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TITLE: Determination of the Dynamics, Structure, and Orientation of the Transmembrane Segment of ErbB2 in Model Membranes Using Solid-State NMR Spectroscopy

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14. ABSTRACT The objective of the proposed research was to investigate the structural properties of the transmembrane helix of the ErbB2 receptor utilizing solid-state nuclear magnetic resonance (NMR) spectroscopy and Molecular dynamics (MD) simulations. 15N Solid-state NMR results demonstrated that TM-ErbB2 has a transmembrane helical domain and that the orientation of the transmembrane domain is $24 \pm 5^\circ$ in shorter chain dimyristoylphosphocholine and $11 \pm 3^\circ$ in palmitoylphosphocholine. The orientation is dictated by the hydrophobic thickness of the synthetic phospholipid bilayers. Molecular dynamics simulations analysis demonstrated that in shorter chain lipids TM-ErbB2 also makes a tilt angle of about $28 \pm 5^\circ$ with respect to the bilayer normal whereas in longer chain lipids, the tilt angle was found to be $14 \pm 4^\circ$. We also conducted dimerization studies with the wild type TM-ErbB2 within the membrane bilayer environments. One of the motifs responsible for dimerization (SAVVG) was mutated to alanines whereas the other motif (GVVFG) was left intact. The GVVFG motif still had the ability to dimerize indicating that homodimerization is dictated by at least one of these motifs in full length receptor.					
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Table of Contents

	<u>Page</u>
Introduction.....	3
Body.....	4
Key Research Accomplishments.....	4
Reportable Outcomes.....	4-7
Conclusion.....	7
References.....	7-8
Appendices.....	n/a

Determination of the Dynamics, Structure, and Orientation of the Transmembrane Segment of ErbB2 in Model Membranes Using Solid-State NMR Spectroscopy.

Introduction: The malignant transformation of a normal cell into a cancer cell requires the activation of multiple oncogenes and the loss of tumor-suppressor genes. Oncogenes encode for proteins that are components of growth factor-activated intracellular signaling pathways. Activation of the epidermal growth factor family (EGFR) of receptor tyrosine kinases, ErbB1, ErbB2, ErbB3 and ErbB4, is due to ligand-induced oligomerization (1). Ligand binding to the extracellular domain of the ErbB receptors is believed to cause receptor homo- and heteroassociation, followed by trans-phosphorylation of tyrosine residues in the activation loop of the kinase domain (2). Many of these combinations preferentially involve ErbB2, a receptor for which no direct ligand has been identified. Instead, ErbB2 is activated by heterodimerization and/or multimerization with other ErbB receptors. Interestingly, ErbB2-ErbB3 heterodimers constitute a potent mitogenic combination, despite the fact that ErbB2 has no known ligand (3, 4).

TM helices are involved in signal transduction, ion transmission and membrane protein folding. Over the years, computational and genetic techniques have been used to engineer antibodies that can target the cytosolic regions of receptor tyrosine kinases with the ultimate goal of disrupting oligomerized partners, but very few techniques are available to target the TM region (5, 6). Studies have shown that the TM domain of the ErbB-receptor family plays essential roles in signal transduction. For example, oligomerization of the transmembrane segment of the ErbB2 oncogene (about 23 amino acids) causes the activation of the ErbB2 receptor, which in turn leads to receptor activation within the oligomers (7, 8). The involvement of the TM domain in oligomerization is still controversial. Sequencing data has demonstrated that oncogenic p185neu differs from c-neu by a single point mutation within the TM region of the glycoprotein (7). Further studies have also provided evidence that specific interactions between the TM helices of ErbB2 can mediate dimerization and receptor activation (8). In contrast to these findings, Tanner and coworkers have argued that interactions between the TM domains do not contribute significantly to ErbB2 receptor dimerization (9). Thus, the structural picture of the TM domain of ErbB2 remains elusive despite the extensive research devoted to this important protein.

The TM domains of the ErbB2 indicate two oligomerization motifs as shown in Figure 1 (10). The underlined sequences represent the Sternberg-Gullick motif and the bold sequences are related to GXXG motif. Sequence-specific association of helices is principally mediated by these two motifs. The types of interactions that can occur within these motifs to create oligomerization need to be thoroughly investigated experimentally. Therefore, integration of several research endeavors is required to understand the complete structural characteristics of the TM helices within the lipid membrane.

hErbB2(653-675)-SIISAVVGILLVVVLG**VV**FGILI

Fig 1: Sequence alignment of the TM domain of the human member of the EGFR family. The motifs identified are the underlined residues representing the Steinberg-Gullick motif and the bolded residues representing the GXXG motif.

The objective of the proposed research is to investigate the structural properties of the transmembrane helix of the ErbB2 receptor utilizing solid-state nuclear magnetic resonance (NMR) spectroscopy and Molecular dynamic (MD) simulation. I am interested in using solid-state and high-resolution NMR to characterize the wild type peptide targeting the transmembrane region of the HER2/neu protein. Solid-state NMR spectroscopy is one of the techniques that can be utilized to probe membrane proteins in lipid bilayers (11, 12). Several literature sources are available that give detailed descriptions of the method and its application to membrane proteins (11-14). Whereas solid-state NMR provides information about the orientation of the protein in the membrane, high-resolution NMR will provide the structure of the protein within the membrane. Both techniques will provide the information needed to define the structural features of the transmembrane segment of ErbB2/neu.

Body:

We plan to study the transmembrane segment of HER2/neu (about 23 amino acid residues) using NMR spectroscopic techniques. The unlabeled peptide will be incorporated into aligned dimyristoylphosphatidylcholine/dihexanoylphosphatidylcholine (DMPC/DHPC) bicelles and the structure analyzed using 2-dimensional NMR. In these studies, the secondary structure of the peptide will be determined. The high-resolution NMR will also include analysis of linewidths for various bicelle sizes. In conjunction with these experiments, solid-state ^2H NMR will be performed to provide the orientation of the peptide with respect to the bilayer normal in both positive and negative aligned bicelles. In cases where we need another label to obtain some missing data, other amino acids such as valine and leucine will be labeled. The ^2H labeled peptides will be synthesized using solid phase peptide synthesis (Molecular Biology Core Facilities, Dana Farber Cancer Institute, Boston). Examination of possible oligomerization will be evaluated after the general peptide structure and orientation are resolved.

Key Research Accomplishments:

We have performed nuclear magnetic resonance (NMR) spectroscopy and Molecular dynamics (MD) simulations in the single membrane-spanning α -helix domain of ErbB2 designated TM-ErbB2. ^2H and ^{15}N Solid-state NMR results demonstrated that TM-ErbB2 has a transmembrane helical domain and that the orientation of the transmembrane domain is $24 \pm 5^\circ$ in shorter chain dimyristoylphosphocholine (DMPC) and $11 \pm 3^\circ$ in palmitoyloleoyphosphocholine (POPC). The orientation is dictated by the hydrophobic thickness of the synthetic phospholipid bilayers. Molecular dynamics simulations analysis demonstrated that in shorter chain lipids TM-ErbB2 also makes a tilt angle of about $28 \pm 5^\circ$ with respect to the bilayer normal whereas in longer chain lipids, the tilt angle was found to be 14 ± 4 . We also conducted dimerization studies with the wild type TM-ErbB2 within the membrane bilayer environments. One of the motifs responsible for dimerization (SAVVG) was mutated to alanines, whereas the other motif (GVVFG) was left intact. The GVVFG motif still had the ability to dimerize. The results demonstrate that the transmembrane region of ErbB2 orientation is lipid environment dependent and that the mutant ErbB2 peptide can still undergo dimerization with only one dimerization motif. The results reveal orientation and structural information about TM-ErbB2 that we intend to investigate in vivo. We are finalizing the structural determination of the wild type ErbB2 peptides in model membrane using the solution NMR techniques. The completed structural studies as well as the in vivo studies involving breast cancer cell lines will result in significant finding suitable for publication.

Reportable Outcomes:

(a) Investigating the Dynamics of the Transmembrane Segment of HER2/neu in Bicelles.

In these preliminary results, the secondary structure of the synthetic, and expressed peptides corresponding to the transmembrane segment of HER2/neu were analyzed by circular dichroism spectroscopy. ^2H Solid-State NMR spectroscopic studies of the HER2 peptides was also conducted in (DMPC/DHPC) bicelles.

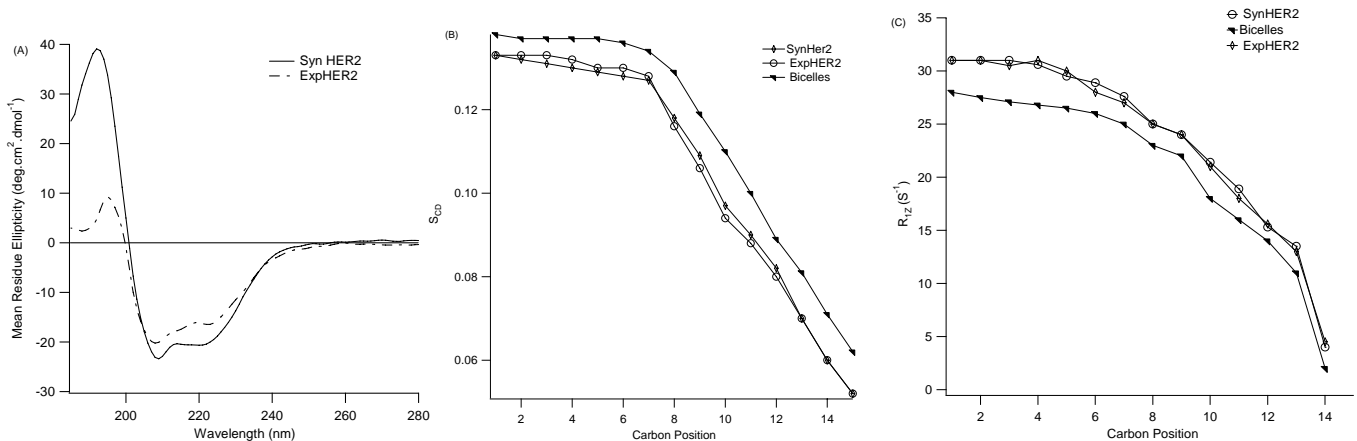


Fig 1: (A) The circular dichroism spectra of expressed/synthetic HER2/neu indicate stable secondary structural characteristics in DMPC/DHPC bicelles. (B) ^2H order parameters indicate that both HER2/neu peptides perturb the bilayer significantly as compared to the pure lipids. (C) Membrane fluidity is also significantly influenced by the HER2/neu peptides. The preliminary results indicate that expressed and synthetic HER2 are indistinguishable in terms of their structure and that both perturb the bilayer to the same extent.

The bicelles of DMPC/DHPC were prepared with a molar q ratio of ~ 3.5 (# of moles DMPC/# of moles DHPC). The peptide concentration was optimized based upon the NMR spectral linewidths. These studies were carried out from the lipid perspective by applying the quad echo pulse sequence on the DMPC- d_{54} lipids. The lipid affinities of the HER2/neu peptides were analyzed based upon their line shapes, order parameters and $T_{1\rho}$ relaxation measurements. Order parameters were then calculated from the quadrupolar splitting of the aligned ^2H spectra and the results compared with the pure lipids. ^2H relaxation measurements of the lipid/peptide mixtures were carried out on the pure DMPC as well as on the lipids containing HER2 peptide to determine membrane fluidity in the presence of the peptides.

(b) Investigating the Orientation of the Transmembrane Segment of HER2/neu in Bicelles.

The next sets of experiments were designed to determine the orientation and structure of the peptides within the bilayers with mechanically aligned phospholipid bilayers. To carry out these sets of experiments, we synthesized specific ^{15}N labeled ErbB2 transmembrane peptide. For the synthetic peptide, residues were chosen such that they represent the hydrophobic region of the peptide, which is supposed to be transmembrane. The ^{15}N -labeled solid-state NMR spectra will reveal the orientation of the protein with respect to the bilayer normal. As shown in Figure 2D, the dynamics of the peptides within the bilayer clearly indicates an alpha helical ErbB2 as demonstrated by the chemical shift anisotropy (CSA) span of about 200 ppm. The aligned spectrum in Figure 3C represents $[^{15}\text{N}\text{-Ala}_{657}]$ ErbB2. The residue was chosen such that the transmembrane region is almost parallel to the bilayer normal. The resonance peak at about 200 ppm indicates that Ala $_{657}$ of ErbB2 is within the phospholipid bilayer environment. Figure 2B indicates that Leu $_{674}$ of $[^{15}\text{N}\text{-Leu}_{674}]$ ErbB2 is outside the phospholipid bilayer.

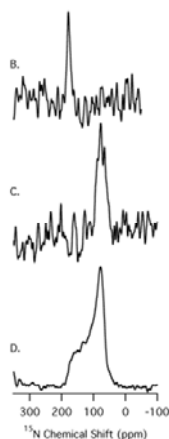


Fig. 2: One-dimensional ^{15}N chemical shift NMR spectra of ErbB2 peptide in oriented phospholipid bilayers. (A) The spectrum corresponding to selectively $[^{15}\text{N}\text{-Ala}_{657}]$ ErbB2 in oriented DMPC phospholipid bilayers. (B) The spectrum corresponding to selectively $[^{15}\text{N}\text{-Leu}_{674}]$ ErbB2 in oriented DMPC phospholipid.

(c) Simulation Results.

A refined model of wild type human ErbB2(653-675) was constructed using the rat ErbB2(650-684) NMR structure (PDB ID: 1IIJ) as a template in prime. Residues S656-G660, forming one of two GXXXG-like recognition motifs, were then mutated to alanine. For each sequence, four peptides were arranged in a square, with 20 Å between them. Each system was solvated with 13246 simple point charge (SPC) water molecules for the wild type and 13700 for the mutant construct. Each system was minimized with steepest descents, to relax unfavorable contacts between molecules. Then, equilibration molecular dynamics (MD) with all the heavy

atoms in the peptide positionally restrained with a force constant of $1000 \text{ kJ mol}^{-1} \text{ nm}^{-2}$, were performed for 200 ps. Unconstrained production MD was carried out for 1200 ps on each system. All simulations were carried out with the GROMACS program version 3.2.1.

In the wild type simulation, after 800 ps all four peptide strands began to oligomerize, self-aggregating via the two GXXXG-like motifs (Fig X a). The mutated peptides also oligomerized after 1000 ps, however only via the remaining GXXXG-like motif (Fig X b). Further work will simulate the mutation of the second GXXXG-like motif, residues G668-G672, to alanine. The double motif mutant will also be modeled, where ten residues S656-G660 and G668-G672 are mutated to alanine. Peptides will be modeled in a solvated lipid bilayer, to mimic their environment in the membrane. Future simulations will be longer in length and will also study the effect of peptide concentration on oligomerization.

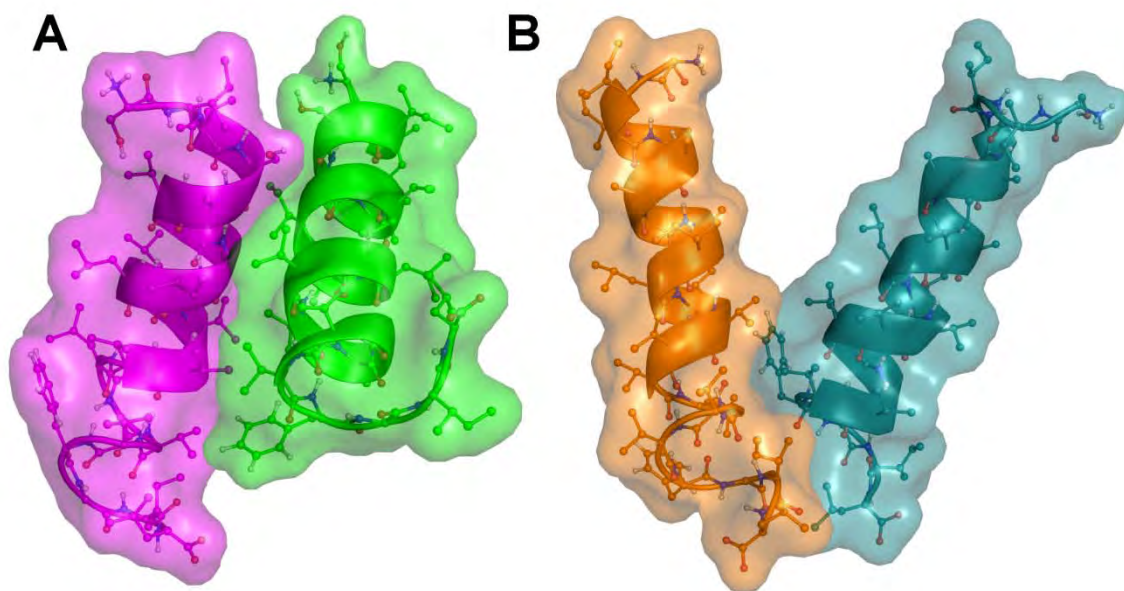


Fig 3. Two of the four (A) wild type and (B) S656-G660 alanine mutant human ErbB2(653-675) peptides following 1200 ps of molecular dynamics.

(d) Structural Determination of ErbB2 in model Membranes.

High-resolution NMR experiments were conducted on synthetic HER2/neu peptides to explore the level of oligomerization. Six residues were labeled with ^{15}N isotopes (^{15}N -Ile₆₅₆, -Val₆₅₉, -Leu₆₆₃, -Val₆₆₅, -Val₆₆₇, -Val₆₇₀). In these studies, isotropic DMPC/DHPC bicelles with a q ratio of ~ 0.5 was used to incorporate the peptide. The cross peaks in the ^{15}N - ^1H HSQC spectra indicate that the peptide is folded into stable secondary structures in the membrane, supporting the circular dichroism spectra in Fig 1(A). ^1H - ^1H NOESY are yet to be assigned together with the TOCSY spectra to determine the structure in the membrane.

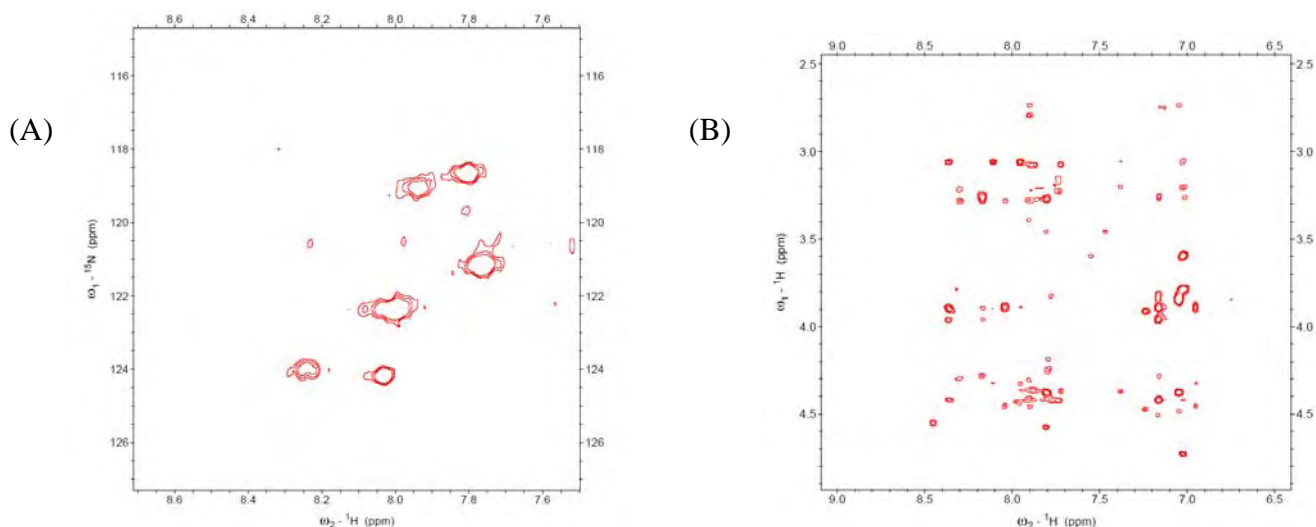


Fig. 4: (A) ^{15}N - ^1H HSQC of selectively labeled synthetic HER2 peptide. (B) ^1H - ^1H NOESY spectra of synthetic HER2 peptide. The spectra were collected in DMPC/DHPC bicelles with a q ratio of ~ 0.5 . The ^{15}N - ^1H HSQC peaks indicate that the peptide is folded into its secondary structure in DMPC/DHPC. The assignment of the cross peaks in 2B will be used to determine the structure of the peptide in the DMPC/DHPC bicelles.

Conclusion:

We have performed nuclear magnetic resonance (NMR) spectroscopy and Molecular dynamics (MD) simulations in the single membrane-spanning α -helix domain of ErbB2 designated TM-ErbB2. ^{15}N Solid-state NMR results demonstrated that TM-ErbB2 has a transmembrane helical domain and that the orientation of the transmembrane domain is $24 \pm 5^\circ$ in shorter chain dimyristoylphosphocholine (DMPC) and $11 \pm 3^\circ$ in palmitoyloleoylphosphocholine (POPC). The orientation is dictated by the hydrophobic thickness of the synthetic phospholipid bilayers. Molecular dynamics simulations analysis demonstrated that in shorter chain lipids TM-ErbB2 also makes a tilt angle of about $28 \pm 5^\circ$ with respect to the bilayer normal whereas in longer chain lipids, the tilt angle was found to be 14 ± 4 . We also conducted dimerization studies with the wild type TM-ErbB2 within the membrane bilayer environments. One of the motifs responsible for dimerization (SAVVG) was mutated to alanines, whereas the other motif (GVVFG) was left intact. The GVVFG motif still had the ability to dimerize. The results demonstrate that the transmembrane region of ErbB2 orientation is lipid environment dependent and that the mutant ErbB2 peptide can still undergo dimerization with only one dimerization motif. We are in the process of conducting solution NMR experiments to elucidate the structure of the peptide within lipid bilayer environments. The present results reveal orientation and structural information about TM-ErbB2 that we intend to investigate in vivo.

Now that we have evidence of the TM-ErbB2's ability to oligomerize, the ultimate goal of this project is to test the refined oligopeptides derived from the experiments above in breast cancer cell lines to block oligomerization. The oligopeptides will be tested in four breast cancer cell lines, BT-474, SK-BR-3, T-47D and MDA-MB-453. Activation states of the HER2 receptor will be evaluated by western blotting with anti-Tyr1248 ErbB2 antibody. Changes in downstream signaling induced by ErbB2 activation will be assessed by Western blotting procedures using anti-phospho-Akt and anti-phospho-Erk1/2 antibodies.

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