Structural Implications of a Val→Glu Mutation in Transmembrane Peptides from the EGF Receptor

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ABSTRACT Certain specific point mutations within the transmembrane domains of class I receptor tyrosine kinases are known to induce altered behavior in the host cell. An internally controlled pair of peptides containing the transmembrane portion of the human epidermal growth factor (EGF) receptor (ErbB-1) was examined in fluid, fully hydrated lipid bilayers by wide-line ^{2}H-NMR for insight into the physical basis of this effect. One member of the pair encompassed the native transmembrane sequence from ErbB-1, while in the other the valine residue at position 627 was replaced by glutamic acid to mimic a substitution that produces a transformed phenotype in cells. Heteronuclear probes having a defined relationship to the peptide backbone were incorporated by deuteration of the methyl side chains of natural alanine residues. ^{2}H-NMR spectra were recorded in the range 35°C to 65°C in membranes composed of 1-palmitoyl-2-oleoyl phosphatidylcholine. Narrowed spectral components arising from species rotating rapidly and symmetrically within the membrane persisted to very high temperature and appeared to represent monomeric peptide. Probes at positions 623 and 629 within the EGF receptor displayed changes in quadrupole splitting when Val^{627} was replaced by Glu, while probes downstream at position 637 were relatively unaffected. The results demonstrate a measurable spatial reorientation in the region of the 5-amino acid motif (residues 624–628) often suggested to be involved in side-to-side interactions of the receptor transmembrane domain. Spectral changes induced by the Val→Glu mutation in ErbB-1 were smaller than those induced by the analogous oncogenic mutation in the homologous human receptor, ErbB-2 (Sharpe, S., K. R. Barber, and C. W. M. Grant. 2000. *Biochemistry.* 39:6572–6580). Quadrupole splittings at probe sites examined were only modestly sensitive to temperature, suggesting that each transmembrane peptide behaved as a motionally ordered unit possessing considerable conformational stability.

INTRODUCTION

Protein receptor tyrosine kinases mediate many of the earliest events in signal transduction across plasma membranes of higher animal cells (van der Geer et al., 1994; Kavanaugh and Williams, 1996). Their common structural features include a single amino acid chain having an external glycosylated portion, a hydrophobic stretch of sufficient length to cross the membrane only once, and an intracellular portion exhibiting phosphorylation sites, docking sites, and protein kinase activity. It is thought that the physical characteristics of the hydrophobic transmembrane portions importantly modulate intermolecular communication and that perturbations of this structure can lead to altered cell growth. Interestingly, receptor tyrosine kinases are often very tolerant of amino acid changes within the membrane-spanning portions. However, certain mutations within these domains have been associated with oncogenic transformation in vitro and in vivo, and are considered examples of receptor modifications that result in excessive stimulatory signal transmission. One such mutation that forms an important basis for structural models of signaling is the increase in cell metabolism and growth that occurs when a specific (hydrophobic) valine is replaced by (polar) glutamic acid in ErbB-2/Neu (Gullick et al., 1992; Brandt-Rauf et al., 1995; Smith et al., 1996; Sajot et al., 1999; Sharpe et al., 2000). Evidence of the same phenomenon has been found in the closely related receptor, ErbB-1 (the human EGF receptor), although the magnitude of the phenotypic effect appears to be less in this case (Miloso et al., 1995). It has been suggested that the oncogenic nature of the Val→Glu substitution in these systems may derive from alteration of the receptor's ability to take part in direct side-to-side associations with other proteins. In keeping with this concept, the valine residue involved is within a five-amino acid "motif" that may mediate contact between receptor transmembrane domains (Sternberg and Gullick, 1990). In designing this model, Sternberg and Gullick (1990) have emphasized the possible role of H-bonding within the motif as a source of the effects of the Val→Glu mutation. Another important proposal has been that conformational changes within the transmembrane domain may play a part in modulating association with neighboring receptors (Brandt-Rauf et al., 1995; Sajot et al., 1999 and references therein). The latter concept relates to suggestions that a precise knobs-into-

Received for publication 26 January 2001 and in final form 25 September 2001.

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Abbreviations used: EGF, epidermal growth factor; ErbB-1_{TM}, 34-amino acid peptide containing the EGF receptor transmembrane domain; ErbB-1_{TM}Mu, the corresponding ErbB-1 peptide having the Val^{627}→Glu substitution associated with aberrant cell behavior; ErbB-2, a human class I receptor tyrosine kinase also known as HER2 or c-erbB-2; ErbB-2_{TM}, 50-residue transmembrane peptide containing the ErbB-2 transmembrane domain; ErbB-2_{TM}Mu, the corresponding ErbB-2 peptide having the Val^{659}→Glu substitution associated with aberrant cell behavior.

0006-3495/01/12/3231/09 $2.00

holes fit of interacting transmembrane peptide surfaces is critical to stable association (Lemmon et al., 1997; White and Wimley, 1999). In the present work we describe an attempt to evaluate, in fluid bilayer membranes at physiological temperature, the direct physical effects that result from the transforming Val→Glu mutation within the ErbB-1 transmembrane domain.

Gullick and colleagues used solution NMR to study a series of soluble peptides possessing key features of the transmembrane domain of the receptor tyrosine kinase, Neu (the rat equivalent of human ErbB-2). They observed similar α-helical geometry in peptides with and without the oncogenic Val→Glu mutation (Gullick et al., 1992). Deber and colleagues examined transmembrane 23-mers of Neu by CD spectroscopy in SDS micelles, and also observed that the wild-type and Val→Glu mutant were both largely helical (Li et al., 1994). Smith et al. (1996) used polarized IR and MAS NMR to study transmembrane 38-mer peptides from Neu in bilayers and lipid films: they concluded that the mutant had measurably less helical fraction and formed dimers of significantly different geometry. Brandt-Rauf and colleagues have noted that conformational energy analyses of ErbB-1 and ErbB-2/Neu predict differences in conformational stability of transmembrane peptides containing the activating Val→Glu mutation (Brandt-Rauf et al., 1994, 1995). Molecular dynamics calculations have suggested differences in conformational flexibility between the two (Sajot et al., 1999). We have recently used wide-line ^{2}H-NMR spectroscopy to explore the effects of this transforming mutation in ErbB-2 (Sharpe et al., 2000) and in Neu (Jones et al., 1998a), demonstrating that spectral differences exist between wild-type and mutant. ^{2}H-NMR is a particularly useful tool for probing the structure and motional characteristics of molecules in fully hydrated membranes that mimic the fluid characteristics of cell membranes. In the present work we applied the same technique to the transmembrane region of wild-type ErbB-1 and its transforming Val→Glu mutant.

Transmembrane peptides were synthesized that contained the natural membrane-spanning sequence of ErbB-1, with and without the Val^{627}→Glu point mutation reported to cause aberrant cell behavior. Alanine residues within the peptides were used as deuterium probe sites. The methyl side chain that characterizes alanine has favorable spectral properties, and its motion and orientation appear to correlate in a straightforward fashion with those of the peptide backbone (Lee et al., 1995; Sharpe et al., 2000). Use of isolated peptides to address issues related to transmembrane domain structure is justified by the observation that such domains typically function as independent units, and that they are transposable cassettes in construction of chimeric receptors (van der Geer et al., 1994; Lemmon et al., 1997). For spectroscopy purposes, peptides were assembled into fluid unsonicated bilayers of 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), a predominant phospholipid in membranes of higher animals. Results of these experiments with ErbB-1 were compared to previous ^{2}H-NMR findings with analogous peptides from the closely related receptor, ErbB-2 (Sharpe et. al, 2000). Additional supporting data from a variety of site-directed ErbB-2 mutants are presented.

MATERIALS AND METHODS

Sources

POPC was obtained from Avanti Polar Lipids (Birmingham, AL). Thin-layer chromatography was performed on silica gel 60 plates (Merck, Darmstadt, Germany), eluting with 55:30:5 (by volume) $CHCl_3/CH_3OH/H_2O$ for lipids and 95:10:3 $CHCl_3/CH_3OH/CH_3COOH$ for amino acids. Deuterium-depleted water and deuteromethyl L-alanine were from Cambridge Isotope Laboratories (Andover, MA). 2,2,2-Trifluoroethanol, NMR grade, bp 77–80°C, was from Aldrich (Milwaukee, WI). FMOC-blocked alanine for peptide synthesis was prepared using standard procedures as described previously (Rigby et al., 1996). Product purity was checked by thin-layer chromatography against an FMOC-derivative standard. ErbB-1 peptides were synthesized by the Peptide Synthesis Laboratory at Queen's University (Kingston, ON) via FMOC solid phase synthesis followed by HPLC purification: wild-type and mutant were produced, isolated, and handled in identical fashion. Expressed ErbB-2 peptides were prepared and purified as described previously (Sharpe et al., 2000). Peptide purity was confirmed by mass spectroscopy.

Polyacrylamide gel electrophoresis was performed using a mini-gel system (Bio-Rad; Hercules, CA). Peptides were run on 16.5% tricine gels, and subsequently stained with Coomassie Brilliant Blue. Molecular weight standards from Gibco (Grand Island, NY) were used, covering the molecular weight range from 3 to 43 kDa. For ErbB-1 peptides, liposome generation was according to the following protocol. The acidic organic solvent TFE was used to prepare solutions of lipid plus peptide that could be dried to form thin films for subsequent hydration with sample buffer. Typically, dry peptide (10 mg) and appropriate amounts of dry lipid were dissolved in 5 ml TFE at 25°C to produce mixtures in which peptide represented 6 mol % of phospholipid. Samples were incubated for at least 30 min after visually apparent 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 25°C under high vacuum with continuous evacuation. Hydration was with 30 mM HEPES with 20 mM NaCl and 5 mM EDTA, pH 7.1–7.3, made up in deuterium-depleted water (vortexing was avoided to minimize production of small vesicles). Generation of liposomes containing ErbB-2 peptides was similar, except that an organic solvent mixture, formic acid/acetic acid/chloroform/trifluoroethanol (1:1:2:1 ratio by volume; Sharpe et al., 2000) was used. Thin-layer chromatography of NMR samples after completion of NMR spectroscopy showed no significant evidence of lipid hydrolysis.

^{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 (Davis, 1991) was used with full phase cycling and $\pi/2$ pulse length of 5–6 μs. Pulse spacing was typically 15–20 μs, and spectral width was 100 kHz. A recycle time of 100 ms was used; recycle times of up to 500 ms did not alter lineshape or relative intensities of the features seen. DePaking was performed by a noniterative method utilizing a nonnegative least squares algorithm (Whittall et al., 1989).

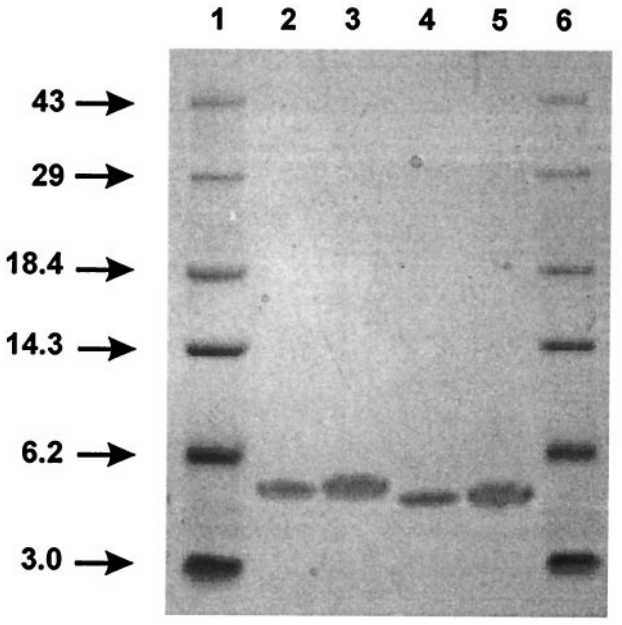

FIGURE 1 Behavior of ErbB-1_{TM} and ErbB-1_{TM}Mu on SDS polyacrylamide gels. Coomassie Blue-stained 16.5% SDS-polyacrylamide gels: *lanes 1* and *6:* molecular weight markers (sizes in kDa listed to the left of panel); *lanes 2* and *3:* ErbB-1_{TM} (4 and 8 μg); *lanes 4* and *5:* ErbB-1_{TM}Mu (4 and 8 μg). Actual M_r is 3.774 kDa for ErbB-1TM and 3.804 for ErbB-1_{TM}Mu.

RESULTS

ErbB-1 transmembrane peptides having the following amino acid sequences were examined in the present work:

ErbB-1_{TM}

KI**A**^{623}TGM**V**^{627}G**A**^{629}LLLLLVV**A**^{637}LGIGLFMRRRHIVRKRT^{654}

ErbB-1_{TM}Mu

KI**A**^{623}TGM**E**^{627}G**A**^{629}LLLLLVV**A**^{637}LGIGLFMRRRHIVRKRT^{654}

The sequence designated ErbB-1_{TM} contains the natural amino acid profile from Ile^{622} to Thr^{654} of the wild-type receptor (single-letter code, N-terminus to the left), with a lysine at position 621. The sequence designated ErbB-1_{TM}Mu is the corresponding profile having the Val^{627}→Glu (V→E) substitution associated with aberrant cell behavior. Putative transmembrane domains, calculated using the method of Rost (1996) based on the entire sequence, are single-underlined. The five-amino acid motif suggested to be involved in side-to-side associations (Sternberg and Gullick, 1990) is double-underlined. Natural alanine residues, which were used as deuterium probe locations, are shown in boldface, as is the site of the transforming mutation within the motif. Note that alanine residues offer probe sites immediately to each side of the five-amino acid motif, and also nine residues downstream of it.

Fig. 1 demonstrates the behavior of ErbB-1_{TM} and ErbB-1_{TM}Mu in SDS detergent micelles: a widely used model of membrane environment which typically preserves transmembrane domain helical structure and in which peptide-peptide hydrophobic associations can occur. Upon polyacrylamide gel electrophoresis in SDS, both native and transformed species ran as single bands of monomer molecular weight. This indicates that, in SDS, any association of the ErbB-1_{TM} peptides is rapidly reversible on the long timescale of gel electrophoresis and/or is of low affinity. Differences have been noted between hydrophobic peptide association in SDS versus lipid bilayers, both in the timescale of interaction and in the sites involved (e.g., Engelman et al., 1995; Brosig and Langosch, 1998).

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 ^{2}H 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 ^{2}H nucleus is attached, and its spatial orientation. Equation 1 describes the quantitative relationship for $\Delta\nu_Q$ measured between the intense "90° orientation" edges. Peptide-peptide interactions, and asymmetric or slowed peptide rotational diffusion, can become evident as perturbations on this general framework. For a deuteron attached to a molecule undergoing fast axially symmetric reorientation, the splitting can be expressed as

$$\Delta\nu_Q = 3/8(e^2Qq/h)\langle 3\cos^2\Theta_i - 1\rangle \qquad (1)$$

where e^2Qq/h is the nuclear quadrupole coupling constant (165–170 kHz for an aliphatic C–D bond (Seelig, 1977; Davis, 1991)) and Θ_i is the orientation of the C–D bond relative to the axis about which the molecule is rotating. The average is taken over all motions that modulate the orientation of the CD bond with respect to the rotation axis. For a deuterated methyl group (three equivalent nuclei) it is convenient to consider Θ_i to be the angle between the C–CD_3 vector and the axis about which the molecule is rotating, while introducing an additional factor of 1/3 to account for rapid rotation of the methyl group about the C–CD_3 axis.

Wide-line ^{2}H-NMR data presented here are for peptides in fully hydrated bilayers of POPC, well above the −3°C gel/fluid phase transition temperature (Davis and Keough, 1985) of this phospholipid. Fig. 2 illustrates temperature effects on spectral lineshape for ErbB-1_{TM} and ErbB-1_{TM}Mu, using peptides deuterated at Ala^{623} and Ala^{637} as examples at a peptide concentration of 10 mol % relative to phospholipid. The prominent spectral features (*arrows*) are motionally narrowed Pake doublets, as expected for molecules undergoing rapid rotational diffusion about the bilayer normal. In the case of the natural or "wild-type" sequence, ErbB-1_{TM}, the Pake doublets from the two deuterated alanine residues overlap and approximate a single Pake doublet of splitting about 7 kHz. In contrast, the mutant peptide, ErbB-1_{TM}Mu, gives rise to two readily resolvable splittings;

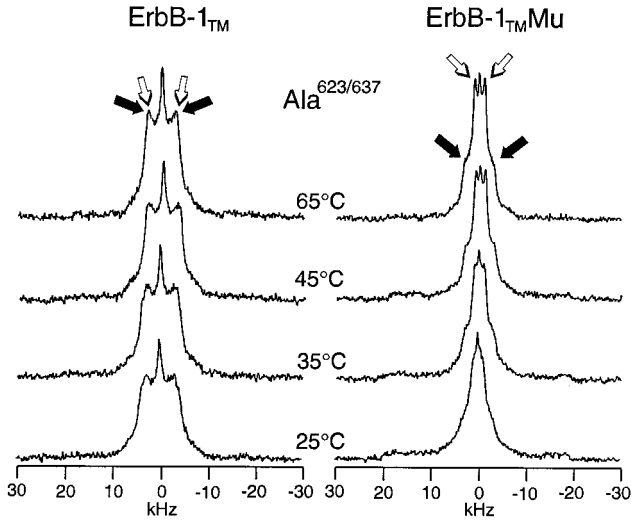

FIGURE 2 ^{2}H-NMR spectra of ErbB-1_{TM} and ErbB-1_{TM}Mu at 10 mol % in POPC. Each peptide contained deuterated amino acids at Ala^{623} and Ala^{637}, and was assembled at 10 mol % into fluid bilayers of POPC. *Left-hand column:* ErbB-1_{TM} (natural or "wild-type" sequence) at the temperatures indicated. *Right-hand column:* ErbB-1_{TM}Mu (mutant sequence, Val^{627}→Glu, characterizing transformed cell behavior) under identical conditions. Hollow arrows identify the splitting corresponding to Ala^{623}; solid arrows identify Ala^{637}. Each spectrum represents 200,000 accumulated transients, processed with a line-broadening of 100 Hz.

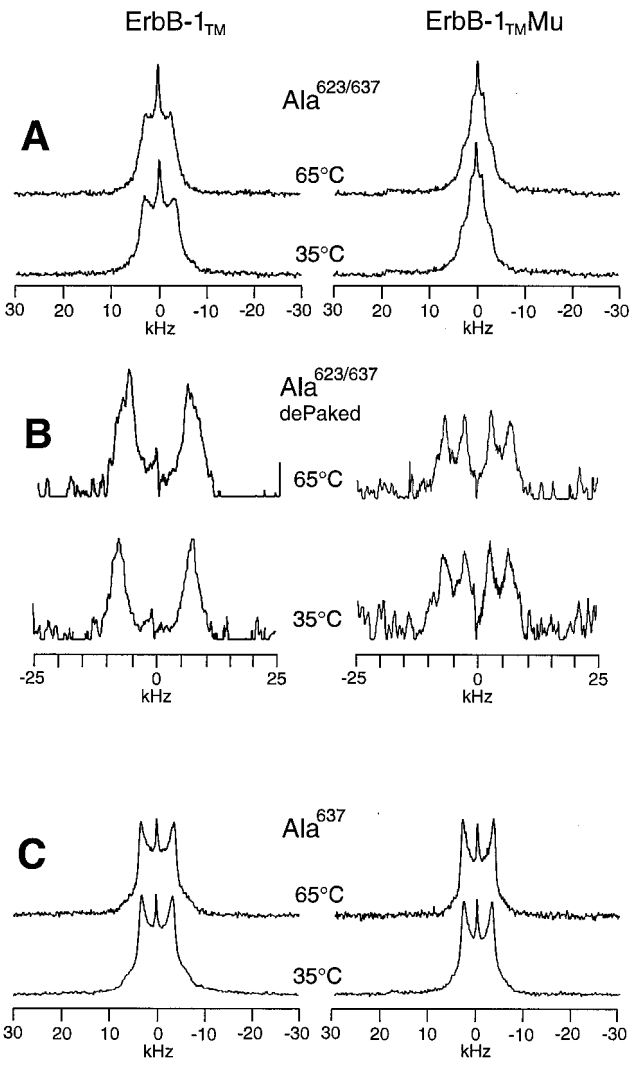

FIGURE 3 Selected ^{2}H-NMR spectra of ErbB-1_{TM} and ErbB-1_{TM}Mu at 6 mol % in POPC. (*A*) ^{2}H-NMR spectra of ErbB-1_{TM} (*left-hand column*) and ErbB-1_{TM}Mu (*right-hand column*) deuterated at Ala^{623} and Ala^{637}, assembled into fluid POPC bilayers. (*B*) DePaked spectra corresponding to the spectra in *A* above. Note that dePaking isolates the "zero degree" components from powder spectra, thus all splittings are twice those measured in the Pake spectra. (*C*) ^{2}H-NMR spectra of ErbB-1_{TM} (*left-hand column*) and ErbB-1_{TM}Mu (*right-hand column*) deuterated at Ala^{637} only and assembled into fluid POPC bilayers. Each spectrum represents 200,000 to 900,000 accumulated transients and was processed with a line-broadening of 100 Hz.

e.g., at 65°C an inner splitting of 2.6 kHz (*hollow arrows*) and an outer splitting of 6.8 kHz (*solid arrows*). The individual contributions from each $-CD_3$ group could be assigned by dePaking and by comparison with spectra of the same peptide labeled at other combinations of sites, as demonstrated in Fig. 3 below. The inner doublet in spectra of the mutant was assigned to Ala^{623}, which is near the membrane surface. Ala^{637} is predicted to be well within the hydrophobic helical domain, a region that should be relatively stable by virtue of backbone i→i + 4 intramolecular H-bonding (White and Wimley, 1999).

Spectra of the mutant peptide also show evidence of a low outer doublet having a splitting close to the 40 kHz value expected for immobilized peptide, particularly at lower temperatures. Under the conditions of our experiments, such a result might be anticipated for large peptide oligomers that have formed via lateral association within the fluid membrane. This observation suggests the possibility of greater self-association of the mutant species; however, this is a complex issue that was not pursued in the present work. The very sharp peak near the center of spectra such as those presented here typically represents two overlapping features. One is a contribution from residual deuterated water (0.2–0.3 kHz downfield of the true spectral center). The other is a peak arising from very small vesicles or highly curved membrane regions for which quadrupole splittings are motionally averaged to zero. Probe nuclei undergoing asymmetric motion can also give rise to intensity in the spectral center; hence one might anticipate a contribution that varies with the state of peptide-peptide interaction and dynamics.

Figs. 3 and 4 present spectra of ErbB-1_{TM} and ErbB-1_{TM}Mu deuterated at various locations and assembled at 6 mol % in POPC bilayers. Approaches used for assignment of peaks to individual alanine residues are indicated. Representative dePaked spectra are included. *DePaking* is a computational manipulation that isolates the "zero-degree orientation" components from powder spectra to optimize resolution of individual contributions; thus spectral split-

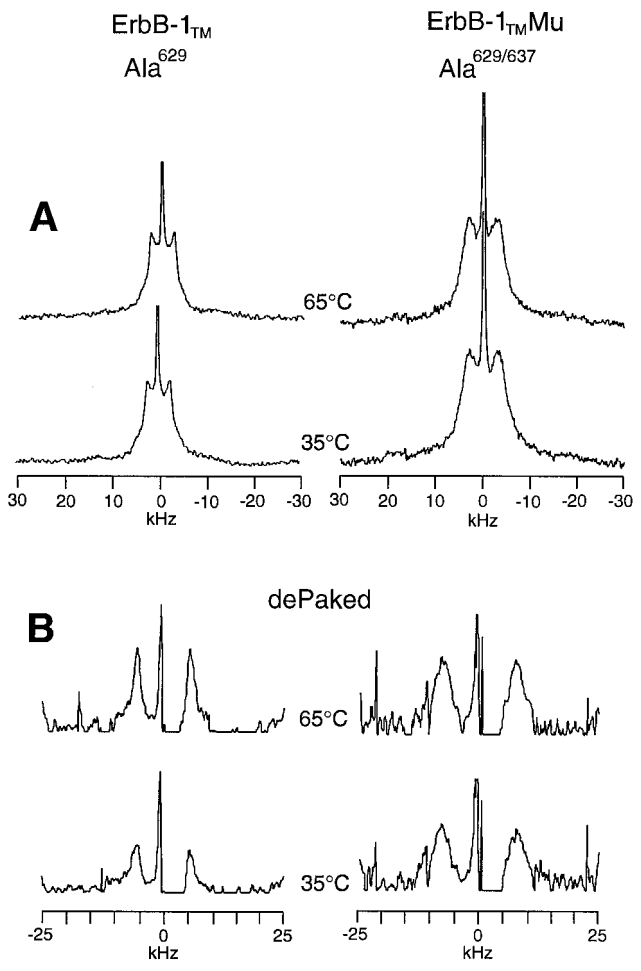

FIGURE 4 Selected ^{2}H-NMR spectra of ErbB-1_{TM} and ErbB-1_{TM}Mu at 6 mol % in POPC. (*A*) ^{2}H-NMR spectra of ErbB-1_{TM} deuterated at Ala^{629} (*left-hand column*) and ErbB-1_{TM}Mu deuterated at Ala^{629} and Ala^{637} (*right-hand column*), assembled into fluid POPC bilayers. (*B*) DePaked spectra corresponding to the spectra in *A* above. Note that dePaking isolates the "zero degree" components from powder spectra, thus all splittings are twice those measured in the Pake spectra. Each spectrum represents 350,000 to 1,200,000 accumulated transients and was processed with a line-broadening of 150 Hz.

tings are twice the value measured from their Pake counterparts. Splittings averaged from a number of experiments have been collected in Table 1 for deuterium probes at the three different natural alanine sites. Results were not significantly affected by pH variation in the range 4.8 to 7.4 (comparison not shown here), and the quadrupole splittings remained within ±0.5 kHz of the values found at 10 mol % peptide concentration (Fig. 2). In general, these splittings, which reflect average probe orientation and order via Eq. 1, were relatively insensitive to temperature over the range examined. There is some evidence that, for probes well within the helical central part of the peptides (629 and 637), spectral splittings tended to decrease slightly with temperature; such an effect is opposite to what would be expected

TABLE 1 Spectral splittings at ^{2}H probe sites in ErbB-1 transmembrane peptides

		Quadrupole Splitting ($\Delta\nu_Q$)(±0.3 kHz)	
	Probe Location	35°C	65°C
Wild-Type	Ala^{623}	7.2	6.3
Mutant		2.6	2.6
Wild-Type	Ala^{629}	5.3	5.4
Mutant		7.8	8.0
Wild-Type	Ala^{637}	7.2	8.0
Mutant		6.6	6.8

All peptides at 6 mol % peptide in POPC bilayers.

from a simple ordering effect of cooling (Eq. 1). Larger temperature effects on spectral splitting are more apparent in the wild-type peptide than in the mutant.

Table 2 presents, for purposes of comparison, the results of previously published experiments analogous to those described above, but carried out using internally controlled pairs of transmembrane peptides from the closely related human protein, ErbB-2 (Sharpe et al., 2000). Like ErbB-1, ErbB-2 is a class I receptor tyrosine kinase comprising a single chain of amino acids that crosses the membrane only once. The Val→Glu mutation within ErbB-2/Neu has been found to produce more marked metabolic effects than that within ErbB-1, and is highly oncogenic in the rat (Miloso et al., 1995; Kavanaugh and Williams, 1996). We have examined the effects of the Val^{659}→Glu transforming mutation in the motif of this receptor, particularly using the following internally controlled pair of expressed peptides. Spectral splittings arising from deuterated alanine residues are compared in Table 2 for peptides with wild-type and mutant motif sequences.

ErbB-2_{TM}

HHHHHHA^{648}SPLTSIV S**A**^{657}V**V** ^{659}GILLVVVLGV**A**^{670}FGILIKRRQQKIRKYTTRRSM^{691}

ErbB-2_{TM}MU

HHHHHHA^{648}SPLTSIV S**A**^{657}V**E**^{659}GILLVVVLGV**A**^{670}FGILIKRRQQKIRKYTTRRSM^{691}

In each case the putative transmembrane domain is single-underlined; double underlining indicates the predicted motif region for dimer formation (Sternberg and Gullick, 1990). Deuteration sites (methyl side chain of alanine residues) and the site of the Val→Glu transforming mutation are shown in bold. Thus, in the case of ErbB-2 peptides, there is one alanine within the motif region, one upstream beyond the membrane surface, and a third alanine 10 residues downstream. Expression of these and related peptides has been described by us previously, as has their behavior in SDS micelles (Sharpe et al., 2000). The magnitudes of the spectral splitting changes arising from the transforming Val→Glu mutation in ErbB-2 are larger than those seen in ErbB-1, while the temperature effects are similarly modest (compare Table 1 with Table 2).

During follow-up of the above ErbB-2 studies we have prepared a number of peptides containing other point mutations that involve changes only to the *size* of amino acid

TABLE 2 Spectral splittings at ^{2}H probe sites in ErbB-2 transmembrane peptides

		Quadrupole Splitting ($\Delta\nu_Q$)($\pm$0.3 kHz)	
	Probe Location	35°C	65°C
Wild-Type	Ala^{648}	4.3	4.4
Mutant		4.9	5.0
Wild-Type	Ala^{657}	7.4	8.2
Mutant		14.2	14.4
Wild-Type	Ala^{670}	5.2	6.4
Mutant		11.6	11.7

All peptides at 6 mol % peptide in POPC bilayers. Data taken from Sharpe et al. (2000).

side chains (as opposed to size and polarity, as in the Val→Glu substitution). In particular, selected leucine or valine residues were replaced with (deuterated) alanine to provide additional ^{2}H probe sites, and in two cases the very small glycine side chain was substituted with a larger group. Table 3 summarizes the resultant effects on probe site quadrupole splittings. It includes limited data from synthetic ErbB-2 transmembrane peptides lacking the hexa-His tag. It is interesting to contrast the relatively small spectral effects that result from increases in amino acid side chain size alone (Table 3), with the larger effects seen to arise from the Val→Glu substitution in which polarity is also increased (Tables 1 and 2). Note, for instance, that Ala^{657} maintains a reasonably well-conserved splitting value of 8–9 kHz throughout Table 3. This result is consistent with the observation that signaling by receptor tyrosine kinases is insensitive to many amino acid point substitutions within their transmembrane domains.

DISCUSSION

It was noted previously (Jones et al., 1998b) that ^{2}H-NMR spectral splittings observed for ErbB-1_{TM} in fluid bilayers are typically larger than might be expected for a polypeptide having ideal α-helical geometry if there is fast rotation about the helix long axis. Jones et al. demonstrated that splittings for deuterated alanine residues in ErbB-1_{TM} could be understood using a model that assumed the peptide to be an ideal α-helix rotating rapidly about an axis from which the helix is tilted 10–14°, with effectively no rotation about the helix axis itself. If the observed splittings are due to this type of motion, quenching of rotation about the helix axis is presumably not due to the formation of stable peptide dimers, because similar results were obtained to 90°C. One cannot, however, discount the possibility that rotation about the helix axis might be hindered by transient peptide-peptide interactions. The possibility of reorientation about the bilayer normal, with the helix axis inclined by an angle of ~10–14° magnitude, has been indicated for other transmembrane polypeptides (see Koeppe II et al., 1994; Prosser et al., 1994; Marassi et al., 1997; Byrström et al., 2000). In addition, it might be possible to account for the observed splittings in the presence of fast rotation about the peptide helix axis if substantial local departures from ideal α-helical geometry are allowed. There is also the possibility that the peptide long axis may be bent or locally deformed. Although such issues are complex and potentially controversial, it is possible to make some general comments based on the observations presented here without presuming a specific model for polypeptide reorientation.

It seems likely that a number of factors contribute to the metabolic effects that arise from the Val→Glu mutation within ErbB-1 and ErbB-2/Neu. Clearly, the phenomenon has a primary origin in the hydrophobic membrane interior because the amino acid substitution involved is well within the single-α-helix transmembrane portion. Workers have noted that hydrophobic transmembrane helices must be seen as intrinsically very stable; the free energy cost of disrupting a single i→i + 4 backbone H-bond is estimated at 4–5 kcal/mol (White and Wimley, 1999), and a transmembrane helix has some 20 such bonds. Nevertheless, the possibility of helix distortion in these systems has been compellingly argued (Brandt-Rauf et al., 1995; Sajot et al., 1999). Even subtle changes in geometry of a transmembrane peptide are thought to have the potential for far-reaching effects on the thermodynamics of its association with neighboring peptides (Gullick et al., 1992; Brandt-Rauf et al., 1995; Smith et al., 1996; Sajot et al., 1999; White and Wimley, 1999). Thus, structural alterations secondary to the mutation may play a role.

TABLE 3 Spectral changes associated with other amino acid substitutions in ErbB-2 peptides

	Probe Location and Quadrupole Splitting ($\Delta\nu_Q$)($\pm$0.3 kHz)					
Peptide	Ala^{648}	Ala^{657}	Ala^{663}	Ala^{664}	Ala^{665}	Ala^{670}
Wild-Type	4.4	8.2				
$L^{663}A$	4.6	9.2	7.8			
$V^{664}A$	5.1	8.8		6.5		
$V^{665}A$	4.5	9.4			14.0	
$A^{648}G,V^{670}A$		7.9				6.4
$G^{660}F,V^{670}A$						6.7*
$L^{663}A,V^{664}A,V^{665}A$			—†	—†	—†	

The amino acid substitution involved is indicated by the single letter code for the natural amino acid followed by the code for the substituted amino acid; e.g., substitution of natural valine at position 664 by alanine is indicated as $V^{664}A$. The simultaneous substitution of natural A^{648} by glycine and natural V^{670} by alanine is written $A^{648}G,V^{670}A$. All peptides were incorporated at 6 mol % in POPC bilayers; data are for spectra run at 65°C.

*Indicates that the peptide involved was a synthetic species, lacking the His tag.

†A synthetic peptide (i.e., lacking the hexa-His tag) with all three large hydrophobic residues, $L^{663}V^{664}V^{665}$, replaced simultaneously by deuterated alanine produced a spectrum with three Pake doublets having splittings 6.0 kHz, 7.7 kHz, and 12.9 kHz at 65°C.

The state of protonation of the glutamic acid that characterizes the mutant has been discussed by others (Gullick et al., 1992; Smith et al., 1996): it is widely acknowledged that amino acid side chain carboxyl groups within the membrane hydrophobic interior are predominantly uncharged for membranes in buffers of neutral pH. This concept has arisen from the known ranges of carboxyl group pK_a as a function of medium dielectric (Ptak et al., 1980; Mathews and van Holde, 1990); and has been borne out by experiments with transmembrane peptides from Neu (Smith et al., 1996) and ErbB-2 (Sharpe et al., 2000). In the present work this same phenomenon was apparently manifest in the ErbB-1 peptides as spectral insensitivity to pH in the range 4.8–7.4. However, there is a modest *size* increase and a considerable *polarity* increase involved in the mutation from valine to glutamic acid, which could contribute to transmembrane domain characteristics. Some insight into the importance of side chain *size* may be obtained from mutation experiments performed on ErbB-2 and listed in Table 3. In these latter experiments, selected nonpolar side chains were substituted by others of very different size but similar (lack of) polarity. In each case there was little effect on the peptide backbone at the deuterium probe sites.

Previous workers have put forward key models as to how the Val→Glu mutation within the putative dimerization motif of class I receptor tyrosine kinases might alter receptor associations. Gullick and colleagues noted that the protonated carboxyl group of the glutamic acid side chain may alter the forces between transmembrane domains by making H-bonding possible between neighboring dimerization motifs (Sternberg and Gullick, 1990). Support for this view has come from several sources. Gullick et al. (1992) synthesized soluble peptides of up to 18 residues, closely resembling portions of the transmembrane sequences of wild-type Neu and its oncogenic mutant. Examination of these by solution NMR demonstrated that wild-type peptides, and mutant peptides having the Val→Glu substitution, shared helical geometry without evidence of disruption. Deber and colleagues used CD spectroscopy to study synthetic 23-mers from the Neu transmembrane domain in SDS micelles; they also observed that the wild-type and mutant were both largely helical (Li et al., 1994). Smith et al. (1996) used IR and MAS NMR to study transmembrane 38-mer peptides from Neu in DMPC bilayers. They concluded that, while both wild-type and mutant were helical, the mutant had measurably less helical fraction, and formed dimers having a larger crossing angle. Brandt-Rauf and colleagues stressed that one must not ignore the possible role of transmembrane peptide conformation. They have noted that conformational energy analyses of ErbB-1 and ErbB-2/Neu predict differences in conformational stability in the motif region when valine is substituted by glutamic acid (Brandt-Rauf et al., 1994, 1995). They suggested that resultant conformational differences could influence the affinity of transmembrane domain side-to-side contacts with neighboring receptors. Our laboratory recorded spectral differences, reflecting limited structural differences, between wild-type and mutant in the ErbB-2/Neu system (Jones et al., 1998a; Sharpe et al., 2000). It has been pointed out by Sajot et al. (1999) (see also Duneau et al., 1997) that molecular dynamics simulations indicate the possibility that the Val→Glu substitution could induce structural effects and H-bonding changes in the motifs. Each of the above experimental approaches, while important to the overall picture, is bounded by its own limitations. Thus Li et al. (1994) and others (Engelman et al., 1995; White and Wimley, 1999) have noted that comparisons in SDS detergent micelles may incompletely reproduce polar amino acid effects and motif contacts that occur within the hydrophobic interiors of membranes. MAS NMR spectroscopy often involves working below the phase transition of the host matrix, while IR spectroscopy studies often rely upon use of dehydrated phospholipid films. An advantage of the ^{2}H-NMR approach used in the present work is the ability to make measurements in fluid fully hydrated lipid bilayers, using "insoluble" peptides containing the natural transmembrane sequence.

The amino acid sequence of ErbB-1 contains alanine residues at positions 623, 629, and 637. Perdeuteration of native alanine side chains afforded $-CD_3$ groups attached directly to the peptide backbone at each of these locations. Upon mutation of Val^{627} to Glu there were significant changes in deuterium quadrupole splitting: by 4.6 kHz at Ala^{623} immediately upstream of the Thr^{624} to Gly^{628} motif region, and by 2.5 kHz at Ala^{629} (immediately downstream of the motif). The most distant probe site (Ala^{637}) showed relatively little change in spectral splitting (within the estimated experimental error of ± 0.3 kHz). This result suggests a localized conformational change in the region of the "motif," as has been indicated by molecular modeling and dynamics calculations (Brandt-Rauf et al., 1994, 1995; Duneau et al., 1997; Sajot et al., 1999). However, it does not exclude the possibility that compensatory changes, e.g., in conformation or in overall peptide long axis tilt and rotational angle, fortuitously leave the spectral splittings from the downstream probe unaltered. The minimum orientational change necessary to produce the spectral effects seen can be estimated from Eq. 1. If the main averaging motion (aside from methyl group rotation) is rotation of the tilted peptide helix about the bilayer normal, replacement of Val^{627} by Glu results in a minimum change in average orientation of the Ala^{623} and Ala^{629} methyls with respect to the bilayer normal of $-5°$ and $-3°$, respectively (and no apparent change at Ala^{637}). If local motions such as peptide wobble, libration, or conformational fluctuations contribute to additional averaging, the observed changes in splitting may reflect slightly larger changes in average orientation. The magnitudes of the changes observed are still consistent with overall helical structure as generally proposed for transmembrane domains.

Analogous, though somewhat larger, spectral effects were seen to arise from the oncogenic $Val^{659} \rightarrow Glu$ mutation within the transmembrane domain of ErbB-2 (Sharpe et al., 2000 (Table 2)). The change in spectral splitting induced at a probe site within the motif region was 6–7 kHz. Interestingly, in ErbB-2 there was also a significant 6–7 kHz change at a probe site 10 residues downstream of the motif (Table 2). These findings are consistent with the concept that the transmembrane structural effects induced by the Val→Glu mutation in ErbB-2 are greater than those induced in ErbB-1, as proposed by Brandt-Rauf and colleagues (Brandt-Rauf et al., 1994, 1995). This might arise from the fact that the polar glutamic acid residue inserted by the mutation is deeper within the hydrophobic domain in the case of ErbB-2 (10 residues for ErbB-2 versus 6 for ErbB-1). It is possible that the measured differences between ErbB-1 and ErbB-2 reflect differences in the direct structural effect, and it is tempting to speculate that they are related to the fact that the biological effect of the mutation in ErbB-2 is apparently greater than in ErbB-1 (Miloso et al., 1995).

The phenomena noted above do not seem to be correlated in a simple fashion with thermal fluctuations of the system. We have noted previously, with regard to transmembrane domains of ErbB-2, that temperature variation between 35°C and 65°C had remarkably little effect on the spectral splittings measured for deuterated alanine probes (Sharpe et al., 2000). In general, one would anticipate that at lower temperature the degree of motional and conformational order of the peptide would be higher, leading to increased spectral splittings as described surrounding Eq. 1. In fact, spectra of the ErbB-2 peptides—and of ErbB-1 peptides in the present work—often displayed no significant change, or had smaller splittings at low temperature. This suggests that the motional order associated with the alanine probes (and thus with the peptide backbone to which they are directly attached) remains high over the considerable temperature range involved. Such an observation is in keeping with a high degree of stability of the transmembrane helix: i.e., input of significant thermal energy had remarkably little effect on peptide average conformation and internal motion. There was measurably greater sensitivity of the wild-type ErbB-1 spectral splittings to temperature, as seen previously for ErbB-2 (Sharpe et al., 2000): in each case the changes for wild-type peptides were up to 1 kHz versus <0.5 kHz for the mutant.

CONCLUSIONS

The transforming effect of a valine→glutamic acid mutation in the membrane-spanning domain of certain class I receptor tyrosine kinases is often considered to arise from alteration of the receptor's associative behavior, such that it is constitutively activated. Using ^{2}H wide-line NMR spectroscopy it was possible to detect structural effects arising from this mutation in the human EGF receptor (ErbB-1), in fully hydrated fluid bilayer membranes of a common natural phospholipid. There were small but significant changes in peptide backbone orientation in the neighborhood of the five-residue motif suggested to play a role in side-to-side associations. Probe orientational changes were not detected at a site nine residues downstream of the motif region. The conformation of the peptide backbone at the sites probed was found to be relatively stable to heating (particularly in the mutant), and to have a high degree of motional order within the fluid membrane. These ^{2}H-NMR results are reminiscent of previous findings for the closely related receptor, ErbB-2, although in the latter case an analogous valine→glutamic acid mutation caused greater spectral changes at a downstream site (Sharpe et al., 2000).

This research was supported by the Natural Sciences and Engineering Research Council of Canada [MRM] and by an operating grant to C.W.M.G. from the Medical Research Council of Canada. NMR spectroscopy was carried out at Memorial University of Newfoundland; and in the McLaughlin Macromolecular Structure Facility at UWO, established with joint grants to the department from the London Life Insurance Co., the R. S. McLaughlin Foundation, the MRC Development Program, and the Academic Development Fund of UWO. S.S. is the holder of an NSERC PGSB scholarship.

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