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ARTICLE

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Farnesylated peptides in model membranes: a biophysical investigation

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Abstract Protein prenylation plays an important role in signal transduction, protein–protein interactions, and the localization and association of proteins with membranes. Using three different techniques, this study physically characterizes the interactions between model dimyristoylphosphatidylcholine membranes and a series of farnesylated peptides. Magic angle spinning nuclear Overhauser enhancement spectroscopy and differential scanning calorimetry reveal that both charged [Ac-Asn-Lys-Asn-Cys-(farnesyl)-OMe and Ac-Asn-Lys-Asn-Cys-(farnesyl)-NH_2] and uncharged [Ac-Cys-(farnesyl)-OMe and farnesol] species partition into dimyristoylphosphatidylcholine bilayers. Calorimetry and vesicle fluctuation analysis of giant unilamellar vesicles show that the charged peptides modestly decrease the main gel–fluid phase transition and markedly increase the bending rigidity of large unilamellar vesicles. Uncharged species, on the other hand, dramatically decrease the main phase transition and modestly decrease the bending rigidity. No difference with carboxyl methylation is detected.

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Keywords Differential scanning calorimetry · Lipid bilayer · MAS-NOESY NMR · Mechanical properties · Vesicle fluctuation analysis

Abbreviations DIEA: *N*,*N*-diisopropylethylamine · DIPCDI: *N*,*N*-diisopropylcarbodiimide · DMF: *N*,*N*-dimethylformamide · DMPC: dimyristoylphosphatidylcholine · DSC: differential scanning calorimetry · GUV: giant unilamellar vesicle · HFIP: 1,1,1,3,3,3-hexafluoro-2-propanol · HOBt: 1-hydroxybenzotriazole · HPLC: high-performance liquid chromatography · MAS-NOESY: magic angle spinning nuclear Overhauser enhancement spectroscopy · T_m: main phase transition temperature · TFA: trifluoroacetic acid · VFA: vesicle fluctuation analysis

Introduction

The post-translational attachment of a lipid chain to proteins is known to promote membrane–protein association (see reviews: Clarke 1992; Zhang and Casey 1996; Sinensky 2000a, 2000b; Silvius 2002). The farnesyl chain, for example, is an isoprenoid molecule which attaches to proteins involved in many biological functions, from signal transduction (γ-G protein subunit) to cell growth (Ras) to nuclear structure (lamin B) (Sinensky 2000b). While not sufficient in itself to bind proteins to membranes, the attachment of the farnesyl chain is necessary for protein–membrane interactions and biological function (Kitten and Nigg 1991; Zhang and Casey 1996; Dong et al. 2002).

A specific role of the farnesyl chain in regulating biological function involves the lateral organization of proteins into membrane domains. Recently, there has been special interest in cholesterol- and sphingolipid-rich membrane domains and evidence that lipids may play a role in determining lateral membrane organization (Ahmed et al. 1997; Rinia and de Kruijff 2001; Samsanov et al. 2001; Zacharias et al. 2002; Bagatolli 2003; Edidin 2003). Such "raft" domains (postulated

to be in the liquid-ordered phase) are important in facilitating signal transduction and protein targeting (Brown and London 2000; Ikonen 2001; Holthuis et al. 2003). Proteins/peptides attached to a prenyl chain (such as farnesyl) are observed to be excluded from these domains (Melkonian et al. 1999; Silvius 1999; Moffett et al. 2000; Wang et al. 2000; Zacharias et al. 2002). A fundamental understanding of how farnesylated peptides affect membrane phase behavior and physical properties could yield more insight into this phenomenon.

In addition to a farnesyl or acyl chain, many proteins are further modified by a carboxyl methyl group. The only reversible step in the post-translational modification pathway, carboxyl methylation has been determined to enhance protein–membrane binding by increasing the hydrophobicity of the lipidated peptide/protein (Black 1992; Parish and Rando 1996; Sinensky 2000b). How carboxyl-methylated modified species affect membrane properties is unclear.

The few existing biophysical studies affirm that farnesylated peptides interact with model membranes and influence membrane curvature and permeability (Epand et al. 1993). Also, peptide–membrane binding energies have been investigated (Silvius and l'Heureux 1994; Ghomashchi et al. 1995; Leventis and Silvius 1998). Interpretations of these results are largely based on a model whereby the farnesyl chain partitions into the hydrophobic acyl chain core of the membrane. While NMR studies of saturated acylated peptides affirm this conformation (Struppe et al. 1998; Huster et al. 2001), evidence of a singly farnesylated peptide has yet to be confirmed.

In this study, we attempt to answer very basic questions about the physical nature of farnesylated peptide–membrane interactions. The complexity of the system is minimized for this purpose: a neutral, single component (dimyristoylphosphatidylcholine, DMPC) lipid bilayer, pure water with no added salt, and minimal models of short-chain farnesylated peptides are used. The synthesized peptides are chosen to be both charged and uncharged. In the absence of added salt, the lysine residue is protonated, rendering the lipopeptides Ac-Asn-Lys-Asn-Cys-(farnesyl)-NH_2 and Ac-Asn-Lys-Asn-Cys-(farnesyl)-OMe positively charged. The interactions of these charged peptides with the lipid bilayer are compared to those of the uncharged species, Ac-Cys-(farnesyl)-OMe and farnesol. Farnesol is an isoprenoid alcohol; the Cys amino acid residue serves as a small, uncharged "headgroup" for the farnesyl chain so that the effect of a neutral headgroup can be determined. The two charged species are distinguished by a carboxyl methyl group.

The interaction of these four peptide species with DMPC bilayers is characterized using three different techniques: (1) magic angle spinning nuclear Overhauser enhancement spectroscopy (MAS-NOESY NMR) is used to identify the location of the peptides in the membrane; (2) differential scanning calorimetry (DSC) provides information on both peptide–membrane binding and effects on the membrane's main phase transition; (3) vesicle fluctuation analysis (VFA) of giant unilamellar vesicles (GUVs) is a non-invasive method to determine how peptide partitioning affects membrane bending rigidity and thereby the mechanical stability of the membrane. Together, these experiments elucidate the effects of this important class of membrane peptides on lipid bilayers. We demonstrate that farnesylated peptides and farnesol partition into the membrane. This partitioning is a function of both the farnesyl chain's affinity for the lipid bilayer as well as charge effects. The uncharged species are found to remain in the membrane, markedly decrease the main phase transition temperature, T_m, and promote the formation of a broad gel–fluid coexistence region, as well as modestly lower the membrane bending rigidity, κ. The charged peptides, on the other hand, are observed to partition between the solvent and the membrane and slightly reduce the temperature of the main phase transition (T_m), which remains cooperative. The membrane bending rigidity (κ) is seen to markedly increase in response to the charged peptides. Our results thus show that farnesylated peptides partition into and affect membrane physical properties, with a strong dependence on electrostatic effects.

Materials and methods

Materials

DMPC was obtained from Avanti Polar Lipids (Alabama, USA). Organic solvents, sugars, and heavy water were products of Sigma-Aldrich (Denmark). DMPC-d_{67} was a gift from Dr. G. Gröbner. All materials were used without further purification.

Peptide synthesis

Farnesylated peptides were obtained by a combination of solid-phase and solution synthesis methods. Based upon the C-terminal sequence of the *Xenopus* lamin B1 protein, the N-terminal acetylated peptide Ac-Asn-Lys-Asn-Cys-(farnesyl)-OMe (Fig. 1A) was synthesized. To elucidate the effects of carboxyl methylation, the corresponding C-terminal amide, Ac-Asn-Lys-Asn-Cys-(farnesyl)-NH_2 (Fig. 1B), was also prepared. As the methoxy moiety in the ester functionality is a competent leaving group (e.g. in diketopiperazine formation at the dipeptide stage), the C-terminal amide is easier to access by synthesis. First, the deprotected peptides were prepared. While the C-terminal peptide amide could be assembled directly by solid-phase synthesis on a PAL-PEG-PS (Albericio et al. 1990) support, the corresponding C-terminal peptide methyl ester was prepared by coupling (DIPCDI, DIEA, HOBt in DMF) of the tripeptide (assembled on a 2-chlorotrityl resin, cleavage with HFIP) with H-Cys-(Acm)-OMe (Kamber 1971) in 73% yield, followed by removal of acid-labile side-chain protecting groups. The subsequent removal of the Acm protecting group by treatment with $Hg(OAc)_2$ yielded the fully deprotected peptides. Finally, treatment of the peptides with *trans*,*trans*-farnesyl bromide in the presence of $Zn(OAc)_2$ to form the thioether (Epand et al. 1995; Liu et al. 1995; Naider and Becker 1997) gave the final farnesylated peptides (Fig. 1A, B). The reaction proceeded under weakly acidic

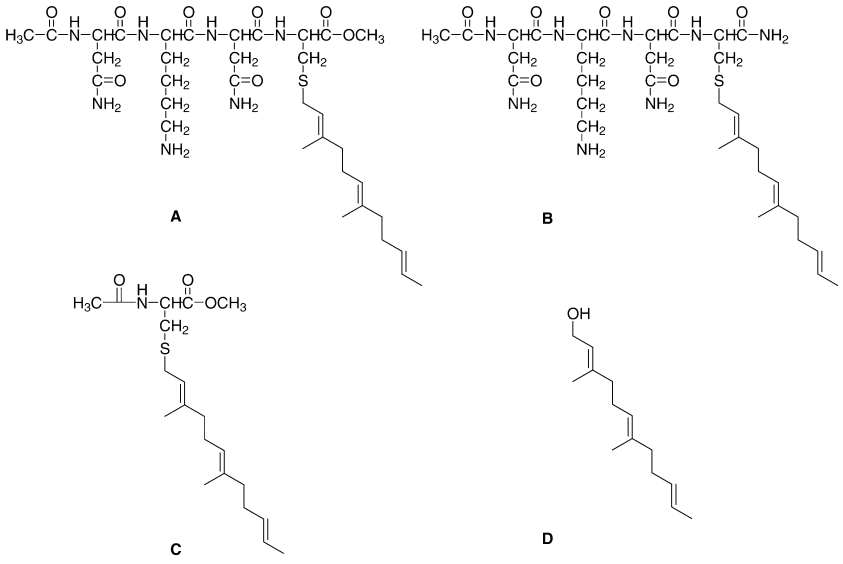

Fig. 1A–D Structures of the synthesized farnesylated peptides. (**A**) Ac-Asn-Lys-Asn-Cys-(farnesyl)-OMe; (**B**) Ac-Asn-Lys-Asn-Cys-(farnesyl)-NH_2; (**C**) Ac-Cys-(farnesyl)-OMe; (**D**) farnesol

conditions to prevent disulfide formation and to protonate the N^{ϵ}-amino group in Lys. The farnesylated amino acid Ac-Cys-(farnesyl)-OMe (Fig. 1C) was also prepared.

Estimate of the partition coefficient: the membrane partitioning assay

Peptides were dissolved together with DMPC in a chloroform/methanol (1:1, v/v) solution. Peptide concentrations of $X_{pep} = 2.5$, 5.0, 7.5, and 10.0 mol% were investigated. After at least 12 h under vacuum to evaporate the organic solvent, the peptide–DMPC blend was resuspended in MilliQ water (Millipore, Bedford, Mass., USA) to yield a final lipid concentration of 1.5 mM. After vigorous shaking and incubation in a water bath at 39 °C for at least 30 min, the lipid dispersions were extruded (Lipex Biomembranes, Vancouver, Canada) through two stacked polycarbonate filters (Nucleopore, 800 nm pore size) to create unilamellar vesicles (Hope et al. 1986; MacDonald et al. 1991). Samples were centrifuged (23,907×*g*; Universal 16, Hettich Zentrifugen, Tuttlingen) at room temperature for 45 min to separate the lipid and water phases. A small aliquot of the water phase was removed and analysed with HPLC (XTerra, C-18, 3.5 μm, 3×50 mm cartridge, Waters HPLC System, Milford, Mass., USA). Peptides were eluted with mixtures of acetonitrile and water, both containing 0.1% TFA. Determining the amount of peptide in the membrane phase relative to the aqueous phase yields a rough estimate of the partitioning coefficient. Similar centrifugation separation procedures have been described in the literature (Epand et al. 1993; Ghomashchi et al. 1995).

MAS-NOESY NMR

Peptide–DMPC-d_{67} mixtures ($X_{pep} = 10$ mol%) were prepared as described above, with the exception that samples were hydrated with 20% (w/w) heavy water and 10% MilliQ water. Samples were repeatedly centrifuged for 15-min intervals at 26 °C to promote homogeneous mixing. After equilibrating at room temperature for at least 48 h, samples were transferred to 2.5-mm MAS rotors. ^{1}H NMR spectra were acquired at 400.51 MHz with a Chemagnetics Infinity 400 spectrometer. Samples were typically spun at 12 kHz (Chemagnetics MAS probe). An airflow temperature controller ensured a sample temperature of 30 ± 1 °C (accounting for MAS frictional heating) (Bielecki and Burum 1995). Two-dimensional NOESY was performed in phase-sensitive mode using hypercomplex acquisition. The utilized pulse sequence (90°–t_1–90°–τ_m–90°–acquire) had 512–750 t_1 values, mixing times (τ_m) in the range of 20–500 ms, and a 90° pulse of 2.4 μs. The spectral width was set to 2/3 of the spinning speed to avoid aliasing of the first-order spinning sidebands back to the centre of the spectrum. The recycle delay was 1.5 s and the number of acquisitions was 32 or 64, resulting in a total experiment time of up to 36 h. For each MAS-NOESY experiment, a corresponding static ^{31}P NMR powder lineshape was acquired to determine the mesophase of the system (Smith 1985; Lindblom 1996). The ^{31}P NMR spectra were acquired at 162.13 MHz using a chemical shift echo sequence (90°–τ–180°–τ–acquire) with continuous-wave proton decoupling during echo delays and acquisition. On the doubly tuned 2.5 mm MAS probe, the ^{31}P 90° pulse was 3.6 μs, the interpulse delay (τ) was 50 μs, the pulse delay was 1.5 s, and the proton decoupler radiation corresponded to 21 kHz.

Differential scanning calorimetry

Varying concentrations of farnesylated peptides ($X_{pep} = 2.5$, 5.0, 7.5, and 10.0 mol%) were dissolved with DMPC in a solution of chloroform, methanol, and water (the proportions of which depend upon each peptide's solubility). The organic solvent was evaporated for at least 12 h under vacuum. MilliQ water was added to yield a typical total lipid concentration of ~1.5 mM. The resulting dispersions were vigorously shaken and placed in a water bath at 39 °C for at least 30 min before extrusion (Lipex Biomembranes, Vancouver, Canada) through two stacked polycarbonate filters (Nucleopore, 800 nm pore size) to create unilamellar vesicles. The presence of vesicles was verified with dynamic light scattering techniques (Malvern Zetasizer 4, UK). Six DSC scans (heating and cooling) were run from 5 to 40 °C at a scanning rate of 10 °C/h (Nano-DSC II, Calorimetry Sciences, Provo, Utah, USA).

T_m is chosen to be the peak of the C_p curve (the temperature of maximal heat absorption). The temperatures of onset and completion of transition, τ_1 and τ_2, are defined as where the C_p peak intersects the baseline. As ΔH is highly dependent upon the choice of baseline, a consistent choice of baseline is necessary for a reliable comparison of transition enthalpies and phase boundaries at differing compositions. The baseline was chosen to begin at the temperature where $\left|\frac{dC_p}{dT}\right|$ is doubled over the temperature range $\Delta t_s = 0.05$ K ($\left|\frac{dC_p}{dT}\right|_{t+\Delta t_s} = 2\left|\frac{dC_p}{dT}\right|_t$) as the transition region is approached from below or above.

Vesicle fluctuation analysis

GUVs were cultivated by swelling dried DMPC–peptide/farnesol films in a ~75 mM sucrose solution (Reeves and Dowben 1968). Suspending the vesicles in a ~76 mM glucose ensured both phase

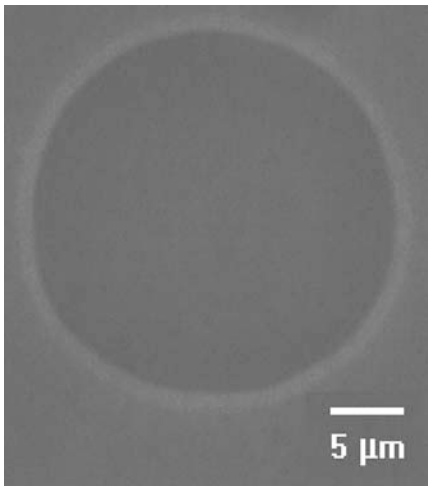

Fig. 2 Vesicle fluctuation analysis (VFA) involves analyzing changes in the contour of giant unilamellar vesicles (GUVs)

contrast and gravitational stability (Henriksen and Ipsen 2003)[1]. Low concentrations of sugar solutions have been shown to have insignificant effects on the membrane's spontaneous curvature (Döbereiner et al. 1999). It is important to note that this series of dilutions renders the actual charged-peptide-to-lipid ratio much smaller than that of the original stock solution owing to the partitioning between the water and membrane phases (this is not the case for the DSC preparation). A freezing-point osmometer (model 3D3, Advanced Instruments, Norwood, Mass., USA) was used to regulate solution osmolarities. Contained within a controlled temperature chamber (37 °C), undulating vesicles were visualized using phase contrast microscopy (Zeiss Axiovert S100, Göttingen, Germany). After obtaining the vesicle's two-dimensional contour in the focal plane of the objective (Fig. 2), a series of 4000 contours was captured and analyzed in real time. A single snapshot required 4 ms to obtain and the time interval between consecutive images was 40 ms. Each determined value for the bending rigidity, κ, is an average of between 3–10 different vesicles of diameter 15–25 µm.

Values for κ were extracted from thermally induced shape undulations of the membrane in the fluid phase (37 °C) (Faucon et al. 1989). The shape of a homogeneous lipid bilayer is dictated by Helfrich's curvature free energy (Helfrich 1973):

$$\mathcal{F} = \sigma A + \frac{\kappa}{2}\int_A \mathrm{d}A\left(\frac{1}{r_1}+\frac{1}{r_2}-2H_0\right)^2 \quad (1)$$

where r_1 and r_2 are local curvature radii of the membrane, and κ is the elastic bending modulus; σ represents the effective surface tension, which is set by the total area and volume of the vesicle; H_0 is the spontaneous curvature of the membrane, which reflects asymmetry in the composition of the two monolayer leaflets of the membrane. As the samples were prepared from a homogeneous mixture of lipids and peptides and considering the rapid transbilayer diffusion exhibited by farnesylated heptapeptides (Schroeder et al. 1997), we assume a homogeneous distribution of peptides in both monolayers ($H_0 = 0$).

The center of mass and the instantaneous vesicle contour in the focal plane at time are obtained and the contour shape is characterized in polar coordinates $r(\phi,t)$. To characterize the thermal fluctuations in shape, the angular correlation function of $r(\phi,t)$ is calculated:

$$C(\psi,t) = \frac{1}{R^2}\left(\frac{1}{2\pi}\int_0^{2\pi}\mathrm{d}\phi\, r(\phi+\psi,t)r(\phi,t) - \left(\frac{1}{2\pi}\int_0^{2\pi}\mathrm{d}\phi\, r(\phi,t)\right)^2\right) \quad (2)$$

where R is the average radius of the contour.

The spectrum of the shape fluctuations of the quasi-spherical vesicle is then obtained by calculation of the time averages of the amplitudes in a Legendre expansion of 2. The average of all coefficients, C_n, is then fit to a theoretical expression for the Legendre amplitudes (derived from Eq. 1):

$$\bar{C}_n(\kappa,\sigma) = \frac{k_\mathrm{B}T}{4\pi\kappa}\frac{2n+1}{(n+2)(\bar{\sigma}+n(n+1))} \quad (3)$$

for $n \geq 2$. Here n is the Legendre mode number and κ and $\bar{\sigma} = \frac{\sigma R^2}{\kappa}$ are fitting parameters obtained by a χ^2 fit of Eq. (3). The procedure is described in detail in Faucon et al. (1989).

Vesicles were selected when $\chi^2_{\min} \approx f$, where $f = n_{\max} - n_{\min}$ is the number of degrees of freedom for the χ^2 fit and κ and σ were not excessively large. High κ values may indicate a multilamellar vesicle, while very large σ values signal a tense membrane which may distort the analysis.

Results and discussion

Peptide–membrane partitioning

The membrane partitioning assay confirms that the peptides partition between the aqueous and lipid phases. Roughly, ~60–70% of the total charged peptides are estimated to associate with the membrane. Neither farnesol nor the lipo-amino acid ester were detected in the aqueous phase. As the centrifugation process may shift the partitioning equilibrium by, for example, altering the degree of hydration, the centrifugation partitioning assay thus yields a rough estimate of the partition coefficient. Other techniques used to determine lipidated peptide–membrane partitioning (isothermal titration calorimetry, fluorescence spectroscopy, equilibrium dialysis measurements) rely on salt and/or a small amount of organic solvent to be incorporated into the aqueous phase. Conducted in a buffer solution (in some cases including an organic solvent), these estimates of the partition coefficient are not representative of the farnesylated peptide–membrane system in this study. Salt screens electrostatic interactions, and organic solvents (such as methanol and ethanol) are known to partition into membranes (Trandum et al. 1999; Westh and Trandum 2000). Such a fluorescence study has estimated that 99% of farnesylated, carboxylmethylated peptides are membrane bound whilst unmethylated peptides exhibit slightly weaker binding (Silvius 2002). Despite the difficulties in determining the partition coefficient, our partition study gives evidence that the farnesyl chain provides affinity for the membrane in an aqueous medium.

Locating the peptide in the membrane

^{31}P NMR confirms that membranes containing the peptides are in the L_α phase at 30 °C (spectra not

[1] Maintaining a 1 mM gradient between sucrose and glucose solutions induces a small excess area and ensures observable thermal fluctuations

shown). Spectra obtained from MAS-NOESY (Figs. 3 and 4) indicate that the peptides are situated in the interfacial region, with the farnesyl chain inserted into the bilayer.

Interactions between amino acids and the lipid headgroup are observed: Lys (H^{δ}), Lys ($H^{\in}$), and Cys (H^{β}) protons all exhibit crosspeaks with glycerol and the upper-chain region of the phospholipids, as well as with water. This substantiates that Lys and Cys side chains (and therefore the peptide headgroup) are located in the interfacial region of the membrane.

The farnesyl chain shows crosspeaks with both lipid headgroups and acyl chains. Farnesyl–acyl chain interactions are more evident in the corresponding MAS-NOESY spectrum using unlabelled DMPC (Fig. 4). This reveals that farnesyl is inserted into the hydrophobic core of the lipid bilayer. Crosspeaks between farnesyl to the DMPC headgroup suggest exchange of peptides between the membrane and aqueous phases. Similar observations of peptide exchange between the membrane and aqueous phases have been made with fluorescence techniques (Ghomashchi et al. 1995; Schroeder et al. 1997).

Numerous intermolecular lipid–lipid and lipid–peptide interactions reflect the high molecular disorder and dynamics in liquid-crystalline bilayers. These interactions may even introduce cross-relaxation between headgroup and hydrocarbon chain protons. In principle, spin diffusion may also contribute to crosspeaks between

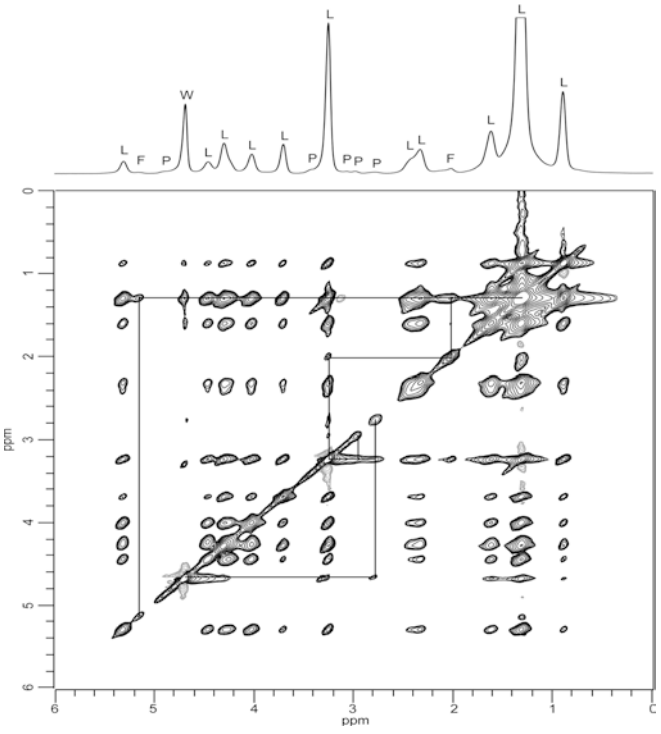

Fig. 4 MAS-NOESY spectrum of 10 mol% Ac-Asn-Lys-Asn-Cys-(farn)-NH_2, DMPC, and 30% heavy water using a 300 ms mixing time. *P* = peptide headgroup, *F* = farnesyl chain, *L* = lipid (DMPC), *W* = water. The *solid lines* above and below the diagonal illustrate, respectively, farnesyl-DMPC and peptide-DMPC crosspeaks

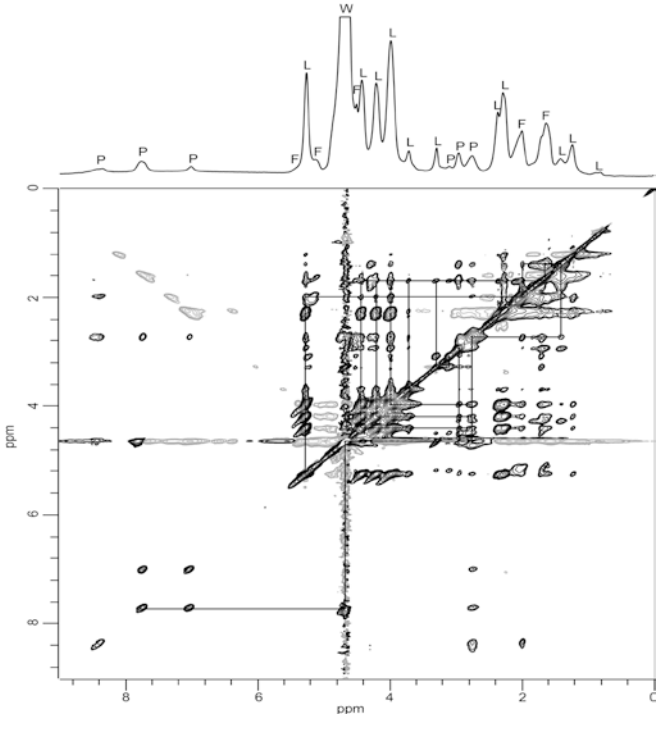

Fig. 3 MAS-NOESY spectrum of 10 mol% Ac-Asn-Lys-Asn-Cys-(farn)-OMe, DMPC-d_{67}, and 30% water using a 300 ms mixing time. *P* = peptide headgroup, *F* = farnesyl chain, *L* = lipid (DMPC), *W* = water. The *solid lines* above and below the diagonal illustrate, respectively, farnesyl-DMPC and peptide-DMPC crosspeaks

apparently distant protons, although this effect has been shown to be small for DMPC at 30 °C and with mixing times below 300 ms (Huster and Gawrisch 1999; Huster et al. 1999).

Our spectra illustrate that the farnesyl moiety partitions into the bilayer, while the peptide headgroup resides at the membrane–water interface. This finding is in accordance with a MAS-NOESY NMR study of a larger lipidated peptide in a DMPC membrane (Huster et al. 2001). Similar to our findings, this model Ras lipopeptide's palmitoyl and hexadecanylate chains were observed to insert into the lipid bilayer interior, while the peptide resides in the interfacial headgroup region. A ^2H NMR study of the myristoylated amine-terminal peptide of the protein kinase A catalytic subunit has also yielded evidence that the *N*-myristoyl group intercalates into the lipid bilayer, with its long axis parallel to the lipid chains (Struppe et al. 1998). Both our results and these observations of saturated acylated peptide conformation validate that the farnesylated peptides participate in the cooperative behavior of the membrane.

Thermodynamic effects: changes in the main phase transition

DSC scans further support that the peptides intercalate into the membrane. All six scans obtained over a period of about 25 hours were identical. The absence of

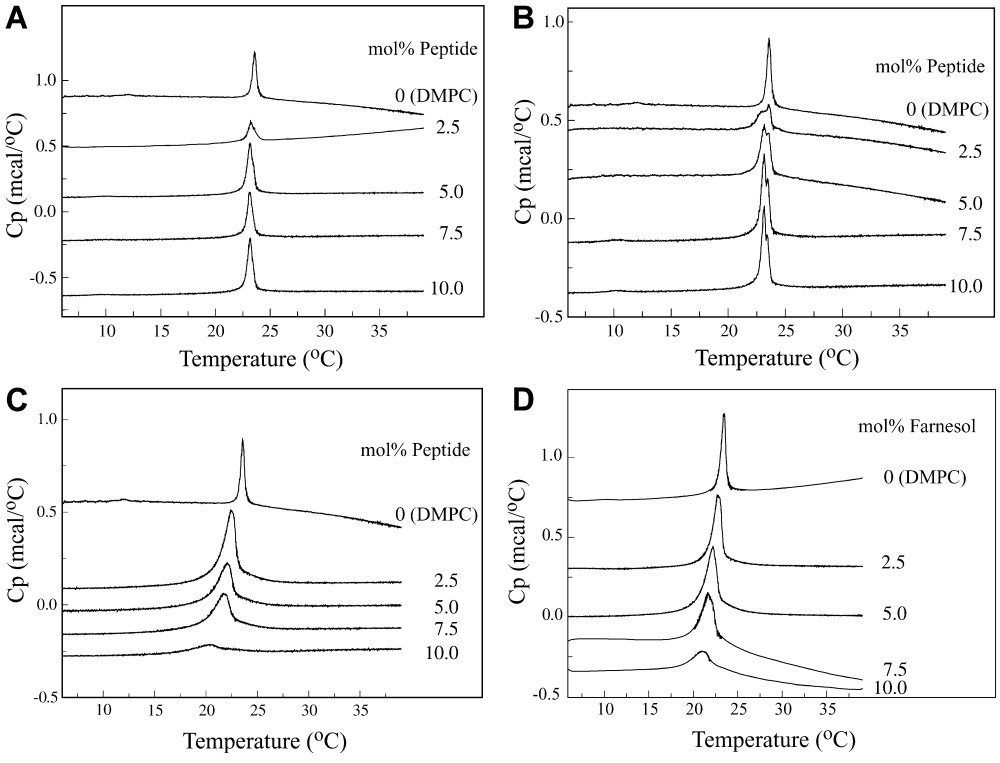

Fig. 5A–D Differential scanning calorimetry (DSC) scans of DMPC unilamellar liposomes containing increasing concentrations (2.5, 5.0, 7.5, and 10 mol%) of peptide. (**A**) Ac-Asn-Lys-Asn-Cys-(farnesyl)-OMe; (**B**) Ac-Asn-Lys-Asn-Cys-(farnesyl)-NH_2; (**C**) Ac-Cys-(farnesyl)-OMe; (**D**) farnesol. The lipid concentration is ~1.5 mM, with the exception of the farnesol scans (~2 mM)

hysteresis effects signals that the system is in thermodynamic equilibrium. Both farnesol and Ac-Cys-(farnesyl)-OMe (Fig. 5d and c, respectively) markedly reduce and broaden the main phase transition. Progressively increasing concentrations of these solutes lower the main phase transition temperature (T_m); these compounds therefore stabilize the fluid phase. This observed behavior is consistent with previous studies of farnesol (Bondar et al. 1994). As the two uncharged species [farnesol and Ac-Cys-(farnesyl)-OMe] exhibit similar trends, the effect of the cysteine residue — and slightly larger headgroup size — must thus be negligible.

Both uncharged compounds induce an opening of the cooperative main phase transition of DMPC into a phase coexistence region. This supports that essentially all of the uncharged species remain in the membrane and the composition can thus be regarded as a conserved property (Gibbs phase rule) (Jorgensen et al. 1991).

The charged peptides, Ac-Asn-Lys-Asn-Cys-(farnesyl)-OMe and Ac-Asn-Lys-Asn-Cys-(farnesyl)-NH_2, also depress T_m (Fig. 5a and b). The reduction in T_m, however, is more subtle than for farnesol and Ac-Cys-(farnesyl)-OMe (Fig. 6). At concentrations above 7.5 mol% for Ac-Asn-Lys-Asn-Cys-(farnesyl)-NH_2 and 5.0 mol% for Ac-Asn-Lys-Asn-Cys-(farnesyl)-OMe, no detectable shift in T_m is observed, indicating a reduction in the membrane's uptake of peptides. This phenomenon is common in the adsorption of ionic surfactants to liquid interfaces (Diamant and Andelman 1996; Evans and Wennerström 1999).

The form of the charged peptide thermograms is also different: whereas increasing concentrations of farnesol and Ac-Cys-(farnesyl)-OMe broaden the main transition, the thermogram peaks of the charged peptides remain narrower and more defined. The narrow transition peak signals that the main transition remains cooperative. The peptide content in the membrane is thus not a conserved quantity, but controlled by a chemical potential set by the bulk solution (Gibbs phase rule again) (Jorgensen et al. 1991).

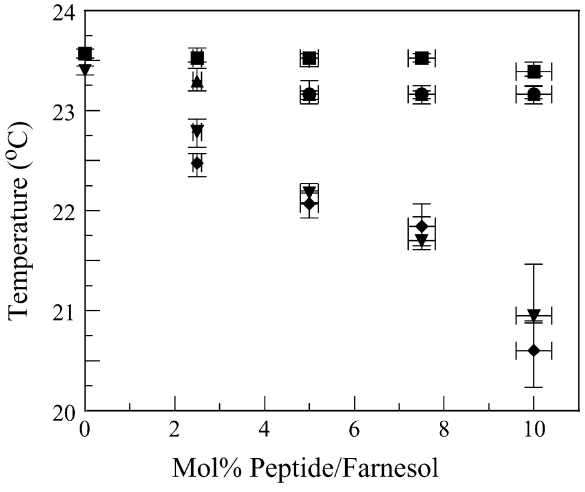

Fig. 6 Plot of the gel–fluid phase transition temperature (T_m) versus peptide concentration [mol%] in DMPC unilamellar vesicles. Ac-Asn-Lys-Asn-Cys-(farnesyl)-OMe (*up triangles*); Ac-Asn-Lys-Asn-Cys-(farnesyl)-NH_2 (*squares*); Ac-Cys-(farnesyl)-OMe (*diamonds*); farnesol (*down triangles*). The emergence of a defined second peak in the Ac-Asn-Lys-Asn-Cys-(farnesyl)-NH_2 scans is also represented (*circles*)

Differences between the two charged peptides, Ac-Asn-Lys-Asn-Cys-(farnesyl)-OMe and Ac-Asn-Lys-Asn-Cys-(farnesyl)-NH_2, are subtle. The main transition peak of Ac-Asn-Lys-Asn-Cys-(farnesyl)-NH_2 is slightly broader than Ac-Asn-Lys-Asn-Cys-(farnesyl)-OMe and exhibits the possible emergence of a second peak. The effects of the carboxyl methyl group on membrane phase behavior are thus determined to be negligible.

At small concentrations of peptide/farnesol, the pre-transition (11 °C) is eliminated. The cooperativity of the pre-transition has been shown to vanish upon addition of membrane solutes (Wang et al. 1993; Pedersen et al. 2002).

Effects on membrane bending rigidity

As a membrane is a free-standing film with vanishing surface tension (in contrast to liquid interfaces), the conformational stability of the membrane is thus mainly determined by the bending elasticity. The bending rigidity, κ, is a key parameter in describing membrane mechanical properties. Despite its importance, few techniques are available to accurately determine κ. With VFA, values of κ in the fluid phase (37 °C) were extracted by fitting the analytical expression for C_n to the experimentally determined values (Fig. 7) (Faucon et al. 1989). The obtained κ values are displayed in Table 1.

Uncharged species: the effects of farnesyl

At a concentration of 5 mol%, Ac-Cys-(farnesyl)-OMe is observed to modestly decrease κ by 6%. Farnesol (5 mol%) exhibits a similar but smaller effect which is not significant within the experimental error. The slight decrease in κ is consistent with the observed tendency of these compounds to promote the fluid phase (Fernandez-Puente et al. 1994). It can thus be concluded that the farnesyl group acts to associate peptides and proteins with the membrane without disrupting membrane integrity. This behavior is in contrast to that of small amphiphatic peptides which destabilize the membrane at very low peptide concentrations (< 1 mol%) (Henriksen et al. 2002).

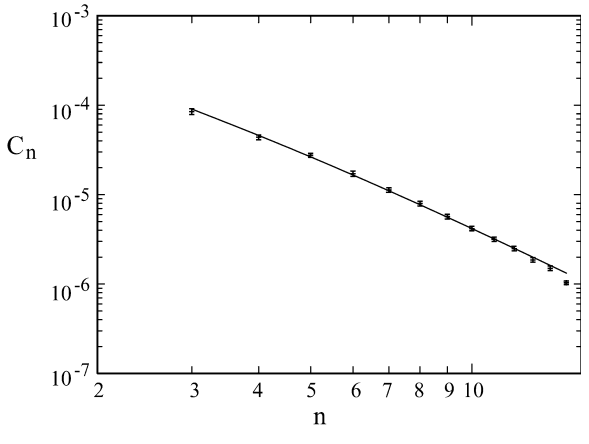

Fig. 7 Representative log–log plot (for DMPC) of C_n versus n, where n is the mode number in the spherical harmonical representation. An analytical expression is best χ^2-fit to the experimental C_n values to yield a value for the bending rigidity, κ

Table 1 Bending rigidity, κ (k_BT), of DMPC GUVs containing farnesylated peptides at 37 °C

0.0 (DMPC)	33.2 ± 0.8
Ac-Asn-Lys-Asn-Cys-(farnesyl)-OMe (5 mol%)	37.3 ± 0.6
Ac-Asn-Lys-Asn-Cys-(farnesyl)-NH_2 (5 mol%)	39.6 ± 0.6
Ac-Cys-(farnesyl)-OMe (5 mol%)	33.1 ± 1.7
Farnesol (5.0 mol%)	32.5 ± 0.9

Charged peptides: electrostatic effects

Despite the fact that the concentration of charged peptides [Ac-Asn-Lys-Asn-Cys-(farnesyl)-NH_2 and Ac-Asn-Lys-Asn-Cys-(farnesyl)-OMe] in the membrane is lower than that of the non-charged species (as discussed in Materials and methods), these charged species markedly increase κ. Such an increase in κ (morphological stabilization) can be attributed to the presence of the electrical double layer. A positive electrostatic contribution to bending rigidity is in accordance with theory (Andelman 1995) and will be the subject of a more thorough investigation.

Conclusions

Based on the synthesis of a series of farnesylated peptides, this study illustrates how lipidated peptides interact with membranes using a variety of biophysical techniques. We have addressed some very basic questions regarding the nature of the interactions between prenylated peptides and the lipid bilayer which have not yet been answered in the literature. Farnesylated peptides are observed to partition into the membrane, lower the phase transition temperature, and affect membrane mechanical properties. The extent to which the peptides partition into the membrane and affect membrane phase behavior and bending rigidity is shown to be dependent upon charge.

Both the partition study and the DSC results show that the presence of charged amino acids dramatically affects the partitioning of the farnesylated peptides into the membrane. In the DSC scans this is reflected in the freezing point depression at low peptide content, which shows that some peptides partition into the membrane. Our VFA results show that there is a significant electrostatic contribution to the bending rigidity: even a small uptake of the charged farnesylated peptides rigidifies the membrane. The electrostatic contribution to bending rigidity has previously been observed only for

multilamellar surfactant and microemulsion systems (Kellay et al. 1994; Fogden and Ninham 1999).

Comparing how charged versus non-charged farnesyl species influence the thermodynamic and mechanical properties of membranes helps to elucidate the role of electrostatics in peptide–membrane adsorption. When contemplating the adsorption of charged peptides to a neutral membrane, some of the various factors involved include: (1) the entropically driven adsorption of peptides to the membrane; (2) interactions amongst peptides and their counterions, both in solution and at the membrane interface; and (3) accumulation of surface charge and electric double layer formation. While the hydrophobic effect (i.e. farnesyl chain's affinity for the bilayer) drives lipidated peptides to partition into the membrane, it is clear that electrostatics also plays a role in this process (Peitzsch and McLaughlin 1993; Seelig 1997; Ladokhin and White 2001).

In principle, the peptide headgroup could induce changes in interfacial packing and thereby influence membrane bending rigidity (Cantor 1999). Considering the small concentration of charged peptide adsorbed to the membrane interface, however, accordingly small changes to packing may be expected. Such contributions to κ must therefore be much smaller than the effects (on the order of k_BT) which we observe. This substantiates the role that electrostatics plays in the mechanical stability of membranes. A more thorough work on this topic is in progress.

Carboxyl methylation has been determined to increase the hydrophobicity of lipidated peptide/proteins and is thereby thought to enhance peptide–membrane interactions (Black 1992; Parish and Rando 1996; Sinensky 2000b). How proteins/peptides modified in this way affect membrane properties has not been documented.

With the resolution of our biophysical techniques, only minor differences in the interactions of DMPC with Ac-Asn-Lys-Asn-Cys-(farnesyl)-OMe and Ac-Asn-Lys-Asn-Cys-(farnesyl)-NH_2 are observed. It is difficult to conclude, however, if such minor differences are an effect of differences in packing caused by the carboxyl methyl group and/or differences in the partitioning coefficients of the two peptides. In enhancing lipidated peptide hydrophobicity, carboxyl methylation may play a role in shifting the partitioning equilibrium of marginally stable farnesylated peptides. It is clear that the amount of peptides adsorbed to the membrane clearly influences the physical properties of the lipid bilayer.

Regarding biological implications, our results are interesting in the context of lipid microdomains (''rafts'') and the role lipidation plays in the lateral organization and sorting of lipidated proteins/peptides in membranes. In particular, it has been shown that proteins/peptides attached to a saturated chain (palmitoyl or myristoyl) have been shown to preferentially partition into ''raft'' lipid domains whereas those modified by a prenyl chain are excluded from these domains (Melkonian et al. 1999; Silvius 1999; Moffett et al. 2000; Wang et al. 2000; Zacharias et al. 2002). These studies are consistent with: (1) our finding that Ac-Cys-(farnesyl)-OMe and farnesol (the uncharged species) stabilize the fluid phase and therefore preferentially partition into a less ordered membrane environment; and (2) previous studies which demonstrate how equivalent concentrations of saturated fatty acids stabilize the gel phase (Schullery et al. 1981; Koynova et al. 1987, 1997) and order lipid membrane acyl chains (Pauls et al. 1983).

Farnesyl's effects on membrane mechanical properties are also in contrast to the polyisoprene membrane component, cholesterol, another molecule which post-translationally attaches to proteins (Mann and Beachy 2000). Promoting the formation of a new fluid yet ordered phase [the liquid ordered phase (l_o) (Ipsen et al. 1987)], cholesterol markedly increases κ (Méléard et al. 1997; Duwe et al. 1990) and is thus a potent membrane rigidifier (i.e. mechanically stabilizes the fluid phase). Farnesyl, on the other hand, may play a role in fluidizing the gel phase (reduces fluorescence anisotropy of the gel phase) (Bondar et al. 1994) (Rowat and Davis, 2003).

While it may be argued that a buffer would create a more biologically relevant investigation, studying prenylated peptide–membrane interactions in the absence of salt facilitates insight into electrostatic effects. As the presence of salt is known to shield electrostatic interactions, the process of charged peptide–membrane adsorption is highly dependent upon salt. Variations in salt, pH, and charge are all important factors in regulating biological function, thus performing the experiments in the presence of a buffer would limit the understanding of the fundamental interactions of the system and applicability of the knowledge to a variety of biological conditions. Simple estimates of the electrostatic enhancement of membrane bending rigidity (Pincus et al. 1990) suggest that this contribution plays a role at physiological salt concentrations and higher surface charge densities. A more extensive study of salt and pH effects on peptide partitioning together with a detailed theoretical interpretation of these effects is currently being developed.

The extent to which the physical effects of prenylated peptide–membrane interactions play a role in biological function remains to be seen.

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