

Interaction between ErbB-1 and ErbB-2 transmembrane domains in bilayer membranes

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Abstract The transmembrane domains of ErbB receptor tyrosine kinases are monotopic helical structures proposed to be capable of direct side-to-side contact with related receptors. Formation of the resulting homo- or hetero-oligomeric complexes is considered a key step in ligand-mediated signalling. ErbB-2, which has not been observed to form active *homo*-dimers in a ligand dependent manner, has been implicated as an important partner for formation of *hetero*-dimers with other ErbB receptors. Recent work has shown that the ErbB-2 transmembrane domain is capable of forming homo-oligomeric species in lipid bilayers, while a similar domain from ErbB-1 appears to have a lesser tendency to such interactions. Here, ^2H nuclear magnetic resonance was used to investigate the role of the ErbB-2 transmembrane domain in hetero-oligomerisation with that of ErbB-1. At low total concentrations of peptide in the membrane, ErbB-2 transmembrane domains were found to decrease the mobility of corresponding ErbB-1 domains. The results are consistent with the existence of direct transmembrane domain involvement in hetero-oligomer formation within the ErbB receptor family. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Deuterium nuclear magnetic resonance; Peptide; Model membrane; Signal transduction; ErbB-2; ErbB-1; Epidermal growth factor; Dimer

1. Introduction

The ErbB family of receptor tyrosine kinases plays an important role in regulation of human growth and development. This family is comprised of four members, and includes the epidermal growth factor (EGF) receptor (ErbB-1) and the ErbB-2/neu proto-oncogene. Each is a single polypeptide chain whose extracellular and cytoplasmic domains are separated by a single membrane-spanning hydrophobic segment of some 22–26 amino acids. Collectively, the ErbB receptors

form a signalling network that regulates cell growth and differentiation in response to EGF and other EGF-like ligands [1,2]. This network is thought to involve both homo-dimeric/oligomeric and hetero-dimeric/oligomeric receptor associations, and to be modulated by ligand binding in normal cells [3]. Such ‘lateral signalling’ appears to widen the range of biological response by enabling different signal cascades from binding of ligands to different pair-wise combinations of ErbB receptor molecules. In this regard, it has been suggested that ErbB-2 is the preferred hetero-dimerisation partner of the other ErbB receptors [4–6]. The intensity of signalling from hetero-oligomers has been postulated to be attributable to increased biological response to these activated hetero-dimers [7] in addition to an observed decrease in ligand dissociation from ErbB-2-containing species [8,9]. It has also been suggested that ErbB-2-containing hetero-oligomers are the high affinity receptors for ErbB ligands, despite the absence of an active ErbB-2 homo-dimer [10]. Many human cancers exhibit high levels of ErbB-2 expression [1,11].

The mechanisms underlying both intrinsic and ligand-induced receptor association are subjects of considerable interest and debate. The transmembrane region is suggested to provide a major level of control over receptor spatial arrangement, associations and dynamics, which in turn regulate signal transduction [1,2]. It has been proposed that one aspect of this control can involve the presence of amino acid motifs which permit close association of transmembrane segments [12,13]. Thus, Gullick et al. have noted that direct interaction could occur between adjacent α -helical transmembrane domains via such association at a non-zero crossing angle [14]. There has been much discussion as to the basis of the attractive forces that might be involved [14–16]: workers appear to favour van der Waals forces, in the context of a knobs-into-holes model [17,18]. Additionally, recent studies have indicated the potential importance of hydrogen bonding between side chains or backbone atoms in stabilising transmembrane domain associations [19,20]. Such concepts have been reinforced by studies of glycoporphin in detergent micelles [21] and lipid bilayers [22]. A wide variety of selective mutations have been performed on cultured cell systems to test the role of ErbB transmembrane motifs. However, while a significant number argue in favour of the associated theories, others do not [13]. Recently, direct measurements have been made on transmembrane peptides from ErbB-2/neu, supporting transmembrane domain involvement in homo-dimeric interactions in dry films and in gel phase bilayers [15,23,24]. Our group has described direct side-to-side homo-oligomer formation by the ErbB-2 transmembrane domain in fluid 1-palmitoyl-2-oleoyl-3-*sn*-

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Abbreviations: ErbB-2, human class I receptor tyrosine kinase also known as HER2 or c-erbB-2 (*neu* in the rat); ErbB-2_{TM}, a 50-residue expressed transmembrane peptide containing Gly⁶⁴⁸ to Met⁶⁹¹ of ErbB-2 plus an N-terminal hexa-His tag; EGF, epidermal growth factor; ErbB-1, human EGF receptor; ErbB-1_{TM}, a 38-residue expressed transmembrane peptide containing Pro⁶²⁰ to Arg⁶⁵¹ of ErbB-1 plus an N-terminal hexa-His tag; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; FACT, formic acid/acetic acid/chloroform/trifluoroethanol (1:1:2:1 ratio by volume)

phosphatidylcholine (POPC) bilayers, while similar peptides derived from ErbB-1 remained primarily monomeric [25]. In the present work, expressed transmembrane peptides from the class I receptor tyrosine kinases ErbB-2 and ErbB-1 were incorporated in fluid membranes and their hetero-oligomeric interactions studied by ^2H nuclear magnetic resonance (NMR) spectroscopy.

2. Materials and methods

2.1. Materials

POPC was obtained from Avanti Polar Lipids (Birmingham, AL, USA). Deuterium-depleted water and deuteromethyl L-alanine were from Cambridge Isotope Laboratories (Andover, MA, USA). 2,2,2-Trifluoroethanol, NMR grade, bp 77–80°C, was from Aldrich (Milwaukee, MI, USA). Deuterated peptides were expressed by general procedures described previously [24,26]. Briefly, His-tagged peptides were produced as TrpE fusion proteins, released from TrpE by cyanogen bromide cleavage, and purified using nickel-chelate chromatography (Ni-NTA resin, Qiagen). Peptide purification was monitored by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and confirmed by electrospray mass spectrometry.

2.2. Preparation of samples

Liposome preparation for NMR spectroscopy was as described recently [25]. Briefly, dry peptide (5–10 mg) and appropriate amounts of dry POPC were dissolved in 5 ml FACT (formic acid/acetic acid/chloroform/trifluoroethanol, 1:1:2:1 ratio by volume) [24] at 25°C to produce clear solutions containing various ratios of peptide (or ErbB-1/ErbB-2 peptide mixtures) to phospholipid. Following complete organic solvent removal, samples were hydrated with 30 mM HEPES containing 20 mM NaCl and 5 mM EDTA pH 7.1–7.3, made up in deuterium-depleted water. The pH of each resulting liposome suspension was adjusted to 7.0 ± 0.2 and it was this entire sample that was transferred to an NMR sample tube and used for spectroscopy. Centrifugation of such suspensions at 23°C and $1500 \times g$ on linear 5–17.5 or 5–30% sucrose density gradients produced single peptide-containing fractions whose ^2H NMR spectra were the same as those of the unspun suspensions.

2.3. NMR experiments

^2H NMR spectra were acquired at 76.7 MHz on a Varian Unity 500 spectrometer using single-tuned Doty 5 and 10 mm static probes with temperature regulation to $\pm 0.1^\circ\text{C}$. A quadrupolar echo sequence [27] was employed with full phase cycling and $\pi/2$ pulse width of 4–5 μs (5 mm probe) or 8–10 μs (10 mm probe). Pulse spacing was 15–20 μs and spectral width was 100 kHz. Recycle delay times of 100 and 500 ms were used. Typically, spectra were processed with 150 Hz line broadening. Spectral simulation was carried out as described previously [25], using the program WSolid1 (K. Eichele and R.E. Wasylshen, Dalhousie University, Halifax, Canada).

3. Results

Expressed peptide sequences produced for this study were as follows:

ErbB-2_{TM}: HHHHHHGSPLTSIVSAVVGILLVVVLGVV-FGLIKRRQKIRKYTTTRSM

ErbB-1_{TM}: HHHHHHPSIATGVVGALLLLLVVALGIG-LFLRRRHIVR

Underlined portions indicate the putative transmembrane domains as predicted using the algorithms developed by Rost [28]. Bold font indicates sites of deuteromethyl probe location (side chains of alanine residues). Each sequence contains a putative Sternberg/Gullick motif [12]: SAVVG in the case of ErbB-2 and TGVVG in ErbB-1. The N-terminal hexa-His tag does not appear to significantly affect peptide behaviour in membranes, as discussed in previous studies of related peptides from neu, ErbB-1 and ErbB-2 [24–26].

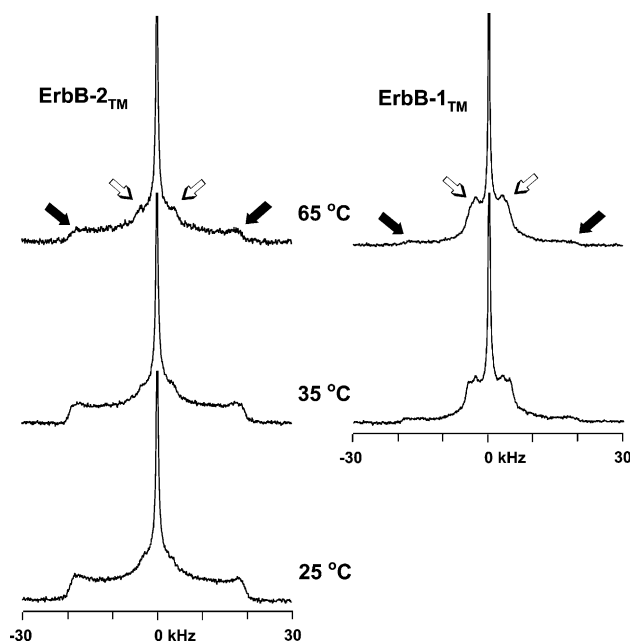


Fig. 1. ^2H NMR spectra corresponding to high receptor concentration. Spectra are displayed for ErbB-2_{TM} and ErbB-1_{TM} at 6 mol% in fluid, fully hydrated bilayers of POPC. Thick solid arrows indicate the 38.5 kHz splitting arising from peptide rotating $< 10^5 \text{ s}^{-1}$ (presumed oligomers); hollow arrows indicate narrowed Pake doublets associated with peptides whose rotational diffusion $> 10^5 \text{ s}^{-1}$ (presumed monomers). 400 000–600 000 transients were accumulated in each case.

Fig. 1 presents illustrative ^2H NMR spectra as a function of temperature for ErbB-2_{TM} and ErbB-1_{TM} assembled individually at 6 mol% in POPC membranes. All measurements were made on fully hydrated samples maintained well above the -3°C gel-to-fluid phase transition temperature [29] of this common natural phospholipid. The spectra contain multiple components, as anticipated, given that on SDS–polyacrylamide gels both transmembrane peptides migrated as monomers in equilibrium with smaller populations of homo-dimers and oligomers [26]. CD spectra were consistent with the transmembrane portions being α -helical in SDS micelles and in fluid bilayers [24,26]; and the features within the liposomal NMR spectra can be understood in this context from an examination of the following relationship between spectral splitting and probe nucleus behaviour.

Elongated amphiphiles dispersed in fluid membranes tend to undergo rapid symmetric rotation about an axis perpendicular to the plane of the membrane. As a result of this motion, each ^2H nucleus in the molecule gives rise to a 'Pake' doublet, whose splitting ($\Delta\nu_Q$) reflects the motional characteristics of the segment of the molecule to which the ^2H nucleus is attached, and its spatial orientation. Eq. 1 describes the quantitative relationship. Peptide–peptide interactions, and asymmetric or slowed peptide rotational diffusion, can become evident as perturbations on this general framework.

$$\Delta\nu_Q = 3/8(e^2Qq/h)S_{\text{mol}}(3\cos^2\theta_i - 1) \quad (1)$$

e^2Qq/h is the nuclear quadrupole coupling constant (165–170 kHz for an aliphatic C–D bond [27,30]) and S_{mol} is the molecular order parameter (assuming axially symmetric order) describing orientational fluctuations of the molecule relative

to the bilayer normal. Θ_i is the orientation of the C-D bond relative to the molecular rotation axis, the average being over reorientation of the CD bond with respect to the molecular symmetry axis. For a deuterated methyl group (three equivalent nuclei) it is convenient to consider Θ_i to be the angle between the C-CD₃ vector and the molecular long axis, while introducing an additional factor of 1/3. A given splitting can be reduced under the conditions of the current experiments by 'wobble' of the entire peptide within the membrane and by finite conformational fluctuations of the peptide backbone. Dominant non-axially symmetric rotation about an axis (e.g. all two-fold 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, local intensity maxima persist at frequencies corresponding to those predicted for symmetric motions about the same axis [31].

For immobilised peptides with little backbone flexibility, in unoriented liposomes each alanine CD₃ group should give rise to a Pake doublet of splitting approximately 40 kHz (since rapid symmetric rotation of the methyl group about its C-CD₃ axis persists until temperatures far below 0°C are reached. Such a spectrum for deuterated transmembrane peptides in fluid bilayer membranes might be anticipated for peptide oligomers that have formed via lateral association within the membrane. However Eq. 1 also dictates that peptide axial rotation and any internal peptide flexibility, leading to a correlation time of $<10^{-5}$ s, would in general lead to reduction of the spectral splitting from 40 kHz to a value determined by backbone orientation and dynamic behaviour.

In the context of the above arguments, there are two apparent features in each spectrum in Fig. 1. Both peptides show spectra indicative of a relatively immobile peptide fraction: a Pake doublet of splitting ~ 38.5 kHz which approaches the 40 kHz value anticipated above. This is much more prevalent in ErbB-2_{TM}, indicating a greater propensity for side-to-side interactions between peptides. The component decreases in intensity at higher temperatures, indicating a significant potential for exchange between mobile and immobile peptide fractions. Additionally, each spectrum contains motionally narrowed Pake doublets indicative of peptides undergoing rapid axial rotation. In the case of ErbB-1_{TM}, three overlapping powder spectra are present, as expected based on past analysis of these label locations [32]. These narrowed doublets are interpretable as representing a mobile, probably monomeric, fraction undergoing rapid symmetric motions in the bilayer. The spectral lines comprising these Pake doublets sharpen appreciably as temperature is increased, consistent with more rapid peptide axial diffusion. A third feature is the narrow peak seen in the centre of each spectrum. We have described the latter in more detail previously as arising from peptide in highly curved vesicles and from peptides whose rotational diffusion is asymmetric in nature [31] (residual HOD contributes a partially resolved additional peak 0.2–0.3 kHz downfield from this).

We have noted previously that lowering the peptide concentration in the membrane leads to a significant reduction of the immobile fractions, as indicated by a relative decrease in intensity of the spectral component with splitting near 40 kHz [25]. This is consistent with greater peptide dispersion. In the case of ErbB-2_{TM}, this decrease is accompanied by the ap-

pearance of intensity in the region of 20–25 kHz (Fig. 2, left-hand column). While less evident at 1 mol% peptide, where there is still a significant presence of immobile oligomers, these new features are clearly seen at 0.25 mol%. This appears to represent an intermediate state of association for ErbB-2_{TM}, which we have suggested may reflect the presence of a significant population of homo-dimers and/or small oligomers [25]. It is probable that such species would be immobile at higher peptide concentration and contribute to the spectral component with splitting near 40 kHz. At higher temperatures there is a decrease in the components with splittings near 20 kHz and those near 40 kHz; and a corresponding increase in the mobile, monomeric fraction. The right-hand column in Fig. 2 shows spectra for ErbB-2_{TM} at 0.25% in the presence of 0.75 mol% ErbB-1_{TM} (unlabelled). These spectra remain very similar to those for 0.25% ErbB-2_{TM} alone, indicating that the aggregation state of this peptide is not changing due to addition of the ErbB-1 transmembrane domain. In all cases, spectra of samples containing lower concentrations of peptide exhibit an increase in the isotropic peak intensity. This has been observed in past [25] and likely reflects increased amounts of residual HOD, in addition to increased populations of peptide undergoing restricted or asymmetric rotation.

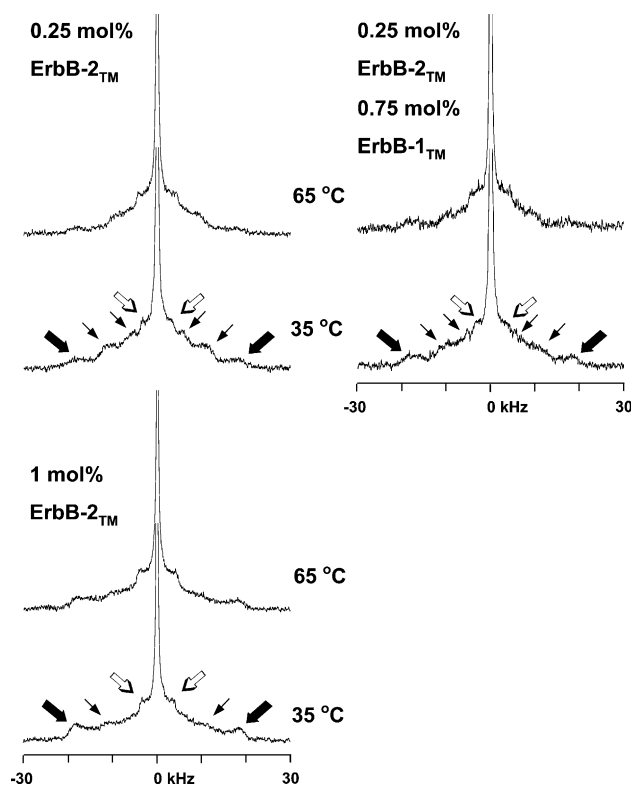


Fig. 2. ²H NMR spectra corresponding to ErbB-2_{TM} at low receptor concentration. Arrayed spectra are displayed for ErbB-2_{TM} at 0.25 and 1 mol% in POPC bilayers (left). Also shown are spectra for ErbB-2_{TM} in POPC bilayers containing 0.75 mol% unlabelled ErbB-1_{TM} (right). Thick solid arrows indicate the 38.5 kHz splitting arising from peptide rotating $\ll 10^5$ s⁻¹ (presumed oligomers); thin arrows indicate spectral features attributed to dimers and/or small oligomers capable of rotation faster than 10^5 s⁻¹; hollow arrows indicate narrowed Pake doublets associated with peptides whose rotational diffusion $\gg 10^5$ s⁻¹ (presumed monomers). Typically 800 000–1 000 000 transients were accumulated for each spectrum.

Fig. 3 demonstrates the result of incorporating *unlabelled* ErbB-2_{TM} and deuterated ErbB-1_{TM} together in POPC bilayers. The left column shows spectra of this peptide at 1 and 0.5 mol%, which demonstrate a decrease in the small immobile peptide fraction (the spectral doublet with splitting near 40 kHz) upon reduction of peptide concentration. There is, however, little evidence of the third spectral component with splitting near 20–25 kHz. This would imply that ErbB-1_{TM} exists primarily as monomers, exhibiting only a weak tendency for oligomerisation in membranes. By comparison to the left column in Fig. 2, it would appear that ErbB-2 transmembrane domains exhibit a greater tendency to self associate in lipid bilayers than ErbB-1.

The right-hand column in Fig. 3 presents spectra of labelled ErbB-1_{TM} at 0.5 mol% in POPC membranes containing 0.5 mol% unlabelled ErbB-2_{TM}. It is apparent that the fraction of immobile ErbB-1_{TM} in this case is greater than in the absence of the ErbB-2 peptide, and is in fact greater than seen at 1 mol% ErbB-1_{TM}. There is evidence of a broadening of the monomer spectrum, indicative of rapidly reversible side-to-side associations, and a marked increase in the magnitude of the immobilised fraction. Also present are features with splitting near 25 kHz reminiscent of features assigned to intermediate motion species in the ErbB-2_{TM} spectrum at 0.25

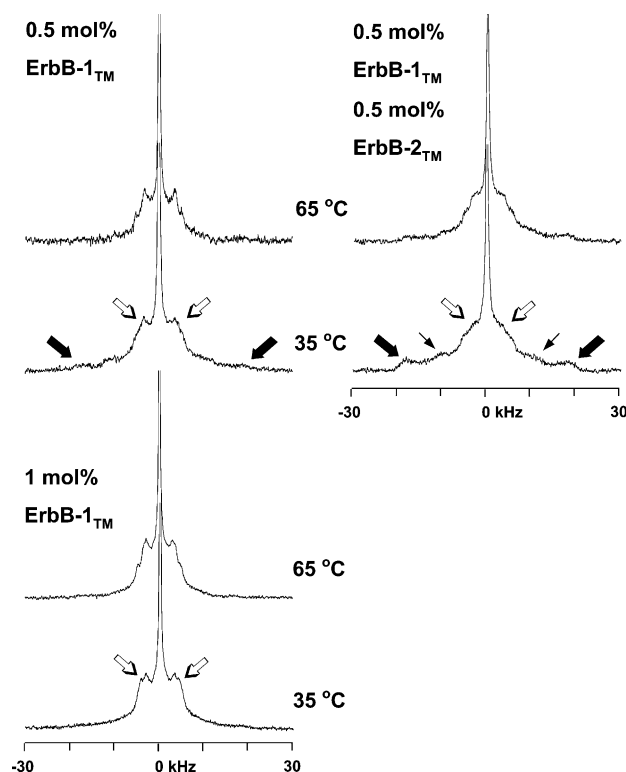


Fig. 3. ^2H NMR spectra corresponding to ErbB-1_{TM} at low receptor concentration. Spectra are displayed for ErbB-1_{TM} at 0.5 or 1 mol% in POPC bilayers (left). Spectra in the right-hand column are of similar samples containing 0.5 mol% labelled ErbB-1_{TM} and 0.5 mol% unlabelled ErbB-2_{TM}. Thick solid arrows indicate the 38.5 kHz splitting arising from peptide rotating $\ll 10^5 \text{ s}^{-1}$ (presumed oligomers); thin arrows indicate spectral features attributed to dimers and/or small oligomers capable of rotation faster than 10^5 s^{-1} ; hollow arrows indicate narrowed Pake doublets associated with peptides whose rotational diffusion $\gg 10^5 \text{ s}^{-1}$ (presumed monomers). Typically 800 000–1000 000 transients were accumulated for each spectrum.

Table 1

Relative amounts of oligomeric ErbB-1_{TM} peptide as estimated by spectral simulation

	% oligomer ($\pm 5\%$)	
	65°C	35°C
ErbB-1 _{TM} concentration		
6 mol%	15	15
2 mol%	17	19
1 mol%	< 5	< 5
0.5 mol%	9	10
ErbB-1 _{TM} +ErbB-2 _{TM} concentration		
1+2 mol%	21	20
1+1 mol%	18	22
0.5+0.5 mol%	21	26

mol%. Clearly, unlabelled ErbB-2_{TM} is directly influencing the mobility of labelled ErbB-1_{TM}, and the 25 kHz spectral intensity can be interpreted in the context of ErbB-2-directed hetero-dimer/oligomer formation.

Simulation of the identifiable ^2H spectral features permitted quantitation of the coexisting peptide populations, as described previously [25]. A summary of oligomer contribution to total spectral intensity is given in Table 1 for a range of ErbB-1_{TM} concentrations, in the presence and absence of ErbB-2_{TM}. It is evident that the greatest ErbB-2_{TM} dependent oligomerisation of ErbB-1_{TM} occurs at 1 mol% total peptide concentrations (0.5 mol% each). At 2 mol% total peptide or higher, the effect is less striking, and increasing the relative population of ErbB-2_{TM} appears to make little difference.

4. Discussion

We have suggested in the past that, even without invoking the presence of specific sites that permit close association (e.g. motifs), transmembrane helical peptides might tend to reversibly associate with one another in bilayer membranes by virtue of behaving as ‘impurities’ in the surrounding liquid-crystal lipid matrix [23,33]. There have been a number of theoretical analyses of this concept [34–36]. Such association would not in general be expected to stop at dimer formation, or necessarily to be of high affinity [18]; although it could provide one basis for the 40 kHz spectral feature seen in the present work. However, the ErbB group of receptors are widely considered to possess sites that may predispose to oligomeric association in response to appropriate extracellular recognition events [1–3,5,6,12,37].

In the work described here, a fraction of dimers and/or small oligomers unable to rotate freely in the membrane may contribute to the observed splitting at $\sim 40 \text{ kHz}$. The observation of distinct smaller spectral splittings associated with a species rotating at an intermediate rate (the Pake feature with 20–25 kHz splitting), would seem consistent with the concept of oligomers having a preferred geometry, as proposed by Gullick et al. for ErbB-2/neu dimers [12,14]. This phenomenon appears to be somewhat ErbB-2 specific, with ErbB-1 exhibiting a lower tendency for self association, even at high peptide concentrations [25]. Here we have observed an ErbB-2 dependent oligomerisation of the ErbB-1 transmembrane domain. It is interesting to note that the phenomenon of heterologous formation seen in this study did not noticeably affect spectra of deuterated ErbB-2_{TM}. This implies that

the ErbB-2 transmembrane domain directs peptide associations, and is the determinant of oligomer formation. Thus, our current data appear to support a significant role for the transmembrane domain of ErbB-2, potentially mediated by the proposed dimerisation motif, in the homo- and hetero-oligomeric association of ErbB receptors. This would be in keeping with its role as the preferred hetero-dimerisation partner for other members of this family, and points to a possible mechanism for dimer or oligomer formation in the absence of an ErbB-2 ligand. ErbB-2_{TM} might then lead to the aggregation of other peptides, while itself being less influenced by their presence. This effect appears to be less striking at higher peptide concentrations. We have previously outlined mechanisms by which peptide perturbation of lipid order may inhibit formation of specific peptide–peptide associations at very high concentration [25]. Alternatively, highly immobile ErbB-2_{TM} may interact less well with other peptides than the monomeric or dimeric species present at low concentrations.

The present spectroscopic observations of receptor transmembrane domain association were made in the absence of ligand. It is not known whether oligomeric species of the intact receptors exist within the cell membrane prior to ligand binding. Previous work by other groups has been unable to exclude the possibility of populations of exchangeable, inactive oligomers since the assays used typically only permit detection of permanent/long-lived species or activated ligand-bound forms [4]. Ligand binding could, for instance, stabilise a particular receptor arrangement leading to a signalling event. This possibility is in keeping with observations that there are two populations of ErbB-1 in many cells, one with a higher affinity for ligand than the other [38]. It has been suggested that the high affinity binding is to preformed oligomers/dimers [6]. In this context, ErbB-2-containing partners may have even stronger affinity for ligand, through an altered spatial arrangement or a different binding site [39]. A recent study has implicated ErbB-2-containing hetero-dimers as the high-affinity EGF receptors in vivo [40]. Related work has suggested that such high affinity receptors may exist as small oligomers with reduced rates of rotational diffusion [41].

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