Organization of Model Helical Peptides in Lipid Bilayers: Insight into the Behavior of Single-Span Protein Transmembrane Domains

ARTICLE *in* BIOPHYSICAL JOURNAL · AUGUST 2002

Impact Factor: 3.97 · DOI: 10.1016/S0006-3495(02)75174-6 · Source: PubMed

5 AUTHORS, INCLUDING:

Kathy Barber
The University of Western Ontario
61 PUBLICATIONS **1,341** CITATIONS
SEE PROFILE

Michael R Morrow
Memorial University of Newfoundland
40 PUBLICATIONS **633** CITATIONS
SEE PROFILE

Organization of Model Helical Peptides in Lipid Bilayers: Insight into the Behavior of Single-Span Protein Transmembrane Domains

Simon Sharpe,* Kathryn R. Barber,* Chris W. M. Grant,* David Goodyear,† and Michael R. Morrow†
*Department of Biochemistry, University of Western Ontario, London N6A 5C1, Canada; and †Department of Physics, Memorial University of Newfoundland, St. John's, Newfoundland A1B 3X7, Canada

ABSTRACT Selectively deuterated transmembrane peptides comprising alternating leucine-alanine subunits were examined in fluid bilayer membranes by solid-state nuclear magnetic resonance (NMR) spectroscopy in an effort to gain insight into the behavior of membrane proteins. Two groups of peptides were studied: 21-mers having a 17-amino-acid hydrophobic domain calculated to be close in length to the hydrophobic thickness of 1-palmitoyl-2-oleoyl phosphatidylcholine and 26-mers having a 22-amino-acid hydrophobic domain calculated to exceed the membrane hydrophobic thickness. ^{2}H NMR spectral features similar to ones observed for transmembrane peptides from single-span receptors of higher animal cells were identified which apparently correspond to effectively monomeric peptide. Spectral observations suggested significant distortion of the transmembrane α-helix, and/or potential for restriction of rotation about the tilted helix long axis for even simple peptides. Quadrupole splittings arising from the 26-mer were consistent with greater peptide "tilt" than were those of the analogous 21-mer. Quadrupole splittings associated with monomeric peptide were relatively insensitive to concentration and temperature over the range studied, indicating stable average conformations, and a well-ordered rotation axis. At high peptide concentration (6 mol% relative to phospholipid) it appeared that the peptide predicted to be longer than the membrane thickness had a particular tendency toward reversible peptide-peptide interactions occurring on a timescale comparable with or faster than $\sim 10^{-5}$ s. This interaction may be direct or lipid-mediated and was manifest as line broadening. Peptide rotational diffusion rates within the membrane, calculated from quadrupolar relaxation times, T_{2e}, were consistent with such interactions. In the case of the peptide predicted to be equal to the membrane thickness, at low peptide concentration spectral lineshape indicated the additional presence of a population of peptide having rotational motion that was restricted on a timescale of 10^{-5} s.

INTRODUCTION

Wide-line ^{2}H nuclear magnetic resonance (NMR) has proven to be a powerful technique for elucidating the structure and behavior of transmembrane peptides and proteins. Its application in this area was developed by a number of groups investigating bacterial and model peptides and proteins (reviewed in Opella, 1986; Macdonald and Seelig, 1988; Davis, 1991; Siminovitch, 1998). The approach permits use of nonperturbing nuclear probes located directly on the molecule of interest in fluid fully hydrated lipid bilayer membranes held at physiological temperatures. We have been extending it to single-span transmembrane receptor proteins from higher animals (Morrow and Grant, 2000; Sharpe et al., 2002 and references therein). ^{2}H NMR may provide important insight, as the concept has evolved that the dynamic behavior of receptors underlies their signaling function and is importantly influenced by the behavior of the transmembrane domain (Kavanaugh and Williams, 1996; White and Wimley, 1999; Ubarretxena-Belandia and Engelman, 2001). A number of workers have proposed that dimer/oligomer formation of receptors (considered a key initial step in signal transduction) can be directed by side-to-side association of their transmembrane portions (Sternberg and Gullick, 1990; Deber et al., 1993; Lemmon et al., 1994; Javadpour et al., 1999; White and Wimley, 1999).

In recent ^{2}H NMR studies of single-span transmembrane peptides from receptor tyrosine kinases we observed that the amino acid sequence of the transmembrane domain appears to influence its orientation and behavior in fluid membranes (Jones et al., 1998; Sharpe et al., 2000). We also observed that features apparently reflecting side-to-side interaction of these transmembrane peptides can be identified in the same spectra (Morrow and Grant, 2000; Sharpe et al., 2002). As part of our investigation of the phenomena underlying spectral features with particular characteristics, we have designed a series of simpler peptides that incorporate key properties of the receptor transmembrane domains. We report here a study of these transmembrane peptides, whose spectra may be expected to reflect phenomena fundamental to the behavior of membrane proteins.

Peptide sequences were constructed from repeating subunits of leucine and alanine (LA). For purposes of NMR spectroscopy, side chains of selected alanine residues were deuterated (i.e., $-CD_3$). This probe location is attractive because the methyl group of alanine is fixed directly to the peptide backbone, thus avoiding complexities arising from incompletely characterized side chain arrangement or motion. Peptides were studied in fluid bilayers of 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), a common major phospholipid of cell membranes. Two primary peptides were examined: a 21-mer whose calculated helical hydro-

Submitted for publication 18 January 2002, received in final form 12 April 2002.

Address reprint requests to Chris W. M. Grant, Department of Biochemistry, University of Western Ontario, London N6A 5C1, Canada. Tel.: 519-661-3065; Fax: 519-661-3175; E-mail: cgrant@uwo.ca

0006-3495/02/07/345/14 $2.00

phobic length was just sufficient to cross the membrane and a 26-mer with hydrophobic length extended five residues beyond this. Transmembrane peptides based upon the LA repeating unit have proven valuable for studies of transmembrane domain effects on membrane lipids (Killian et al., 1996; de Planque et al., 1998; Subczynski et al., 1998; Ren et al., 1999). More recently, similar peptides have been used to investigate the effect of hydrophobic mismatch on peptide location within membranes (Harzer and Bechinger, 2000; de Planque et al., 2001).

MATERIALS AND METHODS

Sources

1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) was obtained from Avanti Polar Lipids (Birmingham, AL), and was used without further purification. 9-Fluorenylmethyl *N*-succinimidylcarbonate (FMOC-OSu) was from Sigma (St. Louis, MO). Deuterium-depleted water and deuteromethyl L-alanine ([d_3]Ala) were from Cambridge Isotope Laboratories (Andover, MA). 2,2,2-Trifluoroethanol, NMR-grade, bp 77–80°C was from Aldrich (Milwaukee, MI). FMOC-blocked alanine was synthesized following standard procedures as described previously (Rigby et al., 1996). Product purity was checked by thin-layer chromatography (Merck, silica gel 60 plates) against an FMOC derivative standard. All peptides were synthesized by the Peptide Synthesis Laboratory at Queen's University (Kingston, ON) and confirmed using high performance liquid chromatography and electrospray mass spectrometry.

Polyacrylamide gel electrophoresis

Polyacrylamide Gel Electrophoresis was performed using a minigel system (Bio-Rad Laboratories, Hercules, CA). Peptides were run on 16.5% tricine gels as described by Schägger (1994), and subsequently stained with Coomassie Brilliant Blue using 3- to 43-kDa markers (Gibco Cleveland). Except where noted otherwise, samples for electrophoresis were pretreated by dissolution in FACT (a 1:1:2:1 by volume mixture of formic acid (90%)/acetic acid/chloroform/trifluoroethanol) for 1 h then dried under N_2 gas followed by vacuum dessication for 12 h to remove any remaining solvent. Samples were then dissolved in a standard loading buffer containing 3% sodium dodecyl sulfate (SDS) and 125 mM dithiothreitol and heated for 30 minutes at 42°C prior to loading onto the gel.

Preparation of samples for NMR spectroscopy

Except where otherwise noted liposome generation was according to the following protocol. The acidic organic solvent FACT (90% formic acid/acetic acid/chloroform/trifluoroethanol) (1:1:2:1 ratio by volume) was added to dry peptide and appropriate amounts of dry lipid, which were dissolved with warming to produce mixtures in which peptide represented from 0.25 to 6 mol% of phospholipid. Samples were allowed to sit for at least 30 min after visually-apparent complete dissolution. Solvent was then rapidly removed under reduced pressure at 55°C on a rotary evaporator to leave thin films in 50 mL round bottom flasks. These were subsequently vacuum desiccated for 18 h at 23°C under high vacuum with continuous evacuation. After vacuum desiccation, samples were hydrated with 30 mM HEPES, 20 mM NaCl, and 5 mM EDTA, pH 7.1 to 7.3, made up in deuterium depleted water and lyophilized for several hours. This hydration/lyophilization step was repeated twice with deuterium depleted water, and sample pH was adjusted to 6.5. At each hydration step, vortexing was avoided to minimize production of small vesicles. TFE (2,2,2-trifluoroethanol) was also tested as a solvent for preparation of solutions of lipid plus peptide and produced similar results.

Spectroscopy

^{2}H NMR spectra were acquired at 76.7 MHz on a Varian Unity 500 spectrometer using either 5- or 10-mm single-tuned Doty solenoid probes with temperature regulation to 0.1 C°. A quadrupolar echo sequence (Davis, 1991) was used with full phase cycling and $\pi/2$ pulse length of 5 to 6 μs (5-mm probe) or 10.3 to 10.7 μs (10-mm probe). Pulse spacing was typically 15 to 20 μs, and spectral width was 100 kHz. A recycle time of 100 ms was used: recycle times of up to 500 ms did not alter lineshape or relative intensities of the features seen. ^{2}H-NMR spectra and quadrupole echo decay times were also acquired at 61 MHz on a spectrometer assembled in house. For this system the $\pi/2$ pulse length was 2.5 to 3 μs (5-mm coil) or 6.5 to 7 μs (10-mm coil). For echo decay measurements, separation of the 90° pulses ranged from 35 μs to 300 μs. ^{31}P-spectra were obtained on a Chemagnetics spectrometer operating at 161.7 MHz. For this purpose the flame ionization detector FID following a single pulse was transformed (e.g., de Planque et al., 1998). A $\pi/4$ pulse of 5 μs was used to minimize saturation (with ^{1}H decoupling and a recycle time of 5 s). ^{31}P NMR spectral width was 50 kHz, and a line broadening of 50 Hz was applied. CD spectra were run on a Jasco J-810 spectropolarimeter in a 1-mm path length water jacketed cell with temperature regulation to ±0.1°C. Samples were produced in a similar fashion to NMR samples, but were hydrated in a 10 mM $NaPO_4$, 10 mM NaCl buffer, pH 7.15; and subsequently bath sonicated and centrifuged for 1 minute at 20,000 × *g* to eliminate large vesicles and reduce light scattering to acceptable levels.

RESULTS

Peptides studied in the present work were the following (N terminus to the left):

LA17-1_N
acetyl-KK-LALALALA**L**ALALALAL-KK-amide
LA17-1_C
acetyl-KK-LALALALAL**A**LALALAL-KK-amide
LA17-2
acetyl-KK-LALALALA**L**A**L**ALALAL-KK-amide
LA22-1
acetyl-KK-LALALALALAL**A**LALALALALA-KK-amide
LA22-2
acetyl-KK-LALALALALAL**A**L**A**LALALALA-KK-amide
LA22-7
acetyl-KK-LALAL**A**L**A**L**A**L**A**L**A**L**A**LALA-KK-amide

This series comprises 21-mers and 26-mers. All peptides were based on repeating LA subunits, with two (+)charged lysine residues at each end. N and C termini were blocked using acetyl and amide groups, respectively. Deuterated alanine residues are indicated by bold font. The first numeral in the identifying acronym refers to the number of hydrophobic amino acids in the membrane-spanning portion, whereas the second refers to the number of deuterated alanine residues present. In the case of the singly-labeled LA-17 species (LA17-1_N and LA17-1_C), the subscript letter in the identifying acronym indicates the location of the deuterated alanine (closer to the N or C terminus) relative to the central leucine. Peptides were designed such that the LA17 species should possess hydrophobic domains equal in length to the (POPC) membrane hydrophobic thickness;

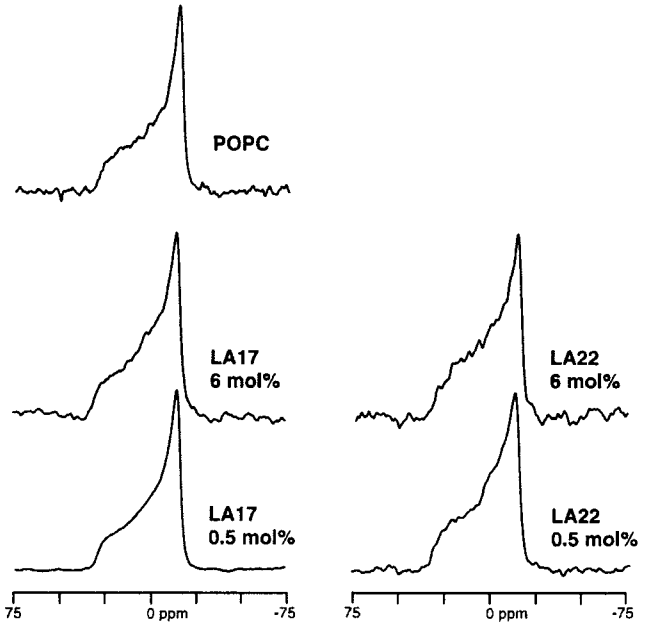

FIGURE 1 ^{31}P NMR spectra of POPC membranes containing LA17 or LA22 peptides. Spectra are shown for POPC alone and for POPC containing LA17 (*left column*) and LA22 (*right column*). Examples are included for POPC containing peptide at concentrations of 6 mol% and 0.5 mol% relative to phospholipid. All experiments were run at 30°C. Spectra represent 6000 to 10,000 accumulated transients and were processed using a line broadening of 50 Hz.

whereas the LA22 species possess hydrophobic lengths greater by five amino acids. Thus, the calculated hydrophobic length of the LA17 peptides is 25.5 Å, presuming classical right-handed α-helical geometry with 3.6 residues per turn and 1.5 Å per amino acid. This corresponds closely to the 25.8-Å hydrophobic thickness of fluid POPC (Nezil and Bloom, 1992). The predicted hydrophobic length of the LA22 peptides was 33.0 Å: i.e., 7.2 Å greater than the membrane hydrophobic thickness. In principle, "snorkelling" of the lysine side chains (Segrest et al., 1990; Killian and von Heijne, 2000) by up to 3 to 4 Å could permit a longer hydrophobic segment in each case; however the LA17 peptides more closely correspond to the membrane hydrophobic dimension and are certainly long enough to span it.

Typical ^{31}P NMR spectra of POPC vesicles prepared in the presence and absence of LA17 or LA22 peptides are shown in Fig. 1. All samples gave powder spectra with chemical shift anisotropy of 45 ppm, indicating fluid lamellar phase phospholipid (Seelig, 1978). As is commonly the case in similar reported spectra, a low intensity component at 0 ppm is present in all peptide-containing samples. This is likely due to the presence of small (rapidly reorienting) vesicles, and/or some fraction of lipid undergoing rapid, large amplitude reorientation, both of which give rise to an isotropic ^{31}P spectral component. Spectral simulation and integration demonstrate that this sharp component at 0 ppm corresponds to only a few % of the lipid in each sample. Because it is generally considered that there are some 12 to 18 lipids in direct contact with a given single-span protein transmembrane domain (Shen et al., 1997; Belohorcová et al., 1997), these spectra demonstrate that the ^{2}H NMR spectral observations described below do not correlate with changes in lipid phase. The ^{31}P spectra also display no obvious correlation with peptide length in the present experiments. A series of CD measurements (not shown here) was made on samples containing 0.5%, 3%, and 6% peptide relative to POPC. In all cases spectra displayed only features typical of peptides having α-helical secondary structure (minima at 208 and 222 nm) with strikingly little change between 30°C and 90°C.

Fig. 2 presents ^{2}H NMR spectra for LA17 peptides at a concentration of 6 mol% in bilayer membranes at 30°C and 60°C. All spectra correspond to samples held well above the −3°C gel-to-fluid phase transition temperature (Davis and Keough, 1985) of POPC bilayers. These spectra can be readily understood in the context that elongated amphiphiles tend to undergo rapid symmetric rotation about an axis perpendicular to the plane of fluid bilayer membranes. For a deuteron attached to a molecule undergoing fast axially symmetric reorientation, the observed doublet splitting, $\Delta\nu_Q$, measured between the prominent 90-degree edges near the spectral maxima, can be written as

$$\Delta\nu_Q = (3/8)(e^2Qq/h)\langle 3\cos^2\Theta_i - 1\rangle \qquad (1)$$

in which e^2Qq/h is the nuclear quadrupole coupling constant (165–170 kHz for a C-D bond) (Seelig, 1977; Davis, 1991) and Θ_i is the orientation of the C-D bond relative to the axis about which the molecule is rotating. The average is taken over all motions that modulate the orientation of the C-D bond with respect to the rotation axis. For molecules having deuterated methyl groups it is convenient to consider Θ_i to be the angle between the C-CD_3 vector and the molecular long axis, and to introduce an additional factor of 1/3 (the additional $|3\cos^2\Theta - 1|/2$ averaging of the quadrupole interaction introduced by methyl group rapid rotation about the C-CD_3 bond in which the C-C-D angle is $\Theta = 109°$). The average in Eq. 1 then accounts for any reduction in splitting due to "wobble" of the entire peptide within the membrane and to conformational fluctuations of the peptide backbone. Interference with peptide rotation, such as might arise from rapidly-reversible peptide-peptide interaction, can lead to spectral broadening and a shift of intensity toward the center, both of which tend to obscure the quadrupole splitting. In addition to the above-described Pake features, a narrow unsplit peak often occurs in the middle of such ^{2}H NMR spectra. It arises from residual deuterated water and from any vesicles with high curvature, for which the quadrupole splittings are motionally averaged to zero. Central spectral intensity can also arise from molecules

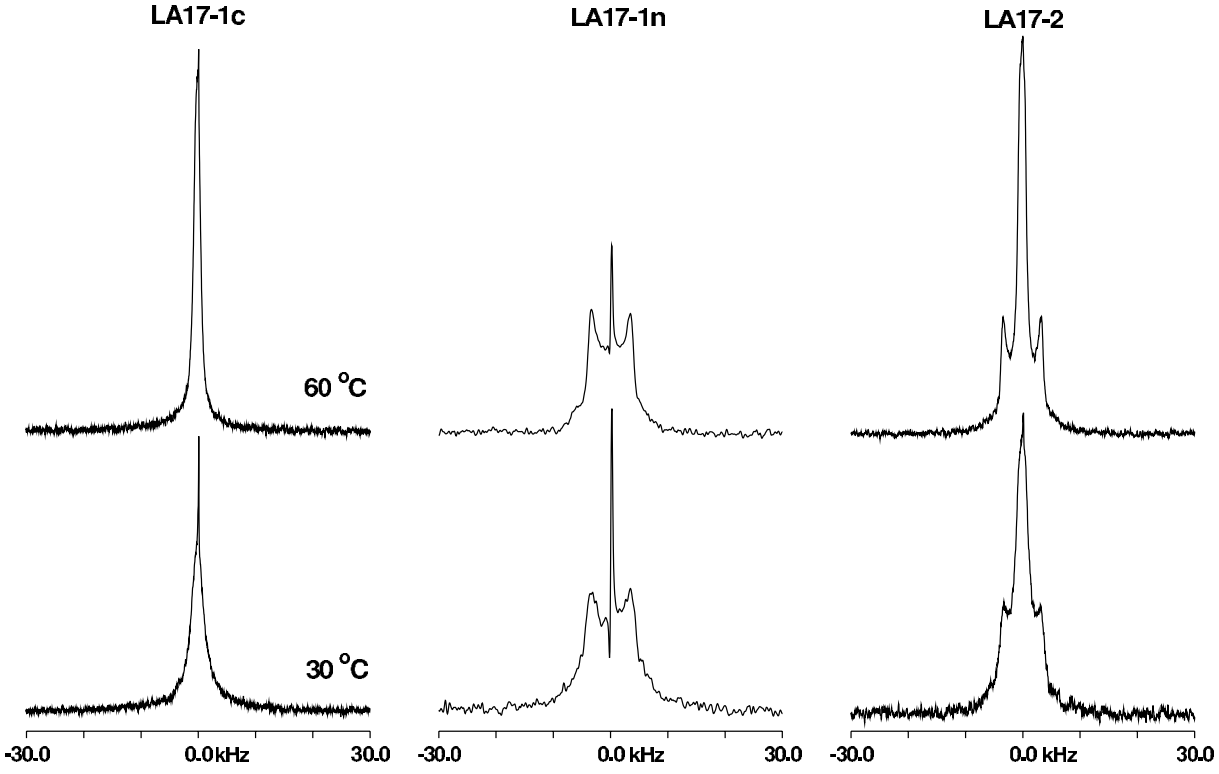

FIGURE 2 ^{2}H NMR spectra of LA17 peptides at 6 mol% in POPC bilayers. Spectra are displayed for LA17-1c (*left column*), LA17-1n (*center*), and LA17-2 (*right column*) in fluid, fully hydrated bilayers. Peptide concentration is quoted relative to phospholipid. LA17-1c and LA17-2 spectra represent 100,000 to 250,000 accumulated transients and were processed with a line broadening of 50 Hz: spectra of LA17-1n reflect 600,000 transients and were processed with a line broadening of 100 Hz.

undergoing asymmetric rotation in membranes (Opella, 1986).

The ^{2}H NMR spectrum of LA17-1_N in fluid membranes (Fig. 2) is essentially a Pake doublet of splitting 7 to 8 kHz. As described surrounding Eq. 1, because the quadrupole splitting is considerably less than 40 kHz, in this situation the peptide is undergoing rotation that is rapid relative to the NMR timescale of 10^{-5} s^{-1}. The Pake doublet width of considerably less than 40 kHz is also good evidence of peptide transmembrane insertion, as transmembrane insertion is by far the most likely basis for dominant rapid axial rotation. Peptide transmembrane insertion (and the peptide helicity measured above by CD) are in agreement with previous reports on $(LA)_n$ peptides having paired lysine residues at each end (e.g., Zhang et al., 1995; Harzer and Bechinger, 2000). Note that the sequence of the LA17 peptides is symmetric about the central leucine and that deuteration in LA17-1_N is on the alanine immediately downstream of this leucine. LA17-1_C is a chemically identical peptide but with deuteration in the side chain of the single alanine residue on the opposite (i.e., C-terminal) side of the central leucine. Interestingly, LA17-1_C gave a quantitatively very different spectrum: a Pake "doublet" of only ~1 kHz splitting, which is unresolved at 6 mol% peptide. The doublet nature of this feature could be appreciated at 30°C with the slightly better-resolved lineshape obtained at lower peptide concentration (described below surrounding Fig. 4). The very different quadrupole splittings seen for alanine residues on either side of the central leucine suggest that, with respect to the axis about which the molecule is reorienting, the time-averaged orientation of these residues differs by ≥6 to 8° and/or that their degree of motional freedom is very different. The latter possibility seems unlikely for two such close and symmetrically located centers. We have noted previously that molecular modeling, presuming standard right handed α-helical geometry with ψ and ϕ angles of -47 and $-57°$, respectively, indicates that the angle between the alanine side chain methyl axis and the helix symmetry axis should be between 56° and 59°. Eq. 1 predicts that a CD_3 group with such an orientation should give rise to a Pake doublet having $\Delta\nu_Q \sim 1.3$ kHz. Thus, it is also noteworthy that the spectral splitting associated with one of these alanine residues differs markedly from the value expected for fast rotation of a standard α-helix about the helix axis. Simultaneous deuteration of both of the above CD_3 groups to produce LA17-2 resulted in a spectrum, which was a simple sum of those arising from LA17-1_C and LA17-1_N (Fig. 2, right hand column).

Fig. 2 also demonstrates that reduction in temperature from 60°C to 30°C led to some broadening of ^{2}H NMR

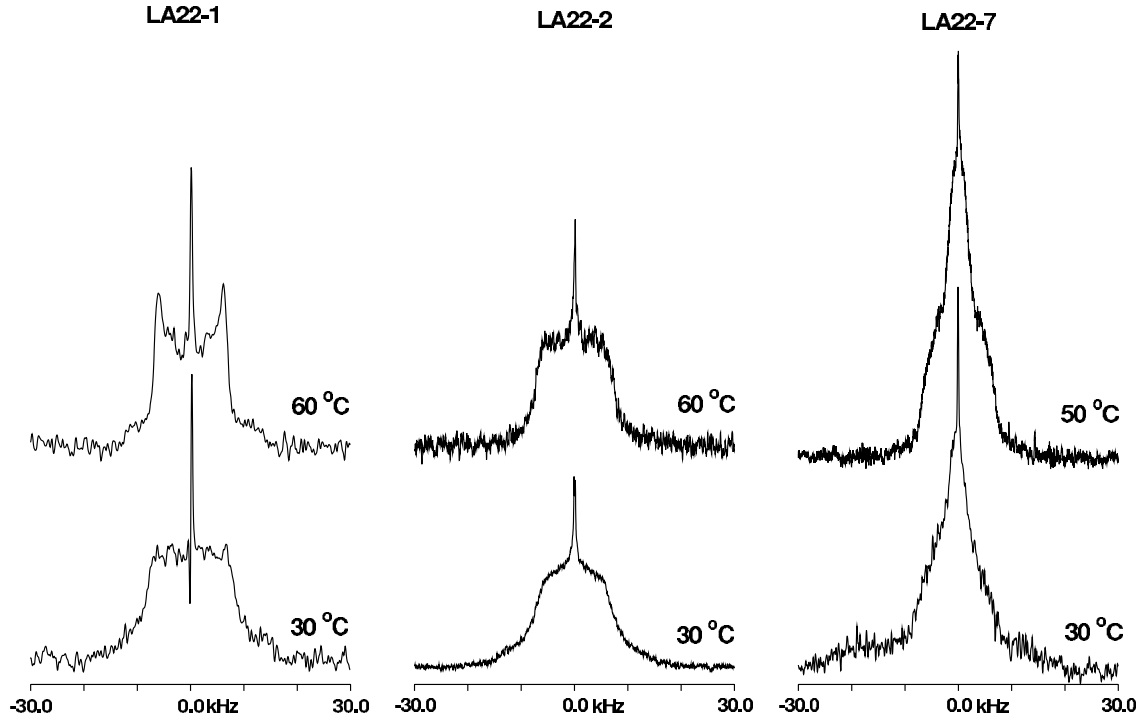

FIGURE 3 ^{2}H NMR spectra of LA22 peptides at 6 mol% in POPC bilayers. Spectra are displayed for LA22-1 (*left column*), LA22-2 (*center*), and LA22-7 (*right column*) in fluid, fully hydrated bilayers. LA22-2 spectra represent 100,000 to 250,000 accumulated transients: spectra of LA22-1 and LA22-7 reflect 200,000 to 400,000 and 600,000 transients, respectively. A line broadening of 100 Hz was applied in each case.

Pake doublet features associated with the 6% LA17 peptide samples. Because 30°C is well above the −3°C fluid/gel transition temperature of POPC, this broadening appears to reflect temperature dependence of peptide-peptide interactions that interfere with rapid rotational diffusion of the peptide about the bilayer normal. Such a view is supported by the relative absence, as described in association with Fig. 4, of temperature-dependent broadening of the same features at lower peptide concentration. The lack of additional spectral features in Fig. 2 (i.e., other than the Pake doublets described) indicates that the peptide is behaving as a single homogeneous population on the timescale involved.

The observations described above are reminiscent of findings in similar experiments on synthetic transmembrane peptides from class I receptor tyrosine kinases, a major class of single-span receptors. For instance in peptides having the natural transmembrane sequence of the EGF receptor and ErbB-2/Neu, each deuterated alanine was found to give rise to a narrowed Pake doublet of splitting ~3 to 10 kHz; and the splitting was dependent on amino acid location within the peptide (Morrow and Grant, 2000; Sharpe et al., 2000). However transmembrane domains of higher animal proteins typically comprise more than 20 hydrophobic amino acid residues and thus, unlike the LA17 model peptides, are considered to be significantly longer than the hydrophobic thickness of natural membranes. Hence in the present work, experiments were also performed on LA-subunit-based model peptides whose calculated hydrophobic lengths exceed the membrane hydrophobic thickness. Fig. 3 shows spectra obtained from such peptides at concentrations of 6 mol% in POPC.

Deuterated peptides giving rise to the ^{2}H NMR spectra shown in Fig. 3 have transmembrane domains estimated to be 7.2 Å longer than the hydrophobic thickness of POPC bilayers. The associated concept of hydrophobic mismatch has been extensively described (Mouritsen and Bloom, 1993; Gil et al., 1998; de Planque et al., 1998; Harzer and Bechinger, 2000). LA22-1, with a single $-CD_3$ group in the central LA subunit, produced a ^{2}H NMR Pake doublet with a splitting of ~14 kHz at 60°C. Cooling to 30°C resulted in a strikingly broadened spectrum of half-height width ~15 kHz. As noted above, such loss of axial symmetry under conditions for which the bilayer is highly fluid suggests the possibility that restriction of peptide rotational diffusion is arising from direct or lipid-mediated peptide-peptide interactions. This interpretation is reinforced by the observation, described surrounding Fig. 5 below, that the same peptide at lower concentration yields a single Pake doublet spectrum with a splitting of ~14 kHz at both 30°C and 60°C. Simultaneous introduction of a second $-CD_3$ group (to produce LA22-2) resulted in ^{2}H NMR spectra, at 6 mol% peptide, which were broadened to an extent that the coexisting spectral components could not be reliably separated. This spectral broadening observed in the case of LA22 pep-

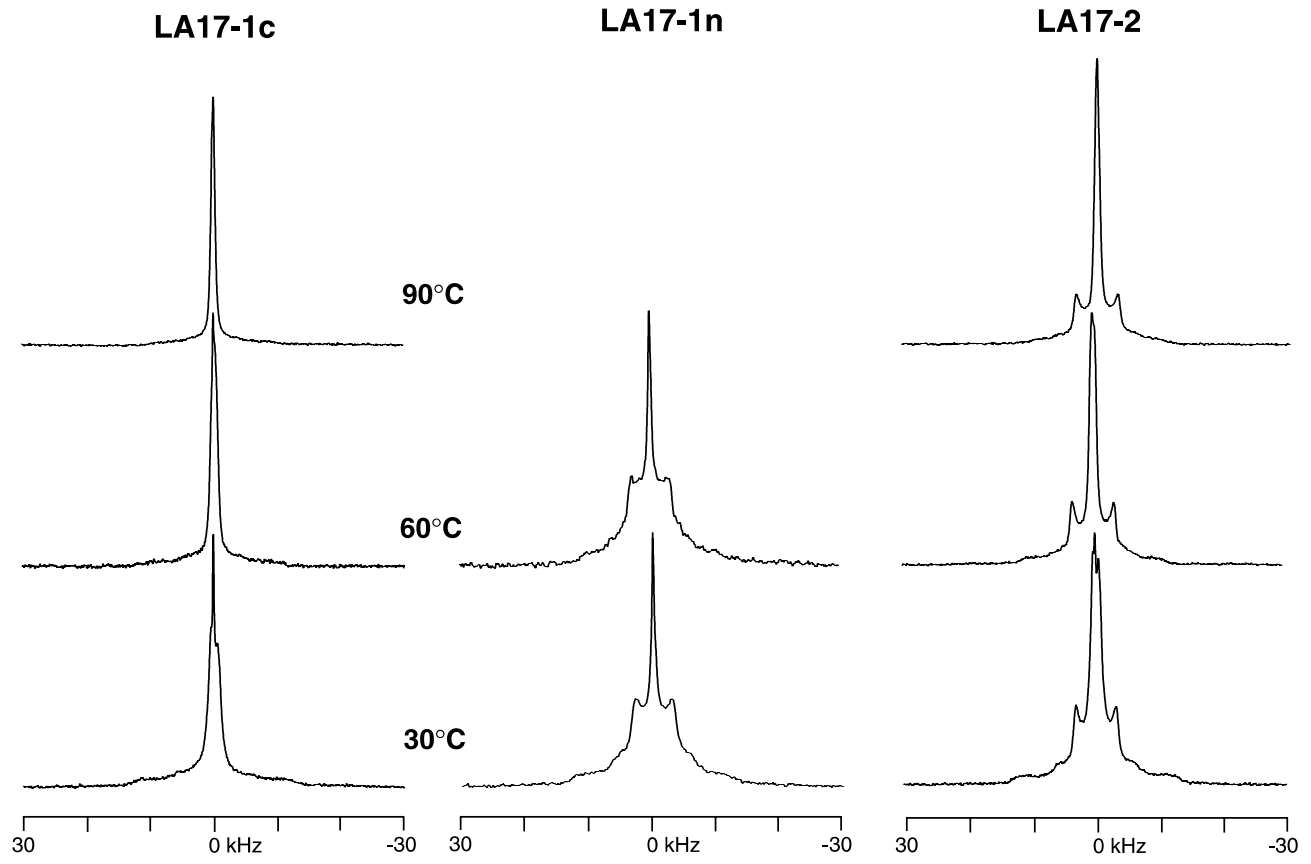

FIGURE 4 ^{2}H NMR spectra of LA17 peptides at 0.5 mol% in POPC bilayers. Spectra are displayed for LA17-1c (*left column*), LA17-1n (*center*), and LA17-2 (*right column*) in fluid, fully hydrated bilayers. Peptide concentration is quoted relative to phospholipid. Typically 500,000 transients were accumulated and spectra were processed with a line broadening of 50 Hz.

tides was much greater than that seen for LA17 peptides, to the extent that even the very large splittings observed for the longer LA22 peptides were not resolved without reducing peptide concentration. This may reflect a greater tendency for the longer peptides to undergo (rapidly-reversible) association.

The peptide, LA22-7, is the same as LA22-1 and LA22-2, but with seven deuterated alanine residues which span the center-most 10.5-Å region of the 25.8-Å membrane hydrophobic interior. Spectra of this species at 6 mol% were also highly broadened and included a feature of lower intensity and smaller splitting. As described below for this peptide at lower concentration, these spectra appear to consist of a number of superimposed doublets with splittings of 12 to 14 kHz and a smaller group of doublets with splittings of less than 5 kHz.

Typical spectra of LA17 and LA22 model peptides dispersed at lower concentration in POPC bilayers are presented in Figs. 4 and 5, respectively. LA22 peptides in particular, produced spectra that were significantly less broadened and which thus gave more clearly defined doublet splittings. The Pake doublets described in Figs. 2 and 3 for samples containing 6 mol% peptide are otherwise preserved. LA17-1_N gives rise to a Pake doublet of splitting 7 kHz, and LA17-1_C gives rise to a poorly-resolved very narrow doublet of splitting $\sim$1 kHz. In LA17-2 these are superimposed. A broad spectral feature of width 20 to 25 kHz is also apparent in LA17 spectra, particularly at lower temperature. The subpopulation of transmembrane peptides giving rise to this new feature are clearly also undergoing rotational diffusion and motional narrowing, as the 90° edges are substantially less than 40 kHz apart. However, the broad and poorly defined shape suggests that this feature arises from molecules for which rotation is significantly restricted and/or asymmetric. A possible source of this motional perturbation could be interference with rotational motion due to partial aggregation of peptide as dimers and/or oligomers. The deviation from Pake spectral shape could thus reflect an increased rotational correlation time for oligomeric species, the coexistence of a mixture of oligomeric species having somewhat different structures, and/or peptides within a given oligomer having orientational differences that persist over the characteristic timescale ($\sim 10^{-5}$ s) of the experiment. The relative disappearance of this feature at 90°C (Fig. 4), with preservation of the features assigned to monomer, is consistent with such in-

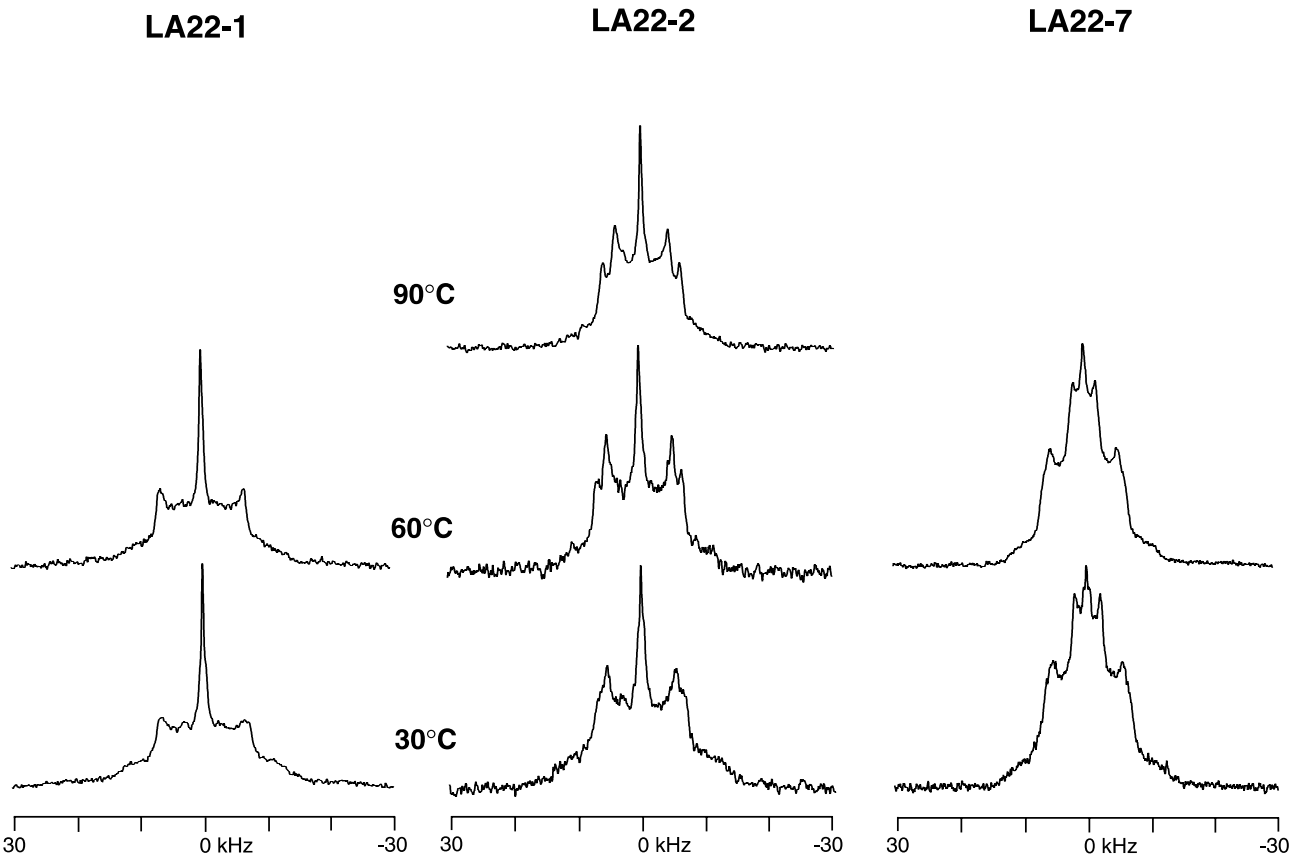

FIGURE 5 ^{2}H NMR spectra of LA22 peptides at 0.5 mol% in POPC bilayers. Spectra are displayed for LA22-1 (*left column*), LA22-2 (*center*), and LA22-7 (*right column*) in fluid, fully hydrated bilayers. 300,000 to 500,000 transients were accumulated, and a line broadening of 150 Hz was applied to all spectra.

terpretations. As discussed later, the apparently counterintuitive appearance at low peptide concentration of a separate spectral component attributed to peptide oligomers may reflect a sensitivity of oligomer formation or lifetime to peptide density in the bilayer.

LA22-1 at low concentration in the membrane gives rise to one doublet with a splitting of ~14 kHz (Fig. 5). Deuteration of a second alanine methyl to produce LA22-2 results in superposition of an additional doublet with a splitting of ~11 kHz. LA22-7, in which the methyl groups of the seven central alanines are deuterated, gives rise to overlapping doublets grouped around 4 to 5 kHz and 11 to 14 kHz. In LA22-7 there is also a small feature with a width of ~1.5 kHz. In the case of the longer peptides, reducing the concentration from 6% peptide to 0.5% has not so obviously caused the appearance of new spectral features identifiable with a long-lived oligomeric state. However, given the larger spectral splittings of the long peptides, a 20 to 25 kHz splitting could be "hidden" under the zero degree edges.

As part of this study, the peptides involved were examined for their behavior in SDS detergent micelles by SDS polyacrylamide gel electrophoresis chromatography. Typical results are illustrated in Fig. 6. The approach takes advantage of the fact that SDS detergent micelles have proven useful membrane models in past studies of protein transmembrane domain self-association (Li et al., 1994; Lemmon et al., 1994; Jones et al., 2000; Sharpe et al., 2000). In each case the LA-based peptides behaved as homogeneous populations on the many-minute timescale characterizing SDS chromatography (i.e., each peptide ran as a single band). It can be difficult to gauge the molecular weight of hydrophobic peptides under such circumstances as they retain their helicity in SDS, however, they tended to behave as higher molecular weight species than predicted by comparison with commercial standards and with the synthetic transmembrane domain of ErbB-1 (e.g., Sharpe et al., 2000). In addition, the LA peptides ran as diffuse bands, further complicating accurate measurement. Such a result is consistent with behavior reported previously by us and others for peptides in rapid monomer-dimer equilibrium (Li et al., 1994; Jones et al., 2000).

The time-dependent decay of the ^{2}H nucleus quadrupolar echo with increasing pulse separation is sensitive to motions that modulate the orientation-dependent quadrupole interaction on the characteristic time scale (10^{-6}–10^{-4} s) of the echo experiment and which thus interfere with refocusing of

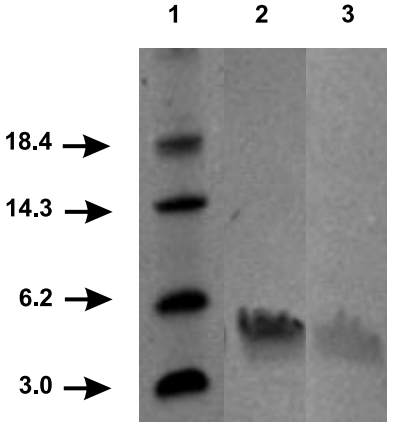

FIGURE 6 Behavior of LA peptides on SDS polyacrylamide gels. Coomassie blue-stained 16.5% Tris-tricine SDS-polyacrylamide gel having the following numbered lanes: (*1*) prestained molecular mass markers (sizes in kDa listed to the left of panel); (*2*) LA22, molecular mass 2602 Da; (*3*) LA17, molecular mass 2165 Da.

the echo (Bloom et al., 1991; Davis, 1991). In the present ^{2}H NMR experiments, quadrupole echoes were formed by applying two $\pi/2$ pulses, shifted in phase by 90°, and separated by an interval, τ. For short τ, the amplitude of echoes formed at time 2τ following the initial pulse decays exponentially with an echo decay rate T_{2e}^{-1}, which reflects an average over all deuterons in a sample. Quadrupole echo amplitudes for the peptides LA17-1$_N$ and LA22-1 were measured using a series of $\pi/2$ pulse separations at selected peptide concentrations and temperatures. Fig. 7*A* to *D* show echo decays at 25°C, 35°C, 45°C, and 55°C for LA17-1$_N$ and LA22-1 at 6 mol% and 0.5 mol% in POPC bilayers. The decays are shown as semilog plots of echo amplitude, normalized to the amplitude of the first echo in each series, versus echo formation time, 2τ. Although the number of pulse separations sampled was necessarily limited by the small signal available from these samples, the observed behavior does approximate exponential decay. The echo decay rates obtained from fitting these decays depend slightly on temperature for the 6 mol% peptide samples and are almost independent of temperature for 0.5 mol% peptide samples.

For a deuteron attached to a rigid molecule undergoing rapid axially symmetric reorientation about a fixed axis, this motion will determine both the observed splitting of the Pake doublet and the reduction, ΔM_2, in the spectral second moment relative to what would be obtained in the absence of motion. The observed spectral splitting and the echo decay rate will thus both depend on $P_2(\cos\beta) = (3\cos^2\beta - 1)/2$ in which β is the angle between the rotation axis and the principal axis of the electric field gradient tensor. Pauls et al. (1985) show how this can be used to obtain the correlation time for reorientation, $\tau_c = (\Delta M_2 \times T_{2e})^{-1}$, from the observed splitting and the echo decay rate, in the short correlation time limit (Abragam, 1961) in which

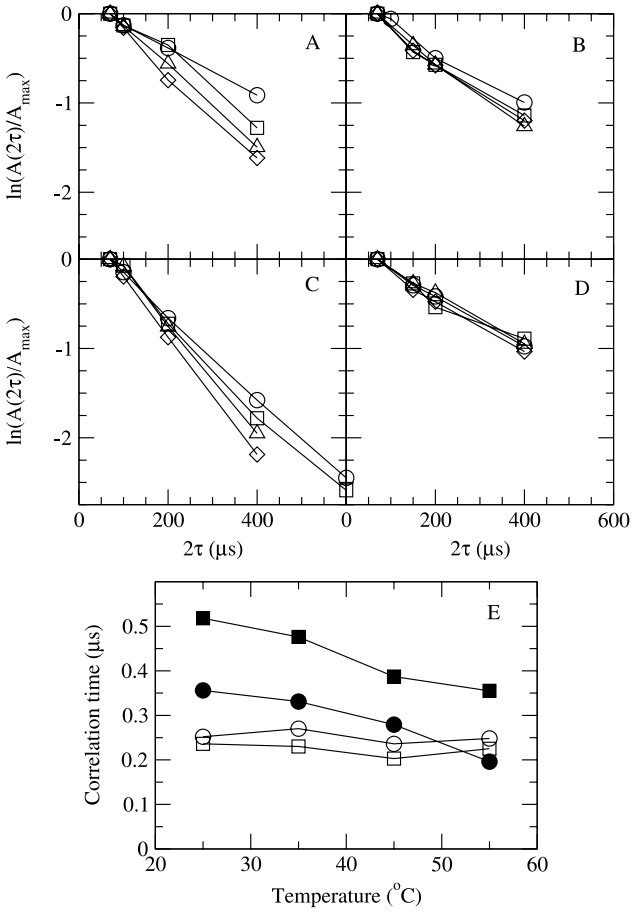

FIGURE 7 Quadrupole echo decays and correlation times (τ_c) for LA peptides. Semilog plots of echo amplitude versus echo formation time 2τ are plotted for LA17-1$_N$ peptides at 6 mol% (*A*) and 0.5 mol% (*B*) and for LA22-1 peptides at 6 mol% (*C*) and 0.5 mol% (*D*) in POPC. In each case, the echo decays are plotted for 25°C (◇), 35°C (△), 45°C (□), and 55°C (○). *E* shows the corresponding correlation times for molecular reorientation (τ_c) for LA17-1$_N$ at 6 mol% (●) and 0.5 mol% (○) in POPC and for LA22-1 at 6 mol% (■) and 0.5 mol% (□) in POPC.

$\Delta M_2 \times \tau_c^2 \ll 1$. Using the analysis introduced by Pauls et al., with a modification to account for additional averaging by fast rotation about the methyl axis as described by Morrow and Grant (2000), correlation times were extracted from echo decay rates. These are plotted, in Fig. 7*E*, as a function of temperature for LA17-1$_N$ and LA22-1 at 6 mol% and 0.5 mol% in POPC. At low concentration, the correlation times display no significant temperature dependence and almost no dependence on peptide length. At the higher concentration, correlation times are higher for the longer peptide and increase with decreasing temperature. The observed correlation times, spanning approximately 0.2 to 0.5 μs, are comparable with but longer than the correlation times (~0.2 μs) reported for the transmembrane segment of epidermal growth factor receptor at 10 mol% and 55°C in POPC/cholesterol (Morrow and Grant, 2000) and the synthetic polypeptide Lys$_2$-Gly-Leu$_{24}$-Lys$_2$-Ala-amide in liquid crystalline DPPC bilayers (Pauls et al., 1985).

DISCUSSION

Although it is clear that extramembranous portions of membrane proteins must play a major role in their behavior and interactions, it is also widely considered that the transmembrane portions contribute importantly to the forces determining dynamic, orientational, and associative characteristics of receptors in higher animal cells (White and Wimley, 1999; Ubarretxena-Belandia and Engelman, 2001). Here we describe the use of nonperturbing deuterium probes located within transmembrane peptides to study the behavioral features of such domains. Of particular interest is the insight offered with regard to fluid fully hydrated membranes at physiological temperatures. Transmembrane hydrophobic peptide helices are thought by many to be very stable and relatively rigid due to the large energy cost of breaking $i - i + 4$ backbone H bonds in a membrane hydrophobic interior (for review, see Lemmon et al., 1997; White and Wimley, 1999). Based upon such logic, it is widely considered that hydrophobic peptides too long for the membrane hydrophobic interior may tilt to avoid unfavorable polar interactions (Shen et al., 1997; Harzer and Bechinger, 2000; concepts reviewed in Killian, 1998). However modeling studies (Brandt-Rauf et al., 1995; Sajot et al., 1999) and molecular dynamics calculations (Belohorcová et al., 1997) have predicted the possibility of significant bends in the helix long axis. It has further been suggested that long-lived bends or kinks could contribute to the affinity of transmembrane domain side-to-side association (Brandt-Rauf et al., 1995; Sajot et al., 1999; Ubarretxena-Belandia and Engelman, 2001).

Pauls et al. (1985) used ^{2}H NMR to study a relatively long synthetic poly-leucine-based transmembrane peptide (Lys_2-Gly-Leu_{24}-Lys_2-Ala-amide deuterated on the exchangeable amide protons) in fluid bilayers of dipalmitoyl phosphatidylcholine. They found that in such a setting this single-span peptide underwent rapid rotation with a correlation time of 2×10^{-7} s, based upon (narrowed) spectral splittings and measurements of T_{2e}. Several groups have performed related NMR experiments on fluid bilayers containing deuterated gramicidin in transmembrane arrangement (i.e., as an end-to-end dimer that represents a "single-span" helical structure). Such experiments have been interpreted in terms of rapid rotational diffusion of the transmembrane species about a bilayer perpendicular (McDonald and Seelig, 1988; Prosser et al., 1994). Cross and colleagues studied gramicidin deuterated on methyl groups in a fluid DMPC matrix (Lee et al., 1993), and Grant and colleagues have examined several ErbB-related peptides with deuterated alanine residues: these CD_3-containing single-span species gave rise to spectra consistent with the motionally-narrowed Pake features recorded in the present work (Jones et al., 1998b, 2000; Sharpe et al., 2000). Killian and colleagues (de Planque et al., 2001) have used different techniques to study a series of LA-based transmembrane peptides closely related to those studied here. These authors have compellingly argued that peptides based on repeating LA subunits ("WALP" peptides) can influence membrane structure, particularly when too short to span the bilayer. As noted surrounding Fig. 1, however, ^{31}P NMR indicates that the great majority of POPC within the samples studied in the present work remains in bilayer phase with little or no cubic or hexagonal phase. This is in keeping with the fact that the LA17 and LA22 peptides are long enough to span the membrane. Killian and colleagues also conclude that at 3.3 mol% peptide (and at higher concentrations) such peptides behave as monomeric species in fluid bilayers, based on the absence of an immobilized (trapped) lipid fraction (de Planque et al., 1998). In a related vein, Subczynski et al. (1998) examined a leucine-based transmembrane peptide (Ac-$K_2L_{24}K_2$-amide) at 2.5 to 9 mol% in fluid bilayers by optical and electron paramagnetic resonance EPR spectroscopy, concluding that it did not form oligomers that were long lived on a timescale of microseconds. Such conditions approach those under which spectra reflecting apparently monomeric peptide were dominant in the present experiments.

The Pake features observed in the present experiments are consistent with the rapid rotational diffusion noted above for a number of transmembrane peptides, including those from single-span signaling proteins of higher animal cells. However, the highly controled nature of the structures studied here permits some insights not previously available. First, there are substantial variations in spectral splittings amongst alanine residues within a given peptide. This was evident in the LA17 peptide, in which (deuterated) alanine residues immediately up- and down-stream of the central leucine gave rise to splittings of ~7 to 8 and ~1.5 kHz, respectively. Yet these deuterated amino acids had the same amino-acid-sequence-neighbors for several helix loops in either direction. Given that the (deuterated) alanine residues involved are symmetrically situated with respect to the peptide midpoint, one would expect them to experience very similar environments. Moreover, for the case of rapid rotation about the axis of a uniform α-helix, the two alanines near the peptide midpoint would be expected to have very similar average C-CD_3 group orientations relative to the axis of rotation and hence nearly identical splittings. Rapid (and symmetric) peptide rotation is clearly occurring, as the splitting of the 90° spectral edges is significantly smaller than the 40-kHz value that characterizes immobilized peptides. To account for the difference between the observed splittings for the two labeled alanines in LA17, the orientations of their methyl symmetry axes with respect to the local rotation axis must differ by $\geq$6 to 8°. An apparently related phenomenon was observed in the LA22 peptide. Thus, for instance, LA22-2 displayed splittings of ~10 and 14 kHz for probes separated by a single residue near the membrane center. The quadrupolar coupling differences observed between neighboring alanine residues near the

bilayer center are probably also too large to be accounted for by variations in peptide local flexibility; although peptide "unravelling" near the ends could account for such an effect for probes nearer the bilayer surface. LA22-7 displayed an apparently wide range of splittings, which is unlikely to be completely accounted for by presuming a flexibility gradient within the rather abbreviated region involved. It follows that there must be some other basis for a different time-averaged spatial orientation of these groups. There seem to be at least two possible sources of this inequivalence: 1) the peptides are not rotating about their long axis but are rotating about some axis that is not coincident with the peptide long axis, 2) if the peptides are rotating rapidly about their long axis they must be fairly sharply bent or deformed. The observation that the splittings for a given peptide display little or no dependence on temperature or peptide concentration suggests that peptide-peptide interaction is not the primary basis for whichever of these behaviors is responsible for the orientational inequivalence. The possibility of reorientation about the bilayer normal, with the helix axis inclined by an angle of ~10 to 14° magnitude, has been indicated for other transmembrane polypeptides (Koeppe II et al., 1994; Prosser et al., 1994; Marassi et al., 1997; Byström et al., 2000).

Issues related to differences in ^{2}H quadrupole splittings for deuterated alanine at different locations within transmembrane peptides have been addressed by us in the past for native transmembrane sequences of receptor tyrosine kinases (Jones et al., 1998; Morrow and Grant, 2000; Sharpe et al., 2000). It has been suggested, based on analysis of analogous ^{2}H NMR data from the EGF receptor and assuming standard α-helical conformation, that the rotational axis of the transmembrane domain of the EGF receptor may not coincide with the molecular symmetry axis (Jones et al., 1998). Jones et al. demonstrated that the splittings could be understood using a model that assumed standard α-helical geometry with fast peptide rotation about an axis tilted 10° to 14° from the helix axis with effectively no rotation about the helix axis itself. They argued that, if fast rotation about the helix axis is allowed, the observed splittings imply substantial local departures from "standard" α-helical geometry.

Although the detailed relationship between molecular rotation axis and the helix axis may be controversial, the concept seems relevant to the present experiments. The LA17 peptides contain 21 amino acids, of which the central 17 form a hydrophobic stretch of repeating LA units beginning and ending with a leucine residue. The calculated total length of these peptides, assuming them to be ideal α-helices of 3.5 residues per turn and 1.5-Å rise per residue is thus 31.5 Å; and the calculated length of the peptide hydrophobic domain is 25.5 Å. The hydrophobic thickness of fluid POPC in bilayer form is 25.8 Å. On this basis the LA17 peptides are predicted to be the correct length to span the hydrophobic region of the membrane with peptide long axis perpendicular to the plane of the membrane. The LA22 peptides have a similarly calculated length of 33.0 Å. Note however that there is a 15-Å-thick "interfacial" region (White and Wimley, 1999) on each side of the hydrophobic region of the bilayer: the two lysine residues and the blocked N and C terminii should extend beyond the hydrophobic region and into (but not across) the interfacial zones. Note too that lysine residues near the boundary between the hydrophobic region and interface may under some circumstances remain within the former with only the polar group "snorkelling" some 3 to 4 Å toward the interfacial region (Segrest et al., 1990; White and Wimley, 1999; Killian and von Heijne, 2000). Such a phenomenon could effectively lengthen a peptide hydrophobic domain but does not alter the fact that the LA17 peptides more closely mimic the membrane hydrophobic thickness.

Based upon the present experiments and our previous work with transmembrane peptides from receptor tyrosine kinases, inequivalence of alanine methyl deuterons within the transmembrane domains appears to be a general phenomenon. As noted above, there are several situations that might give rise to such observations. If the peptide is straight and uniform, the observed inequivalence would imply that rotation about the helix axis is slow, and that fast rotation occurs about an axis (presumably the bilayer normal) from which the helix axis is tilted by a finite angle. If this is the case, the observation of distinct, inequivalent alanine deuteron splittings on a given peptide suggests that the apparent absence of fast rotation about the helix axis is due to the peptide assuming a preferred orientation in the bilayer rather than being kinetically trapped in an arbitrary orientation by interactions between the peptide sidechains and surrounding lipids. Alternatively, the inequivalence could arise from rotation of a bent polypeptide. If the latter is the case, the observation of unique splittings for most labels would imply that all peptides experienced the same average bend over the experimental characteristic time (~10^{-5} s). A third possibility is that the peptide might be rotating about the long axis of a helix, which contains persistent local departures from an average α-helical geometry, although this may be more difficult to rationalize for the potentially symmetrical alanine residues of LA17-2. We have noted above that, based on standard length calculations, one would not anticipate the tilt or bending implied by the observed inequality of alanine deuteron splittings for LA17 peptides in fluid POPC; however such calculations are recognized approximations.

The geometry of the α-helix is defined in terms of the dihedral angles ψ and ϕ, which are in the neighbourhood of $\psi = -47°$ $\phi = -57°$ for a right-handed helix with 3.6 residues per turn. For this structure, the angle between the alanine methyl symmetry axis and the helix axis is ~56° and thus very close to the magic angle: orientation of the CD_3 group at this angle relative to the axis of rotation would produce spectral splittings near zero. The distal ends of

helical transmembrane peptides tend to "unravel" (i.e., they are less conformationally stable than the central regions (e.g., Gullick et al., 1992; Zhang et al., 1995)). Belohorcová et al. (1997) have suggested, based on molecular dynamics studies, that the polypeptide helix may be a dynamic structure with the capacity to bend and to accommodate perturbations of the dihedral angles. Shen et al. (1997) drew similar conclusions from molecular dynamics calculations on a 32-residue, transmembrane polyalanine in a DMPC bilayer with a hydrophobic thickness of 22.8 Å. Although they observed that the portions of the peptide in water were random coil and that there was some destabilization of the helix in the interfacial region, the central 12 residues (length 18 Å) were found to maintain a very stable helical geometry. There appears to be little evidence of departures, on average, from a uniform helix in the bilayer interior. Indeed, recent studies of ^{15}N-labeled proteins in oriented bilayers (Byström et al., 2000; Marassi and Opella, 2000; Wang et al., 2000) have indicated unique tilt and rotational orientation of transmembrane polypeptides and little evidence of nonuniformity along the helix. Accordingly, the most reasonable explanation for the alanine methyl deuteron inequivalence observed in this work would seem to be fast rotation of a tilted, uniquely oriented, uniform helix about the bilayer normal.

The seven label locations on LA22-7 provide some opportunity to test the extent to which such rotation of a uniform, uniquely oriented helix might account for the observed distribution of splittings. The alanine methyl deuteron splitting for a given helix tilt and orientation is given by Jones et al. (1998). At low peptide concentration, LA22-7 gives rise to distinct groups of splittings in the ranges 4 to 5 kHz and 11 to 14 kHz. Because of the manner in which the seven alanines are situated around the helical wheel, the observed range of splittings can be used to constrain the tilt angle. The orientation about the helix axis is constrained by knowledge of the splittings for LA22-1 and LA22-2. If the rotation angle about the helix axis is taken to be 0° when the alanine that is labeled in LA22-1 is farthest from the bilayer normal, the observed spectrum is most closely approximated by a tilt of 17° and a rotation of ~200°. At this orientation, three splittings are calculated to have a magnitude of ~14 kHz, two have a magnitude of ~12 kHz, and two have a magnitude of ~5 kHz. While the spectrum is only approximated, the fact that a uniform, tilted helix rotating about the bilayer normal can be oriented so as to give rise to a roughly bimodal distribution of splittings reinforces the plausibility of accounting for the observed splittings with simple rotation of a uniform helix.

The effect of peptide length on peptide orientation and peptide-peptide interaction, is another issue that arises in the present experiments. Thus, both the range of splittings seen for a given peptide and the extent to which the spectra were broadened at high peptide concentration were influenced by peptide length. It has been noted that assembling a transmembrane peptide of greater hydrophobic length than the bilayer hydrophobic thickness might be expected to lead to increased transmembrane domain tilt or conformational change of lipid or protein (for review, see Gil et al., 1998; Killian, 1998). The larger maximal splittings seen in the LA22 peptides are consistent with such a concept. Ren et al. (1999) recently examined a series of poly-leucine TM peptides by fluorescence and CD spectroscopy, concluding that the secondary structure of (helical) peptides was not sensitive to bilayer hydrophobic thickness. This would lead one to interpret differences between LA17 and LA22 in terms of phenomena other than peptide deformation. Ren et al. further suggest that their measurements are consistent with the possibility of peptide oligomerization; and point out that peptides that were "too long" for the membrane might consequently associate differently. Hellstern et al. (2001) have used chemical crosslinking to examine the phenomenon of reversible oligomer formation amongst TM peptides from sarcolipin: they note that there is a greater tendency to higher oligomer formation in phospholipid bilayers than in detergent micelles. Since this manuscript was submitted, a report has appeared of fluorescence energy transfer evidence for association between hydrophobic peptides of leucine and alanine in fluid POPC lipid bilayers (Yano et al., 2002). While such observations might suggest a possible mechanism for quenching of rotation about the helix axis, peptide-peptide interaction alone, as noted above, appears inconsistent with the observed temperature dependence and peptide-concentration dependence of the splittings.

Spectral evidence of peptide-peptide interaction at 6 mol% is perhaps not surprising. At this concentration, there are only 7 to 8 lipids per protein in each leaflet of the bilayer, and encounters between neighboring peptides must be frequent. The larger splittings seen for the longer peptide seem consistent with a concept discussed above, that peptides too long for the membrane may tend to tilt to accommodate their length. Hence, the greater concentration-induced broadening seen for LA22 may simply reflect a greater tendency for tilted helices rotating about the bilayer normal to transiently interfere with each other, either directly or through lipids surrounding the helices. Such an interpretation is supported by the observation that the correlation time for reorientation of the tilted peptide about the bilayer normal increases with increasing concentration and that this increase is more pronounced for the longer peptide.

A more puzzling aspect of the LA17 behavior is the appearance, at low peptide concentration, of a broadened feature ~25 kHz in width. The fact that this feature is narrowed below the ~40 kHz splitting expected for methyl groups attached to nonrotating molecules indicates that it arises from peptides, which are still reorienting. Whereas it may in fact represent a superposition of features with a range of splittings, the larger maximum splitting may reflect a greater angle, for at least some molecules, between the (local) helix axis and the peptide rotation axis, which pre-

sumably remains the bilayer normal. The broadening suggests that the reorientation is more restricted. Taken together, these observations are consistent with the existence of a fraction of the peptides associating and rotating as long-lived dimers and/or oligomers and this long-term association being less favorable at 6 mol% peptide. The likelihood that this feature reflects peptide association is supported by the observation, illustrated in Fig. 4, that its amplitude decreases substantially as temperature is increased to 90°C. If a similar feature is present in the spectra of the LA22 peptides, it would be somewhat obscured by the larger splittings of the monomer in this case.

One possible explanation of the observed concentration dependence of the 25 kHz feature seen at low mol% peptide could be that high peptide concentrations alter the bilayer properties and thus the extent to which peptide association is driven by energetically unfavorable lipid-peptide interactions. For instance Rinia et al. (2000) have demonstrated aggregation of transmembrane peptides of repeating LA subunits in pure highly ordered phospholipids below their phase transition, which is consistent with relative disfavor of lipid-peptide interactions versus lipid-lipid and peptide-peptide. In this scenario, the peptides can be considered as impurities that raise the bilayer free energy either by elastic distortion or by disrupting favorable lipid-lipid interactions. The free energy of the bilayer might then be reduced by associating some of the peptides and reducing the number of lipids directly influenced. High peptide concentrations might reduce the energetic advantage of peptide association, for example by decreasing membrane order, or by altering the bilayer thickness or the dielectric environment. The thermodynamics of possible association of transmembrane peptides has been considered extensively by workers over the years. Contributions from lipid-peptide interactions and from peptide-peptide interactions have both been predicted to be important (Lemmon et al., 1997; Gil et al., 1998; White and Wimley, 1999; Morrow and Grant, 2000). Huschilt et al. (1985) observed that for polyleucines of 16 and 24 leucine residues (the former just long enough to cross the bilayer), there was only a modest difference in lipid ordering by the longer peptide. White and Wimley (1999) touch on this concept, pointing out as have many others that there is likely to be a monolayer of lipids in close contact with a given monomeric TM peptide at any one time, the lipids being "soft" and the helices "hard". The acyl chains of lipids in the immediate vicinity are generally considered to be strongly perturbed. Nevertheless, in the present samples ^{31}P NMR demonstrated that bilayer nature was preserved.

An alternate possibility could be that peptides are sterically hindered from associating or "organizing" at high peptide concentration. For instance, under appropriate conditions the peptide surfaces may favor a loose correlation as multimers with a nonzero peptide-peptide crossing angle, such as the 40 to 50° suggested by studies of soluble helical bundles (Chothia et al., 1981). Such an oblique crossing might permit closer approach than a lengthy parallel interaction, without reducing the projection of the peptide on the bilayer normal by more than ~6% (less than 2 Å). Whether or not peptides associate in this way would depend on how the energetic cost of distorting the bilayer interface to accommodate this tilt compares with the energy released by association. The peptide crowding at 6 mol%, with only 7 to 8 lipids per peptide in a given leaflet, may preclude accommodation of the very different orientations necessary to permit associations characterized by a large crossing angle.

These experiments suggest the possibility of both long-lived and short-lived peptide-peptide association amongst single-span transmembrane peptides. This is an important concept as it is a key underpinning of major models of signaling in higher animals. It is also a major concept in structure and function of multispan membrane proteins (White and Wimley, 1999). Although there is considerable interest in the possibility of amino acid motifs that may predispose to side-to-side association of peptides, as discussed below, the potential importance of less specific interactions has also been widely noted. Thus, Lemmon et al. (1997) discuss viable bases for promiscuity of single-span transmembrane domains. Orzáez et al., (2000) suggest "a global contribution of the TM fragment, and probably the flanking region, in the dimer formation process more than a very specific and local interaction." White and Wimley (1999) have recently reviewed the thermodynamics of transmembrane peptide associations in bilayer membranes, favoring the conclusion that nonspecific interactions are not likely to be of sufficient affinity to cause long-term peptide-peptide association. However, the latter workers hold out the probability that, where tight knobs-into-holes fit can occur (without leaving unfilled defects in the lipid matrix) significant association is likely. Related logic underlies considerable current interest in the possibility of "motifs" (amino acid sequences) that might encourage side-to-side association (Sternberg and Gullick, 1990; Lemmon et al., 1994; Gurezka et al., 1999; Javadpour et al., 1999; Choma et al., 2000). It has been suggested that one aspect of transmembrane domain association may involve close contact made possible by the existence of amino acids including alanine and glycine (Sternberg and Gullick, 1990; Deber et al., 1993; Javadpour et al., 1999; White and Wimley, 1999). If precise fit is an important aspect of peptide-peptide association, factors that modulate orientation of the transmembrane segments involved could then be expected to modulate this association.

CONCLUSIONS

^{2}H NMR of synthetic peptides in fluid bilayer membranes permitted consideration of the possible role of transmembrane domains in membrane protein behavior. The approach allowed direct measurement of mismatch effects on peptide spatial arrangement and motional characteristics. Lengthen-

ing the hydrophobic domain beyond the membrane hydrophobic thickness appeared to result in increased peptide tilt and alterations in peptide-peptide interaction. However, it was clear that the behavior of even simple transmembrane peptides is not trivial: there were restrictions upon peptide free rotation, and/or significant conformational deviation from classic helical parameters. The results reaffirmed expectations of transmembrane peptide rapid rotational diffusion as monomers at high peptide concentration in the membrane. The present work also suggests that dimer/oligomer stability may be influenced by lipid-mediated effects and that there can be inherent differences in the timescale and/or stability of such interactions at different peptide-to-lipid ratios.

This research was supported by a grant from the Medical Research Council of Canada to C.W.M.G. and from the Natural Science and Engineering Research Council of Canada to M.R.M. NMR spectroscopy was carried out in the Department of Physics and Physical Oceanography, Memorial University of Newfoundland and in the McLaughlin Macromolecular Structure Facility, established with joint grants to the department from the R. S. McLaughlin Foundation, the London Life Insurance Co., the MRC Development Program, and the Academic Development Fund of University of Western Ontario. S.S. is the holder of an NSERC PGSB scholarship.

REFERENCES

Abragam, A. 1961. Principles of Nuclear Magnetism. Clarendon Press, Oxford. 1–599.

Belohorcová, K., J. H. Davis, T. B. Woolf, and B. Roux. 1997. Structure and dynamics of an amphiphilic peptide in a lipid bilayer: a molecular dynamics study. *Biophys. J.* 73:3039–3055.

Bloom, M., E. Evans, and O. G. Mouritsen. 1991. Physical properties of the fluid lipid-bilayer component of cell membranes: a perspective. *Q. Rev. Biophys.* 24:293–397.

Brandt-Rauf, P. W., M. R. Pincus, and R. Monaco. 1995. Conformation of the transmembrane domain of the c-erbB-2 oncogene-encoded protein in its monomeric and dimeric state. *J. Protein Chem.* 14:33–40.

Byström, T., E. Strandberg, F. A. Kovacs, T. A. Cross, and G. Lindblom. 2000. Influence of transmembrane peptides on bilayers of phophatidylcholines with different acyl chain lengths studied by solid-state NMR. *Biochim. Biophys. Acta.* 1509:335–345.

Choma, C., H. Gratkowski, and W. F. DeGrado. 2000. Asp-mediated self-association of a model transmembrane helix. *Nat. Struct. Biol.* 7:161–166.

Chothia, C., M. Levitt, and D. Richardson. 1981. Helix to helix packing in proteins. *J. Mol. Biol.* 145:215–250.

Davis, J. H. 1991. ^{2}H NMR spectroscopy in partially ordered systems. *In* Isotopes in the Physical and Biomedical Science, Vol 2. E. Buncel and J. R. Jones, editors. Elsevier, Amsterdam. 99–157.

Davis, P. J., and K. M. W. Keough. 1985. Chain arrangements in the gel state and the transition temperatures of phosphatidylcholines. *Biophys. J.* 48:915–918.

Deber, C. M., A. R. Khan, Z. Li, C. Joensson, M. Glibowicka, and J. Wang. 1993. Val-Ala mutations selectively alter helix-helix packing in the TM segment of M13 coat protein. *Proc. Natl. Acad. Sci. U.S.A.* 90: 11648–11652.

de Planque, M. R. R., E. Goormaghtigh, D. V. Greathouse, R. E. Koeppe II, J. A. W. Kruijtzer, R. M. J. Liskamp, B. de Kruijff, and J. A. Killian. 2001. Sensitivity of single membrane spanning α-helical peptides to hydrophobic mismatch with a lipid bilayer: effects on backbone structure, orientation, and extent of membrane incorporation. *Biochemistry.* 40:5000–5010.

de Planque, M. R. R., D. V. Greathouse, R. E. Koeppe II, H. Schäfer, D. Marsh, and J. A. Killian. 1998. Influence of lipid/peptide hydrophobic mismatch on the thickness of diacylphosphatidylcholine bilayers: a ^{2}H NMR and ESR study using designed α-helical peptides and gramicidin A. *Biochemistry.* 37:9333–9345.

Gil, T., J. H. Ipsen, J. H. Mouritsen, M. C. Sabra, M. M. Sperotto, and M. J. Zuckermann. 1998. Theoretical analysis of protein organization in membranes. *Biochim. Biophys. Acta.* 1376:245–266.

Gullick, W. J., A. C. Bottomley, F. J. Lofts, D. G. Doak, D. Mulvey, R. Newman, M. J. Crumpton, M. J. E. Sternberg, and I. D. Campbell. 1992. Three dimensional structure of the transmembrane region of the proto-oncogenic and oncogenic forms of the *neu* protein. *EMBO J.* 11:43–48.

Gurezka, R., R. Lage, B. Brosig, and D. Langosch. 1999. A heptad motif of leucine residues found in membrane proteins can drive self-assembly of artificial transmembrane segments. *J. Biol. Chem.* 274:9265–9270.

Harzer, U., and B. Bechinger. 2000. Alignment of lysine-anchored membrane peptides under conditions of hydrophobic mismatch: a CD, ^{15}N and ^{31}P solid-state NMR spectroscopy investigation. *Biochemistry.* 39: 13106–13114.

Hellstern, S., S. Pegoraro, C. B. Karim, A. Lustig, D. D. Thomas, L. Moroder, and J. Engel. 2001. Sarcolipin, the shorter homologue of phospholamban, forms oligomeric structures in detergent micelles and in liposomes. *J. Biol. Chem.* 276:30845–30852.

Huschilt, J. C., R. S. Hodges and J. H. Davis. 1985. Phase equilibria in an amphiphilic peptide-phospholipid model membrane by ^{2}H NMR difference spectroscopy. *Biochemistry.* 24:1377–1386.

Javadpour, M. M., M. Eilers, M. Groesbeek, and S. O. Smith. 1999. Helix packing in polytopic membrane proteins: role of glycine in transmembrane helix packing. *Biophys. J.* 77:1609–1618.

Jones, D. H., K. R. Barber, and C. W. M. Grant. 1998a. Sequence-related behavior of transmembrane domains from class I receptor tyrosine kinases. *Biochim. Biophys. Acta.* 1371:199–212.

Jones, D. H., E. H. Ball, S. Sharpe, K. R. Barber, and C. W. M. Grant. 2000. Expression and assembly of a transmembrane region from neu. *Biochemistry.* 39:6572–6580.

Jones, D. H., K. R. Barber, E. W. VanDerLoo, and C. W. M. Grant. 1998b. The EGF receptor transmembrane domain: ^{2}H NMR implications for orientation and motion in a bilayer environment. *Biochemistry.* 37: 16780–16787.

Kavanaugh, W. M., and L. T. Williams. 1996. Signaling through receptor tyrosine kinases. *In* Signal Transduction. C.-H. Heldin and M. Purton, editors. Chapman & Hall, London. 3–18.

Killian, J. A., I. Salemink, M. R. R. de Planque, G. Lindblom, R. E. Koeppe II, and D. V. Greathouse. 1996. Induction of nonbilayer structures in diacylphosphatidylcholine model membranes by transmembrane α-helical peptides: importance of hydrophobic mismatch and proposed role of tryptophans. *Biochemistry.* 35:1037–1045.

Killian, J. A. 1998. Hydrophobic mismatch between proteins and lipids in membranes. *Biochim. Biophys. Acta.* 1376:401–416.

Killian, J. A., and G. von Heijne. 2000. How proteins adapt to a membrane-water interface. *Trends Biol. Sci.* 25:429–434.

Koeppe II, R. E., J. A. Killian, and D. V. Greathouse. 1994. Orientations of the tryptophan 9 and 11 side chains of the gramicidin channel based on ^{2}H NMR spectroscopy. *Biophys. J.* 66:14–24.

Lee, K.-C., W. Hu, and T. A. Cross. 1993. ^{2}H NMR determination of the global correlation time of the gramicidin channel in a lipid bilayer. *Biophys. J.* 65:1162–1167.

Lemmon, M. A., K. R. MacKenzie, I. T. Arkin, and D. M. Engelman. 1997. Transmembrane α-helix interactions in folding and oligomerization of integral membrane proteins. *In* Membrane Protein Assembly. G. von Heijne, editor. R. G. Landes Co. 3–23.

Lemmon, M. A., H. R. Treutlein, P. D. Adams, A. T. Brünger, and D. M. Engelman. 1994. A dimerization motif for transmembrane helices. *Nat. Struct. Biol.* 1:157–163.

Li, S.-C., C. M. Deber, and S. E. Shoelson. 1994. Glu-mediated dimerization of the transmembrane region of the oncogenic neu protein. *In* Peptides: Chemistry, Structure and Biology. R. S. Hodges and J. A. Smith, editors. ESCOM, Leiden.

MacDonald, P. M., and J. Seelig. 1988. Dynamic properties of gramicidin A in phospholipid membranes. *Biochemistry.* 27:2357–2364.

Marassi, F. M., and S. J. Opella. 2000. A solid-state NMR index of helical membrane protein structure and topology. *J. Mag. Res.* 144:150–155.

Marassi, F. M., A. Ramamoorthy, and S. J. Opella. 1997. *Proc. Natl. Acad. Sci. U.S.A.* 94:8551–8556.

Morrow, M. R., and C. W. M. Grant. 2000. The EGF receptor transmembrane domain: peptide-peptide interactions in fluid bilayer membranes. *Biophys. J.* 79:2024–2032.

Mouritsen, O. G., and M. Bloom. 1993. Models of lipid-protein interactions in membranes. *Annu. Rev. Biophys. Biomol. Struct.* 22:145–171.

Nezil, F. A., and M. Bloom. 1992. Combined influence of cholesterol and synthetic amphiphilic peptides upon bilayer thickness in model membranes. *Biophys. J.* 61:1176–1183.

Opella, S. J. 1986. Protein dynamics by solid-state NMR. *Methods Enzymol.* 131:327–361.

Orzáez, M., E. Pérez-Payá, and I. Mingarro. 2000. Influence of the C-terminus of the GpA transmembrane fragment on the dimerization process. *Protein Sci.* 9:1246–1253.

Pauls, K. P., A. L. MacKay, O. Soderman, M. Bloom, A. K. Tanjea, and R. S. Hodges. 1985. Dynamic properties of the backbone of an integral membrane polypeptide measured by ^{2}H-NMR. *Eur. Biophys. J.* 12:1–11.

Prosser, R. S., S. I. Daleman, and J. H. Davis. 1994. The structure of an integral membrane peptide: a ^{2}H NMR study of gramicidin. *Biophys. J.* 66:1415–1428.

Ren, J., S. Lew, J. Wang, and E. London. 1999. Control of the transmembrane orientation and interhelical interactions within membranes by hydrophobic helix length. *Biochemistry.* 38:5905–5912.

Rigby, A. C., K. R. Barber, G. S. Shaw, and C. W. M. Grant. 1996. Transmembrane region of the epidermal growth factor receptor: behavior and interactions via ^{2}H NMR. *Biochemistry.* 35:12591–12601.

Sajot, N., N. Garnier, and M. Genest. 1999. Dimer models for ErbB-2/neu transmembrane domains from molecular dynamics simulations. *Theor. Chem. Acc.* 101:67–72.

Seelig, J. 1977. Deuterium magnetic resonance: theory and applications to lipid membranes. *Q. Rev. Biophys.* 10:353–418.

Seelig, J. 1978. ^{31}P-NMR and the headgroup structure of phospholipids in membranes. *Biochim. Biophys. Acta.* 525:105–140.

Segrest, J. P., H. De Loof, J. G. Dohlman, C. G. Brouillette and G. M. Anantharamaiah. 1990. Amphipathic helix motif: classes and properties. *Proteins.* 8:103–117.

Schägger, H. 1994. A Practical Guide to Membrane Protein Purification. Chpt. 3. H. Schägger and G. Von Jagow, editors. Academic Press, San Diego, CA.

Sharpe, S., K. R. Barber, and C. W. M. Grant. 2000. $Val^{659} \rightarrow$ Glu mutation within the transmembrane domain of ErbB-2: effects measured by ^{2}H NMR in fluid phospholipid bilayers. *Biochemistry.* 39: 6572–6580.

Sharpe, S., K. R. Barber, and C. W. M. Grant. 2002. Evidence of a tendency to self-association of the transmembrane domain of ErbB-2 in fluid phospholipid bilayers. *Biochemistry.* 41:2341–2352.

Shen, L., D. Bassolino, and T. Stouch. 1997. Transmembrane helix structure, dynamics, and interactions: multi-nanosecond molecular dynamics simulations. *Biophys. J.* 73:3–20.

Siminovitch, D. J. 1998. Solid-state NMR studies of proteins: the view from static ^{2}H NMR experiments. *Biochem. Cell Biol.* 76:411–422.

Sternberg, M. J. E., and W. J. Gullick. 1990. A sequence motif in the transmembrane region of growth factor receptors with tyrosine kinase activity mediates dimerization. *Prot. Eng.* 3:245–248.

Subczynski, W. K., R. N. A. H. Lewis, R. N. McElhaney, R. S. Hodges, J. S. Hyde, and A. Kusumi. 1998. Molecular organization and dynamics of 1-palmitoyl-2-oleoylphosphatidylcholine bilayers containing a transmembrane α-helical peptide. *Biochemistry.* 37:3156–3164.

Ubarretxena-Belandia, I. and D. M. Engelman. 2001. Helical membrane proteins: diversity of functions in the context of simple architecture. *Curr. Opin. Struct. Biol.* 11:370–378.

Wang, J., J. Denny, C. Tian, S. Kim, Y. Mo, F. Kovacs, Z. Song, K. Nishimura, Z. Gan, R. Fu, J. R. Quine, and T. A. Cross. 2000. Imaging membrane protein helical wheels. *J. Mag. Res.* 144:162–167.

White, S. H., and W. C. Wimley. 1999. Membrane protein folding and stability: physical principles. *Ann. Rev. Biophys. Biomol. Struct.* 28: 319–365.

Yano, Y., T. Takemoto, S. Kobayashi, H. Yasui, H. Sakurai, W. Ohashi, M. Niwa, S. Futaki, Y. Sugiura, and K. Matsuzaki. 2002. Topological stability and self-association of a completely hydrophobic model transmembrane helix in lipid bilayers. *Biochemistry.* 41:3073–3080.

Zhang, Y-P., R. N. A. H. Lewis, R. S. Hodges, and R. N. McElhaney. 1995. Peptide models of helical hydrophobic transmembrane segments of membrane proteins: 2. DSC and FTIR spectroscopic studies of the interaction of Ac-K_2-$(LA)_{12}$-K_2-amide with phospatidylcholine bilayers. *Biochemistry.* 34:2362–2371.