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# Sequence-related behaviour of transmembrane domains from class I receptor tyrosine kinases

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**Abstract**

$^2$H NMR spectroscopy and freeze-fracture electron microscopy were used to compare the transmembrane domains of two Class I protein receptor tyrosine kinases (the EGF receptor and Neu/erbB-2) regarding overall behaviour in fluid lipid bilayer membranes. The 34-residue peptide, $EGFR_{tm}$, was synthesised to contain the 23 amino acid hydrophobic stretch ($Ile_{622}$ to $Met_{644}$) thought to span the membrane of the human EGF receptor, plus the first 10 amino acids ($Arg_{645}$ to $Thr_{654}$) of the cytoplasmic domain. Deuterium probes replaced selected $^1$H nuclei at sites corresponding to $Ala_{623}$, $Met_{644}$, and $Val_{650}$. The 38-residue peptide, $Neu_{tm}$, was synthesised having the 21 residue hydrophobic stretch ($Ile_{660}$ to $Ile_{680}$) calculated to span the membrane in rat Neu/erbB-2, plus residues $Lys_{681}$ to $Thr_{691}$ of the contiguous cytoplasmic domain. Deuterium probes replaced selected $^1$H nuclei at $Ala_{661}$, $Leu_{667}$, and $Val_{676}$. A third peptide, $Neu_{tm}$ *, was also prepared, corresponding to the transmembrane domain of a constitutively-activating Neu/erbB-2 transformant in which $Val_{664}$ is replaced by Glu: it was deuterated in a manner identical to $Neu_{tm}$. Peptides were studied by $^2$H NMR spectroscopy at 1 mol% and 6 mol% in unsonicated fluid bilayers of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and in POPC containing 33 mol% cholesterol, over the range 12° to 65°C. Overall motion was found to be different for each of the three peptides under a given set of conditions. $EGFR_{tm}$ spectra were characteristic of axially symmetric motion in membranes of POPC alone, and in POPC/cholesterol at 35°C and above. In contrast, spectra of the transmembrane peptides, $Neu_{tm}$ and $Neu_{tm}$ *, were characteristic of significantly axially asymmetric motion under all conditions studied (and regardless of sample preparation method). Addition of 33% cholesterol to membranes was accompanied by spectral changes consistent with increased formation of peptide dimers/oligomers in all cases. The transformant peptide, $Neu_{tm}$ *, showed greater spectral evidence of immobilisation than did the wild type - probably reflecting a greater tendency to form large oligomers. Sequence-related details within the transmembrane domains of Class I receptor tyrosine kinases appear to exert important control over their associations within membranes. Freeze-fracture electron microscopy of the NMR samples demonstrated their liposomal nature. Peptide-related intramembranous particles (IMPs) were present which likely represent oligomers of the transmembrane peptide. IMP size and distribution were similar under a given set of conditions for all three peptides,

Abbreviations: RTK, receptor tyrosine kinase; EGF, epidermal growth factor; $EGFR_{tm}$, 34-amino-acid peptide corresponding to the human EGF receptor transmembrane domain plus 10 residues of the cytoplasmic domain; $[d_4]Ala_{623}$ $[d_3]Met_{644}$ $[d_8]Val_{650}$, deuterated amino acids corresponding to the indicated positions in the human EGF receptor; Neu, rat protein also known as Neu/erbB-2 or Neu/HER2; $Neu_{tm}$, synthetic transmembrane 38 mer from Neu; $[d_3]Ala_{661}$ $[d_3]Leu_{667}$ $[d_8]Val_{676}$, deuterated amino acids corresponding to the indicated positions in Neu; $Neu_{tm}$ *, $Neu_{tm}$ having $Val_{664}$ replaced with Glu—an oncogenic mutation; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; TFE, trifluoroethanol

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suggesting that the differences seen by NMR spectroscopy reflect structures smaller than the 2 nm resolution limit of freeze-fracture EM and peptide relationships within its 20 nm accuracy of identifying lateral position. 

*Keywords:* Deuterium NMR; Peptide; Model membrane; Signal transduction; Cholesterol; Freeze-fracture

## 1. Introduction

The epidermal growth factor receptor (also called c-erbB-1 in humans) and Neu/erbB-2 (also referred to as Neu/HER2 {c-erbB-2 in humans}) are important Class I receptor tyrosine kinases (RTKs) [1–7]. They are highly homologous proteins that coexist within the plasma membrane of a given cell and are thought to have different, yet somewhat interdependent, signalling functions. Each possesses an external glycosylated portion responsible for the earliest events in recognition; a hydrophobic stretch of sufficient length to cross the bilayer membrane once; and an intracellular portion exhibiting phosphorylation sites, docking sites, and protein kinase activity. Considerable attention has focused on these and related species with regard to the molecular mechanism of signal transduction.

It is now widely considered that the transmembrane domains of RTKs can form meaningful side-to-side associations that are critical early events in signal transduction pathways [5–12]. The Class I species, Neu/erbB-2 and the EGF receptor, have served as platforms for the development of innovative and compelling mechanisms for such effects [13–21]. In these models the concept that transmembrane domains of Class I RTKs are capable of direct homodimeric interaction is clearly a central one, and one which has become increasingly validated with the emergence of evidence that single amino acid substitutions in the transmembrane domain of the EGF receptor [22] as well as in Neu/erbB-2 [13], can trigger abnormal cell behaviour. Nevertheless, controversy surrounds the details of the proposed mechanisms—including the role of conformation, motifs, and non-covalent bond formation. Moreover, while receptor dimerisation is often assumed to be the operational triggering event, workers have also suggested that monomers may be active forms in the same or related pathways and that dimerisation is in itself not sufficient as a trigger (e.g., [23–29]).

Wideline $^2$H NMR spectroscopy lends itself well to studying structural aspects of such issues since the technique utilises non-perturbing probes localised to the molecule of interest, which can then be dispersed as a minor component in multi-component membrane assemblies. One can work at physiological temperatures, and without the need to sonicate or to add detergent-like molecules. The approach has benefited from extensive development by other workers [30–40]. Recently, we reported $^2$H NMR experiments on the transmembrane domain of the human EGF receptor (c-erbB-1), demonstrating that the spectra obtained could be understood in terms of previous $^2$H NMR studies on model hydrophobic peptides and ones from prokaryotic organisms, and that the results provided a measure of homodimeric interactions between the transmembrane domains [41,42]. Neu/erbB-2 is a potentially interesting comparator for the EGF receptor. The two have considerable homology, yet Neu/erbB-2 is often viewed as an RTK that is especially sensitive to alterations within the transmembrane domain [10]: a single amino acid alteration of $Val_{664}$ to Glu leads to cell transformation [13] (it should be noted however that an analogous $Val_{627} \rightarrow$ Glu substitution in the EGF receptor has also been recently claimed to have metabolic effects [22]). In the present work, we tested for sequence-related aspects of transmembrane domain behaviour that might direct the function of these two Class I receptors.

The peptide, $EGFR_{tm}$, corresponding to residues 621 to 654 of the human EGF receptor, was prepared by solid phase synthesis. This sequence represents the putative transmembrane region (residues 622 to 644) and a 10-residue stretch of the cytoplasmic domain including the $Thr_{654}$ residue which is phosphorylated (apparently as a regulatory mechanism) during EGF-mediated signal transduction [43]. Three different deuterated amino acids were incorporated at topographically distinct sites within $EGFR_{tm}$. Methyl groups were chosen as deuteron locations because of

the relatively good signal-to-noise ratio afforded by three equivalent nuclei. The same approach was taken to Neu/erbB-2. $Neu_{tm}$ was synthesised to contain the 21 residue hydrophobic stretch thought to span the membrane ($Ile_{660}$ to $Ile_{680}$ of rat Neu) and residues $Lys_{681}$ to $Thr_{691}$ of the contiguous cytoplasmic domain. Deuterium probes replaced selected $^1H$ nuclei at $Ala_{661}$, $Leu_{667}$, and $Val_{676}$. $Neu_{tm}*$ was also synthesised, corresponding to the transmembrane domain of the constitutively-activating Neu/erbB-2 transformant in which $Val_{664}$ is replaced by Glu.

The study of transmembrane subunits of RTK's for insight into their mechanistic function in signalling seems justified by the 'two step' model of membrane protein biogenesis, whereby transmembrane $\alpha$-helices are thought to insert across the membrane and subsequently associate to form functional multi-subunit complexes [44]. It has been generally observed that, in folded membrane proteins, the conformation of transmembrane segments is little altered from that which would be predicted based on the conformation and transbilayer locus of the separate entities [9]. Moreover, numerous functional chimeric Class I receptors have now been produced in which the transmembrane domain is employed as a separate cassette (e.g., [45,46]). The phospholipid selected for peptide reassembly, 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC), reflects the monounsaturated nature, chain length, and low phase transition temperature of common naturally-occurring species in higher animal plasma membranes. POPC has a gel/fluid phase transition temperature of $-3$°C [47], and the phase behaviour of its mixtures with cholesterol has been characterised [48]: all samples studied in the present work represented fluid membranes at the temperatures investigated.

## 2. Materials and methods

1-Palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC), 1,2-3-*sn*-dimyristoyl phosphatidylcholine (DMPC) and L-$\alpha$-phosphatidylserine (beef brain, > 99%) were obtained from Avanti Polar Lipids (Birmingham, AL) and were used without further purification. 9-Fluorenylmethyl *N*-succinimidylcarbonate (FMOC-OSu) and cholesterol were from Sigma (St. Louis, MO). Deuteromethyl L-methionine ([$d_3$]Met), perdeuterated L-alanine ([$d_4$]Ala), and deuterium-depleted water (< 0.5 ppm $^2H$), were from Isotec (Miamisburg, OH). Perdeuterated L-valine ([$d_8$]Val), deuteromethyl L-alanine ([$d_3$]Ala), and deuteromethyl L-leucine ([$d_3$]Leu), were from Cambridge Isotope Laboratories (Andover, MA).

FMOC-blocked amino acids were synthesized following standard procedures as described previously [41]. 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 (Clayton, Australia), and sequence confirmed by mass spectroscopy and amino acid analysis, purity 85–95% [41]. $Neu_{tm}$ and $Neu_{tm}*$ were also synthesised by Chiron. Initial purity was 60%. This was improved by HPLC to 80% as follows. 20 mg peptide was dissolved in 500 $\mu$l of 1,1,1,3,3,3-hexafluoro-2-propanol (Aldrich, '99 + %') and filtered through an Acrodisc CR PFTE 0.45 $\mu$m minispike (Gelman Sciences, Ann Arbor, MI). A 50-$\mu$l volume was applied to a Waters $\mu$Bondapak $NH_2$ Radial-Pak cartridge (8 × 100 mm, 10-$\mu$m particle size, 125 Å pore size), eluting at 2 ml/min with a 0.25% $min^{-1}$ gradient of 0.1% TFA/$CHCl_3$ to 0.1% TFA/$CH_3OH$. The Neu peptides have proven very difficult to isolate in highly pure form. Contaminants of synthetic peptides were species differing from the target sequence by one or two amino acids. Residual impurities in each case consisted of small amounts of a variety of peptides of similar size.

Except where noted otherwise, liposome generation was according to the following 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 55°C to produce mixtures in which peptide represented 6 mol% of phospholipid. Samples were allowed to sit at this temperature for at least 30 min after visually-apparent complete dissolution. Solvent was then 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-both made up in deuterium-depleted water. Samples were warmed to 55°C, without

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 $\pm 0.1$ C°. A quadrupolar echo sequence ('SSECHO' from the Varian pulse library) was employed with full phase cycling and $\pi/2$ pulse length of 5–6 $\mu$s. Pulse spacing was typically 30 $\mu$s, and sweep width was 100 kHz.

Samples for freeze-fracture electron microscopy were withdrawn directly from the samples prepared for $^2$H NMR or were prepared in the same fashion. Specimens were frozen from room temperature in liquid freon, and were fractured and Pt-shadowed at $-110$°C in a Balzers BAF 301 apparatus equipped with electron beam guns. Replicas were cleaned initially in $NaClO_4$, rinsed with distilled water, and immersed in 1/1 acetone/ethanol to remove residual lipid.

## 3. Results

Amino acid sequences for the 34-mer, $EGFR_{tm}$, and the 38-mers, $Neu_{tm}$/$Neu_{tm}$*, are shown in Fig. 1. In each case they comprise the putative transmembrane domain and 10 or 11 residues of the cytoplasmic domain from the parent glycoprotein. The suggested transmembrane portion calculated using the method of Rost [49] has been underlined.

$EGFR_{tm}$ † KI**A**TGMVGALLLLLVVALGIGLF**M**RRRHI**V**RKRT

$Neu_{tm}$ KPVTFII**A**TVVGV**L**LFLILVVV**V**GILIKRRRQKIRKYT

$Neu_{tm}$* ↑ $E_{664}$

Fig. 1. Amino acid sequence of $EGFR_{tm}$ and of $Neu_{tm}$/$Neu_{tm}$*. The putative transmembrane domain has been underlined (cytoplasmic domain to the right). Deuterated amino acids are indicated by bold letter symbol: perdeuterated alanine was substituted for $Ala_{623}$ ([$d_4$]$Ala_{623}$), methionine deuterated selectively in the methyl group for $Met_{644}$ ([$d_3$]$Met_{644}$), and perdeuterated valine for $Val_{650}$ ([$d_8$]$Val_{650}$). In $Neu_{tm}$ and $Neu_{tm}$* alanine deuterated selectively in the methyl group was substituted for $Ala_{661}$ ([$d_3$]$Ala_{661}$), leucine deuterated selectively in the methyl group for $Leu_{667}$ ([$d_3$]$Leu_{667}$), and perdeuterated valine for $Val_{676}$ ([$d_8$]$Val_{676}$). $Neu_{tm}$* is identical to $Neu_{tm}$ but with a $Val_{664} \rightarrow$ Glu substitution. A biotinylated lysine residue (†) replaced the extracellular $Ser_{621}$ in the case of $EGFR_{tm}$. This was also the case for $Neu_{tm}$ in the samples in which only $Ala_{661}$ was deuterated.

Elongated amphiphiles 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 relating $^2$H NMR spectral splittings ($\Delta\upsilon_Q$) to molecular orientation and motional characteristics.

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

$e^2Qq/h$ is the nuclear quadrupole coupling constant (165–170 kHz for a C–D bond) [30–32], $S_{mol}$ is the molecular order parameter (assuming axially symmetric order) describing orientational fluctuations of the C–D bond relative to the bilayer normal, and $\Theta_i$ is the average orientation of each C–D bond relative to the bilayer normal. For deuterated methyl groups, which undergo rapid rotation about the 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 in Eq. (1) by considering $\Theta_i$ to be the angle between the C–$CD_3$ vector and the molecular long axis, and introducing an additional factor of 1/3. Due to the $S_{mol}$ term in Eq. (1) a given splitting can be 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 twofold rotational jumps, and three-{or higher} fold jumps with unequal population weighting of available conformers), can cause a shift in intensity toward the spectral centre, leading to significant obscurement of the Pake splitting; although quite commonly intensity persists at frequencies corresponding to those predicted for symmetric motions about the same axis [33,50–55].

Eq. (1) dictates that a $CD_3$ group on an immobilised peptide side chain should give rise to a single Pake doublet of about 40 kHz splitting (this presumes rotational motion about the C–$CD_3$ axis, which is valid except at temperatures far below 0°C). In the present experiments it was considered that the intensity advantage and favourable motional character-

istics of the deuteromethyl groups could permit identification of up to four intense Pake doublets in the spectra, and subsequent relation to overall peptide behaviour in the membrane.

Typical spectra of $EGFR_{tm}$, $Neu_{tm}$, and $Neu_{tm}*$ in POPC bilayers are displayed in Fig. 2. With some samples precautions were taken to avoid cooling below room temperature prior to spectroscopy; however, this was not seen to change the overall spectral result. A sharp peak in the middle of each spectrum could be separately resolved on an expanded frequency axis into two components (not shown here). One of these, offset about 0.3 kHz downfield from the powder spectrum midpoint, represents residual deuterated water; the other, about which the powder pattern is symmetric, reflects the presence of some vesicles with high curvature for which the quadrupole splittings are motionally averaged to zero. These general features of $^2H$ NMR powder spectra of amphiphiles in liposomes will not be further considered here.

For $EGFR_{tm}$ at 6 mol% in bilayer membranes we have demonstrated previously that the dominant spectral features approximate a superposition of single Pake doublets from each $CD_3$ group [41]. Thus the spectral feature contributed by $[d_4]Ala_{623}$ was one Pake doublet, while $[d_8]Val_{650}$ gave a pair of doublets (one for each valine methyl group), and $[d_3]Met_{644}$ produced a doublet with evidence of spectral asymmetry manifest as an additional central peak. The positions of these four doublets are indicated by arrows in Fig. 2 (at 65°C the quadrupole splittings are 4.7 kHz {Ala}, 2.6 kHz {Met}, 1.1 and 4.7 kHz {Val}). Where non-methyl deuterons existed these produced only minor features due to their low relative numbers and to relaxation effects (see also Ref. [56]). Spectral widths were less than 40 kHz, yet spectra were essentially Pake doublet in nature at physiological temperatures; hence, it is clear that deuterated $EGFR_{tm}$ undergoes rapid axial rotation in these membranes.

Spectra for $Neu_{tm}$ and $Neu_{tm}*$ in POPC (Fig. 2) were similar in overall width to those obtained for $EGFR_{tm}$, but with most of the intensity present as a 'pyramidal' shape suggesting axial asymmetry of probe motion. This was true even in highly fluid

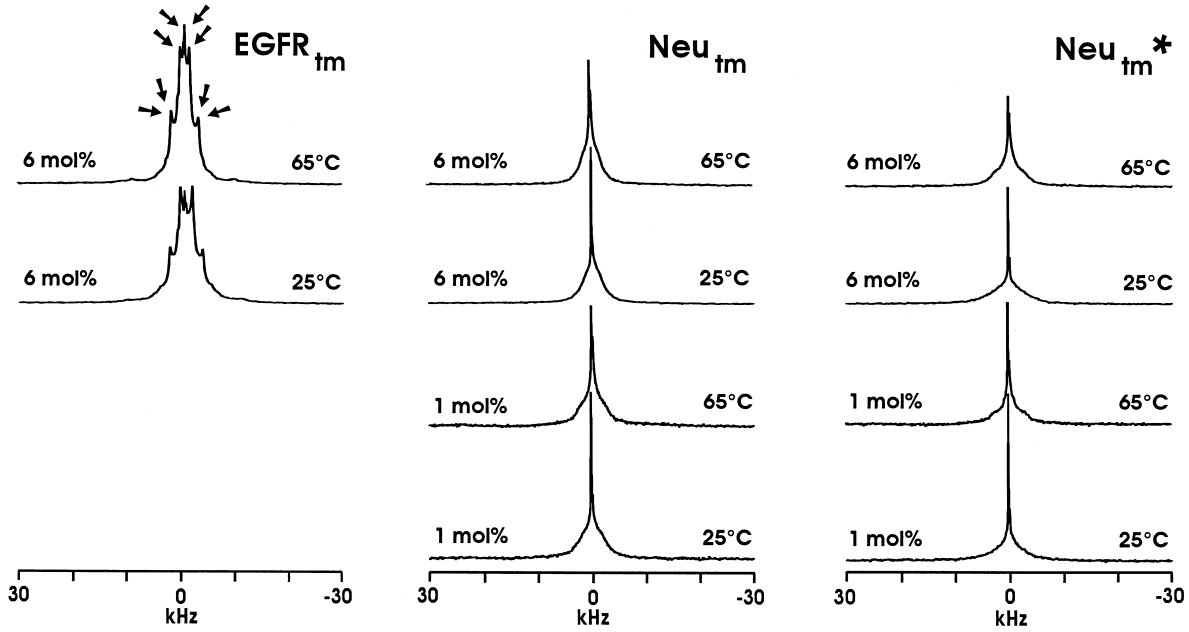


Fig. 2. $^2H$ NMR spectra corresponding to $EGFR_{tm}$, $Neu_{tm}$ and $Neu_{tm}*$ in POPC bilayers. Each peptide contained three deuterated amino acids as described in Fig. 1. Spectra are shown for peptide/phospholipid mol ratios of 6:100 and 1:100. The number of accumulated transients represented by each spectrum is 400,000, 500,000 and 300,000 for $EGFR_{tm}$, $Neu_{tm}$ and $Neu_{tm}*$, respectively. Spectra have been normalised within each column, without allowing for possible intensity loss based on peptide immobilisation at low temperatures. Arrows in the upper left spectrum indicate peak locations for the four deuteromethyl groups in $EGFR_{tm}$ as assigned previously [41].

membranes—i.e., 65–75°C, and with dilution of the peptide down to 1 mol% relative to phospholipid. A very narrow central doublet (0.2 kHz splitting) with relatively low intensity also existed. Spectra of $Neu_{tm}$ and $Neu_{tm}*$ displayed poorly-defined shoulders (total width 3–6 kHz) in the most fluid membranes. In the case of $Neu_{tm}*$ these tended to disappear with temperature reduction. Spectra corresponding to $Neu_{tm}*$ at 6 mol% were generally wider than those of comparable $Neu_{tm}$ samples.

We noted previously with regard to $EGFR_{tm}$ that as sample temperature was decreased the Pake-doublet spectral shape could become progressively obscured by a shift of intensity from the 90° spectral edges toward the spectral centre - presumably due to increased peptide–peptide association and increasingly asymmetric motion [41]. In membranes containing cholesterol the effect was dramatic. Fig. 3 illustrates typical spectral features observed after addition of 33 mol% cholesterol to the membranes of samples such as those described in Fig. 2: the temperature-and-cholesterol-related effects on $EGFR_{tm}$ can be seen in comparing the left hand columns of Figs. 2 and 3. Gradual appearance of broad spectral peaks at $+20$ kHz and $-20$ kHz (i.e., quadrupole splitting $\Delta V_Q = 40$ kHz) in the spectra of cholesterol-containing samples is associated with peptide that has been slowed to the point that it is not undergoing rotational motions of frequency greater than about $10^4$ $s^{-1}$. These phenomena seen for $EGFR_{tm}$ were reflected in the experimental results for $Neu_{tm}$, but were particularly notable in the case of $Neu_{tm}*$. Thus when temperature was reduced for membranes containing $Neu_{tm}*$ spectral shoulders at about $+3$ kHz and $-3$ kHz tended to become obscured, especially in the presence of cholesterol. Obvious outer spectral peaks appeared at $+20$ kHz and $-20$ kHz which increased in intensity at lower temperature. These peaks (corre-

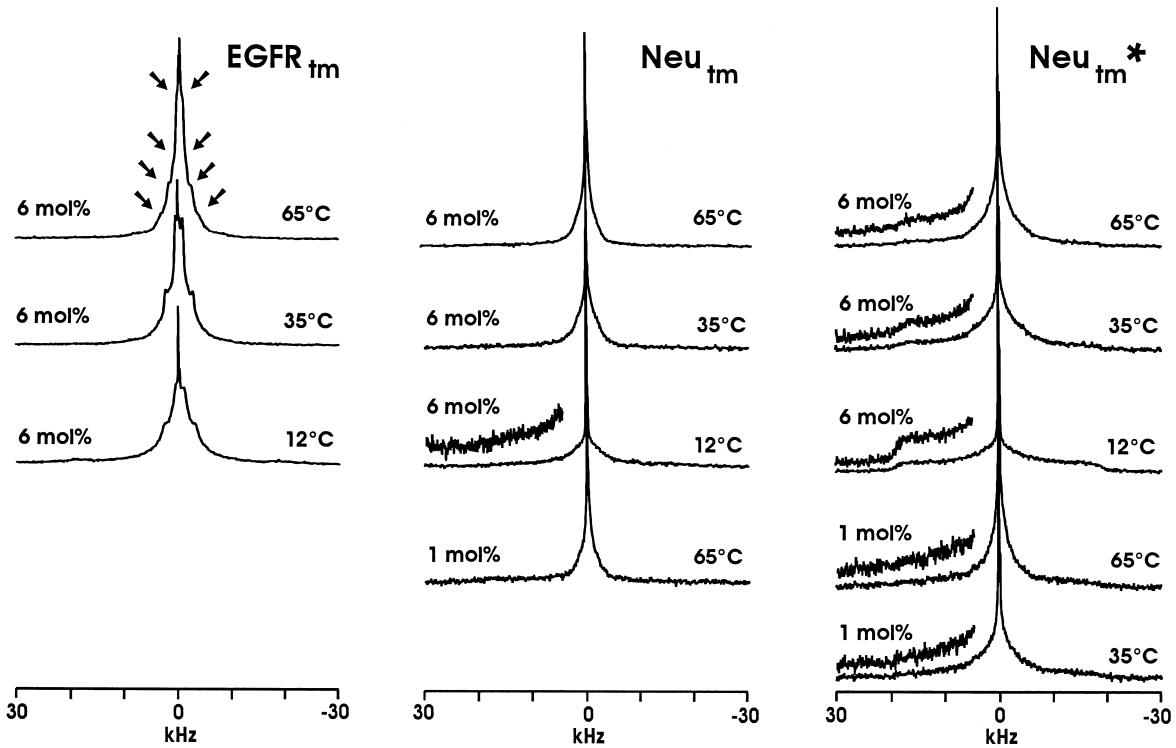


Fig. 3. $^2H$ NMR spectra corresponding to $EGFR_{tm}$, $Neu_{tm}$ and $Neu_{tm}*$ in POPC/cholesterol bilayers. Each peptide contained three deuterated amino acids as described in Fig. 1. Spectra are shown for peptide/phospholipid mol ratios of 6:100 and 1:100. The number of accumulated transients represented by each spectrum is 400,000 to 600,000. Vertically expanded regions are shown as inserts to permit more accurate comparison of low intensity outer spectral features. Spectra have been normalised within each column, without allowing for possible intensity loss based on peptide immobilisation at low temperatures. Arrows in the upper left spectrum indicate peak locations for the four deuteromethyl groups in $EGFR_{tm}$ as assigned previously [41].

sponding to 40 kHz splitting) were relatively reduced in intensity in membranes having low peptide concentration, whereupon spectra of $Neu_{tm}*$ became similar to those of $Neu_{tm}$.

A variety of alternative approaches to sample preparation were tested in an effort to ensure that the generally asymmetric nature of the peptide motion suggested by spectra of $Neu_{tm}$ and $Neu_{tm}*$ was not a function of sample preparation technique. Selected results of such experiments are presented in Fig. 4. The rapid dilution method used by Killian et al. [57] to incorporate a variety of hydrophobic peptides into bilayer membranes was tested by dissolving $Neu_{tm}$ in TFE and adding the solution to an aqueous suspension of POPC which was then further diluted with water (Fig. 4A). Replacement of TFE with hexafluoroisopropanol (HFI—a particularly good solvent for hydrophobic peptides) for solubilising lipid and peptide prior to drying and subsequent hydration produced identical spectra (Fig. 4B). This was also true when formic acid/acetic acid/$CHCl_3$/ethanol (1:1:2:1) was substituted for TFE (spectra not shown). Detergent dialysis with subsequent collection on sucrose density gradients—an approach used by Smith et al. [21] in dealing with similar peptides—did not alter the result (spectra not shown). Addition of the negative-charged phospholipid, phosphatidylserine (PS), and sonication produced similar spectra: e.g., PS, was added to POPC/cholesterol bilayers containing 1 mol% $Neu_{tm}$ (producing a final lipid ratio of 1:7:3.5 PS/POPC/cholesterol); the sample was vortexed and then probe sonicated to the point of significant suspension clearing; and spectra were run before and after freeze–thawing (the latter to ensure a larger liposome population) (Fig. 4D). Assembly of $Neu_{tm}$ into fluid bilayers of the 14-carbon-fatty-acid host matrix, DMPC, produced the result shown in Fig. 4C. Fig. 4 also includes spectra of $Neu_{tm}$ deuterated

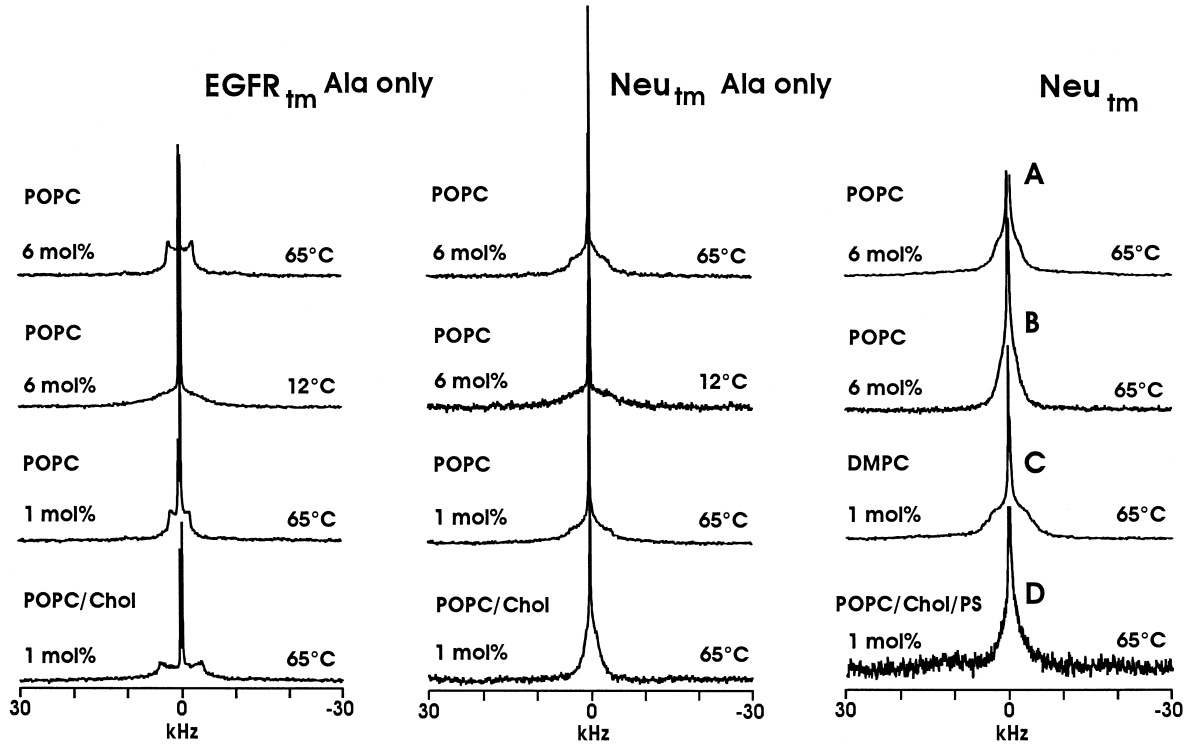


Fig. 4. $^2H$ NMR spectra corresponding to peptides deuterated only in alanine residues ($EGFR_{tm}$ at $Ala_{623}$ and $Neu_{tm}$ at $Ala_{661}$—left hand and middle columns, respectively), and for selected $Neu_{tm}$ samples with deuteration at $Ala_{661}$, $Leu_{667}$ and $Val_{676}$ but prepared by alternative routes (right hand column). Spectra are shown for peptide/phospholipid mol ratios of 6:100 and 1:100. Right hand column: (A) dilution method of Killian et al. [57]; (B) hexafluoroisopropanol substituted for TFE in the preparation method described in Section 2; (C) DMPC substituted for POPC in the preparation method described in Section 2; (D) phosphatidylserine added to a 1 mol% $Neu_{tm}$ sample as described in Section 3 to give a mol ratio of 7:3.5:1 POPC/cholesterol/PS. Spectra have been normalised within each column.

selectively at $Ala_{661}$ for comparison with $EGFR_{tm}$ deuterated selectively at $Ala_{623}$ (middle and left hand columns, respectively). Once again the $Neu_{tm}$ spectra suggest axially asymmetric motion. CD spectra of $Neu_{tm}$ in the TFE solvent generally employed for the present work demonstrated features very similar to those for $EGFR_{tm}$ and characteristic of predominantly $\alpha$-helical array (spectra not shown).

Fig. 5 presents freeze-fracture electron micrographs of the typical structures found in the samples

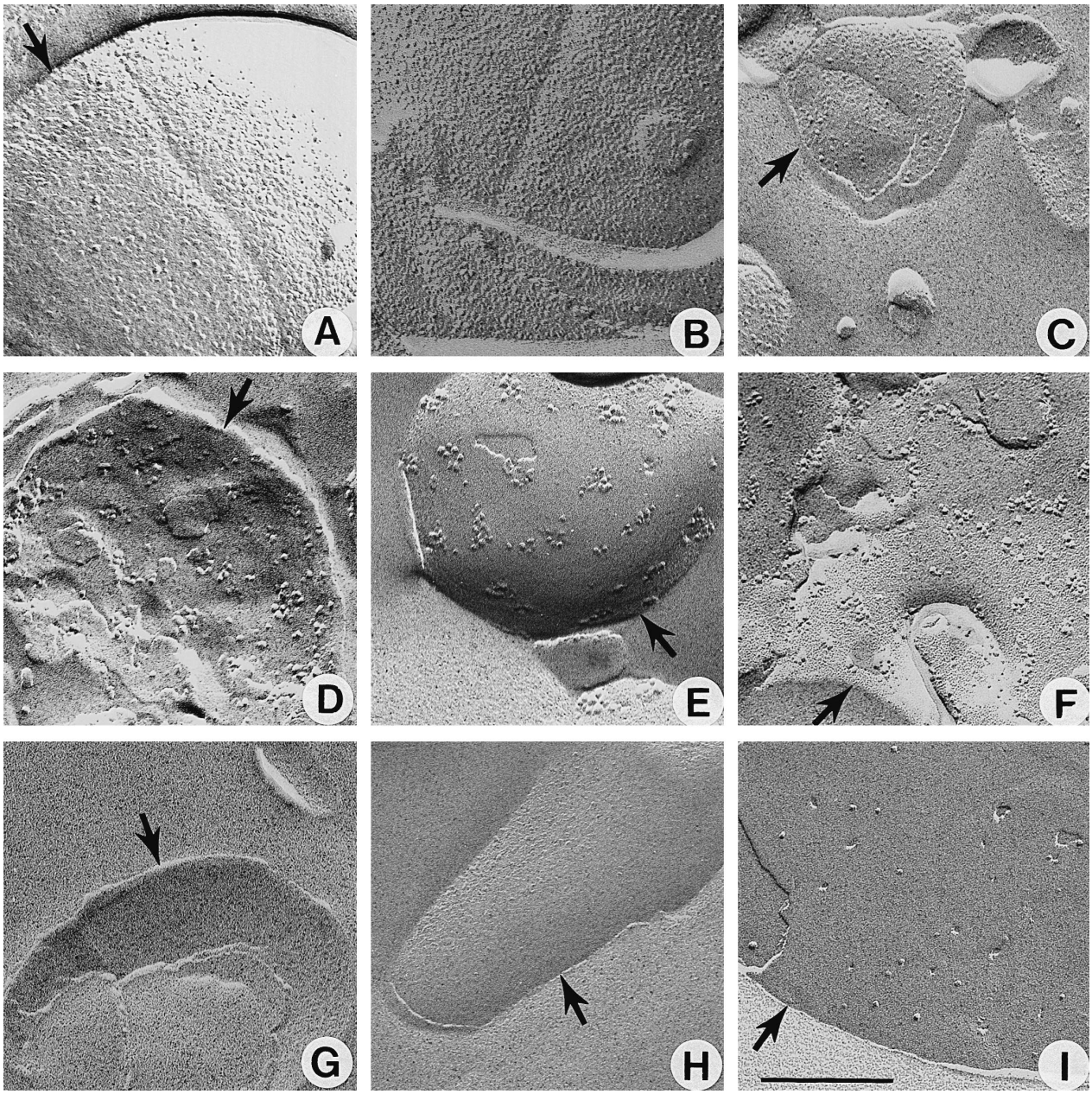


Fig. 5. Freeze-fracture electron micrographs of typical NMR samples. In the top row samples contained 6 mol% peptide in POPC alone: (A) $EGFR_{tm}$, (B) $Neu_{tm}$, (C) $Neu_{tm}*$. In the middle row samples contained 6 mol% peptide in POPC/cholesterol: (D) $EGFR_{tm}$, (E) $Neu_{tm}$, (F) $Neu_{tm}*$. The effect of reduction in peptide concentration to 1 mol% is shown in the bottom row: (G) $EGFR_{tm}$ in POPC, (H) $Neu_{tm}*$ in POPC, and (I) $Neu_{tm}*$ in POPC/cholesterol. Arrows point from ice to liposome edge. Bar indicates 200 nm. Shadow direction is from bottom to top of page.

used for $^2$H NMR spectroscopy. In this technique a rapidly-frozen specimen is fractured in vacuum at −110°C and the fracture face is immediately shadowed with an atomic layer of platinum and carbon. The fracture plane is known to split bilayer membranes between their component monolayers. The hydrophobic membrane interior exposed by the fracture plane shows the presence of particulate discontinuities ('bumps'). Such peptide-related features in bilayer model membranes are thought to be closely analogous to the 'intramembranous particles' that characterise fracture faces of cell membranes [58] and model membranes containing transmembrane proteins [59]. The particle size distribution (measured on the basis that a *single* 'particle' is a bump without visible internal fine structure—i.e., which cannot be further subdivided visually) ranged from 4.6 to 6.9 nm in the absence of cholesterol, and from 4.6 to 9.2 nm in the cholesterol-containing membranes. The particles seen in liposomes without cholesterol (Fig. 5A,B,C) appeared less sharply resolved than those in membranes with cholesterol (Fig. 5D,E,F), and tended to be less clustered. Note too that obvious IMPs were strikingly (disproportionately) reduced in number in POPC membranes when the peptide concentration was reduced to 1 mol% (Fig. 5G,H); while this was less the case in membranes containing cholesterol (Fig. 5I). Under comparable conditions the appearance of the fractured samples was essentially indistinguishable amongst the three peptides studied.

## 4. Discussion

We have previously reported wideline $^2$H NMR spectra of $EGFR_{tm}$ in lipid bilayers, with emphasis on characterisation of deuteration sites [41,42]. In the present work, we attempted a comparison of overall motional behaviour amongst membrane-associated portions of $EGFR_{tm}$ and two other Class I RTKs. In each case the peptide studied comprised the putative transmembrane domain plus an additional 10–11 amino acids extending into the (C-terminal) cytoplasmic domain. Relevant levels of comparison include: $EGFR_{tm}$ to $Neu_{tm}$, 6% peptide to 1% peptide, POPC host matrix to POPC/cholesterol, and normal phenotype ($Neu_{tm}$) to transformed phenotype ($Neu_{tm}*$). Based on past experience, the primary sample preparation technique used was hydration of thin films dried down from peptide/lipid solutions made in the solvent, trifluoroethanol (TFE). TFE is a 'lipomimetic' solvent known to support $\alpha$-helical arrangement in peptide segments for which this secondary structure is the native form [60–63]. Killian et al. [57] have cautioned that hydrophobic peptide aggregation can occur after prolonged incubation in TFE (and HFI), however we have not observed that spectra of samples prepared in this manner are influenced by sample history; and we can produce the same 1 mol% peptide spectrum by making a fresh sample or by redissolving and 'diluting with lipid' a previous 6 mol% sample. Bouchard et al. [64] concluded that organic solvent choice (ethanol vs. TFE) influenced the initial dimer type for gramicidin in DMPC bilayers, although within hours the ethanol form adopted the more natural form produced using TFE. In the present work, we screened a very wide variety of sample preparation techniques for Neu peptides, and observed no important effect on our results.

The Neu peptides gave spectra indicative of significantly axially asymmetric motion in all samples studied; whereas for $EGFR_{tm}$ such spectra were associated with the presence of cholesterol, reduced temperature, and higher peptide concentrations [42]. Spectra reflecting axially asymmetric motion are in general not seen for deuterated hydrophobic peptides that have been previously reported in fluid bilayer membranes at such low peptide/lipid ratios (e.g., [41,65–70]). However they are common for deuterated molecules whose rotational rates are slowed to the range $10^3$–$10^4$ $s^{-1}$ or less in gel phase membranes: in such cases spectral intensity typically shifts from a Pake doublet powder pattern into a more centrally-peaked pattern (i.e., like the Neu peptide spectra) as the motion becomes more asymmetric. The membranes examined in the present work were all fluid, and most spectra were narrower than the 40 kHz value expected for immobilised peptide $CD_3$ groups. For $\alpha$-helical peptides in fluid bilayer membranes a major axis of motional averaging is the membrane perpendicular. These results for the Neu peptides might well be anticipated for molecules whose axial diffusion is interfered with by intermolecular associations that lead to asymmetric rotation about the membrane perpendicular and/or to

slowed rotation about this axis. $^2$H NMR spectra which are apparently highly analogous to those seen here for Neu peptides (and to $EGFR_{tm}$ spectra seen at low temperature in cholesterol-containing membranes) have been observed by Mueller et al. [66] for a hydrophobic dipeptide subjected to a major reduction in membrane fluidity. Very similar spectra have also been described by MacDonald and Seelig [67] in association with peptide–peptide (gramicidin) interactions brought about by raised peptide concentration. Lee et al. [71] have suggested that slow gramicidin axial diffusion in fluid membranes can arise from intermolecular interactions.

It might be presumed that elevated temperatures and highly fluid membranes would tend to reduce inter-peptide associations, leading to maximal peptide dispersion in the membrane and resultant axially symmetric motion. Certainly reducing peptide concentration in the membrane can be expected to reduce peptide–peptide association. However even at 65–75°C and 1 mol% in POPC, spectra of $Neu_{tm}$ remained characteristic of significantly axially asymmetric motion. As mentioned above, the fact that $Neu_{tm}$ spectral widths were much less than the 40 kHz anticipated for highly immobilised $CD_3$ groups indicates that the labelled species is in fact undergoing axial diffusion within the membrane at rates greater than $10^4$ $s^{-1}$. Thus, our working hypothesis is that, for peptides corresponding to the Neu/erbB-2 transmembrane domain, dimer-/oligomerisation is a high probability event-leading to asymmetric rotation within the membrane. Ideally, one could test this by further reduction of the peptide concentration, but the NMR technique used is not sensitive enough to detect significantly smaller sample quantities with present equipment. The existence of a difference between $EGFR_{tm}$ and $Neu_{tm}$ with regard to self-association affinity is consistent with the concept that transmembrane sequence can determine RTK self-association [16,17].

Spectra of $Neu_{tm}*$ were wider than corresponding spectra of $Neu_{tm}$ in membranes containing 6 mol% peptide. Moreover, incorporation of cholesterol into the membrane led to the appearance of considerable spectral intensity at $+20$ kHz and $-20$ kHz for $Neu_{tm}*$ (i.e., spectral splitting some 40 kHz—indicative of strong peptide immobilisation). The immobilising effect of cholesterol was much less obvious in spectra of $Neu_{tm}$. In the absence of cholesterol, reduction in peptide concentration within the limits possible (6 to 1 mol%) had little effect on spectra of $Neu_{tm}$ or $Neu_{tm}*$; however, in membranes containing cholesterol, reduction in peptide concentration from 6 to 1 mol% dramatically narrowed spectra of $Neu_{tm}*$—i.e., reversed the cholesterol-induced peptide immobilisation. These observations may be seen as indicative of a greater tendency to extensive oligomerisation in the case of $Neu_{tm}*$.

Sternberg and Gullick have developed a structural basis as to how Neu/erbB-2 monomers might associate within the membrane ([18,19,72] and references therein). They suggest that a motif of some five amino acids is involved in homodimerisation of a number of monomeric receptors, and that the transmembrane domain is intrinsically $\alpha$-helical. They have applied this logic in particular to the $Val_{664} \rightarrow$ Glu oncogenic transformation which underlies the present $Neu_{tm}/Neu_{tm}*$ comparison. Their suggestion has been that, based on a greater tendency of the transmembrane domain of the transformant to self-associate, signalling is constitutively activated by the $Val_{664} \rightarrow$ Glu substitution. Such a concept is very exciting as a possible molecular basis for loss of growth control. Smith et al. [21] have favoured a related model for Neu/erbB-2, and have demonstrated consistent behaviour of transmembrane peptides by optical spectroscopy and magic angle spinning NMR. Another important model for the Neu transformant studied in the present work is that, in order to dimerize, the transmembrane domain must be fully $\alpha$-helical and that this represents a higher energy state for the native species than for the transformant ([17,20] and references therein; see also Ref. [73]).

In the modelling studies mentioned above, the homodimer of the transmembrane domain is typically seen as a (right handed) pair crossing at a significant angle [10]. MacKenzie et al. [74] demonstrated via high resolution NMR spectroscopy that this is the case for the transmembrane $\alpha$-helical domains of the glycophorin A dimer in detergent solution (crossing angle $\{-\}40°$), and that stabilisation is by van der Waals forces. Smith et al. [21] have performed optical spectroscopy and magic angle spinning NMR studies on a transmembrane peptide from Neu incorporated into bilayer membranes, finding evidence of

a crossing angle of about 50° and of measurably different orientation for the analogous peptide from the $Val_{664} \rightarrow Glu$ transformant. Our results offer some support for such concepts. In principal, Eq. (1) permits absolute correlation of spectral splittings with orientation of the C–D bond; although this presumes knowledge of the $S_{mol}$ term. Assuming that the peptides studied here take on a helical secondary structure, then existing literature observations suggest that $S_{mol}$ for the overall helix would be in the range of 0.9 ([38,69], see also Ref. [21]). The $CD_3$ group of alanine can be expected to be rigidly affixed to this. We have previously pointed out with regard to modelling of $EGFR_{tm}$ that for a standard right handed $\alpha$-helix the C–$CD_3$ bond of the deuterated alanine should be directed near the magic angle (54.7°) with respect to the helix long axis [41]. Thus, the spectral quadrupole splitting for the methyl deuterons should approach zero if the helix axis is perpendicular to the membrane bilayer. In fact the spectral width observed for this alanine in $EGFR_{tm}$ and $Neu_{tm}$ is some 6–10 kHz (Fig. 4)—i.e., up to 25% of the theoretical maximum of 40 kHz. While this is hardly compelling evidence of a given peptide orientation, it is certainly consistent with expectations that the angle between the peptide long axis and the membrane perpendicular is considerably removed from 0°. Moreover, in our hands there are basic differences in behaviour between $Neu_{tm}$ and $Neu_{tm}*$—particularly in membranes containing cholesterol. It is interesting that the transformant peptide demonstrated evidence of greater tendency to form large oligomers (greater apparent immobilisation), and that the spectral difference was reduced at low peptide concentration—consistent with the concept that interactions of higher order than dimerisation may occur in signalling pathways. Our results cannot however be seen as evidence for greater *dimerisation* (as opposed to *oligomerisation*) of $Neu_{tm}*$ vs. $Neu_{tm}$ (although this may be the case) since all spectra of both Neu peptides were measurably asymmetric.

Freeze-fracture electron microscopy of the NMR samples demonstrated the liposomal nature of the material studied, and that the same features existed for all three peptides. It is important to note that this technique (i) utilizes atomic shadowing to produce a Pt/C replica some 2 nm thick, and (ii) relies upon rapid freezing that can induce lateral rearrangement of receptors over at least 10–20 nm in the model membrane [59]. It is generally considered that in freeze-fracture electron micrographs of cell membranes IMPs represent the locations of one or more transmembrane proteins [58]. Given that in our experiments there is one peptide per 15–16 phospholipids at 6 mol% peptide, the density of obvious particles in the membrane fracture faces is less than that which would be expected if each represented a monomeric peptide. The possibility that the IMPs seen represent peptide oligomers in reversible equilibrium with ('invisible') monomers or dimers is further born out by the freeze-fracture appearance of the 1 mol% samples: reduction in peptide concentration led to a disproportionate reduction in the particle density in membranes with cholesterol and to virtually complete disappearance in the membranes of POPC without cholesterol (Fig. 5). Such results are highly consistent with the conclusions of Segrest et al. [75] surrounding a proteolytic fragment containing the transmembrane domain of glycophorin in lipid bilayers: this peptide gave rise to freeze-fracture IMPs of the size described in the present work, and the peptides appeared to represent oligomers (see also Ref. [59]). It seems reasonable to conclude that the similarity in freeze-fracture appearance amongst $EGFR_{tm}$, $Neu_{tm}$, and $Neu_{tm}*$, in the face of their very different $^2H$ NMR behaviour, indicates that the spectral differences recorded reflect processes occurring on a smaller scale than the resolution limit of freeze-fracture EM.

Killian et al. [57] have described a series of experiments highlighting the important topic of mismatch in hydrophobic length between transmembrane peptide and membrane [76]. The concept exists that a peptide might be induced to cluster as a result of such mismatch: this might at first sight be considered the source of differences in behaviour between $EGFR_{tm}$ and $Neu_{tm}$ in the present work. Presuming a right handed $\alpha$-helix of 3.6 residues per turn, the calculated length of the 23-residue putative hydrophobic domain displayed for $EGFR_{tm}$ is 34.5 Å [77]. This compares with a calculated hydrophobic membrane thickness for POPC bilayers of 25.8 Å at 25° (vs. 29.9 Å for the same membranes containing 30 mol% cholesterol) [78]. The relevant peptide hydrophobic dimension (perpendicular to the plane of the bilayer) would be reduced to 31.3 Å if the $EGFR_{tm}$ $\alpha$-helix

were oriented at 25° to the bilayer normal (for instance if dimers were formed having a crossing angle of −50°). One might conclude therefore that $EGFR_{tm}$ is slightly 'too long' for the membrane, that greater hydrophobic mismatch [57,76,79,80] occurs in the case of membranes without cholesterol, and that this mismatch will increase at higher temperatures. For $Neu_{tm}$, which has only 21 residues in the putative transmembrane region calculated via the Rost approach [49], the relevant length of the hydrophobic stretch is 31.5 Å (28.5 Å at a 25° angle to the bilayer normal). It seems that in the system described in the present work the Neu peptides represent if anything a closer match to the membrane thickness than does $EGFR_{tm}$, and thus that hydrophobic length mismatch may not be a driving force for the spectral differences seen here. DMPC (hydrophobic thickness of fluid bilayers 22.8 Å [57]) provides some potential for testing this conclusion in that the ratio of $Neu_{tm}$ hydrophobic domain to DMPC hydrophobic thickness is 1.38, while the comparable ratio for $EGFR_{tm}$ to POPC is 1.34: i.e., the hydrophobicity mismatch of $Neu_{tm}$ in fluid DMPC might be seen as the same as that of $EGFR_{tm}$ in POPC. Spectra of $Neu_{tm}$ in DMPC remained intrinsically typical of axially asymmetric motion (Fig. 4 C) (although there may be some evidence of more clearly identifiable shoulders than in POPC). We have noted previously [41] that $EGFR_{tm}$ in fluid DMPC gave rise to Pake spectra qualitatively similar to those found in POPC.

## 5. Conclusions

In fluid phospholipid membranes at physiological temperatures there is a measurable tendency for the isolated transmembrane domain of the EGF receptor to form homodimers or oligomers. This tendency is significantly increased in the presence of cholesterol, although peptide–peptide association remains reversible and is most marked at low temperatures. The transmembrane domain of another Class I receptor tyrosine kinase, Neu/erbB-2, appears to have a greater tendency to homodimer/-oligomer formation in fluid membranes. There are very significant intrinsic differences in the motional behaviour of the membrane-spanning peptides from these two Class I RTKs at the peptide/lipid ratios studied. The transmembrane domain of the oncogenic Neu/erbB-2 mutant in which there is a $Val_{664} \rightarrow Glu$ substitution has a greater tendency toward formation of large oligomers than does the corresponding peptide from the normal phenotype.

We suggest that, in general, transmembrane $\alpha$-helices such as those studied here will tend to reversibly associate with one another in bilayer membranes due to relatively poor fit into the surrounding liquid crystal host matrix. For fluid membranes, we expect this to be particularly true in the presence of cholesterol (see also Ref. [81]). The affinity of peptide–peptide association in such a context should be influenced by amino acid sequence via its determination of how closely and with what orientation(s) the peptides can pack to optimize inter- or intra-peptide bonding while minimising disruption of lipid–lipid interactions. For full-length RTKs this process will be further affected by the bulky portions extending beyond the membrane surface.

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