The EGF Receptor Transmembrane Domain: Peptide-Peptide Interactions in Fluid Bilayer Membranes

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ABSTRACT A peptide containing the transmembrane domain of the human EGF receptor was studied in fluid lipid bilayers for insight into receptor tyrosine kinase lateral associations in cell membranes. The peptide comprised the 23-amino acid hydrophobic segment thought to span the membrane (Ile^{622} to Met^{644} of the EGF receptor), plus the first 10 amino acids of the receptor's cytoplasmic domain (Arg^{645} to Thr^{654}). Probes for solid-state NMR spectroscopy were incorporated by deuteration of the methyl side chains of alanine at positions 623 and 637. ^{2}H-NMR spectra were recorded from 25 to 65°C in membranes composed of 1-palmitoyl-2-oleoyl phosphatidylcholine, with and without 33% cholesterol, and relaxation times were measured. Peptide concentration ranged from 0.5 to 10 mol %. The peptide behaved as predominant monomers undergoing rapid symmetric rotational diffusion; however, there was evidence of reversible side-to-side interaction among the hydrophobic transmembrane domains, particularly at physiological temperatures and in the presence of natural concentrations of cholesterol. The results of these experiments in fluid membranes are consistent with the existence of lipid-protein interactions that would predispose to receptor microdomain formation in membranes of higher animal cells.

INTRODUCTION

Current models of signaling at cell surfaces raise important issues concerning the motional and associative properties of the receptor tyrosine kinases—major transmembrane proteins of higher animal cells. Typically, these receptors comprise a single chain of amino acids having an external glycosylated portion, a hydrophobic stretch of sufficient length to span the membrane only once, and an intracellular portion that communicates directly with cytoplasmic elements (van der Geer et al., 1994). Their lateral redistribution in the membrane, and side-to-side associations among them, are considered to be fundamental events in signaling. Such proteins are often found to be overexpressed in human tumors: i.e., to exist at increased concentration within the membrane, such that tumor growth may be driven by stimulatory signaling arising from excessive side-to-side receptor contacts (Alroy and Yarden, 1997; Gullick and Srinivasan, 1998). It is increasingly suggested that direct interactions between receptor hydrophobic transmembrane domains modulate receptor association (Lemmon et al., 1997), yet very few measurements of this phenomenon have been made. Membranes of higher animal cells are viewed biophysically as planar liquid crystals in which lateral diffusion of proteins is importantly regulated by thermodynamic processes. In the present work we examined associative behavior of the transmembrane portion of the human EGF receptor—a prototypic example of receptor tyrosine kinases—in fluid bilayer host matrices mimicking key lipid features of cell plasma membranes.

The peptide studied contained the natural sequence from residues 622 to 654 of the human EGF receptor. This represents the putative transmembrane region (residues 622 to 644) and a 10-residue stretch of the cytoplasmic domain including a threonine residue (Thr^{654}) that is phosphorylated during EGF-mediated signal transduction. Deuterium probe nuclei were located on the (methyl) side chains of alanine residues (Ala^{623} and Ala^{637}). Because the alanine side chain is a single methyl group, internal probe motion is limited to well-understood rapid rotation about the C-CD_3 axis, and spectral features can be fairly cleanly interpreted in terms of peptide backbone behavior. ^{2}H-NMR spectra and relaxation times were recorded for peptides assembled into bilayers of POPC, a predominant phospholipid in plasma membranes of higher animals. POPC bilayers have a fluid/gel phase transition of −3°C (Davis and Keough, 1985), well below the temperatures of experiments described here. Cholesterol was added at 33 mol % to mimic natural concentrations. Use of a transmembrane peptide, rather than the intact receptor, is justified by the "two step" model of membrane protein biogenesis: that transmembrane α-helices are capable of independent insertion into the membrane, and subsequent association to form functional multi-subunit complexes (reviewed in Lemmon et al., 1997).

MATERIALS AND METHODS

POPC was obtained from Avanti Polar Lipids (Birmingham, AL) and was used without further purification. Cholesterol was from Sigma (St. Louis, MO). Deuterated alanine was from CIL (Andover, MA). Peptides were

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Abbreviations used: EGF, epidermal growth factor; $EGFR_{TM}$, 34-amino-acid peptide corresponding to the EGF receptor transmembrane domain plus 10 residues of the cytoplasmic domain; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; [d_3]Ala^{623} [d_3]Ala^{637}, deuterated amino acids numbered to correspond to their position in the human EGF receptor.

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synthesized as described elsewhere and had a purity of >85–95% (Rigby et al., 1996).

NMR samples were prepared as unsonicated liposomes via the following general protocol. Dry peptide (up to 10 mg) with appropriate amounts of dry lipid were dissolved with warming to 55°C in 2,2,2-trifluoroethanol [4 ml (Aldrich, Milwaukee, WI), NMR grade, bp 77–80°C)] a solvent in which it is dispersed as α-helical monomers (Rigby et al., 1998). Samples were allowed to sit at 55°C for at least 30 min after visually apparent dissolution. Solvent was then rapidly removed under reduced pressure at 45–55°C on a rotary evaporator to leave thin films in 50 ml round bottom flasks. These were subsequently held for 18 h at 23°C under high vacuum. Hydration was with 30 mM HEPES containing 20 mM NaCl and 5 mM EDTA, pH 7.1–7.3, made up in deuterium-depleted water. Samples were warmed to 35°C without vortexing during hydration to minimize production of small vesicles. NMR spectra were run from high to low temperature after preincubation for at least 15 min at 65°C.

Specific labeling of the minority component in such small samples resulted in very weak signals and the need for extensive averaging. Complementary experiments were run on two spectrometers operating at different fields. ^{2}H-NMR spectra obtained at 3.55 T were acquired using a locally constructed spectrometer. For these spectra, the $\pi/2$ pulse length was typically between 4 and 5 μs and the pulse separation was varied between 55 μs and 300 μs. Because of the unusually low level of signal from these samples, shorter pulse separations were avoided in the long acquisitions at this field to ensure the absence of any possible effects from residual coherent interference. Free induction decays were collected with a digitizer dwell time of 2 μs and oversampling by a factor of 2 (Prosser et al., 1991) to obtain an effective dwell time of 4 μs. Spectra were obtained by averaging between 400,000 and 600,000 free induction decays with a repetition time of 450 ms, and line broadening was not applied. Echo amplitudes were used in quadrupole echo decay and inversion recovery experiments, collecting between 40,000 and 100,000 transients and averaging up to 5 points around each echo peak.

^{2}H-NMR spectra at 11.7 T were acquired on a Varian Unity 500 spectrometer using a single-tuned Doty 5 mm solenoid probe 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 5–6 μs. Pulse spacing was 20–30 μs. A repetition time of 100 ms was chosen to optimize signal while avoiding spectral distortion and saturation after comparison with results obtained at values of 50 and 500 ms; 600,000 to 1,200,000 FIDs were averaged and a line broadening of 100 Hz was applied to the transformed spectra. During long acquisitions spectra were routinely compared at long and short time intervals, and in several cases fresh samples were made to check results from samples that had been run extensively at high temperature: spectra were found to be unchanged.

RESULTS

The amino acid sequence of the peptide, $EGFR_{TM}$, is displayed in Fig. 1. The suggested transmembrane portion calculated using the method of Rost (1996) is underlined. Locations of alanine residues with deuterated side chains (—CD_3) are indicated by bold letters.

*KI**A**^{623}TGMVGALLLLLVV**A**^{637}LGIGLFMRRRHIVRKRT-COO$^-$

FIGURE 1 Sequence of the 34-amino acid peptide, $EGFR_{TM}$, representing residues Ile^{622}-Thr^{654} of the human EGF receptor, with a biotinylated lysine (*K) at position 621 (single-letter code has been used for individual amino acids). The putative transmembrane domain has been underlined (cytoplasmic domain to the right). Deuterated amino acids are indicated by boldface type.

For molecules undergoing rapid axially symmetric rotation, the spectrum of a given deuterium nucleus is characterized by a "Pake doublet" whose prominent 90° edges (arising from molecules reorienting about an axis perpendicular to the field) are split by

$$\Delta\nu_Q = \tfrac{3}{8}(e^2Qq/h)S_{mol}\langle 3\cos^2\Theta_i - 1\rangle \quad (1)$$

In this relationship, e^2Qq/h is the nuclear quadrupole coupling constant (165–170 kHz for an aliphatic C–D bond (Seelig, 1977; Davis, 1991)), S_{mol} is the molecular order parameter (assuming axially symmetric order) describing orientational fluctuations of the molecule relative to the bilayer normal, Θ_i is the orientation of the C–D bond relative to the molecular rotation axis, and the average is over reorientation of the C–D bond with respect to the molecular symmetry axis. If reorientation is not axially symmetric, the prominent singularities in the spectrum move toward the center and the result is a more "pyramidal" shape (see below) (Huang et al., 1980; Meier et al., 1986; Siminovitch et al., 1988; Auger et al., 1990; Morrow et al., 1995). Sharp single peaks occurring in the middle of spectra of samples such as those studied here are attributable to residual deuterated water and to the presence of some very small vesicles for which the quadrupole splittings are motionally averaged to zero.

Probe nucleus reorientation about the methyl group symmetry axis (i.e., rotation of the alanine methyl group) remains in the fast limit except at temperatures well below 0°C. Hence, under the conditions of our experiments, ^{2}H-NMR spectra of the CD_3 side chain of alanine within an *immobilized* peptide should consist of a single Pake doublet having 90° edges separated by close to 40 kHz (Seelig, 1977; Davis, 1991). However, we have demonstrated that, for a given deuterated alanine within $EGFR_{TM}$ in fluid bilayers containing up to 6 mol % peptide, the dominant spectral feature approximates a single Pake doublet of width considerably less than 40 kHz (Jones et al., 1998). Thus it is clear that the peptide undergoes rapid axial rotation in such membranes.

It has been suggested, based on analysis of the splittings and assuming standard α-helical conformation, that the $EGFR_{TM}$ helix backbone axis of rotation may not coincide with the molecular symmetry axis (Jones et al., 1998). Jones et al. demonstrated that splittings for several deuterated residues 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 and 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 the molecular rotation axis and the helix axis may be controversial, it is possible to make some general comments based on the observations presented here without presuming a specific model for polypeptide reorientation.

Fig. 2 displays stacked spectra as a function of temperature for the deuterated transmembrane peptide from the EGF receptor at high concentration (10 mol %) in bilayers of POPC. These spectra were acquired in a field of 3.55 T. At 65°C, the spectrum is a superposition of a Pake doublet with 90° edges separated by ~4.6 kHz, and a feature with a width of ~10.5 kHz. The wider feature appears to reflect probes experiencing less axially symmetric reorientation of the methyl group symmetry axis. Based on previous studies of this peptide and selective deuteration experiments (Jones et al., 1998), the narrower feature is identified with the deuterated methyl group of Ala^{623}. This residue is situated close to the bilayer surface and to the N-terminus, where there may be more freedom for peptide backbone departure from α-helical conformation and "unraveling," or where the axis for reorientation may coincide more closely with the helix axis. The wider feature is associated with the deuterated methyl group of Ala^{637}, which is situated well within the bilayer interior: a region of the peptide considered to be α-helical (Lemmon et al., 1997).

As the temperature is lowered both spectral components lose the prominent 90° edges, and the distribution of spectral intensity becomes increasingly characteristic of axially asymmetric reorientation of the methyl symmetry axis—presumably due to the onset of axially asymmetric rotation of the peptide in the membrane. The splitting of the feature assigned to Ala^{623} increases significantly as temperature is lowered (e.g., by over 1 kHz or 24% at 45°C), while the change in width of the Ala^{637} feature is within experimental uncertainty (e.g., increased by ~0.5 kHz or 5% at 45°C): this difference between the two probe locations was more marked at lower peptide concentration (described in association with Fig. 5). The loss of axial symmetry with decreasing temperature, displayed in Fig. 2, is probably not simply a consequence of lipid ordering: the liquid-crystal-to-gel transition in POPC occurs at ~−3°C, and for saturated phosphatidylcholines addition of an amphiphilic polypeptide to the bilayer generally lowers the temperature at which ordered phase lipid is first formed (Rice and Oldfield, 1979; Huschilt et al., 1985; Macdonald and Seelig, 1988; Mouritsen and Bloom, 1993; Zhang et al., 1995). An important possibility suggested previously (Rigby et al., 1996; Jones et al., 1997) is that such lineshape changes may reflect an increase in peptide-peptide interaction as temperature is reduced.

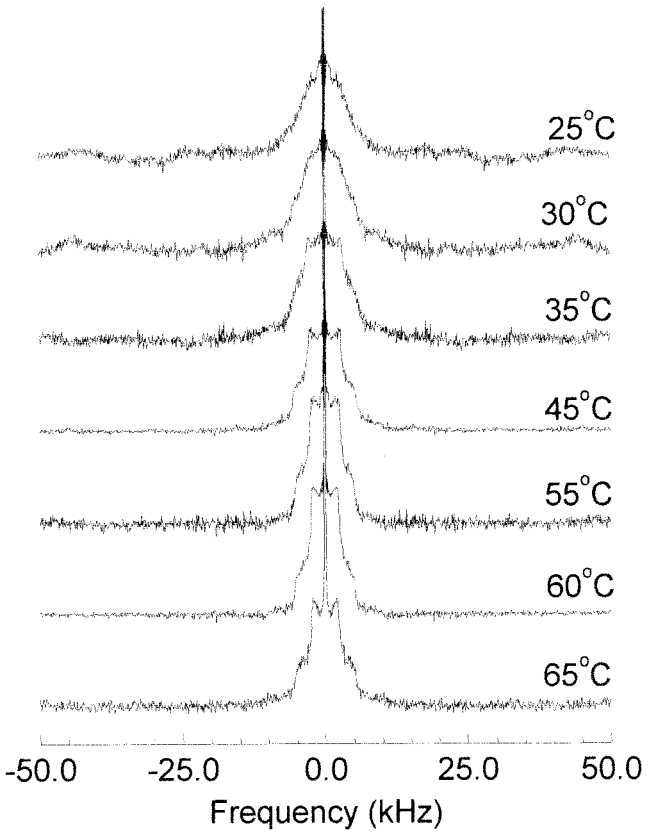

FIGURE 2 ^{2}H-NMR spectra of the deuterated peptide, $EGFR_{TM}$, at 10 mol % in POPC bilayers for selected temperatures from 65 to 25°C. Spectra were obtained at 3.55 T using a quadrupole echo sequence with pulse separation $\tau = 55$ μs, and have been symmetrized.

In the 25°C spectrum of Fig. 2 there appears to be a small step in intensity near ±20 kHz. Although this is difficult to distinguish from a baseline artefact, it is interesting to note that this is the (40 kHz) splitting that would be expected for methyl groups in the absence of rotational diffusion of the peptide backbone within the membrane.

Fig. 3 shows ^{2}H-NMR spectra of the deuterated peptide for a range of temperatures and peptide concentrations in POPC membranes containing cholesterol at a concentration thought to be typical of eukaryote plasma membranes. Peptide concentrations examined ranged from 10 to 4 mol % relative to phospholipid. As in Fig. 2, spectra were acquired at a field of 3.55 T. There was no evidence of hysteresis. Addition of cholesterol to membranes generally increases orientational order of the lipid bilayer environment without greatly restricting lateral diffusion or lipid rotation (reviewed in Davis, 1993; McMullen and McElhaney, 1995). The spectra in Fig. 3 demonstrate that cholesterol reduces the distinction between the Ala^{623} and Ala^{637} methyl deuteron splittings. Thus, between 65°C and 45°C the spectra approximate a Pake doublet with prominent 90° edges separated by ~7.7–8.0 kHz. These observations are consistent with previously reported studies on this transmembrane peptide (Rigby et al., 1996; Jones et al., 1997, 1998).

Fig. 3 shows that, in POPC/cholesterol bilayers, the splitting of the 90° edges in the prominent doublet (a superposition of the Ala^{623} and Ala^{637} methyl deuteron spectra) depends only weakly on temperature and polypeptide concentration. This is similar to the behavior, displayed in Fig. 2, of the Ala^{637} feature for bilayers of POPC without cholesterol. Also, as observed for deuterated peptide in bilayers of POPC, there is marked broadening of spectral lineshape

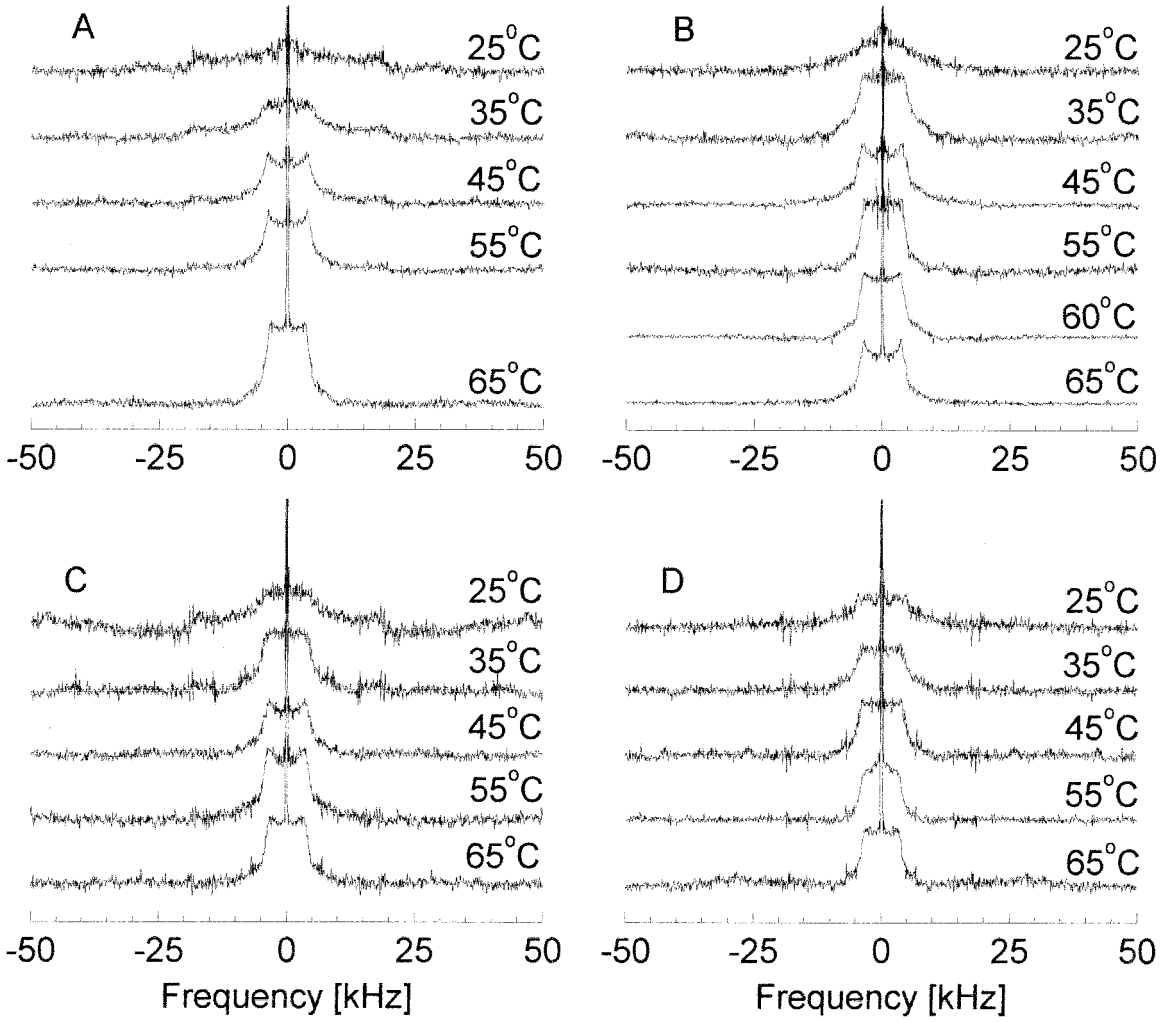

FIGURE 3 ^{2}H-NMR spectra of the deuterated peptide, $EGFR_{TM}$, at 10 mol % (*A*), 8 mol % (*B*), 6 mol % (*C*), and 4 mol % (*D*), in bilayers of 2:1 POPC/cholesterol, for selected temperatures from 65 to 25°C. Spectra were obtained at 3.55 T using a quadrupole echo sequence with pulse separation τ = 55 μs, and have been symmetrized.

with temperature reduction, despite the fact that the lipid membranes are highly fluid under all conditions examined. It can be seen that, for membranes containing cholesterol, this temperature-dependent broadening is less marked at lower peptide concentrations. It would appear that in the presence of cholesterol the spectral splitting associated with Ala^{623} has increased to ~8 kHz, while that of Ala^{637} has decreased slightly and is manifest as subtle shoulders near 9 kHz. It is significant that the Ala^{637} splitting does not *increase* with addition of cholesterol to the bilayer: if fluctuations of the peptide rotation axis orientation were contributing significantly to narrowing of the Ala^{637} methyl deuteron splitting (through the S_{mol} term in Eq. 1), cholesterol-induced ordering might be expected to constrain such fluctuations and so increase S_{mol} and the splitting.

^{2}H-NMR spectra observed in this work were obtained using a quadrupole echo sequence consisting of two $\pi/2$ pulses, shifted in phase by 90° and separated by an interval, τ. The echo is formed at time 2τ following the initial pulse and the spectrum is formed by Fourier transformation of the free induction decay starting from $t = 2\tau$. Motions that modulate the orientation-dependent quadrupole interaction during this sequence interfere with refocusing of the echo. The dependence of the echo amplitude on pulse separation, for short τ, has the form

$$A(2\tau) = A(0)\exp(-2\tau/T_{2e}) \quad (2)$$

where T_{2e}^{-1} is the effective echo decay rate averaged over all deuterons in the sample. Motions with correlation times in the range 10^{-6} to 10^{-4} s contribute strongly to quadrupole echo decay—motions that are substantially faster or slower contribute less effectively.

Fig. 4 *A* shows decays of quadrupole echo amplitude at 55°C for 10 mol % peptide in fluid bilayers of POPC, and

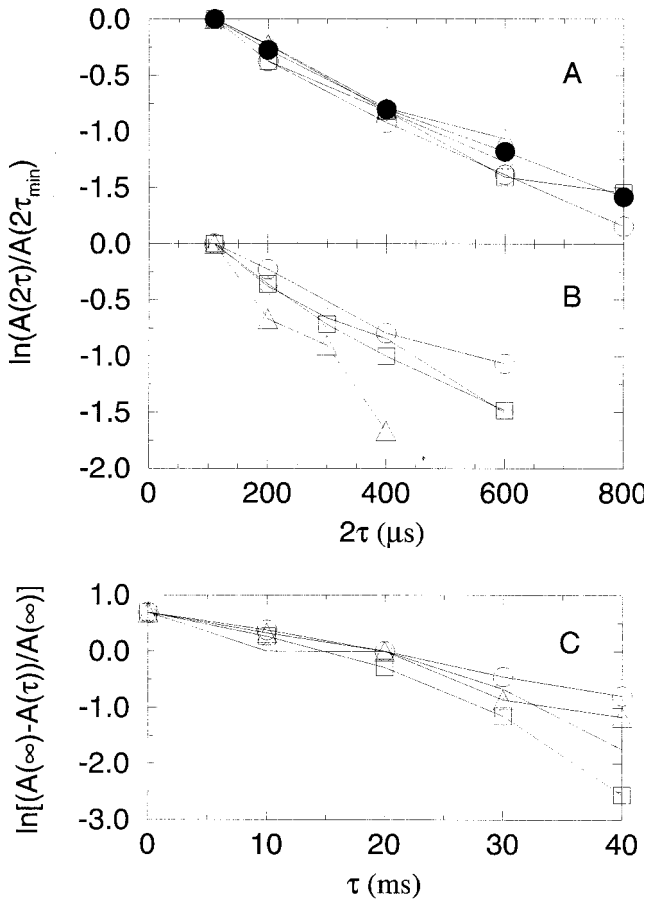

FIGURE 4 Relaxation time measurements on $EGFR_{TM}$ in fluid lipid bilayers. (*A*) Quadrupole echo decay curves for deuterated peptide in POPC bilayers at 10 mol % (●); and for the same peptide in bilayers of 2:1 POPC/cholesterol at 10 mol % (○), 8 mol % (□), 6 mol % (◇), and 4 mol % (△): all at 55°C. (*B*) Quadrupole echo decay curves for deuterated peptide at 4 mol % in bilayers of 2:1 POPC/cholesterol at 55°C (○), 45°C (□), 35°C (◇]), and 25°C (△). (*C*) Inversion recovery curves for deuterated peptide in bilayers of 2:1 POPC/cholesterol at 10 mol % (○), 8 mol % (□), 6 mol % (◇), and 4 mol % (△): all at 55°C. Relaxation time experiments were carried out at 3.55 T using echo amplitudes.

for a range of peptide concentrations in POPC/cholesterol bilayers. Data are displayed in 4 *B* for temperatures from 25 to 55°C for peptide at 4 mol % in the POPC/cholesterol host matrix. Also shown are the results of inversion recovery curves (Fig. 4 *C*), from which longitudinal relaxation times (T_1) may be inferred, for a range of polypeptide concentrations at 55°C. In these experiments the signals from Ala^{623} and Ala^{637} are averaged. More extensive relaxation and echo decay studies were precluded by the limited signal-to-noise ratio involved. Nevertheless, it was possible to draw some insight from the results. At 55°C the echo decay times, T_{2e}, and thus the correlation times for motions that modulate the quadrupole interactions at the Ala^{623} and Ala^{637} residues, appear to be insensitive to polypeptide or cholesterol concentration. At the fixed polypeptide concentration of 4 mol %, the echo decay time is weakly dependent on temperature, decreasing from ~450 μs at 55°C to ~190 μs at 25°C. Although there is considerable uncertainty in echo decay time determination from these data, particularly at 25°C, this change corresponds to a loss in total signal intensity of only ~30% for echoes acquired with a pulse separation of 55 μs, the condition used for acquisition of spectra in Fig. 3. T_1 values obtained from the initial decays range from 20 to 30 ms; thus the faster motions that determine longitudinal relaxation rate also appear to be relatively insensitive to polypeptide concentration in the POPC/cholesterol matrix studied.

In the spectra of Fig. 3 the apparent intensity of the prominent doublet with edges at ~±4 kHz decreases substantially with decreasing temperature for all peptide concentrations studied. Even at 4 mol % peptide (Fig. 3 *D*), the loss in apparent intensity is greater than can be accounted for by the reduction in echo decay time for the corresponding peptide concentration displayed in Fig. 4 *B*. The shortest decay in Fig. 4 *B* thus appears to reflect a total intensity comprising contributions from the prominent doublet (which decays rapidly at lower temperature), and another spectral component with a significantly longer echo decay time. A likely candidate would be a spectral component with edges near ±20 kHz arising from immobilized peptide, evidence for which was described earlier (Fig. 2). Weak components near ±20 kHz can also be seen in some spectra of Fig. 3, particularly at low temperature. Intermediate degrees of peptide immobilization producing broad spectra of width approaching 40 KHZ can also be seen in spectra reported previously at 12–25°C at a field of 11.7 T for the same peptide deuterated at other sites (Jones et al., 1997). Immobilization of the peptide would constrain modulation of the motionally averaged methyl deuteron quadrupole interaction and would be expected to result in a longer echo decay time. The presence of such a slowly decaying component could mask the effect of temperature on the shorter echo decay time of the narrower doublet. Unfortunately, the low signal-to-noise ratio in these spectra at 25°C precluded separation of echo decay times for these two spectral components. Nevertheless, the results obtained suggest that, at higher temperatures, the motion of the polypeptide results in a rapid reorientation of the alanine methyl group symmetry axis (i.e., rapid rotational diffusion of the peptide) and that, with decreasing temperature, a small fraction of the polypeptide molecules are effectively immobilized. This immobilized fraction is most apparent at higher polypeptide concentration.

Fig. 5 presents spectra obtained in a magnetic field of 11.7 T for $EGFR_{TM}$ at 6 and 0.5 mol % in fluid bilayers. The higher field offers better signal-to-noise for the same number of transients, and the shorter pulse separation minimizes differential decay of the echo associated with different spectral components. It is interesting, therefore, that in these spectra, even at 6 mol % peptide, the major Pake

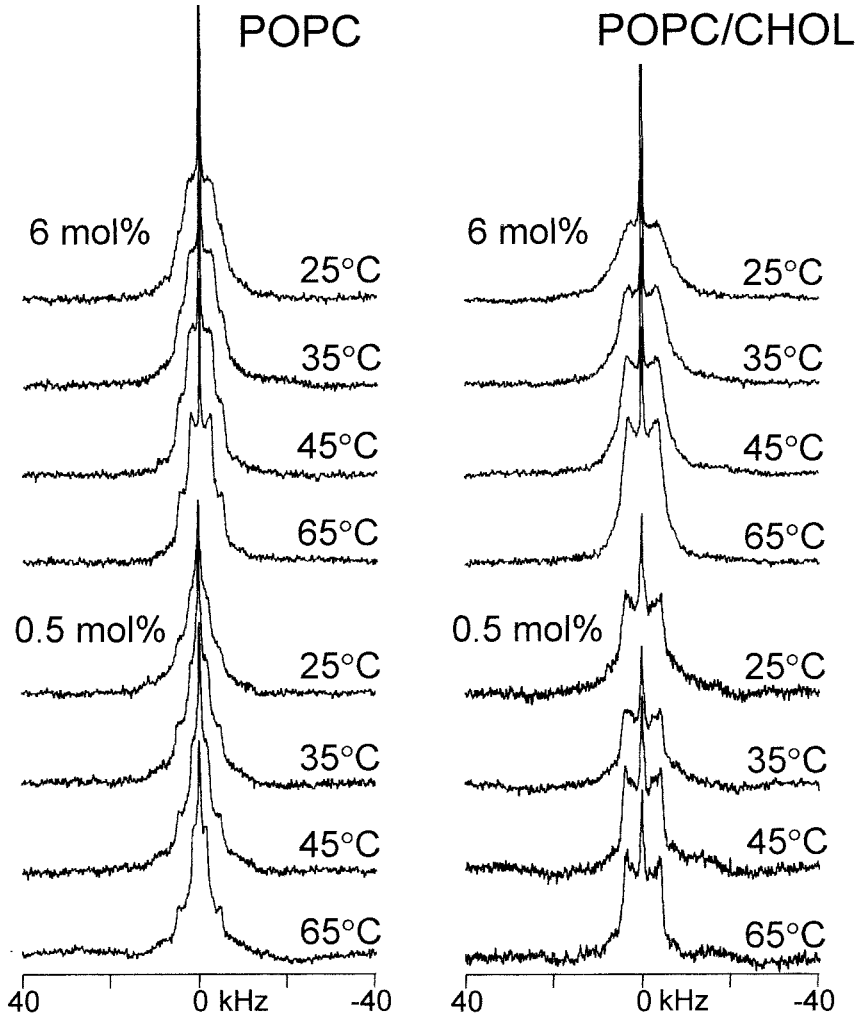

FIGURE 5 Unsymmetrized ^{2}H-NMR spectra at 11.7 T. Spectra are shown for $EGFR_{TM}$ at 6 mol % and 0.5 mol % in bilayers of POPC and 2:1 POPC/cholesterol for selected temperatures from 65°C to 25°C. Spectra were obtained using a quadrupole echo sequence with pulse separation $\tau = 20$ μs. Accumulated transients for each spectral group were 455,000 for 6% in POPC (except at 65°C the number was 237,000); 1,226,000 for 0.5% in POPC (except at 35°C 45°C the number was 600,000); 424,000 for 6% in POPC/cholesterol; 823,000 for 0.5% in POPC/cholesterol. A line broadening of 100 Hz was applied.

features retain their prominent edges down to 25°C, and the spectral component arising from effectively immobilized polypeptide is almost indistinguishable from the wings of the narrower doublet. This result reinforces the fact that only a small fraction of polypeptide is immobilized. As determined at lower field, spectra of the samples having higher peptide concentration (6 mol % here) demonstrate less prominent (less vertical) spectral edges, particularly in the presence of cholesterol. Taken together, the spectra of $EGFR_{TM}$ in fluid bilayers are broadened and decreasingly characteristic of axially symmetric reorientation as temperature is lowered. This tendency is reduced at lower peptide concentration, suggesting that it may reflect peptide-peptide interaction.

DISCUSSION

In the present experiments, replacement of ^{1}H by ^{2}H in alanine methyl groups permitted solid-state NMR of a receptor tyrosine kinase transmembrane domain in fluid bilayers possessing fundamental characteristics of cell membranes. Effects of temperature and polypeptide concentration were considered for insight into receptor dynamics and interactions—factors thought to modulate transmembrane signaling by these receptors of higher animal cells.

Quadrupole splittings of the dominant spectral feature were considerably smaller than the value of ~40 kHz expected for deuterated alanine methyl groups on immobilized peptides. The observation of well-defined edges in the spectra at higher temperatures implies that the dominant motion of the peptide is fast axially symmetric rotation under these conditions. However, the distribution of intensity across this feature was flatter than would be expected for a Pake doublet, suggesting that there may be a distribution of motional parameters. One possibility is that rotational diffusion of some fraction of the polypeptides departs significantly from axial symmetry as a result of reversible peptide side-to-side association.

In POPC membranes without cholesterol, two doublets of equal intensity were observed for the transmembrane peptide containing two deuterated alanine residues. The inner splitting was assignable to Ala^{623}, which is near the amino

terminus of the peptide: its smaller splitting has been suggested to be due to departures from α-helical conformation (Rigby et al., 1996; Jones et al., 1998). The doublet with the larger splitting was assigned to Ala^{637}. In bilayers of POPC containing 33 mol % cholesterol a single doublet was observed, and the methyl deuterons on the two alanines are presumed to give rise to nearly identical splittings. It was previously suggested that the cholesterol-induced increase in splitting displayed by the Ala^{623} methyl deuterons reflects stabilization of the α-helical conformation near that residue in response to cholesterol-induced bilayer thickening (Jones et al., 1997). It is significant, therefore, that the splitting for Ala^{637}, which is located well within a stable α-helical transmembrane segment, did not increase when cholesterol was added: a manipulation that increases bilayer chain order. This suggests that even in POPC without cholesterol, the axis about which the polypeptide reorients is almost fully ordered along the bilayer normal (as has been suggested for the bacterial peptide, gramicidin (Koeppe II et al., 1994; Prosser et al., 1994)).

In membranes of POPC the Ala^{623} splitting increased as temperature was reduced, while that of Ala^{637} did not. In POPC/cholesterol, the splitting of the prominent doublet (and thus of both Ala^{623} and Ala^{637}) were only weakly dependent on temperature and polypeptide concentration. Such weak dependence on temperature is consistent with a situation in which motional narrowing of the doublet in question is dominated by rotation about an axis that is ordered along the bilayer normal. If such behavior is characteristic of labeled alanines in stable α-helical transmembrane regions, as wobbling of the peptide rotation axis about the bilayer normal would be expected to make a temperature-dependent contribution to S_{mol}, these observations seem to imply that the amplitude of peptide motion is not changing substantially with temperature reduction, and thus once again that the peptide rotation axis is ordered.

Although the resolved splitting of the Ala^{637} doublet for $EGFR_{TM}$ in POPC and the common Ala^{637}/Ala^{623} doublet in POPC/cholesterol were largely insensitive to temperature and polypeptide concentration, other aspects of the observed spectra changed. As temperature was lowered, the edges corresponding to molecules rotating about an axis perpendicular to the applied field became less vertical, indicating that the rotation became increasingly axially asymmetric. The extent to which this happened was sensitive to polypeptide concentration—being more marked at higher peptide concentrations in the membrane. This is highly significant in that it demonstrates that the spectral changes observed with decreasing temperature cannot be attributed primarily to coupling of individual polypeptides to temperature-dependent properties of the bilayer interior. The effect of peptide concentration on spectral lineshape is most striking in the membranes containing physiological amounts of cholesterol. This may indicate that the extent to which neighboring transmembrane peptides interact is sensitive to lipid chain order within the bilayer.

The observed quadrupole echo decay times, T_{2e}, at 55°C for a range of $EGFR_{TM}$ concentrations in POPC/cholesterol and for 10 mol % $EGFR_{TM}$ in POPC were all close to 450 μs. It is interesting to compare the rotational correlation time implied by this result with that reported for a synthetic amphiphilic polypeptide, Lys_2-Gly-Leu_{24}-Lys_2-Ala-amide, in liquid crystal phase DPPC bilayers (Pauls et al., 1985).

For an aliphatic C–D bond, e^2qQ/h is ~170 kHz, and η, the asymmetry parameter of the quadrupole interaction, can be neglected. If the molecule containing this bond undergoes rapid rotation about an axis having spherical polar coordinates (β, α) within the principal axis system of the electric field gradient tensor, the spectrum of the deuteron will be a doublet with prominent edges, arising from molecules rotating about an axis perpendicular to the applied field, separated by

$$\Delta\nu = (\omega_Q/2\pi)P_2(\cos\beta) \tag{3}$$

where $P_2(\cos\beta) = (3\cos^2\beta - 1)/2$ and $\omega_Q/2\pi = (3/4)(e^2qQ/h)$. Pauls et al. (1985) show that the second moment of the resulting motionally narrowed powder spectrum will be smaller than that of the powder spectrum in the absence of motion by

$$\Delta M_2 = (1/5)\omega_Q^2\{1 - [P_2(\cos\beta)]^2\}. \tag{4}$$

If the correlation time for the rotation, τ_c, satisfies the condition $\Delta M_2 \cdot \tau_c^2 \ll 1$, the echo decay time, T_{2e}, is given by (Abragam, 1961)

$$(T_{2e})^{-1} = \Delta M_2 \cdot \tau_c \tag{5}$$

and the motion is said to be in the short correlation time limit.

For a deuterated methyl group attached to a rigid molecule, we can define θ_{methyl} as the angle between the C–D bond and the methyl group symmetry axis, and β as the angle between the methyl group symmetry axis and the axis about which the molecule as a whole is rotating. Modulation of the quadrupole interaction by rapid rotation of the methyl group about its symmetry axis does not, of itself, contribute significantly to the observed echo decay rate but does, in effect, partially average the quadrupole interaction and thus reduces both the splitting and the change in apparent second moment attributable to rotation of the molecule as a whole. Taking rapid methyl group rotation into account gives

$$\Delta\nu = (\omega_Q/2\pi)P_2(\cos\theta_{methyl})P_2(\cos\beta) \tag{6}$$

and

$$\Delta M_2 = (1/5)\omega_Q^2[P_2(\cos\theta_{methyl})]^2\{1 - [P_2(\cos\beta)]^2\} \tag{7}$$

where θ_{methyl}, the angle between the CD bond and the methyl group symmetry axis, is ~109°, so that $P_2(\cos\theta_{methyl}) \approx (1/3)$.

At 55°C, the alanine methyl deuteron spectra reflect, primarily, fast rotation of the methyl group about its symmetry axis and fast rotation of the entire molecule. We suggest that the rotation axis of the molecule is well-ordered in the bilayer, with S_{mol} close to the value of 0.9 found for gramicidin (Koeppe II et al., 1994; Prosser et al., 1994). We can then use Eq. 6 to determine $P_2(\cos\beta)$ from the observed splitting, where β is now the angle between the methyl group symmetry axis and the rotation axis of the molecule. In doing so, we neglect S_{mol}, which may result in an underestimate of $P_2(\cos\beta)$ by up to 10%. A splitting of 8 kHz, as is observed for $EGFR_{TM}$ in POPC/cholesterol at 55°C, corresponds to $P_2(\cos\beta) \approx 0.2$. The reduction in apparent second moment of the powder spectrum resulting from rotation of the molecule as a whole is thus estimated to be $\Delta M_2 \approx 1.4 \cdot 10^{10}\ s^{-2}$. From Eq. 5 the observed echo decay time of 450 μs at 55°C thus corresponds to a correlation time of $\sim 1.6 \cdot 10^{-7}$ s. This is very similar to the correlation time of $2 \cdot 10^{-7}$ s obtained by Pauls et al. (1985) for the synthetic amphiphilic polypeptide Lys_2-Gly-Leu_{24}-Lys_2-Ala-amide in liquid crystalline DPPC.

Lowering the temperature appears to substantially shorten the quadrupole echo decay time of the prominent doublet component. This was most apparent as a loss of doublet intensity displayed in the spectra obtained with a quadrupole echo sequence pulse separation of 55 μs. Although decay of the quadrupole echo can be affected by motions that are too slow to contribute to motional narrowing (Bloom and Sternin, 1987), such motions would also be slow enough that an increase in correlation time, as expected for cooling, would reduce the contribution to echo decay rate. The simplest explanation for the observed temperature dependence is that the interactions affecting the axial symmetry of polypeptide rotation are also increasing rotational correlation time as temperature is reduced. Once again, the extent to which the echo decay time decreases with temperature appears to be sensitive to polypeptide concentration.

Another effect of temperature on the observed spectra was the appearance of intensity at increasingly greater spectral width as temperature was lowered (out to as much as $\pm$ 20 kHz for some polypeptide concentrations when the membranes were cooled into the range of 25–35°C). Such an effect is characteristic of a deuterated methyl group that is effectively immobilized on the time scale of the NMR experiment—while still undergoing rapid rotation about the C-CD_3 axis attaching it to the peptide backbone. It suggests the presence of polypeptide molecules whose rotation has been severely curtailed, presumably as a result of strong polypeptide-polypeptide interaction or aggregation. It is significant that this occurs under conditions for which the lipid/cholesterol component of the bilayer matrix is still very fluid (Thewalt and Bloom, 1992). The fraction of peptide immobilized under the conditions of these experiments is small. Variability in the amount of spectral intensity observed at $\pm$20 kHz may indicate that any formation of some highly immobilized fraction is slow and sensitive to aspects of thermal history.

Our findings relate to a point emphasized in a theoretical description of membrane protein-lipid interactions by Sperotto and Mouritsen (1991). Based on simulation studies of a small transmembrane protein in pure DPPC bilayers, they note the distinction that: "Phase separation and bulk protein segregation/crystallization are macroscopic phenomena, which indicate the coexistence of two macroscopically distinct phases in the lipid-protein system. Protein aggregation, on the other hand, corresponds to formation of a new type of microscopic or mesoscopic super-particle or complex consisting of a cluster of proteins which, in general, will be associated with a certain size distribution."

CONCLUSIONS

NMR spectroscopy of deuterated alanine residues provided a sensitive method of considering the dynamics of the transmembrane portion of the human EGF receptor in fluid bilayers. By working well above the host matrix fluid/gel cooperative transition and with membranes containing 33 mol % cholesterol, it was possible to mimic the lack of lipid cooperativity characterizing cell plasma membranes. The molecule appeared to undergo rapid rotation about a highly ordered membrane director axis. Observed effects of temperature and polypeptide concentration on spectra and relaxation times are suggested to have an important basis in interference with polypeptide rotational diffusion by peptide-peptide interactions. Such interactions could come about via direct contact of neighboring molecules or through lipid-mediated interaction. In the fluid membranes studied, peptide behavior seemed better characterized by microdomain formation (as often anticipated in models of higher animal cell plasma membranes (Jacobson et al., 1995; Holowka and Baird, 1996)) than by phase separation. The possibility that transmembrane hydrophobic domains influence reorientation of neighboring receptors may be significant to our understanding of transmembrane signaling. ^2H wideline NMR relaxation times of higher animal receptor proteins have not to our knowledge been reported previously: the approach seems particularly appropriate to examination of signaling protein interactions in fluid membranes.

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