Epidermal Growth Factor Receptor Transmembrane Domain: ^{2}H NMR Implications for Orientation and Motion in a Bilayer Environment†

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ABSTRACT: As part of a study of receptor tyrosine kinase behavior in membranes, we have collected extensive NMR data from three well-defined probe locations within the transmembrane region of the human EGF receptor. Spectra were obtained for selectively deuterated alanine residues in a series of peptides corresponding to the putative transmembrane domain (with short extramembranous extensions). Peptides were incorporated into fluid unsonicated liposomes of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and POPC containing 33 mol % cholesterol to mimic common lipid composition of cell plasma membranes. The peptide concentration was in the range of 1−6 mol % relative to that of phospholipid. Data acquired at 35 °C have been analyzed quantitatively to determine their implications to receptor spatial orientation and dynamics. If it is presumed that the single transmembrane portion approximates an α-helix of 3.6 residues per turn, this helix was found to be tilted away from the membrane perpendicular, about which there was rapid axial diffusion. However, rotation about the peptide long axis was static on the NMR time scale of 10^{-4} s, and the peptide appeared to have a preferred direction(s) of lean. The results for this peptide, whose hydrophobic length is greater than the membrane hydrophobic thickness, were very similar between membranes of POPC and membranes of POPC containing 33 mol % cholesterol, despite considerable host matrix differences in thickness and order. Allowed values of peptide tilt occupied a narrow range: between 10 and 14° in POPC and between 10 and 12° in POPC/cholesterol. Although the existence of some preferred direction(s) of lean was demanded by the results, the direction of lean was not uniquely determined. We have interpreted these results, which were essentially unchanged at 65 °C, as reflecting the behavior of peptide monomers undergoing rapidly reversible peptide−peptide interactions. For transmembrane monomers, interference with rotation about the peptide long axis might be understood to arise from an energy benefit (in a tilted peptide) to prevention of particular amino acid side chains near the membrane surfaces from moving in and out of hydrophobic or hydrophilic environments. It will be desirable to test the conclusion of preferential lean of a monomeric receptor since such behavior could provide a mechanism for modulating monomer association with other species (i.e., signal transduction).

Receptor tyrosine kinases (RTKs)[1] play key roles in signaling across cell membranes, being involved in control of metabolic state, differentiation, and morphogenesis. There is a general perception that lateral associations, orientation, conformation, and motional characteristics of these membrane proteins, provide the "vocabulary" for communication between the extracellular environment and the cell's internal machinery (reviewed in refs *1*−*5*). Compelling models describing how this vocabulary may operate at the molecular level have been devised, particularly with regard to members of the class I RTK family such as the EGF receptor and erbB-2/Neu (*6*−*10*, and references therein). These models place considerable emphasis on the physical orientation and behavior of the transmembrane domains as primary factors that mediate side-to-side associations within the membrane and specific interactions at membrane surfaces. Direct associations of transmembrane domains are generally thought to require receptor tilt. Class I RTKs provide an ideal system for study since they have transmembrane segments long enough to traverse the membrane only once. Their short hydrophobic transmembrane domains have been suggested to undergo highly specific homo- and heterodimer/oligomer interactions that dictate the outcome of extracellular contacts in health and disease (*11*). However, there have been very few direct measurements made on these structures in membranes or on the factors to which they are sensitive. In this work, we have used wideline ^{2}H NMR to address this issue quantitatively for the human EGF receptor.

Like other class I RTKs, the EGF receptor has only one polypeptide chain (*3*, *5*). There is an external glycosylated portion responsible for the earliest events in recognition, a

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[1] Abbreviations: EGF, epidermal growth factor; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; TFE, trifluoroethanol; Ala^{623}, Ala^{629}, and Ala^{637}, deuterated amino acids corresponding to the indicated position in the human EGF receptor; RTK, receptor tyrosine kinase.

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*K**I**A**623**TGM**V**G**A**629**LLLLL**V**V**A**637**LGIGLFMRRRHIVRKRT654 -COO⁻

FIGURE 1: Typical amino acid sequence of EGF receptor transmembrane peptides. The putative transmembrane domain has been underlined (cytoplasmic end to the right). Deuterated alanine residues corresponding to Ala[623], Ala[629], and Ala[637] of the human EGF receptor are bold. Experiments were carried out with six different peptides based on the structure shown; variants included biotinylation (*) of the N terminus, amidation of the C terminus, phosphorylation of T[654], and lengthening of the N terminus by three residues while shortening the C terminus by seven residues (H_2N-K[618]IPSI**A**[623]T...R[647]-COOH).

single transmembrane stretch believed by many workers to participate at a variety of levels in signal transduction, and an intracellular portion exhibiting docking sites, protein kinase activity, and both tyrosine and threonine phosphorylation sites. The transmembrane segment is generally viewed as being a continuous α-helix (*12*−*14*). In developing a system that permits ^{2}H NMR measurement of phenomena that regulate receptor behavior, we have experimented with liposomal model membranes containing RTK transmembrane peptides having selectively deuterated amino acids. We demonstrated previously that ^{2}H NMR spectra of the EGF receptor transmembrane domain can be interpreted in terms of reversible formation of homodimers and oligomers within lipid bilayers (*15*, *16*). In this work, we focused on spectral data from three alanine residues within the putative transmembrane domain. ^{1}H nuclei in methyl groups were replaced with ^{2}H at positions corresponding to Ala[623], Ala[629], and Ala[637] of the human EGF receptor. The restricted and well-defined side chain internal motions of alanine residues permit clear insight into the peptide orientation and dynamics. 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) was chosen for liposome formation as it engenders structural features of the predominant natural phospholipids in higher animal cells: a saturated 16-carbon fatty acid at position 1 of the glycerol backbone and a cis-monounsaturated 18-carbon fatty acid at position 2. Corresponding membranes containing 33 mol % cholesterol were also studied since this reproduces a key plasma membrane characteristic and provides a method of altering membrane thickness in a well-characterized fashion.

MATERIALS AND METHODS

1-Palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) was obtained from Avanti Polar Lipids (Birmingham, AL) and was used without further purification. Cholesterol was from Sigma (St. Louis, MO). Deuterated alanine (d_3 and d_4) were from CIL (Andover, MA). Peptides were prepared as described elsewhere and had a purity of >85−95% (*15*). Tricine SDS−PAGE gels (4% stacking gel, 16.5% separating gel with 3% cross-linker) were run according to the method of Schägger and von Jagow (*17*). Amino acid sequences of the transmembrane peptides studied were typified by the 34-mer shown in Figure 1. Variants included lengthening of the N terminus, and shortening and charge modification of the C terminus as described in the caption of Figure 1.

Samples were prepared as unsonicated liposomes via the following general protocol. Dry peptide (up to 10 mg) with appropriate amounts of dry lipid was dissolved while the solution was warmed to 55 °C in 2,2,2-trifluoroethanol (TFE, 4 mL, Aldrich, NMR grade, bp of 77−80 °C). 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 carried out with 30 mM HEPES containing 20 mM NaCl and 5 mM EDTA (pH 7.1−7.3) in deuterium-depleted water. Samples were warmed to 35 °C, with minimal vortexing during hydration to minimize production of small vesicles.

^{2}H NMR spectra were acquired at 76.7 MHz 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 (*18*) ("SSECHO" from the Varian pulse library) was employed with full phase cycling and a $\pi/2$ pulse length of 5−6 μs. Pulse spacing of 20−30 μs and a repetition time of 100 ms were chosen to optimize the final *S*/*N* ratio while avoiding spectral distortion and saturation, after experimentation with longer and shorter values. The spectral sweep width was 100 kHz. Molecular modeling was achieved via Insight II (Biosym Technologies, San Diego, CA) and WebLab Viewer (MSI).

Calculations described in the text, from which are derived predicted spectral splittings at "all possible" values of peptide helix orientation and which subsequently compare these with experimental values to a select subset of allowed orientations, were performed using a program written in "C". However, the equations given here also produced the same result, albeit more tediously, using a standard spread sheet such as Excel.

RESULTS

Elongated amphiphiles, when dispersed in fluid membranes, tend to undergo rapid symmetric rotation about axes perpendicular to the bilayer. For such molecules containing deuterium nuclei, eq 1 is useful in that it relates ^{2}H NMR spectral splittings ($\Delta\nu_Q$) to molecular orientation and motional characteristics.

$$\Delta\nu_Q = {}^3/_8\,(e^2Qq/h)\,S_{mol}\,|3\cos^2\Theta_i - 1| \qquad (1)$$

where e^2Qq/h is the nuclear quadrupole coupling constant [165−170 kHz for a C−D bond (167.5 kHz was used in these calculations)] (*18*−*20*), S_{mol} is the molecular order parameter (assuming axially symmetric order) describing orientational fluctuations of the C−D bond relative to the bilayer normal, and Θ_i is the orientation of the C−D bond relative to an axis of rotation being considered. For deuterated methyl groups, which undergo rapid rotation about the attaching bond axis even at temperatures well below 0 °C, it is convenient to consider a resultant C−D vector directed along the C−CD_3 bond. This can be dealt with by considering Θ_i to be the angle between the C−CD_3 vector and the molecule's rotational axis, and introducing an additional factor of $^1/_3$ into the right-hand side of eq 1.

In using eq 1 to assess the implications of our ^{2}H NMR spectral data, we made several initial assumptions: (i) That the transmembrane peptides exist as straight right-handed α-helices of 3.6 amino acids per turn. (ii) That S_{mol} for the transmembrane domain as a unit is high, with a value of 0.9 being used on the basis of literature observations (*21*, *22*). (iii) That the alanine residues at positions 629 and 637 (Ala[629] and Ala[637]) are in regions relatively resistant to α-helix

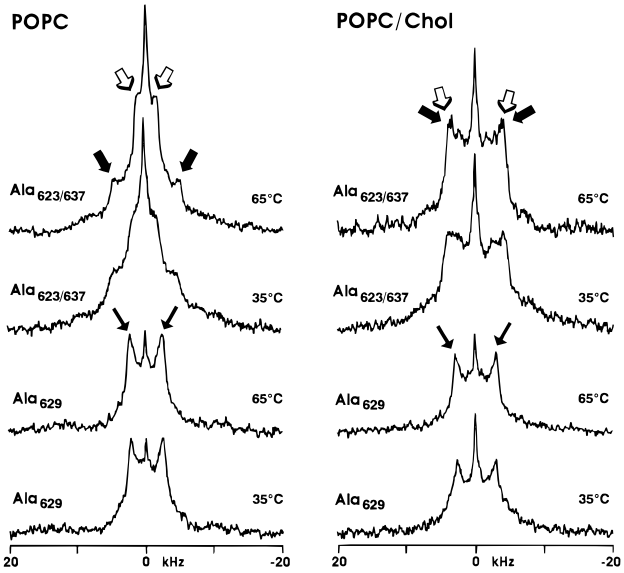

FIGURE 2: Typical ^{2}H NMR spectra of transmembrane peptides from the human EGF receptor. The left-hand column corresponds to the peptide sequence illustrated in Figure 1 in unsonicated liposomes of POPC alone, while the right-hand column represents membranes having 33 mol % cholesterol in addition to POPC. Arrows denote the assigned positions of the Pake doublets associated with deuteromethyl groups at the three distinct locations: Ala^{623} (hollow arrows), Ala^{629} (thin filled arrows), and Ala^{637} (wide filled arrows). Subtraction of spectra run on samples labeled only at Ala^{623} (spectra not shown) confirmed the assignments of Ala^{637} in samples deuterated at both locations. Spectra are shown for peptide/phospholipid molar ratios of near 1/100 (1 mol % peptide). The number of accumulated transients represented by each spectrum is 280000 for Ala^{629} and 680000−1220000 for Ala^{623} and Ala^{637}. Spectra have been normalized to a constant area per deuterated methyl group.

unraveling. (iv) That the alanine residue at position 623 (Ala^{623}) is in a peptide region within which helix destabilization may be of concern.

Representative spectra obtained for peptides in fluid liposomes of POPC and POPC/cholesterol are displayed in Figure 2 to demonstrate peak assignments. Some of these peptides deuterated at Ala^{623} have been reported previously by our lab (*15*, *16*); however, this is the first report of data associated with positions Ala^{629} and Ala^{637}, which were critical to measurement of the peptide orientation. The very sharp peak in the middle of each spectrum is a general feature of ^{2}H NMR powder spectra of amphiphiles in liposomes; it arises from residual deuterated water and from vesicles with high curvature for which quadrupole splittings are motionally averaged to zero, and will not be considered here. For a peptide rotating rapidly and symmetrically about an axis perpendicular to the membrane, each alanine CD_3 group should give rise to a pair of broad spectral lines (the so-called Pake doublet, arrows in Figure 2), whose separation ("spectral splitting") describes peptide orientation via the Θ_i term of eq 1 (although the rapid internal rotation intrinsic to methyl groups necessitates a factor of $^1/_3$ on the right-hand side of the equation as noted above). If rotation of the peptide as a unit is significantly asymmetric, there can be a shift in intensity toward the spectral center and a rounding of the Pake doublet edges (*23*−*29*). There is some evidence of this in the lower-temperature spectra of Figure 2, and we have previously demonstrated association of the phenomenon with peptide−peptide homodimer/oligomer formation (*15*, *16*).

Table 1: Spectral Splittings at Alanine ^{2}H Probe Sites[a]

membrane composition		spectral splittings ($\Delta\nu_Q$) (±0.5 kHz)
6 mol % peptide in POPC	Ala^{623}	6.0 (5.5[b])
	Ala^{629}	5.3−5.4
	Ala^{637}	10.6
6 mol % peptide in POPC/cholesterol	Ala^{623}	8.8 (7.8[b])
	Ala^{629}	7.4
	Ala^{637}	8.4
1 mol % peptide in POPC	Ala^{623}	4.2
	Ala^{629}	5.3
	Ala^{637}	10.1
1 mol % peptide in POPC/cholesterol	Ala^{623}	8.3 (7.3[b])
	Ala^{629}	6.2
	Ala^{637}	8.9

[a] Spectral splittings ($\Delta\nu_Q$) correspond to deuterated CD_3 groups on the three alanine residues within the transmembrane domain of peptides from the human EGF receptor. Values quoted are for samples held at 35 °C after pre-equilibration at 65 °C, and generally reflect averages over several samples in different peptide preparations. Values were derived by inspection; the estimated uncertainty takes into account variability found for samples prepared on different occasions, and interobserver variability. Peptide structures are described in Figure 1. In each case, the peptide was assembled into bilayers of POPC or POPC containing cholesterol at 33 mol % relative to POPC. All samples were prepared by hydration of films dried down from a TFE solution. At 65 °C, measured spectral splittings were within experimental error of values found at 35 °C with the exception of that for Ala^{623} in peptides dispersed in pure POPC; in the latter case, spectral splittings were typically reduced by 17−20% at the higher temperature, consistent with some conformational flexibility at this location. [b] Small amounts of a peptide with a longer N terminus (extending to K^{618} of the natural sequence as described in the text) were available. Values in parentheses illustrate the effect on the Ala^{623} splitting of this lengthening.

The S_{mol} factor in eq 1 dictates that the Pake doublet peak separation can be further reduced by additional spatial fluctuations of the CD_3 group. This is the basis of one attractive aspect of using alanine residues as probe locations: the methyl group is firmly fixed to the peptide backbone so that additional spatial fluctuations are limited to conformational instability of the peptide at the site of a given alanine residue and to wobble of the peptide as a whole. Table 1 contains a listing of measured spectral splittings acquired at 35 °C from a wide variety of samples. These values remained the same within experimental error at 65 °C, with the exception of values for Ala^{623} in peptides dispersed in pure POPC. In the latter case, spectral splittings were typically reduced by 17−20% at the higher temperature; this is consistent with some conformational flexibility in the N-terminal region of the peptide backbone such that local order is decreased with temperature (i.e., S_{mol} is reduced as the temperature rises).

For a standard α-helix, the angle measured (using Insight II) between the alanine C−CD_3 bond and the peptide long axis is 56° (Figure 3). Substituting this value for Θ_i in eq 1 (while incorporating the factor of $^1/_3$ for a methyl group and using a value of 0.9 for S_{mol}) yields a predicted value of only 1.2 kHz for the spectral splitting, $\Delta\nu_Q$. This very small value reflects the fact that 56° is close to the "magic angle" of 54.7°, at which quadrupolar splittings collapse to zero. It follows that, if the transmembrane region is a standard α-helix, rapid axial rotation about its long axis would collapse the spectral splitting almost to zero. Thus, on the basis of

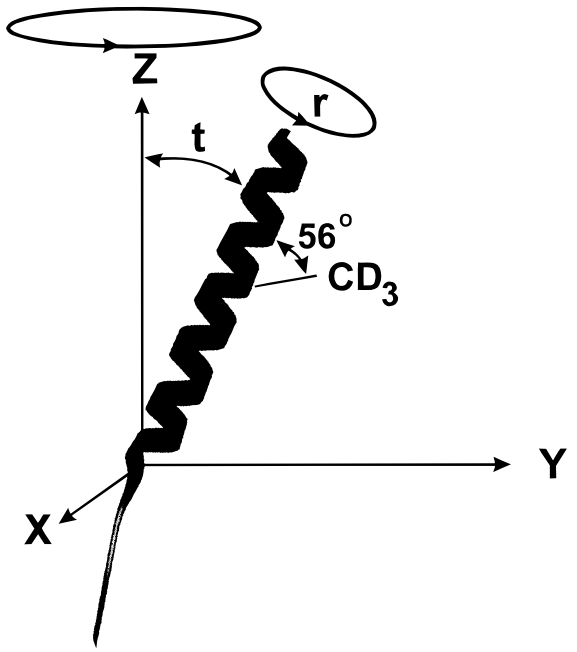

FIGURE 3: Diagramatic illustration of transmembrane peptide orientation and motion. The putative transmembrane domain of the peptides studied is represented by an α-helix (N terminus directed "up"), while the cytoplasmic portion of the C terminus is shown unstructured and extended (directed "down"). For illustrative purposes, an axis system has been centered at the bottom end of the putative transmembrane domain. The *z*-axis corresponds to the membrane perpendicular, about which rapid axial rotation was found to occur. The angle of tilt of the helix long axis away from the membrane perpendicular is represented by *t*. *r* is the degree of rotation (preferred rotational position) about the peptide helix long axis. *r* has been taken to be zero when C_α of Ala^{623} is directed along the (+) *y*-axis and is positive into the page (counterclockwise as viewed from above).

the large spectral splitting values actually observed (Table 1), it would seem that rotation faster than 10^4-10^5 s^{-1} about the peptide long axis is not taking place in our system. Yet rotation on this time scale about an axis perpendicular to the membrane is taking place because the spectra are Pake doublets of width corresponding to rapid axial rotation (e.g., Figure 2) (*23*). These facts, taken in combination, preclude the possibility that the peptide exists primarily as a standard α-helix standing straight up in the membrane. If it is a helix of 3.6 residues per turn, it must have an overall tilt ("lean") away from the membrane perpendicular. Following this logic further, the peptide helix appears to have a preferential direction of lean in the membrane such that any given amino acid has a preferred orientation (e.g., toward the membrane interior or away from it). If this were not the case, all alanine residues would have the same spectral splitting in a given membrane. This is clearly not the case even for Ala^{629} and Ala^{637} in the central region of the peptide.

Continuing with the assumption that the transmembrane domain is a straight α-helix, the above findings led us to express peptide orientation and rotational diffusion in terms of an angle of tilt, *t*, between the peptide long axis and the membrane perpendicular, and a fixed angle of rotation, *r*, about the peptide long axis; i.e., the peptide leans by an unknown angle toward an unknown preferred side (Figure 3). The approach is analogous to that taken by Kovacs and Cross (*30*) in dealing with spatial characterization of the transmembrane domain from influenza A virus M2 protein. As an arbitrary reference, we took *r* equal to 0 to be the point at which Ala^{623} would face the positive *y*-axis when the transmembrane domain is a perfect α-helix (the arrangement shown in Figure 3). By reference to the peptide sequence and a standard helical wheel, this reference point places Ala^{629} at $r + 120°$ and Ala^{637} at $r + 40°$. It then remains to relate the orientation, Θ_i, of each alanine $C-CD_3$ bond to the membrane perpendicular in terms of *t* and *r*, and to use the observed spectral splittings to solve for *t* and *r*. If we recall that the $C-CD_3$ bond itself is tipped 56° from the helix axis, a straightforward trigonometric conversion results in the following:

$$\text{for } Ala^{623},\ \cos\Theta_i = -\sin 56°\cos(r)\sin(t) + \cos 56°\cos(t)$$

$$\text{for } Ala^{629},\ \cos\Theta_i = -\sin 56°\cos(r + 120°)\sin(t) + \cos 56°\cos(t)$$

$$\text{for } Ala^{637},\ \cos\Theta_i = -\sin 56°\cos(r + 40°)\sin(t) + \cos 56°\cos(t)$$

These values were substituted into eq 1 (containing the factor of $^1/_3$ described above), and all possible spectral splittings were generated as a function of *t* (varied in 0.1° increments from 0 to 50°) and *r* (varied in 0.5° increments from 0 to 360°). Subsets of *t* and *r* values were then sought that were consistent with the experimentally observed spectral splittings. Since alanine CD_3 groups are directed 37.4° in the negative *r* direction (by the conventions selected above) rather than straight away from the helix axis, final *r* values reported here have been increased by this amount over the values calculated.

Given the proximity of Ala^{623} to the N terminus of the peptides and experimental evidence of greater conformational flexibility at this point, in seeking values of *t* and *r* that are consistent with observed spectral splittings, we tested first for a fit to splittings associated with Ala^{629} and Ala^{637} only. The resultant ranges of peptide tilt, *t*, and fixed rotation, *r*, are shown graphically in Figure 4A for each of the lipid compositions and peptide concentrations examined. These ranges were then screened for points fitting the experimental splittings measured for Ala^{623}, and the resulting subset of *t* and *r* values is shown in Figure 4B. The process is described in the caption of Figure 4. In each case, the average of each circumscribed range was calculated, and this value is recorded as a {*t*,*r*} vector beside the corresponding plotted range of *t* and *r* values.

The conservative approach of using experimental data from Ala^{629} and Ala^{637} (ignoring Ala^{623}) produced four separate circumscribed ranges of peptide *t* and *r* values (only one of which is likely to be "correct") for a given membrane composition. The four corresponding values of {*t*,*r*} so obtained for a given membrane differ primarily in the value of fixed rotation, *r*, about the peptide long axis (the preferred direction of lean). Increasing peptide concentration had no significant effect on the four possible *r* values, while it increased *t* by about 0.5°, both in the absence and in the presence of cholesterol. Cholesterol addition had an up to 20° effect on *r*, and decreased tilt (*t*) by up to 1°. While differences of this order were statistically significant as judged by the paired T-test, the overall experimental

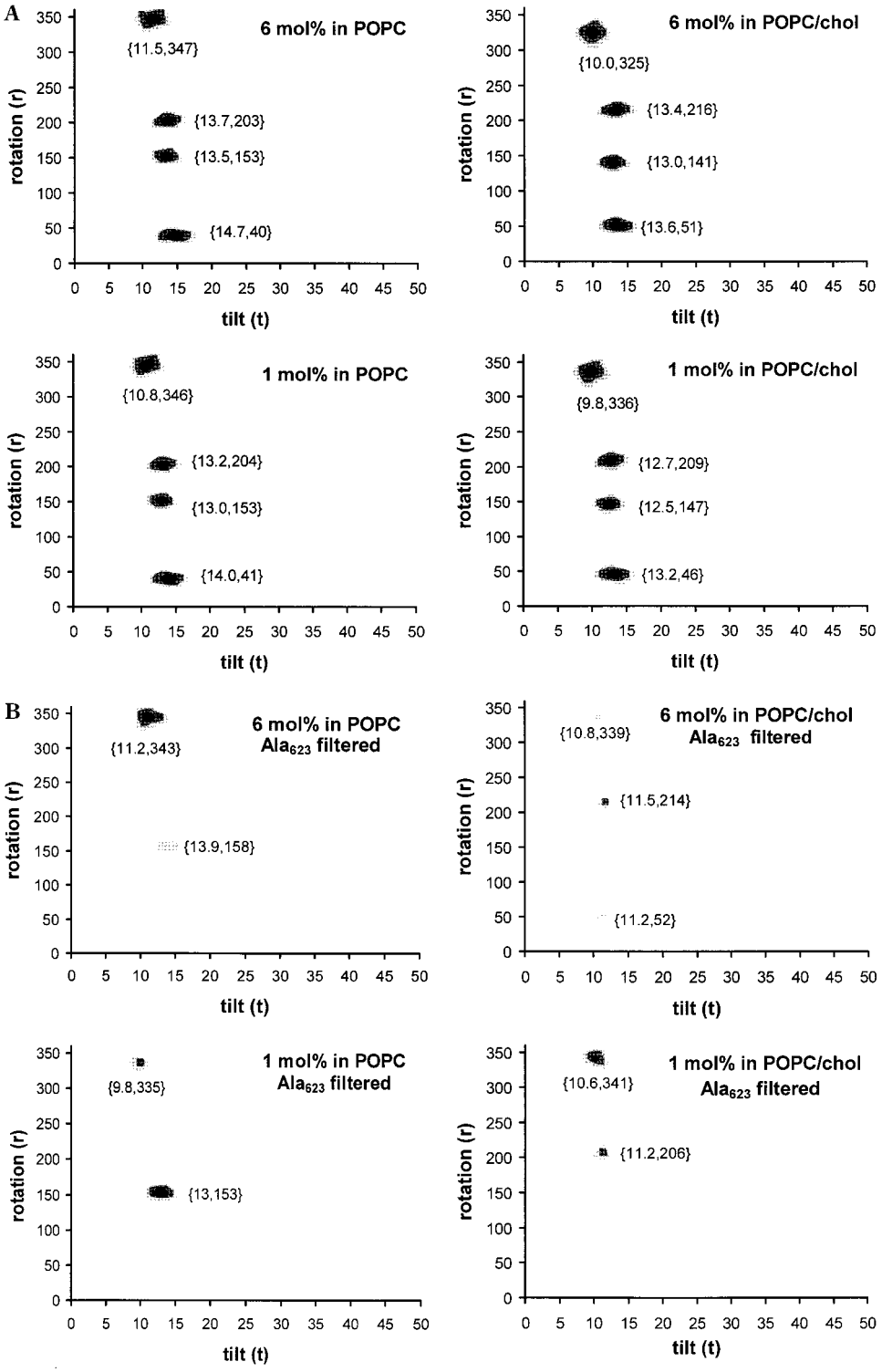

FIGURE 4: Acceptable ranges of peptide long axis tilt (t) and rotational orientation (r). In each case, the $\{t,r\}$ vector corresponding to the average of each range was calculated and is recorded beside the plotted range. t is the angle between the EGF receptor transmembrane helix long axis and the membrane perpendicular, while r is the apparently preferred degree of angulation about the peptide long axis. In establishing ranges of t and r, the approach taken was to solve eq 1 for spectral splitting ($\Delta\nu_Q$) using "all possible" values of t and r and then to screen (filter) these values for combinations of t and r that gave results consistent with experimental ^{2}H NMR spectral splittings listed in Table 1. t was varied in 0.1° increments from 0 to 50°, and r was varied in 0.5° increments from 0 to 360°. Data were for fluid bilayers of POPC and POPC/cholesterol at 35 °C. In panel A, experimental values of spectral splittings associated with Ala^{629} and Ala^{637} deep within the putative transmembrane sequence were used. Subsets of orientations were sought that were consistent with experimental splittings: agreement within ±0.5 (solid black regions), ±1 (dark gray), and ±1.5 kHz (light gray). In panel B, the solutions in panel A were subsequently screened for a subset of solutions which also agreed with experimental data associated with Ala^{623} in the human EGF receptor transmembrane domain. Since there was potential for S_{mol} (motional order) to be reduced by instability of the helix near this probe location, the ranges plotted include wider filters which extend twice as far above the experimental value as below it. Thus, subsets of orientations were sought that were consistent with Ala^{623} data within 2 kHz above and 1 kHz below (solid black regions), 2.5 kHz above and 1 kHz below (dark gray), and 2.5 kHz above and 1.5 kHz below (light gray).

uncertainty as discussed below dictates that the differences be seen as remarkable primarily for their small size.

Since the concern with data from Ala^{623} hinged partly on this residue's nearness to the N terminus of most of the peptides examined, we considered using only the (limited) Ala^{623} data available from the single peptide with the longer N terminus (sequence beginning several residues farther upstream at K_{618}; see Figure 1). However, for membranes without cholesterol, all peptides studied gave the same ranges for *t* and *r*. For membranes containing cholesterol, the peptide with the longer N terminus produced a difference of about 0.5° of tilt; hence, the plots shown for cholesterol-containing membranes in Figure 4B were derived using Ala^{623} data from this peptide only. As with the results from Figure 4A, the most appropriate interpretation of these numbers is likely that the values indicate a remarkably tight range of peptide tilt centered around 12° for POPC membranes and 11° for POPC/cholesterol membranes, and that there is a preferred rotational position (*r*) which is very consistent within 20° among membranes but incompletely determined.

DISCUSSION

In this work, we have reported for the first time use of wideline ^{2}H NMR spectroscopy in quantitating the spatial arrangement of a higher-animal transmembrane peptide sequence in lipid bilayer environments. Lipid matrixes were chosen to mimic fluidity characteristics, common fatty acid nature, and high cholesterol content of plasma membranes. Nonperturbing deuterium probes were located at three well-defined sites within the EGF receptor transmembrane domain. Ala^{623} is in a region that Brandt-Rauf and Pincus and colleagues (*31*) have considered to be potentially unstable as an α-helix, with implications for signal transduction. It is also near the membrane surface, and Shen et al. (*32*) have emphasized the possibility that the secondary structure of hydrophobic peptides may fluctuate on a 10^{-9} s time scale (*32*) near the surface of the bilayer. Moreover, in most of the peptides studied in this work, Ala^{623} is close to the N terminus, and the ends of helical peptides are known to have a greater tendency to unravel (*7*, *33*−*35*). The fact that heating-induced conformational flexibility was observed only at Ala^{623} in these experiments (decreased spectral splitting in going to 65°) is consistent with these suggestions. On the other hand, recent calculations by Belohorcová et al. (*36*) indicate that, in a phospholipid environment, the extent of hydrophobic peptide end unraveling may be limited to a slight average extension of the terminal loop. In keeping with such a possibility, using a peptide that included the residues from K^{618} to R^{647} of the natural EGF receptor sequence (H_2N−K^{618}IPSIA^{623}T...R^{647}-COOH), we observed Ala^{623} splittings of 5.5 kHz at 6 mol % in POPC and 7.8 kHz in POPC/cholesterol, versus values of 6.0 and 8.8 kHz, respectively, for peptides in which Ala^{623} was only two residues from the N terminus. In the final analysis, orientations derived using Ala^{629} and Ala^{637} data proved to be compatible with an approximation to helicity at Ala^{623} on the long (10^{-4} s) time scale of the NMR experiment.

The spectral splittings observed experimentally for Ala^{629} and Ala^{637}, when used to solve eq 1 for acceptable values of *t* and *r*, generated remarkably tight ranges for the peptide angle of tilt in the membrane. Angles compatible with peptide NMR spectra ranged from 10 to 14° in POPC and from 10 to 12° in membranes with added cholesterol. The fixed angle of rotation, *r*, about the peptide long axis was much less well defined; four distinct ranges of *r* were obtained for each membrane composition. However, these values calculated for *r* were also very consistent (within 10−20°) among the membranes and peptides studied. Data filtration using the Ala^{623} splittings served to rule out some of the possible *r* values identified for each membrane in Figure 4A. It also led to some minor refinement of the *t* values. However, concern over conformational flexibility at this position would lead us to avoid exclusive reliance on the solutions that depend on Ala^{623}. There were small quantitative differences among the different membrane compositions studied, but overall experimental uncertainty dictates that the differences in *t* and *r* among the different membranes should be seen as remarkable primarily for their small size. This can be appreciated in considering the source of the finite peptide tilt and fixed rotational orientation.

For POPC bilayers, the thickness of the hydrophobic region has been reported to be 25.8 Å at 25 °C and 29.9 Å for the same membranes containing 33 mol % cholesterol (*37*). Presuming a right-handed α-helix of 3.6 residues per turn, the calculated length of the 23-residue hydrophobic transmembrane domain predicted for the EGF receptor by the algorithm of Rost and colleagues [Figure 1 (*38*)] is 34.5 Å (*39*). Tipping the peptide helix would reduce its axial length measured perpendicular to the membrane, but a tilt of 41.6° would be necessary to completely bury a helix central axis 34.5 Å long in POPC (30° in POPC/cholesterol). The van der Waals diameter of the peptide helix is about 12 Å; thus, a given hydrophobic side chain makes contact with its lipid surroundings at a distance of 6 Å from the helix axis and could be buried more readily by being tilted directly toward the membrane, effectively reducing the necessary tilt angle by up to 20°. Predictions of the membrane-buried lengths of transmembrane proteins can vary by one or two amino acids depending upon the algorithm used. Thus, depending upon the disposition of hydrophobic amino acids about the periphery of the transmembrane domain, if there is no rotation about the peptide long axis, a modest tilt angle such as the 10−14° tilt observed could result in adequate burial of the EGF receptor hydrophobic transmembrane portion. Such calculations are further complicated, although the restrictions may be eased, by the possibility that lipid adaptation minimizes peptide mismatch (*40*, *41*, and references therein).

One conclusion that emerged early in our calculations was that the peptide axis appears to be tilted from the membrane perpendicular. The suggestion that a monomeric single-α-helix transmembrane peptide can lean in the membrane is not extraordinary; in this case, it could be rationalized by invoking an entropic need to bury as much as possible of the peptide's 34.5 Å hydrophobic domain in a membrane with a hydrophobic thickness of only 25.8−29.9 Å. Indeed, key recent molecular dynamics studies (*32*, *36*) predict such behavior for model hydrophobic peptides. The concepts of slow rotation about the peptide long axis and preferential lean of a monomer are more unexpected, however, and necessitate examination of our hypotheses regarding peptide helicity and order. We have demonstrated by high-resolution

NMR that even in the organic solvent used to prepare the samples the peptide studied has stable α-helical structure from residue Met^{626} to Arg^{647} (*42*). The premise that the peptide is an α-helix of 3.6 residues per turn in membranes seems further justified by numerous observations on related systems (*9*, *12*, *15*, *21*, *30*, *32*, *34*, *35*, *43*−*47*). One might note that molecular dynamics simulations in vacuo of the transmembrane peptide from a related class I RTK, c-erbB-2, demonstrated the possibility that loops of the α-helix can adopt π-helix (4.4 residues per turn) on a time scale of 10^{-9} s (*10*). However, in a π-helix, the angle the C−CD_3 bond makes with the helix axis becomes 53.4°, which is still very close to the 54.7° magic angle. As a result, reprocessing of our data presuming even a complete π-helix led to the same major conclusions. The value of 0.9 used for S_{mol} also seems defensible on the basis of existing literature observations (*21*, *22*; see also ref *9*). Values close to 1 have been measured for the transmembrane domain of phage coat protein (*48*). Substituting an S_{mol} value of 0.95 instead of 0.9 decreased the calculated tilt by 0.8°, while choosing an S_{mol} value of 0.85 produced a 0.9° increase in calculated tilt; neither affected the calculated values of *r*. Thus, although this range of uncertainty in S_{mol} has little impact on the basic conclusions drawn in this work, it does underscore the prematurity of attributing significance to the calculated 1° difference in peptide tilt between POPC and POPC/cholesterol membranes. One might attempt to explain the preferential lean of a monomeric peptide and slow rotation about the peptide long axis by suggesting that these features make it possible to avoid exposing certain side chains near the membrane surfaces to an unfavorable environment (e.g., exposing a hydrophobic side chain to water as indicated above with regard to calculation of requirements for membrane thickness).

It is appropriate to consider briefly the possibility of a bend in the α-helix long axis; for instance, this could produce better hydrophobic burial in the relatively thin membranes of POPC (see also ref *41*). Structures of α-helical domains in globular proteins readily demonstrate bends of 10° over a distance of four helix turns. Recent molecular dynamics calculations on transmembrane peptides suggest that modest bends are energetically tolerable, at least with a lifetime of 5×10^{-10} s (*32*, *36*), although fluctuations occurring on such a (short) time scale would only serve to reduce S_{mol} in our experiments. The presence of a long-lived bend in the peptide helix however ($>10^{-4}$ s) could potentially relax the apparent demand of our data that there be no rotation about the peptide long axis, since rapid rotation of a bent helix about some average peptide long axis could place the alanine C−CD_3 bond directions at angles significantly different from the 56° measured for a straight helix. It would take an angular deviation of some 6° below the magic angle, or 8° above, to result in an alanine CD_3 group splitting of 6 kHz for a peptide rotating rapidly about its long axis. None of the three alanine residues probed here had spectral splittings close to the zero kilohertz value expected for a 54.7° orientation of the CD_3 axis relative to the peptide axis of rapid rotation. Thus, all three alanine residues would have to be in significantly bent regions of helix, a situation that would likely require a bend of greater than 20° over the four helix turns separating Ala^{623} and Ala^{637}. Moreover, bending might be expected to be less energetically favorable in the cholesterol-containing membranes in view of their greater stiffness and hydrophobic thickness. We have not observed major orientational changes at probe locations from Ala^{629} to Val^{650} in any of our experiments upon addition of 33% cholesterol (*15*, *16*) or upon altering the temperature between 25 and 65 °C. It seems unlikely that a monomeric peptide would have a large bend that remains largely unchanged in the face of major changes in membrane order and thickness.

To this point, we have interpreted our findings in terms of a primarily monomeric peptide, albeit undergoing rapidly reversible homodimeric interactions. Compelling evidence of monomeric behavior for single-transmembrane α-helices even at high concentrations in bilayer membranes has been discussed recently (*40*, *41*). However, an important model in which class I RTKs can form dimers by direct side-to-side contact of their transmembrane domains exists. Gullick and colleagues noted that a sequence of five amino acids can be found as a "motif" in the transmembrane domain of many RTKs (*6*). They suggested that homodimers could arise via association of these motifs at a non-zero crossing angle (see also ref *31*). MacKenzie et al. (*46*) demonstrated by high-resolution NMR spectroscopy that a reminiscent arrangement occurs for the transmembrane α-helical domains of the glycophorin A dimer in detergent solution (crossing angle of 40°) and that stabilization is by van der Waals forces. Smith et al. (*9*) have performed optical spectroscopy and magic angle spinning NMR studies on a transmembrane peptide from Neu (a class I RTK like the EGF receptor), incorporated into bilayer membranes: they found evidence of dimers with a crossing angle of about 50°. The dimerization motif originally predicted for the human EGF receptor by Gullick and colleagues is T^{624}GMVG^{628}. This is close to the N terminus of the peptides studied here, a location which shows evidence of conformational instability when warmed to 65 °C as discussed above. We suggest that stable dimers of this particular peptide are unlikely to be the predominant form at such a high temperature and that, since our spectral splittings for Ala^{629} and Ala^{637} were largely unchanged between 35 and 65 °C, our calculations are appropriate for a peptide behaving primarily as a monomer. We also suggest that clean Pake spectra indicative of axially symmetric rotation (Figure 2) would be surprising in a side-to-side dimer. Thus, we have in past interpreted the spectral evidence of axially asymmetric peptide rotation which appears with temperature reduction as reflecting dimer and oligomer formation (*15*, *16*). Certain transmembrane peptides and proteins, including glycophorin A, migrate as dimers on SDS−PAGE gels (*49*, *50*). On SDS−PAGE gels, the EGF receptor transmembrane peptide (molecular mass of 4 kDa) migrated with standards with a molecular weight of 4 kDa (our unpublished observation). The implication would be that in SDS micelles the peptides studied here exist as predominant monomers, in agreement with the observations of others on the transmembrane domain of the EGF receptor (*50*). It is interesting that the crossing angle of some 23° generally expected for a left-handed dimer (*51*) would require no change in the peptide tilt of 10−14° measured in this work.

CONCLUSIONS

If one requires that the transmembrane domain of the EGF receptor be a right-handed α-helix of 3.6 residues per turn,

our data strongly imply that the peptide long axis tilts at an angle of between 10 and 14° in fluid membranes of POPC and between 10 and 12° in POPC/cholesterol. Peptide rotation about the membrane perpendicular is axially symmetric and faster than 10^4-10^5 s^{-1} at 35 °C and above. However, rotation about the long axis of the peptide itself is slow or nonexistent on this time scale. There is a preferred rotational position about the peptide long axis; i.e., any given amino acid appears to be nonrandomly oriented with regard to the membrane hydrophobic interior. Lack of rapid rotation about the long axis of an elongated monomeric peptide in a fluid membrane is an unexpected observation. However, for a peptide whose putative hydrophobic length is greater than the hydrophobic thickness of the membranes studied, these findings could be collectively rationalized as reflecting free energy minimization through preservation of side chain hydrophobic and/or hydrophilic environment for amino acids at the membrane surfaces. For a peptide dispersed at 1−6 mol % in fluid bilayers of POPC and POPC/cholesterol, the orientation appeared to be only modestly sensitive to membrane thickness or lipid order. An alternative interpretation of the peptide as a dominant homodimer was seen as less likely. It will be desirable to test the possibility of preferential lean in a monomeric transmembrane peptide, since such an arrangement offers a mechanism whereby a monomeric receptor could regulate the accessibility of a binding site (on steric grounds) without conformational change.

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