Transmembrane Region of the Epidermal Growth Factor Receptor: Behavior and Interactions *via* ^{2}H NMR†

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ABSTRACT: The first wide-line ^{2}H NMR investigation of a receptor tyrosine kinase is reported. Selectively deuterated peptides from the membrane-associated portion of the human epidermal growth factor (EGF) receptor were synthesized for examination in lipid bilayers mimicking certain natural membrane features. The peptide sequence included the 23-amino acid hydrophobic stretch thought to span the membrane (Ile_{622}−Met_{644} of the EGF receptor), plus the first 10 amino acids of the receptor's cytoplasmic domain (Arg_{645}−Thr_{654}). Dispersion of the peptide with lipid in the lipomimetic solvent, trifluoroethanol (TFE), was found to be a very useful initial step for sample preparation. TFE readily dissolved all components and was then easily removed *in vacuo* to yield thin films which could be subsequently hydrated to produce bilayers incorporating homogeneously dispersed peptide. Samples extensively studied consisted of 6 mol % peptide in multilamellar liposomes of 1-palmitoyl-2-oleoylphosphatidylcholine and similar liposomes containing cholesterol. ^{2}H NMR spectra of the resulting unsonicated model membranes indicated the existence of peptide monomers undergoing rapid axially symmetric diffusion. It was possible to examine structural and behavioral effects of events often suggested as pivotal in signaling mechanisms and to consider by wide-line NMR for the first time the effect of cholesterol on hydrophobic peptides. When it was incorporated into bilayers by an alternative method involving dialysis of aqueous solutions prepared using a cationic detergent, spectra suggested that the peptide existed primarily as irreversibly aggregated oligomers which were relatively immobile on a time scale of 10^{-3}−10^{-4} s. For liposomes prepared by hydration of thin films, deuterated methyl groups on the peptide at locations corresponding to Ala_{623}, Met_{644}, and Val_{650} of the human EGF receptor were individually distinguishable. In highly fluid matrices, spectra suggested the presence of peptide monomers, diffusing symmetrically about axes perpendicular to the membrane. Studied as a function of temperature, ^{2}H NMR spectra of such samples permitted independent consideration of membrane/peptide relationships at separate locations in the receptor tyrosine kinase. None of the locations probed demonstrated significant conformational sensitivity to temperature over a wide range. Effects seen at Ala_{623} and Met_{644}, at opposite ends of the putative membrane-spanning domain, suggested slight increases in motional order with decreasing temperature. Addition of 33% cholesterol to the membrane caused little apparent conformational change at Val_{650} or Met_{644}. However, in the presence of the sterol, Met_{644} and Ala_{623} exhibited nonaxially symmetric motion at low temperatures, perhaps as a result of peptide oligomerization. Moreover, the presence of cholesterol led to considerable change in spatial arrangement or order at Ala_{623}. There was little evidence to support transmission of conformational changes along the peptide segment probed.

The molecular basis of signal transduction has become of direct interest to investigators in many fields. In recent years, our laboratory has been attempting to define early events in chains of communication that begin at the cell surface. We have used wide-line ^{2}H NMR spectroscopy to study membrane receptors containing deuterium probes substituted for selected hydrogen nuclei (Barber et al., 1994; Singh et al., 1995; Jones et al., 1996). This is a technique of choice for such work, as it is nonperturbing and highly sensitive to orientational and motional characteristics of molecular subdomains and can be applied to intact membranes (Seelig, 1977; Davis, 1983; Smith, 1984). Thus, in principle, one may assess communication among different regions in macromolecular membrane assemblies by measuring the alteration at one site induced by events at a distant site. The method does however require substantial quantities of material and heteronuclear labeling pathways that are often challenging. In the present work, we have attempted to test the approach on the membrane-spanning portion of the human EGF receptor, the first such study of a receptor tyrosine kinase (RTK).[1]

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[1] Abbreviations: EGF, epidermal growth factor; $EGFR_{tm}$, synthetic EGF receptor transmembrane 34mer; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; DTAB, dodecyltrimethylammonium bromide; TFE, trifluoroethanol; DMPC, 1,2-dimyristoylphosphatidylcholine; RTK, receptor tyrosine kinase; [d_4]Ala_{623}, [d_3]Met_{644}, and [d_8]Val_{650}, deuterated amino acids corresponding to the indicated positions in the human EGF receptor.

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The EGF receptor is an example of class I RTKs (Marchesi, 1986; Brandl et al., 1988; Yarden & Ullrich, 1988; Hollenberg, 1991; Fantl et al., 1993). It is a single polypeptide chain with an M_r of 170 kDa, possessing a glycosylated extracellular ligand binding domain, a single hydrophobic membrane-spanning region, and an intracellular domain containing intrinsic protein tyrosine kinase activity (Ullrich et al., 1984; Carpenter et al., 1991). The cytoplasmic domains of this class of receptor tyrosine kinase have an ATP binding site some 50 residues from the inner membrane surface, and several phosphorylation sites. The transmembrane segments are likely α-helical (Deber & Li, 1995; Smith et al., 1996). EGF binding to the extracellular domain induces a variety of responses, which are to some extent dependent on cell type. One of the most rapid responses is stimulation, within seconds in some preparations, of a tyrosine-specific kinase within the receptor cytoplasmic domain [reviewed in Staros et al. (1985) and Hollenberg (1991)]. This has been hypothesized to be the primary signaling event at the inner surface. It has also become widely accepted that the subsequent chain of events for the human EGF receptor includes phosphorylation of other proteins and also autophosphorylation (Davis, R. J., 1988; Hollenberg, 1991). Little is known on the molecular level about the EGF receptor membrane arrangement and interactions or about the mechanism by which information is transferred to the cytoplasmic surface. This reflects the difficulty involved in isolating individual contributory factors in cells, and even in fragmented cells (Wofsy et al., 1992; Bormann & Engelman, 1992; Fantl et al., 1993).

Wide-line NMR spectroscopy has become a key developmental area (Smith & Peersen, 1992; Cross & Opella, 1994; Henry & Sykes, 1994; Prosser et al., 1994; Opella et al., 1994; Smith & Bormann, 1995). Recent advances in ^{2}H NMR techniques and understanding lay the necessary foundation for the approach taken in the present work [reviewed in Opella (1986), Opella and Stewart (1989), and Cross (1993)]. Replacement of selected protons with deuterons has been applied to model hydrophobic peptides, including a membrane-spanning polyleucine having polar ends (Pauls et al., 1985) and a hydrophobic trimer (Mueller et al., 1986). The bacterial hydrophobic peptide gramicidin A has been a test system for a number of important studies in model membranes. Thus deuteration of amide linkages and alanine residues made it possible to measure orientation and dynamics of the α-helical backbone (Datema et al., 1986; Davis, J. H., 1988; Hing et al., 1990; Prosser et al., 1991, 1994; Lee et al., 1993). Spectra of other deuterated side chains in model peptides and gramicidin can involve more complex interpretations but have great potential to offer additional information related to intermolecular effects (Opella, 1986; Mueller et al., 1986; MacDonald & Seelig, 1988; Killian et al., 1992; Hu et al., 1993; Ketchem et al., 1993; Koeppe et al., 1994; Hu & Cross, 1995; Lee & Cross, 1994; Lee et al., 1995). Bacteriophage coat protein has provided a source of insight into the applicability of ^{2}H wide-line NMR to eukaryote proteins that span the membrane (Shon et al., 1991).

For the present experiments, the peptide, $EGFR_{tm}$, corresponding to residues 621−654 of the human EGF receptor, was synthesized. This comprises the putative transmembrane region (residues 622−644) and a 10-residue stretch (residues 645−654) of the cytoplasmic domain. It thus included Thr_{654}, which is known to be phosphorylated during EGF-mediated signal transduction, perhaps as a regulatory mechanism (Hunter et al., 1984). A biotinylated lysine residue was substituted for the (extracellular) Ser_{621} residue to permit future studies of material selectively immobilized in bilayers. Three different deuterated amino acids were incorporated, conferring the potential to monitor topographically distinct sites within the receptor. Methyl groups were chosen as deuteron locations because of the relatively good signal-to-noise ratio afforded by three equivalent nuclei. Perdeuterated alanine at the position corresponding to Ala_{623} of the human EGF receptor ([d_4]Ala_{623}) and methionine deuterated selectively in the methyl group at the position corresponding to Met_{644} of the human EGF receptor ([d_3]Met_{644}) provided probes within the putative transmembrane region. The former was close to the "extracellular" surface and the latter near the "cytoplasmic" surface. A perdeuterated valine ([d_8]-Val_{650}) was located six residues external to the membrane cytoplasmic surface in the aqueous compartment, occupying the position of Val_{650} in the natural receptor sequence.

MATERIALS AND METHODS

1-Palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) and 1,2-dimyristoyl-3-*sn*-phosphatidylcholine (DMPC) were obtained from Avanti Polar Lipids (Birmingham, AL) and were used without further purification. 9-Fluorenylmethyl *N*-succinimidylcarbonate and cholesterol were from Sigma (St. Louis, MO). Deuteromethyl-L-methionine ([d_3]Met) and perdeuterated L-alanine ([d_4]Ala) were from Isotec (Miamisburg, OH). Perdeuterated L-valine ([d_8]Val) was from Cambridge Isotope Laboratories (Woburn, MA).

FMOC-blocked amino acids were synthesized following standard procedures (Atherton & Sheppard, 1989). Product purity was checked by TLC (Merck, silica gel 60 plates) against an FMOC derivative standard. The $EGFR_{tm}$ peptide was synthesized by Chiron Mimotopes Pty. Ltd. (Clayton, Australia) and the sequence confirmed by mass spectroscopy and amino acid analysis with purity >90−95%. Analytical HPLC was rechecked locally using a Zorbax SB-C18 reversed phase column (4.6 mm inside diameter × 25 cm, 5 μm particle size) run with a gradient of 0.05% trifluoroacetic acid/water to 0.05% trifluoroacetic acid/acetonitrile (2%/min with a flow rate of 1 mL/min) monitoring absorbance at 210 nm.

Liposomes containing 6 mol % $EGFR_{tm}$ were initially prepared by detergent dialysis using dodecyltrimethylammonium bromide (DTAB, Sigma) recrystallized from acetone/methanol. Typically, 20 mg of peptide was combined with 60 mg of POPC in 1 mL of 300 mM DTAB and incubated at 33 °C for 120 h. The clear, viscous solution was diluted with the addition of 1 mL of 300 mM DTAB and returned to 33 °C for several hours. The sample was exhaustively dialyzed over 7 days *vs* 5 mM HEPES buffer (pH 7.0) containing 20 mM NaCl and 5 mM EDTA at 33 °C (total volume of 4 L). The volume of the final sample was reduced *via* vacuum dialysis at room temperature, and the sample was maintained at room temperature until NMR spectra were recorded. To study the effect of freezing, some of the same material was frozen and thawed three times and the NMR spectra were recorded. A similar sample was frozen and repeatedly lyophilized from deuterium-depleted water, prior to hydration with deuterium-depleted water and warming to

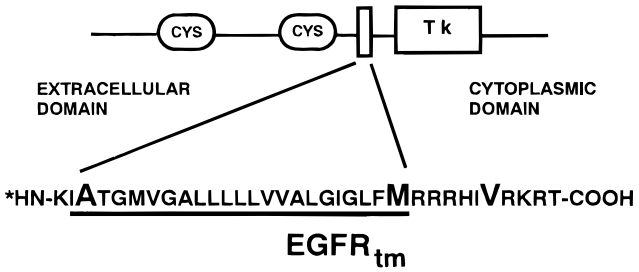

FIGURE 1: Sequence of the 34-amino acid peptide $EGFR_{tm}$ representing residues Ile_{622}−Thr_{654} from the human EGF receptor. A biotin (*)-substituted lysine residue was added (replacing Ser_{621}) at the N-terminus. The putative α-helical hydrophobic transmembrane segment is underlined, and its relationship to the class I receptor tyrosine kinase structure is indicated pictorially above (cystine-rich and tyrosine kinase domains noted). Deuterated amino acids are indicated by bold type (**A** = [d_4]Ala_{623}, **M** = [d_3]Met_{644}, **V** = [d_8]Val_{650}; values in brackets denote the number of deuterons on the amino acid).

45 °C (final buffer concentration of 30 mM HEPES with 20 mM NaCl and 5 mM EDTA at pH 7.1).

Liposome generation by hydration of thin films was according to the following general protocol. TFE (2,2,2-trifluoroethanol, 4 mL, Aldrich, NMR grade, bp 77−80 °C) was added to dry peptide (10 mg), and appropriate amounts of dry lipid were then weighed in and dissolved with warming to 50 °C to produce mixtures in which peptide represented 6 mol % of the phospholipid. Samples were allowed to sit at this temperature for at least 30 min after visually apparent complete dissolution. Solvent was rapidly removed under reduced pressure at 45 °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. Hydration was with 25 mM HEPES (pH 7.1) or 30 mM HEPES with 20 mM NaCl and 5 mM EDTA (pH 7.1−7.3). Samples were warmed to 55 °C without vortexing during hydration.

^{2}H NMR spectra were acquired at 76.7 MHz on a Varian Unity 500 spectrometer using a singly tuned Doty 5 mm solenoid probe, with temperature regulation to ±0.1 °C. A quadrupolar echo sequence (SSECHO from the Varian pulse library) was employed with full phase cycling and a $\pi/2$ pulse length of 5.2 μs. Pulse spacing was typically 30 μs. Molecular modeling was with Insight II (Biosym Technologies, San Diego) using the method of steepest descents and a dielectric constant of 1.

RESULTS

The primary structure and deuteration sites of the peptide, $EGFR_{tm}$, studied in the present work are indicated in Figure 1 [generally accepted membrane-spanning portion underlined (Carpenter et al., 1991)]. The purified peptide was found to be water soluble. However, high-resolution ^{1}H NMR spectra of the aqueous solutions were characterized by extremely broad lines, indicating that the material existed as microaggregates of considerable size, and presumably reflecting oligomerization *via* peptide hydrophobic domains.

We have in the past successfully incorporated receptor glycoproteins with hydrophobic domains into liposome membranes by dialysis of aqueous phospholipid/glycoprotein solutions made with the cationic detergent DTAB (Grant & McConnell, 1974; Ketis & Grant, 1980). Hence, the same approach was tested in dealing with $EGFR_{tm}$. Concern that lipid rigidification or sample freezing might predispose the material to irreversible aggregation of the peptide, or to its extrusion from the membrane, prompted us to avoid low temperatures for liposome preparation and storage. Samples were dialyzed at 37 °C and maintained at room temperature until their wide-line ^{2}H NMR spectra could be run. Subsequently, such samples were also frozen and rewarmed. These variations in sample history were found not to significantly affect the peptide spectral features. Furthermore, liposomes could be lyophilized and rehydrated without spectral alteration.

^{2}H NMR spectra were interpreted employing the following well-documented general approach. Elongated amphiphiles dispersed in fluid membranes tend to undergo rapid symmetric rotation about axes perpendicular to the bilayer. If the molecules in question are deuterated, eq 1 is useful in relating ^{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) (Seelig, 1977; Davis, 1983; Smith, 1984), 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 average orientation of each C−D bond relative to the bilayer normal. For deuterated methyl groups, which undergo rapid rotation about the C−CD_3 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. The latter situation can be dealt with in eq 1 by considering Θ_i to be the angle between the C−CD_3 vector and the molecular long axis and introducing an additional factor of $^1/_3$. The same approach is appropriate to S−CD_3 groups. Thus, for a single CD_3 group directly attached to a completely immobilized peptide backbone above 0 °C, the powder spectrum expected is a Pake doublet with quadrupole splitting approaching 40 kHz (Opella, 1986; Lee & Cross, 1994; Tamura et al., 1996). Conceptually, *via* the S_{mol} term, this value for a given CD_3 group could be further reduced under the conditions of the current experiments by "wobble" of the entire peptide within the membrane, by finite conformational fluctuations of the peptide backbone, and by side chain internal motions. Dominant nonaxially symmetric rotation about an axis [e.g. all 2-fold rotational jumps, and 3 (or higher)-fold jumps with unequal population weighting of available conformers] can give somewhat triangular spectra, having overall widths similar to those predicted for symmetric motions about the same axis (Huang et al., 1980; Opella, 1986; Beshah & Griffin, 1989; Auger et al., 1990; Lee & Cross, 1994).

A number of samples were prepared containing $EGFR_{tm}$ labeled with deuterium on all three of the selected amino acids. It was hoped that the intensity advantage and favorable motional characteristics of the four deuteromethyl groups per peptide would permit assignment of up to four intense Pake doublets in the spectra, which could then be interpreted with recourse to eq 1. However, motions which are slowed to the range 10^3−10^4 s^{-1}, and nonaxially symmetric motions, could shift the intensity from a standard Pake doublet powder pattern into central buildup. Important in the latter category would be peptide that cannot pirouette

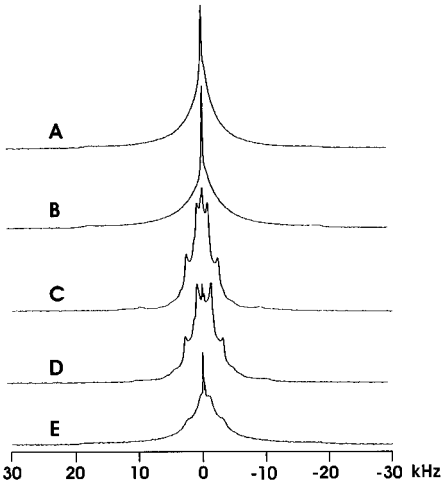

FIGURE 2: Selected ^{2}H NMR spectra for $EGFR_{tm}$ containing all three deuterated amino acids. The peptide:phospholipid mole ratio was 3:50 in all cases, and spectra have been normalized. (A and B) Prepared by detergent dialysis followed by freeze-drying and rehydration; spectra run at 65 and 30 °C, respectively (520 000 accumulated transients). (C and D) Sample and conditions similar to those in A and B, but sample prepared by hydration of thin films dried from the TFE solutions of lipid and peptide. In all samples, the peptide:POPC ratio was 3:50 (150 000 accumulated transients). (E) Spectrum with features reminiscent of those in A and B, but liposomes produced from thin films as in C and D above. The sample was $EGFR_{tm}$ at 12 °C in POPC/cholesterol (150 000 accumulated transients).

rapidly and symmetrically about an axis perpendicular to the membrane, as a result of being part of an oligomer or/and because it is not well-assembled as rod-shaped transmembrane amphiphiles.

Typical wide-line ^{2}H NMR spectra of liposomal structures obtained *via* detergent dialysis from aqueous solution are shown in Figure 2A,B (6 mol % $EGFR_{tm}$ in POPC bilayers). Results are presented for 65 °C and following sample cooling to 30 °C [the fluid−gel phase transition temperature of POPC is −3 °C (Davis & Keough, 1985)]. There is evidence of increased spectral intensity near $\Delta\nu_Q = 36$ kHz. This is close to the 40 kHz value expected for CD_3 groups undergoing no motion other than that associated with axial rotation about the C−CD_3 (or S−CD_3) bond. However, the major spectral feature characteristic of samples prepared by dialysis was the intense, unsplit, broad central peak suggestive of non-axially symmetric motion. Both of these features persisted to high temperatures in spite of the very fluid nature of the membranes involved. In contrast, model peptides and gramicidin with deuterated side chain methyls have been recorded to give axially symmetric Pake spectra when dispersed in fluid bilayers [e.g. Pauls et al. (1985), Mueller et al. (1986), Prosser et al. (1991), Killian et al. (1992), Koeppe et al. (1994), and Hu and Cross (1995)]. Mueller et al. (1986) used deuterated hydrophobic tripeptides to demonstrate that peptide immobilization in membranes can lead to conversion of Pake-doublet-type spectra to immobilized/nonaxially symmetric spectra. Since the ^{2}H NMR spectrum in Figure 2A persisted over the temperature range studied, and given the peptide's aggregated state in aqueous media, the spectrum would appear to be most consistent with $EGFR_{tm}$ being in a highly and irreversibly aggregated state (thus unable to undergo axially symmetric motion) when associated with lipid bilayers by the dialysis procedure described. The bulk of our work was subsequently performed on samples prepared using the thin film approach as described below.

TFE is a "lipomimetic" solvent known to support α-helical arrangement in peptide segments for which this secondary structure is the native form (Nelson & Kallenbach, 1989; Segawa et al., 1991; Dyson et al., 1992; Sönnichsen et al., 1992). $EGFR_{tm}$ dissolved readily in TFE. Peptide backbone amide ^{1}H NMR resonances of $EGFR_{tm}$ in this solvent containing up to 25% H_2O had overall line widths of 16 Hz, indicating that the peptide existed as monomers or very small oligomers. Study of $EGFR_{tm}$ by two-dimensional high-resolution ^{1}H NMR in the same solvent was highly consistent with the presence of α-helical character involving residues Met_{626}−Arg_{647}, and CD spectroscopy demonstrated a shift from unstructured in H_2O to α-helical in TFE (unpublished results). Since the lipids tested in the present work were also soluble in TFE, it provided a logical solvent for achieving molecular codispersions which could be dried to thin films for subsequent hydration. As with the dialyzed structures described above, precautions were initially taken to avoid cooling hydrated bilayers below the host lipid phase transition; however, sample cooling was again found not to irreversibly affect spectral results. Illustrative spectra of $EGFR_{tm}$ in hydrated membranes prepared *via* the TFE solvation approach are included as Figure 2C,D for direct comparison with samples prepared by dialysis. "Pake-type" features typical of molecules undergoing rapid axially symmetric motion in membranes are readily identified. Spectral features more reminiscent of those in Figure 2A,B could however be observed in samples prepared from thin films if peptide motion was severely restricted by very low temperatures and the presence of cholesterol (Figure 2E; see below, Figure 5).

The small sharp peak in the middle of Pake doublet spectra could be separately resolved on an expanded frequency axis into two components (not shown here). One was offset about 0.3 kHz downfield from the powder spectrum midpoint and represents residual HOD; the other, about which the powder pattern is symmetric, reflects the presence of some vesicles with a high curvature for which the quadrupole splittings are motionally averaged to zero. These general features of ^{2}H NMR powder spectra of amphiphiles in liposomes will not be further considered here. Examination of the effects of temperature on spectra of $EGFR_{tm}$ labeled on single amino acids (Figures 3 and 5) made it possible to assign identities to the Pake doublets in Figure 2C,D and to begin to interpret their implications for lipid/peptide interactions. All such studies were performed as a function of decreasing temperature, and spectra have been normalized within each group.

The deuterated alanine residue ($[d_4]Ala_{623}$) in $EGFR_{tm}$ carries a single labeled methyl group and is close to one end of the putative membrane-spanning domain near the peptide amino terminus. Thus, for membranes permitting peptide axial rotation, one might anticipate a single Pake doublet associated with the alanine CD_3 group, whose splitting is particularly sensitive to lipid effects on peptide whole body axial rotation. Figure 3 shows that spectra of POPC bilayers containing $EGFR_{tm}$ with deuterated Ala_{623} only are characterized by an intense Pake doublet whose splitting increased from 4.7 to 6.4 kHz with decreasing temperature (Table 1 and Figure 4). A broader and less intense doublet with a 21 kHz splitting at 65 °C seems likely to represent the α-deuteron. At the lowest temperatures

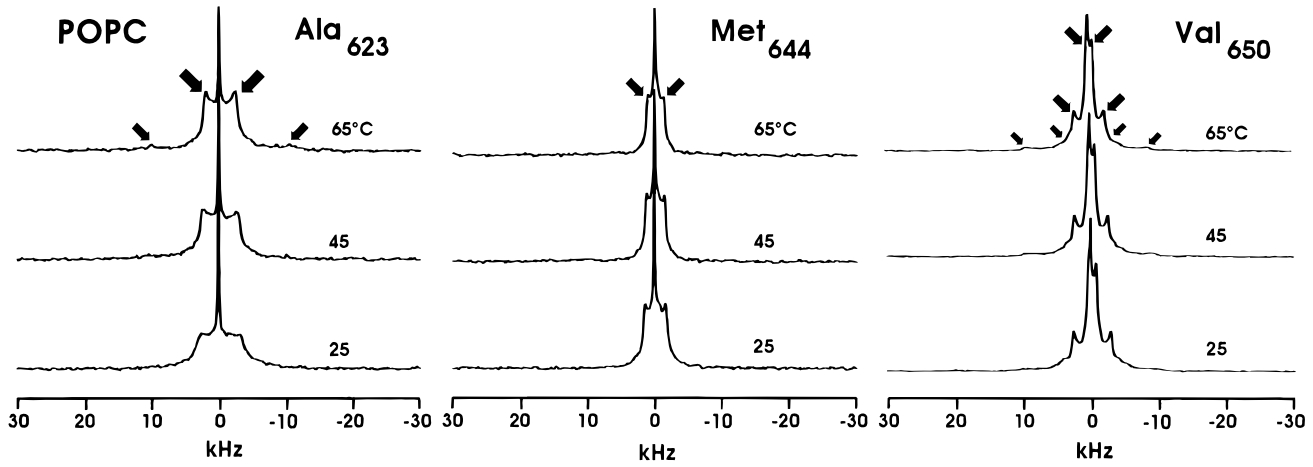

FIGURE 3: Selected stacked ^{2}H NMR spectra corresponding to $EGFR_{tm}$ having only single deuterated amino acids in POPC bilayers. The peptide:POPC mole ratio was 3:50. Peptide was assembled into unsonicated membranes by hydration of films dried down from TFE solution. From left to right: alanine ([d_4]Ala_{623}) (large arrows in the top spectrum indicate the location of the Pake doublet assigned to the CD_3 group, while smaller arrows indicate the apparent location of the Pake doublet associated with the α-deuteron; there is some axial asymmetry at low temperatures), methionine ([d_3]Met_{644}) (arrows in the top spectrum indicate the location of the Pake doublet assigned to the CD_3 group; the central region suggests some degree of nonaxially symmetric motion over the full temperature range, likely associated with side chain internal flip averaging), and valine ([d_8]Val_{650}) (large arrows in the top spectrum indicate the location of the two Pake doublets assigned to the two CD_3 groups, while smaller arrows indicate possible locations of Pake features associated with the α- and/or β-deuterons). All spectra were recorded as a function of decreasing temperature and are normalized within each stack. Each spectrum represents 150 000 accumulated transients (Ala) or 200 000 transients (Met and Val).

Table 1: ^{2}H NMR Spectral Splittings ($\Delta\nu_Q$) Corresponding to Deuterated CD_3 Groups in $EGFR_{tm}$[a]

deuterated amino acid	spectral splittings ($\Delta\nu_Q$) (±0.2 kHz)					
	12 °C	25 °C	35 °C	45 °C	55 °C	65 °C
[d_4]Ala_{623}	**nd**	**6.4**	**6.0**	**5.6**	**5.1**	**4.7**
	−	9.4	8.8	8.9	nd	8.3
[d_3]Met_{644}	**nd**	**3.4**	**3.1**	**2.9**	**2.8**	**2.6**
	5.0	4.3	3.8	3.6	nd	2.9
[d_8]Val_{650} inner splitting	**nd**	**1.3**	**1.2**	**1.2**	**1.1**	**1.1**
	1.0	0.8	0.7	0.7	nd	0.7
[d_8]Val_{650} outer splitting	**nd**	**5.8**	**5.4**	**5.2**	**4.9**	**4.7**
	6.1	5.6	5.2	4.9	nd	4.4

[a] The upper number in each row: peptide assembled into POPC bilayers without cholesterol (bold numbers). The lower number is for POPC bilayers containing 33 mol % cholesterol. All samples were prepared by hydration of films dried down from TFE solution, studied as a function of decreasing temperature. Data correspond to spectra in Figures 3 and 5. The quoted estimated uncertainty reflects the typical maximal variability found for samples prepared on different occasions and the interobserver variability.

studied (but still well above the −3 °C phase transition temperature of POPC), there is a distinct change in line shape as intensity shifts more centrally to produce an apparent nonaxially symmetric spectrum. Measured spectral splittings for a complete range of temperatures are listed in Table 1 and plotted in Figure 4.

The CD_3 group on methionine ([d_3]Met_{644}) is at the opposite (COOH-terminal) side of the hydrophobic, putative transmembrane region (Figure 1). Selected spectra of $EGFR_{tm}$ deuterated only at this location are shown for direct comparison with the above alanine spectra in Figure 3. Each consists of a single Pake doublet with centrally peaked infilling. There was very little change in the spectral shape over the complete temperature range examined. The doublet splitting increased from 2.6 to 3.4 kHz with decreasing temperature.

[d_8]Val_{650} is in the cytoplasmic portion of the peptide, six amino acid residues from the putative membrane surface. The two CD_3 groups of a given valine residue in a peptide with secondary structure, undergoing rapid rotation within a membrane, should in general be spectrally distinct (Lee & Cross, 1994). Typical results for $EGFR_{tm}$ deuterated only at this location are included in Figure 3. As anticipated, two intense Pake doublets are apparent for the CD_3 groups. Their line shapes remained very much unchanged over the range of temperatures studied. Quadrupole splittings increased modestly, from 1.1 to 1.3 kHz for the inner doublet and from 4.7 to 5.8 kHz for the outer doublet as temperature was decreased. As for [d_4]Ala above, there was some evidence of spectral intensity that seems likely to be associated with the α-deuteron and/or in this case with the additional presence of a β-deuteron; this was most apparent at higher temperatures [see Lee at al. (1995)].

Figure 5 illustrates the effect of cholesterol incorporation on spectra of $EGFR_{tm}$ in POPC. It will be seen that for the more fluid membranes (i.e. at the higher temperatures studied) the qualitative features described above for sterol-free membranes are preserved. Quantitative effects may be appreciated from Table 1 (upper number in each row is without cholesterol) and by comparing the POPC to POPC/cholesterol plots in Figure 4. Clearly, the measured [d_8]-Val_{650} spectral features were affected little by cholesterol at any temperature. [d_3]Met_{644} showed some increase in spectral splitting in cholesterol-containing membranes and increased spectral asymmetry at the lowest temperatures which had not been noted in the absence of cholesterol. The [d_4]Ala_{623} splitting almost doubled with the addition of cholesterol, and its spectrum largely disappeared at the lowest temperature. Spectral disappearance has been reported in deuterated lipids for motions slowed to the intermediate time scale (approximately 10^4 s^{-1} in this case) (Haberkorn et al., 1977; Oldfield et al., 1978; Griffin, 1981; Meier et al., 1986; Renou et al., 1989; Hamilton et al., 1994).

To begin to widen our understanding of RTK sensitivity to local molecular events, a system having 1,2-dimyristoylphosphatidylcholine (DMPC) as the host matrix phos-

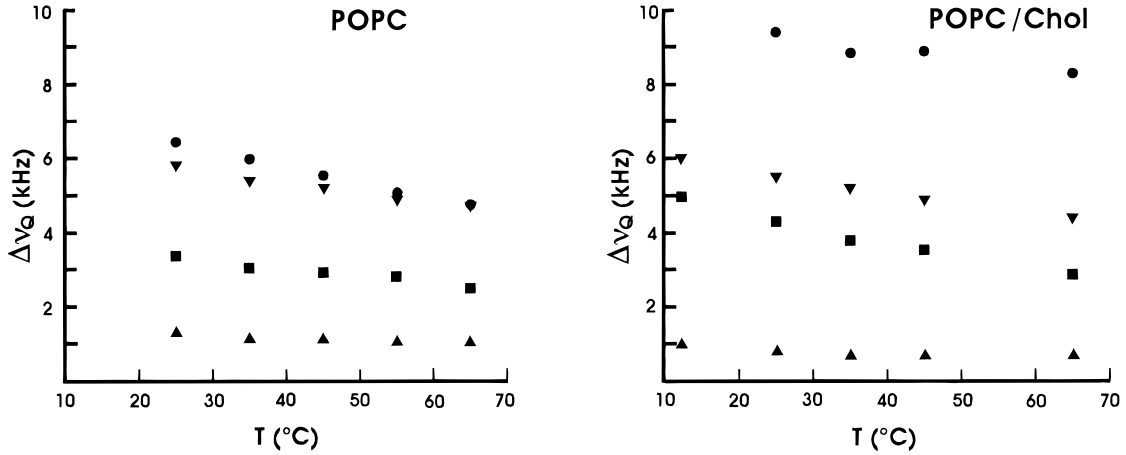

FIGURE 4: Plots of quadrupolar splitting *vs* temperature for $EGFR_{tm}$ in POPC bilayers (A) and in POPC bilayers containing 33 mol % cholesterol (B) (values shown in Table 1). ▼ and ▲ have been used to indicate splittings for the outer and inner Val−CD_3 Pake doublets, respectively; ■ denotes the Met−CD_3 splitting, and ● represents Ala−CD_3. All data were recorded as a function of decreasing temperature. The peptide:phospholipid mole ratio was 3:50.

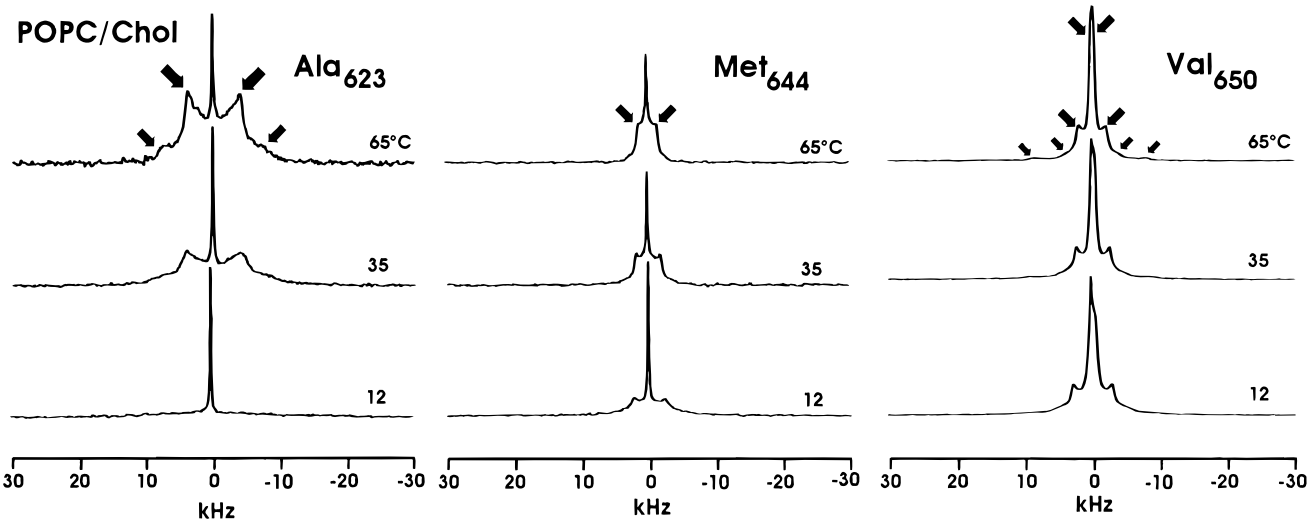

FIGURE 5: Cholesterol effects on ^2H NMR spectra of $EGFR_{tm}$ having only single deuterated amino acids, for direct comparison to Figure 3. Peptide was assembled into POPC/33 mol % cholesterol bilayers by hydration of films dried down from TFE solution; the peptide:POPC mole ratio was 3:50. From left to right: alanine ([d_4]Ala_{623}) [large arrows in the top spectrum indicate the location of the (single) Pake doublet assigned to the CD_3 group, while smaller arrows indicate a possible location of the Pake doublet associated with the α-deuteron; there is marked intensity loss at low temperatures], methionine ([d_3]Met_{644}) (arrows in the top spectrum indicate the location of the Pake doublet assigned to the CD_3 group; the central region suggests some degree of nonaxially symmetric motion over the full temperature range, likely associated with side chain internal flip averaging, and there is some intensity loss at low temperatures), and valine ([d_8]Val_{650}) [large arrows in the top spectrum indicate the location of the (two) Pake doublets assigned to the two CD_3 groups, while smaller arrows indicate possible locations of Pake features associated with the α- and/or β-deuterons].

pholipid was compared to that with 1-palmitoyl-2-oleoylphosphatidylcholine, while maintaining a cholesterol content reflective of plasma membranes. $EGFR_{tm}$ with all three deuterated amino acids was employed (Figure 6). DMPC has only 14-carbon saturated fatty acids, while POPC contains only 16- and 18-carbon acyl chains (of which the latter is monounsaturated). The phase transition temperature is 23 °C for pure DMPC (Shimshick & McConnell, 1973) *vs* −3 °C for POPC. A notable aspect of Figure 6 is the similarity of the spectra obtained at any given temperature, in spite of the different fatty acids and the different phase transition temperatures of the host phospholipids. Nevertheless, there are apparent quantitative spectral differences which in principle can be related to conformational and motional distinctions.

Although the phenomenon was not systematically studied in the present work, it has been our experience that lowering the peptide concentration in the membrane altered spectral appearance toward that characterizing higher peptide concentrations at higher temperatures. An example of this is included in Figure 6: lowering the $EGFR_{tm}$ concentration to 1 mol % based on total lipid (lowest left-hand spectrum) produced a spectrum at 12 °C which more resembled the Pake doublet shape and measured values seen for 4 mol % peptide at 35 °C (stacked spectra in the left-hand column above it).

DISCUSSION

We are not aware that trifluoroethanol (TFE) has been used previously for preparing wide-line NMR samples of transmembrane peptides. Hydration of thin films, produced by evaporation of lipid/peptide mixtures in this solvent, proved to be a very convenient method for generating unsonicated bilayer membranes into which a hydrophobic, membrane-spanning portion of the EGF receptor was incorporated in dispersed and uniform fashion. ^2H NMR spectral charac-

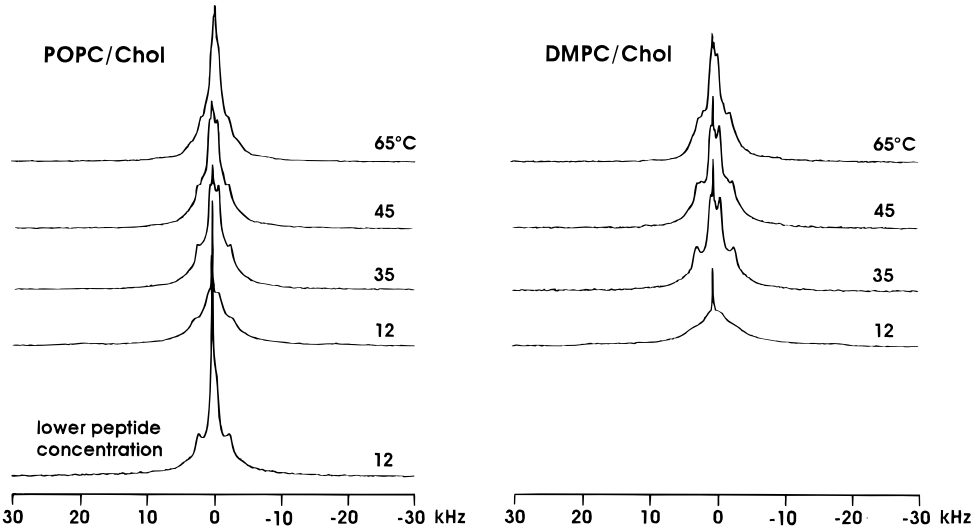

FIGURE 6: ^{2}H NMR spectra corresponding to $EGFR_{tm}$ having all three deuterated amino acids, assembled into POPC/cholesterol (left-hand column) and DMPC/cholesterol bilayers (right-hand column). Samples made by hydration of films dried down from TFE solution. Each spectrum represents 150 000 accumulated transients. The peptide:phospholipid mole ratio was 3:50. The bottom left-hand spectrum shows the effect of decreasing peptide content by a factor of 4.

teristics of this system were highly stable and independent of sample history. TFE carries the additional benefit that it is a widely preferred lipomimetic solvent, generally considered to support naturally occurring α-helical secondary structure while contributing minimally to generation of inappropriate secondary structural elements (Nelson & Kallenbach, 1989; Segawa et al., 1991; Dyson et al., 1992; Sönnichsen et al., 1992).

At (and above) physiological temperatures, ^{2}H NMR spectra were obtained for $EGFR_{tm}$ which displayed distinct features attributable to the three different deuterated amino acid residues in the peptide sequence. These spectra could be understood in terms of concepts derived from the extensive earlier work of others on different systems. Thus, Pauls et al. (1985) incorporated a deuterated α-helical polyleucine 24mer with several polar residues at each end into DPPC bilayers, *via* an approach similar to that used in the present work. They employed the organic solvent mixture 50:1 $CH_3OH/CHCl_3$. Pauls et al. concluded that ^{2}H NMR wide-line spectra of their peptide reflected the presence of α-helical structures spanning the bilayer membrane and demonstrated high sensitivity to peptide rigid-body rotational diffusion. They estimated peptide rotational correlation times of 2×10^{-7} s in phospholipid host matrices above their phase transition temperatures and motions slower than 10^{-5} s below T_t. MacDonald and Seelig (1988) obtained highly consistent results for gramicidin A with deuterium-labeled tryptophan side chains, noting effects of peptide/peptide side-to-side interaction at higher peptide concentrations in DMPC membranes. Lee et al. (1993) studied gramicidin A bearing deuterated alanine in DMPC bilayers. They found that the measured correlation time of 3.6×10^{-7} s for the peptide in fluid membranes was longer than they had expected and concluded that this might reflect the formation of lipid/peptide aggregates.

In the present work, one intense Pake doublet was identified for each CD_3 group of the deuterated amino acids in $EGFR_{tm}$. Other, less intense, spectral features were readily accounted for by the C_α and C_β deuterons in [d_8]Val and by the C_α deuteron of [d_4]Ala. These non-methyl features were not extensively considered here but could prove very valuable for future studies. Lee et al. (1995) have noted though that the intensity of such resonances can be reduced due to unfavorable relaxation properties. Our observation that each nonequivalent deuteron location produced (only) one Pake doublet argues that either there is a single physical arrangement for $EGFR_{tm}$ under a given set of conditions or if there is more than one arrangement then these are interconverting rapidly on the experimental time scale of some $10^{-3}-10^{-4}$ s. Taken together, our results suggest that, in the physiological temperature range, $EGFR_{tm}$ monomers undergoing rapid axial diffusion about their long axes in the membrane exist. Reversible side-to-side dimerization or oligomerization likely gives rise to a subpopulation exhibiting more significant nonaxially symmetric motions and/or slower axial diffusion. This latter aspect was reflected in broadening of Pake features and in increasingly nonaxially symmetric spectral shapes at low temperatures. Peptide aggregates, and peptide simply associated with the membrane surface, would be expected to give rise to less discrete spectral features, as seen for the samples prepared by detergent dialysis, due to loss of rapid axially symmetric motion about axes perpendicular to the plane of the membrane.

Examination of $EGFR_{tm}$ spectra as a function of temperature permitted several important comparisons. A variety of distinct phenomena determine spectral shape. In particular, eq 1 describes quadrupole splitting in terms of a C−D bond motional order term (S_{mol}) and a spatial orientation term ($3 \cos^2 \Theta_i - 1$). In general, for fluid membranes, molecular order is expected to increase as temperature is reduced and as surrounding lipid becomes more organized. Thus, for POPC bilayers [phase transition at −3 °C (Davis & Keough, 1985)], there was a very modest linear increase in spectral splittings for each of the CD_3 groups with reduction in temperature. This likely reflects a small increase in S_{mol} for the peptide, brought about by decreasing thermal energy and increasing lipid order. The slight downward slope of the plots in Figure 4 is most noteworthy for its proportionate constancy among the different locations probed, even comparing probes inside and outside the hydrophobic domain. This similarity in slope extended to membranes containing cholesterol (see below). Such a result argues that

the sites probed retained their average conformations and orientations over a wide range of temperatures and associated variations in lipid fluidity and that the peptide behaved as a conformational unit.

Since, of the three deuterated residues, only [d_8]Val_{650} was outside the putative membrane-spanning region, its mobility might be expected to be the least affected (constrained) by the state of the lipid. However, our high-resolution NMR study in TFE/H_2O indicated that the α-helical region extended as far as Arg_{647} so that we did not anticipate a high degree of peptide backbone internal mobility at [d_8]Val_{650}. Spectral inequivalence of the two side chain CD_3 groups was encountered, resulting from different average orientations relative to the axis of peptide rotation (Opella, 1986). Beshah and Griffin (1989) and Lee and Cross (1994) have published ^2H NMR analyses of valine side chain motion for pure valine and for valine residues in gramicidin, respectively. Rotation about $C_\alpha-C_\beta$ was found by these workers to be among three unequally populated sites, with no additional large amplitude motion. It was in the fast exchange limit for fluid membranes but was measurably slowed below the host matrix phase transition in pure DMPC bilayers (Lee & Cross, 1994). Their spectra for deuterated methyl groups of valine residues in gramicidin dispersed in fluid DMPC bilayers were very similar to those described in the present work.

As indicated above, temperature responsiveness at the location, Val_{650}, was minimal. Met_{644}, whose deuteromethyl group is within the putative hydrophobic region, showed parallel (lack of) responsiveness to temperature in POPC bilayers. Tamura et al. have analyzed the methyl group motion for methionine residues in aqueous solutions of the protein *Streptomyces* subtilism inhibitor (1996) [and see Opella (1986)]. They were able to approximate these motions by modeling the $S-CD_3$ group as if it were attached to a leash which described a cone in space. Tamura et al. further noted that the degree of mobility depended on the location of methionine within the protein, the hydrophobic interior location showing high immobilization. Opella (1986) has demonstrated experimentally and theoretically that rotational (nonaxially symmetric) "flips" about the methionine side chain internal bonds can produce a less defined (unsplit) triangular central region in Pake doublet spectra [e.g. 2-fold flips about the $C-SCD_3$ bond; see also Huang et al. (1980) and Auger et al. (1990)]. This latter phenomenon was well-exhibited in the present work, which demonstrated a line shape very reminiscent of that described by Opella (1986) [see also Tamura et al. (1996)].

[d_4]Ala_{623} possesses a CD_3 group attached directly to the peptide backbone. The potential of this deuteron location for studies of membrane-associated peptide dynamics has been discussed by Lee et al. (1993) [see also Beshah and Griffin (1987)]. The splitting associated with the Ala_{623} CD_3 group was only marginally more sensitive to temperature than were those of Val_{650} or Met_{644} (note the slightly steeper slope associated with the Ala spectral splitting in Figure 4). Its spectrum did however show markedly greater alterations in line shape than did those of the other amino acids, changing from axially symmetric to clearly nonaxially symmetric as the temperature dropped below the physiological range. This phenomenon was repeated in cholesterol-containing membranes (see below). As mentioned above, in TFE/H_2O solutions, the α-helical stretch of $EGFR_{tm}$ commenced shortly after Ala_{623} (at Met_{626}). Thus, as in the case of Val_{650}, at Ala_{623}, the peptide backbone should not exhibit high internal mobility. Our wide-line NMR result seems very likely to reflect the fact that, of the three amino acids deuterated in the present work, motions of the Ala_{623} side chain are the most completely dependent on peptide whole-body axial rotational diffusion [see also Lee et al. (1993)].

In $EGFR_{tm}$, the Ala_{623} residue was very close to the NH_2 terminus and to the biotin residue. Both of these factors may have contributed to the fact that our high-resolution ^1H NMR study in TFE/H_2O did not demonstrate α-helicity at this site. In a related vein, Smith et al. (1994) concluded that α-helical secondary structure in a 30mer corresponding to the hydrophobic transmembrane domain of glycophorin in DMPC likely "unraveled" near the membrane surface. When $EGFR_{tm}$ was modeled as a perfect α-helix, an angle approaching 125° was measured between the $C-CD_3$ bond and the peptide long axis (an angle of 54° was measured for C_α-D). These relationships to the peptide long axis are near the magic angle. Hence, for rapid rotation of $EGFR_{tm}$ in α-helical form, about its long axis oriented 90° to the plane of the membrane, spectral splittings approaching 0 kHz should be observed for [d_4]Ala_{623} (eq 1). Measured quadrupole splittings for this alanine deuteromethyl group in POPC bilayers were 5−6 kHz (maximum possible value is about 40 kHz). The addition of cholesterol increased the observed values by 50−80% (see below). The latter would seem at first sight to indicate significant deviation from α-helical secondary structure at this location. However, it has been pointed out that hydrophobic transmembrane domains may well have an axis tilt of 20−25° to the membrane perpendicular (Arkin et al., 1995), as noted by Smith et al. (1996) for a neu/erbB-2 peptide. If this is the case, our observed splittings are consistent with $EGFR_{tm}$ adopting an α-helical form at Ala_{623} in the presence of lipid, although our data certainly do not require such an arrangement.

To our knowledge, NMR spectroscopy has not been used previously to study peptides in membranes containing cholesterol, a critical component of higher-animal cell plasma membranes. Phospholipids in fluid membranes are generally considered elongated surface-fixed amphiphiles that undergo rapid rotational diffusion about their long axes. The effect of cholesterol on fluid membrane lipids is to leave whole-body rotational diffusion largely unaltered while greatly decreasing acyl chain flexibility (Oldfield & Chapman, 1972; Demel & De Kruyff, 1976; Yeagle, 1985; Vist & Davis, 1990; McMullen & McElhaney, 1995). A peptide transmembrane α-helical domain is very similar in cross-sectional dimensions to a phospholipid and is seen as undergoing analogous rotational diffusion. Thus, it is noteworthy that in the present work there was close similarity in spectral splittings for [d_3]Met_{644} in membranes with and without cholesterol. For instance, the presence of cholesterol increased the quadrupole splitting from 2.6 to 2.9 kHz at 65 °C and from 3.4 to 4.3 kHz at 25 °C. Clearly, however, the presence of cholesterol reduced the motional symmetry of Met_{644} at low temperatures. For [d_8]Val_{650}, the effect of cholesterol was to marginally reduce both outer and inner quadrupole splittings, by an amount that was essentially within experimental error (Figure 4 and Table 1), and there was no effect on spectral symmetry. One must conclude that there was little cholesterol influence on peptide confor-

mation at these locations, but there was some influence on motional symmetry at low temperatures.

In contrast, with addition of cholesterol, the CD_3 group splitting of [d_4]Ala_{623} increased from 4.7 to 8.3 kHz at 65 °C and from 6.4 to 9.4 kHz at 25 °C. Without being able to confidently assign the weaker alanine α-deuteron resonances, we could not determine whether this effect was primarily due to a change in peptide conformation or order. It is tempting to suggest a conformational rearrangement at the NH_2 terminus of the peptide (perhaps toward α-helix formation near the membrane surface), since modest or no ordering effects by cholesterol were seen at the other deuterated sites, and only modest temperature-induced ordering effects were seen at any of the deuterated sites. The observation that the changes seen at this site were not transmitted to distant deuterated sites is similar to our experience with deuterium-labeled complex oligosaccharide chains attached to glycosphingolipids in fluid membranes (Barber et al., 1994; Singh et al., 1995).

The phenomenon of disappearance of spectral features upon lowering of membrane temperature has been recorded previously for deuterated lipids whose rigid-body rotational diffusion slows to the time domain of $10^{-4}-10^{-5}$ s (Haberkorn et al., 1977; Oldfield et al., 1978; Meier et al., 1986; Renou et al., 1989; Hamilton et al., 1994). In the present work, spectral disappearance was observed for [d_4]Ala_{623} in POPC/cholesterol at low temperatures, and to a lesser extent for [d_3]Met_{644} (Figure 5). This was somewhat unexpected since, as mentioned above, cholesterol is generally seen as leaving rotational diffusion rates of lipids largely unaffected in fluid membranes [and the temperature range investigated was well above the phase transition temperature of POPC [see also Thewalt and Bloom (1992)]]. Interestingly, cholesterol has been suggested to lead to reduced motional freedom of the integral membrane glycoprotein, band 3, by causing it to aggregate (Muhlebach & Cherry, 1982). The possibility of specific interactions between sterols and membrane proteins has been discussed in detail (Yeagle, 1985; Tampé et al., 1989). Peptide oligomerization at low temperatures in the cholesterol-containing membranes would certainly adequately explain the unexpectedly large peptide immobilization seen here, and this interpretation was borne out by reduction of peptide concentration (Figure 6 and unpublished observations). Increased peptide oligomerization might result from reduced lateral diffusion rates if there were significant interpeptide attractive forces. Indeed, Siminovitch et al. (1988) have suggested that cholesterol can reduce lipid lateral diffusion in fluid bilayers. It would be surprising if there were not frequent peptide/peptide collisional events in the systems studied here, since they contained 4−6 mol % peptide. Clearly though, as mentioned above, if there is a tendency for the membrane-spanning portion of $EGFR_{tm}$ to self-associate, the process is rapidly reversible on the NMR time scale of 10^{-4} s. A somewhat semantic alternative is that peptide oligomerization is driven by a tendency to be excluded (phase separated) from the surrounding bilayer matrix. It is interesting that Jans et al. (1989) have noted greatly decreased (vasopressin) receptor mobility in membranes, not attributable to a membrane phase transition, below physiological temperatures [see also Jans (1992)].

The shift in spectral shape from Pake-doublet-type to triangular, suggested in the present work to be associated with oligomerization and resultant slowed/asymmetric motion, has been described by Mueller et al. (1986). They found the same phenomenon for a hydrophobic tripeptide with deuterated tyrosine in DPPC bilayers upon cooling through the lipid phase transition temperature. They concluded that the effect arose from restriction of peptide reorientation. A phenomenon apparently closely analogous to that seen in the present work is found in spectra recorded by MacDonald and Seelig (1988) for deuterated gramicidin A in fluid DMPC bilayers. They recorded a shift toward central buildup and loss of Pake features in association with an increased peptide:lipid ratio (from 1:15 to 1:10) under conditions of side-to-side peptide oligomerization.

Interaction with lipid is thought to be an important determinant of protein structure within membranes (Wooley & Deber, 1987; Cramer et al., 1992; Deber & Li, 1995). Apart from the cholesterol and temperature effects described, an initial experiment was performed in which the host phospholipid was switched from POPC (16- and 18-carbon acyl chains of which the latter is monounsaturated) to DMPC (all 14-carbon saturated fatty acids). The phase transition temperature of pure DMPC bilayers is 23 °C (Shimshick & McConnell, 1973) vs −3 °C for POPC. $EGFR_{tm}$ with all three deuterated amino acids was used, and peak assignment in the DMPC-rich matrix was not proven. It is interesting though that spectra of $EGFR_{tm}$ in the DMPC/cholesterol host matrix were close to being superimposable on those in POPC/cholesterol at a given temperature (Figure 6). The modest nature of host matrix effects on $EGFR_{tm}$ conformation and order is in keeping with the suggestion by Mouritsen and Bloom (1993) that lipid/peptide interactions in the membrane hydrophobic interior may be minimized in fluid membranes.

One may briefly consider how the current observations reflect upon possible mechanisms of signal transduction. An important theory has been that ligand binding to a receptor extracellular domain induces a conformational change, which in turn results in transmission of a conformation-altering "force" *via* the transmembrane segment to the cytoplasmic domain [e.g. Staros et al. (1985), Gill (1990), Bormann and Engelman (1992), and Fantl et al. (1993)]. Doubts have been expressed that external binding events produce sufficient force to induce conformational change at a distant site [Bormann & Engelman, 1992; but see Otoda et al. (1993)]. A variant of this mechanism suggests that signaling *via* transmitted conformational change would depend upon association of the receptor with another membrane protein (perhaps other copies of the same receptor). A second major candidate for an initial physical trigger in signaling is altered intermolecular association of the receptor (e.g. formation or dissociation of dimers). There is of course no need to exclusively adopt either theory. Our observations appear to favor mechanisms that involve signaling by changes in association or dynamics, rather than by conformational sensitivity.

Receptor association and dynamics at the cell surface are not known to an accuracy of molecular dimensions. In unstimulated intact cells, EGF receptors are widely held to be randomly distributed (Schlessinger, 1988; Jans, 1992) and perhaps subject to a process of reversible dimerization that is fundamental to activation [reviewed in Bormann and Engelman (1992) and Fantl et al. (1993)]. Involvement of the hydrophobic domain in dimerization for class I RTKs has been specifically claimed (Bormann et al., 1989). Dimerization as an activating event for the EGF receptor is

a well-known postulate. However, this has been difficult to prove categorically [Spaargaren et al., 1990; Wofsy et al., 1992; Mohammadi et al., 1993; see also Hynes and Stern (1994)]. Indeed, the possibility that EGF binding in fact leads to reversal of receptor dimerization has also been raised [Biswas et al., 1985; reviewed in Bormann and Engelman (1992)]. It would appear that the great majority of EGF receptors, in the widely studied A431 cultured cell line, are low-affinity and highly mobile, while the high-affinity subpopulation does not diffuse rapidly over distances defined by the limitations of fluorescence photobleaching experiments (thousands of angstroms) (Schlessinger et al., 1978; Rees et al., 1984). Thus, EGF receptors involved in physiological stimulatory events have been referred to as "immobile" (over distances of several thousand angstroms), while allowing for possibly rapid movement over shorter distances (Jans, 1992). The binding of EGF to its receptor is generally seen as 1:1, with some question as to the role of cooperativity (Wofsy et al., 1992; Bormann & Engelman, 1992). Our results suggest that, at least in the presence of cholesterol, the transmembrane segment was involved in reversible oligomer formation, which became more pronounced below physiological temperatures. Apparently, this associative effect had very little influence on orientation or order at any of the sites probed since their spectral splittings were notably insensitive to temperature, which has a strong effect on association.

In viewing the present work, the one factor that clearly communicated across the membrane was the rotational diffusion rate of the peptide. One might speculate that, for an intact receptor, immobilization by a macromolecular binding event at the surface could very much alter axial rotation of distant domains, perhaps determining their ability to translate weak associative forces into long-lived and enzymatically functional interactions.

CONCLUSIONS

^{2}H wide-line NMR spectroscopy has considerable potential for testing models of eukaryote signal transduction at the membrane level. CD_3 groups offered the combined advantages of spectral interpretability and good signal-to-noise ratio. Dialysis of lipid/peptide solutions prepared using a cationic detergent was not a successful method in our hands for dispersing the hydrophobic peptide in bilayer membranes. However, the α-helix-stabilizing lipomimetic solvent, TFE, proved to be very satisfactory for producing thin lipid/peptide dry films for subsequent hydration. The transmembrane domain of the EGF receptor incorporated into bilayer membranes could be studied in apparent monomer/oligomer equilibrium. It was possible in the present experiments to simultaneously probe three topographically distinct locations within membrane-associated regions of a receptor tyrosine kinase. The deuterated alanine residue was particularly sensitive to peptide rigid-body diffusion. The methionine CD_3 exhibited intrinsically asymmetric motion but offered another well-characterized monitor of lipid environment. Extramembranous valine CD_3 groups provided insight into the degree of communication between the hydrophobic transmembrane portion and the cytoplasmic domain.

In fluid membranes, peptide conformation and orientation were notably insensitive to temperature and associated variations in lipid fluidity over a wide range. Addition of cholesterol reduced the rate of peptide axial diffusion at temperatures below the physiological level, likely as a result of increased reversible oligomerization. Peptide conformation and order at Val_{650}, the sixth residue from the putative cytoplasmic membrane surface, and at Met_{644} were largely unaffected by the addition of cholesterol. The alanine residue near the opposite surface was significantly altered in conformation and/or order. The only "signaling event" that was certainly transmitted from the intramembrane region to the cytoplasmic domain was rotational immobilization.

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