it appears from the results in Tables 1 and 2 that proteins bearing a single geranylgeranyl chain, whether methylated or not, or a farnesyl chain and a C-terminal methyl ester may be fully bound to membranes. Proteins that are farnesylated but not methylated may exist as a mixture of membrane-bound and soluble forms. Of course this analysis does not take into account the possible existence in cell membranes of protein receptors for prenylated proteins.

A prenylated peptide may be viewed as a ligand with two domains: (1) The prenyl domain that inserts into the hydrophobic region of the membrane; (2) a polybasic peptide, which interacts with acidic phospholipids. Page and Jencks (1971) have developed a theoretical treatment that describes the binding of a ligand with multiple domains to a receptor. If a ligand with two domains, A and B, binds to a receptor in a way that both the A and B parts contact the receptor, then the binding constant for the ligand A−B interacting with the receptor can be up to 10^8-fold larger than the product of the individual binding constants for the separate pieces A and B. Large binding synergism effects approaching 10^8 have been reported [for example, Nakamura and Abeles (1985)]. This large effect stems from the fact that when A and B bind separately, they each must give up considerable rotational and translational entropy (disfavoring binding), whereas if A is tethered to B in an ideal way, only the molecule A−B must give up its rotational and translational entropy upon receptor binding. Or said another way, the perfect tether for binding enhancement is one that allows the A part to bind and then the B part to bind without any additional loss in rotational and translational entropy (or vice versa). Application of this analysis to the present study shows that the binding constant for ^{F}K-Ras-COOH to POPC/POPG LUVs [$K_{A\text{-}B} = 1/(70 \times 10^{-6}) = 1.4 \times 10^4$ M^{-1}] and the product of the binding constants of the two domains of this peptide (K-Ras-COOH, $K_A \approx 200$ M^{-1} and *N*-acetyl-*S*-farnesylcysteine, $K_B \approx 200$ M^{-1}, $K_A K_B \approx 4 \times 10^4$ M^{-1}) are similar in magnitude, i.e., well shy of the theoretical maximum ratio of 10^8. Thus, it appears that when the prenyl group inserts into the membrane, the polybasic peptide portion has to give up considerable rotational and translational entropy to bind to the membrane (or vice versa). It is tempting to suggest that the peptide tethered to the membrane by the prenyl group has a random coil conformation and then must assume a much less disordered conformation as its multiple lysines lay down onto the acidic phospholipids in the membrane. Such a process requires a considerable loss in peptide translational and rotational entropy. The circular dichroism spectrum of ^{GG}K-Ras-COOMe in solution shows that the peptide adopts primarily a random coil conformation. The conformation of the membrane-bound peptide is either a random coil or more likely a mixture of conformations in which the ϵ-amino groups of the lysines are bound close to the membrane surface.

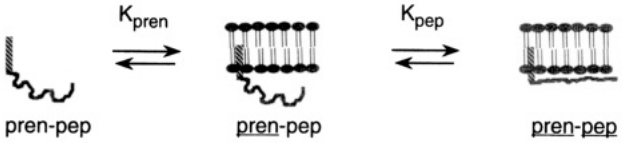

FIGURE 6: Interaction of prenylated peptides with membranes. The lipidated peptide consists of a prenyl chain (striped rectangle) and a peptide (thick line). The process of membrane binding is visualized in two steps: (1) the insertion of the prenyl chain into the lipid bilayer; (2) the interaction of the polybasic peptide with the anionic headgroups of acidic phospholipids. The definition of the equilibrium constants is as follows: $K_{pren} = [\underline{pren} - pep]/([pren - pep][PL]_G)$ and $K_{pep} = [\underline{pren} - \underline{pep}]/([\underline{pren} - pep][PL]_L)$, where $[PL]_G$ is the global phospholipid concentration (moles of phospholipid available for peptide binding divided by the total system volume) and $[PL]_L$ is the local phospholipid concentration (see text).

In a second approach for understanding the role that the prenyl chain and polybasic peptide contribute to the membrane affinity of prenylated peptides, the binding of the prenylated peptide to LUVs is again visualized in two steps (Figure 6): (1) Insertion of the prenyl group into the membrane; (2) binding of the peptide portion to the membrane surface. Once the peptide is anchored to the membrane via its prenyl group (step 1), the binding of the peptide portion (step 2) is enhanced by the high local concentration of membrane that the peptide portion "sees". This idea has been recently invoked to quantitatively account for the binding of myristoylated, polybasic src peptides to membranes (Buser et al., 1994). The local concentration of phospholipid that the peptide portion of the lipid-anchored peptide "sees" can be, to a first approximation, calculated (Buser et al., 1994) to be 3 M using a radius for ^{F}K-Ras-COOH of 15 Å and the surface area of a phospholipid of 50 Å^2. In terms of the equilibrium constants defined in Figure 6, the equilibrium constant for the binding of the prenylated peptide to LUVs can be calculated with the equation $K_B = K_{pren}(1 + K_{pep}[PL]_L)$ (Buser et al., 1994), where $[PL]_L$ is the local concentration of phospholipid that the bound peptide "sees" (3 M). Using the values of K_{pren}, and K_{pep}, of $\approx$200 M^{-1} and $\approx$200 M^{-1}, respectively (Table 2), a value of $K_B = 1.2 \times 10^5$ M^{-1} is obtained, which is within an order of magnitude of the experimental value of 1.4×10^4 M^{-1}. These

arguments suggest that it is the local concentration effect and not the rotational and translational entropy arguments that better explains the synergistic effects on membrane binding of attaching a prenyl chain to a polybasic peptide.

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