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Coupling of Transmembrane Helix Orientation To Membrane Release of the Juxtamembrane Region in FGFR3

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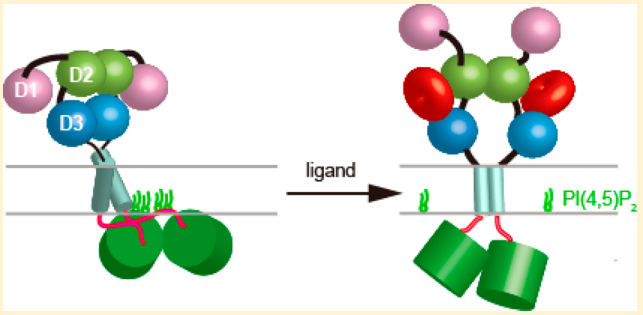
Supporting Information

ABSTRACT: Activation of the protein tyrosine kinase receptors requires the coupling of ligand binding to a change in both the proximity and orientation of the single transmembrane (TM) helices of receptor monomers to allow transphosphorylation of the receptor kinase domain. We make use of peptides corresponding to the TM and juxtamembrane (JM) regions of the fibroblast growth factor receptor 3 to assess how mutations in the TM region (G380R and A391E), which lead to receptor activation, influence the orientation of the TM domain and interactions of the intracellular JM sequence with the membrane surface. On the basis of fluorescence and Fourier transform infrared spectroscopy, we find that both activating mutations change the TM helix tilt angle relative to the membrane normal and release the JM region from the membrane. These results suggest a general mechanism regarding how the TM−JM region functionally bridges the extracellular and intracellular regions for these receptors.



FGFRs constitute one of several subfamilies in the RTK family of membrane receptors. Four FGFRs, namely, FGFR1, FGFR2, FGFR3, and FGFR4, are known to mediate a variety of cellular responses during embryonic development. Dysregulation of FGFR signaling is associated with many developmental disorders and cancer.[1] The prototypical FGFR consists of an extracellular ligand binding domain, a single TM domain, and a cytoplasmic tyrosine kinase domain. The crystal structures of the soluble extracellular[2,3] and the intracellular[4,5] regions of FGFR3 provide a considerable amount of information that aids in understanding the mechanism underlying receptor signaling; however, there are still no high-resolution structures of the full-length receptors of any RTK.

The mechanism that emerges from structural and biochemical studies is one where the FGF ligand binds to the extracellular domain of the receptor in a 1:1 complex. This complex is thought to form a symmetric dimer in the active state.[6] The ligand binds to two of the immunoglobulin-like extracellular subdomains, D2 and D3, and these two subdomains along with the D2−D3 linker are necessary and sufficient for ligand binding. The D1 subdomain together with the D1−D2 linker have an autoregulatory role in receptor function.[6] High resolution structures of activated kinase domains for FGFR1[4] and FGFR2[5] have been shown to be asymmetric dimers. Importantly, Bae et al.[4] demonstrated that the formation of the asymmetric dimer between the activated FGFR1 kinase domains is required for the transphosphorylation of the FGFR in FGF-stimulated cells.

The activation mechanism proposed on the basis of FGFR crystal structures builds on that proposed by Kuriyan and coworkers for the EGFR.[7,8] An open question has been how a symmetric extracellular domain dimer leads to asymmetric interactions between the intracellular domains. For the EGFR family, the question of how ligand binding is coupled to receptor activation has been of intense interest since the intracellular JM domain is allosterically coupled to the ligand-binding site.[9,10] We have addressed how the TM and intracellular JM regions of the rat ErbB2/Neu receptor might transmit the extracellular signal induced by ligand binding to the intracellular domain.[11] We took advantage of a constitutively active mutation (V664E) in the TM region of the Neu receptor and compared the structures of the TM−JM sequences of the wild-type and V664E mutant. The results of solid-state NMR and fluorescence studies on these peptides reconstituted into lipid bilayers showed that the TM helices of the V664E mutant dimerized more tightly than did those of the wild-type Neu construct. In addition, the unstructured JM region of the wild-type Neu TM−JM peptide was found to be bound to acidic membranes containing the phosphoinositide, PIP2. In contrast, the JM region of the mutant was not bound to the membrane, and we further showed that the binding affinity of the JM sequence for the membrane was dependent

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on the relative orientation of the TM helices. We concluded that the TM helices in the active dimer conformation could release the JM region from the membrane surface, which would then allow for the formation of an asymmetric dimer by the intracellular kinase regions. Endres and co-workers proposed a similar model on the basis of detailed biochemical and structural analyses of the EGFR.[7,8]

The similar structures of receptors in the EGFR and FGFR families suggest a common mechanism involving the TM region for transmitting the structural changes from the outside to the inside of a cell. There are two pathogenic mutations, G380R and A391E, in the TM region of FGFR3 that lead to activation. The A391E mutation appears to be similar to the V664E mutation in the Neu receptor (i.e., substitution of a hydrophobic residue with glutamic acid). A391E is the genetic cause of Crouzon syndrome[12] and has also been identified as a somatic mutation in bladder cancer.[13] This mutation can enhance FGFR3 activation in the absence of the ligand, which results in an increase in the dimerization propensity of the TM sequence alone in lipid bilayers[14] and of the full-length receptor in the cell membrane.[15] The G380R mutation is related to achondroplasia,[16] which is the most common form of dwarfism. Hristova and colleagues reported that the TM region containing the G380R mutation did not alter the dimerization energetics of the TM helices in lipid bilayers.[17] Later, they showed that the G380R mutation had only a modest effect on receptor dimerization in plasma membrane vesicles derived from HEK293T cells, which suggests that the kinase activity due to the G380R mutation is caused by a mutation-induced structural change rather than an increase in dimerization.[18]

Here we compare the structure of the TM−JM region of wild-type FGFR3 peptides reconstituted into lipid bilayers to that of FGFR3 peptides containing the G380R and A391E mutants. The results from fluorescence, polarized FT-IR, and solid-state NMR spectroscopy show that the introduction of active mutations in the TM sequence changes the TM helix tilt angle relative to the membrane normal and releases the JM region from the membrane. Additionally, we examined the correlation between the TM helix orientation and the JM release from the membrane and found that the JM sequence of FGFR3 was released from the membrane as the TM helix tilt decreased relative to the bilayer normal. The results obtained in this study provide a framework for the general structural and functional understanding of how the TM−JM region bridges the extracellular and intracellular regions in RTKs.

EXPERIMENTAL PROCEDURES

Materials. ^{13}C-labeled amino acids and (5,5,5-d3) leucines were purchased from Cambridge Isotope Laboratories (Andover, MA) and derivatized using standard methods. Other amino acids were purchased from the Peptide Institute (Osaka, Japan). Solvents and other agents for peptide synthesis were purchased from Wastanabe Chemicals (Hiroshima, Japan). Octyl-β-glucoside was obtained from Nacalai Tesque (Kyoto, Japan). DMPC, DMPG, POPC, and POPS were obtained from Avanti Polar Lipids (Alabaster, AL) as lyophilized powders and used without further purification. Alexa Fluor 568 C_5-maleimide was purchased from Invitrogen (Eugene, OR).

Peptide Synthesis and Purification. Peptides corresponding to the TM and intracellular JM regions of the FGFR3 (369−407) for solid-state NMR and FT-IR experiments were synthesized by solid-phase methods with the following sequence.

RRAGSVYAGILSYG(/R)VGFFL$_{385}$FIL$_{388}$VVA(/E)-AVTLCRLRSPPKKGLG. Two arginine residues were added to the N-terminus of the peptide following the suggestion from You et al.[17] to eliminate complementary electrostatic interactions as a driving force for dimer formation. The C-terminus was amidated. The synthetic peptides were purified by reverse phase HPLC on a C4 column with a gradient of formic acid/1-propanol (4:1) over formic acid/water (2:3).[19] The purity was confirmed with MALDI mass spectrometry and analytical RP-HPLC.

For fluorescence experiments that focused on the JM region, peptides with a longer sequence were synthesized using native chemical ligation.[20] The sequence corresponds to FGFR3(367−422).

DEAGSVYAGILSYGVGFFLFILVVAAVTL-CRLRSP-PKKGLGSPTVHKISRFPLKRC. The ligation site was located between Leu395 and Cys396. Two peptide fragments, FGFR3(367−395)-thioester and FGFR3(396−422), were chemically synthesized using solid phase t-Boc and Fmoc chemistry, respectively. For the C-terminal fragment, a thiazolidine ring was introduced at the N-terminus to allow specific fluorescence labeling on a thiol group at the C-terminal end of the fragment. The thiazolidine ring was opened using methoxyamine to recover the thiol group for the ligation reaction. For this reaction, the fragment with the TM sequence was dissolved with *n*-octyl-β-D-glucoside following the procedure reported previously.[21] The ligation reaction was monitored using RP-HPLC and SDS-PAGE. The final product was characterized by mass spectrometry and SDS-PAGE.

For the Trp fluorescence experiment, we chemically synthesized the FGFR3(367−422)-Trp sequence with microwave-assisted Fmoc peptide synthesis using a peptide synthesizer, Liberty Blue (CEM Corporation, NC, U.S.A.).

Reconstitution of Peptides into Membrane Bilayers for NMR Experiments. The FGFR3 receptor peptides were cosolubilized with DMPC, DMPG, and *n*-octyl-β-D-glucoside in trifluoroethanol. The peptide/lipid molar ratio was 1:50; the molar ratio between DMPC/DMPG was 10:3. The solution was incubated for 90 min at 37 °C, after which the solvents were removed under a stream of argon gas and then under vacuum. MES buffer (50 mM MES, 50 mM NaCl, 5 mM DTT, pH 6.2) was added to the solid from the previous step, and they were mixed at 37 °C for 6 h. The *n*-octyl-β-D-glucoside was removed by dialysis. The reconstituted membranes were pelleted and loaded into NMR rotors.

Fluorescence Spectroscopy. Fluorescence experiments were carried out on a Hitachi F-2500 fluorescence spectrophotometer. After the reconstitution, vesicles were formed via extrusion through a 200 nm polycarbonate filter. POPC and POPS (or otherwise mentioned) in a ratio of 10:3 were employed for the lipid bilayers in the fluorescence experiments. The peptide/lipid ratio depended on the fluorescence probe that we used. The ratio was set to 1:1000 for observing the fluorescence from Alexa568. For experiments measuring the fluorescence from tryptophan, the ratio was set to 1:100. The lipid concentration was 200−250 μM in MOPS buffer (1 mM MOPS, 0.1 M KCl, pH 7.0). For experiments with PIP2, the PIP2 was introduced into the membranes by the addition of PIP2 micelles to the vesicle solution.[22] The PIP2 concentration ranged from 0.05−4 μM, which corresponded to the PIP2/peptide ratios of 1:50−2:1. For the experiments monitoring Alexa568, the excitation wavelength was 568 nm. To monitor

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the fluorescense of tryptophan, the excitation wavelength was 295 nm.

Solid-State NMR Spectroscopy. Solid-state NMR MAS experiments were performed on Varian Infinity-plus 500, 600, and 700 spectrometers operated at 11.74, 14.09, and 16.44 T, respectively (Palo Alto CA). Broadband triple resonance MAS probes for 3.2 mm and 4.0 mm rotors were used. For the deuterium-observe experiments, the MAS frequency was maintained at 5 kHz and a single pulse excitation was employed with a 5 μs, 90° pulse length, followed by a 10 μs delay before data acquisition. A total of ~600 000 transients were averaged for each spectrum. The probe temperature was maintained at 25 °C.

Polarized IR Spectroscopy. Polarized attenuated total reflection FT-IR spectra were obtained on a Hitachi FT-730 spectrometer. Membranes containing FGFR3 TM−JM peptides (~1−2 mg) were layered on a germanium internal reflection element using a slow flow of nitrogen gas directed at an oblique angle to the IR plate to form an oriented multilamellar lipid−peptide film. One thousand scans were acquired and averaged for each sample at a resolution of 4 cm^{-1}. The absorption of polarized light by the amide I bond yields the dichroic ratio defined as a ratio of absorption intensity for parallel, relative to perpendicular, polarized light. From the dichroic ratio, we estimated the tilt angle of the TM helix relative to the membrane normal based on the method described by Smith and co-workers[23,24] using a value of 41.8 for angle α between the helix director and the transition-dipole moment of the amide I vibrational mode. For our ATR FT-IR experiment, the amount of lipid used per experiment was ~4 mg. Considering the area of the ATR plate covered (~500 mm^2), the films on the plate in our experiments were assumed to be greater than ~10 μm. For calculating the dichroic ratio, the thick film limit is applicable.[25] Equations that we used for the calculation of the dichroic ratios were based on this assumption.

RESULTS

The JM Sequence of the TM−JM (G380R) and TM−JM (A391E) Peptides Is Not Associated with the Membrane Surface. We first show that peptides corresponding to the JM sequence alone bind to the negatively charged surface of membrane vesicles, and an increase in the concentration of the highly negatively charged phosphoinositide, PIP2, leads to the association or aggregation of these peptides on the membrane surface. The intracellular JM sequence of FGFR3 has four positively charged residues that would be expected to bind to the negatively charged surfaces of plasma membranes. Previous studies by McLaughlin and co-workers[26] showed that model polylysine peptides with 3 and 5 residues bind to model membranes having 33% negative charge and 100 mM monovalent salt with free energies of 3 and 5 kcal/mol. Our system, with 4 positive charges, 23% negatively charged lipids, and 100 mM monovalent salt is similar. Furthermore, it was shown that the addition of polyvalent PIP2, but not monovalent PS lipids, leads to the aggregation of positively charged peptides on the membrane surface.[22]

Figure 1A presents the results of the fluorescence experiments on the intracellular JM sequence with the fluorescence label, Alexa568, attached to the C-terminus. The peptides were added to large unilamellar vesicles formed by extrusion and composed of POPC and POPS in a 10:3 molar ratio. PIP2 was incorporated into the preformed vesicles by being mixed with a

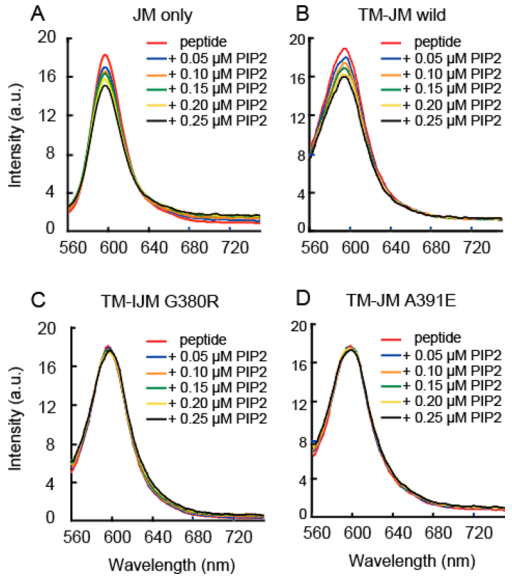

Figure 1. Comparison of fluorescence spectra of the wild-type FGFR3 TM−JM peptide with the G380R and A391E mutants. The influence of PIP2 on JM−JM interactions was measured for TM−JM sequences of the isolated JM domain (A) and the TM−JM peptides corresponding to the wild-type (B), G380R mutant (C), and A391E mutant (D) sequences. The fluorescence measurements in panels A−D are representative of three independent reconstitutions.

solution of PIP2 micelles, and the fluorescence intensity of the Alexa568 label was measured as a function of the amount of PIP2 in the bilayer. McLaughlin and co-workers have shown that PIP2 monomers can be incorporated into the outer leaflet of preformed vesicles by exposing them to micelles of PIP2;[22] fluorescence correlation spectroscopy measurements showed that PIP2 diffuses with other lipids in the membrane (giant unilamellar vesicles) after incorporation.

The fluorescence emission band at 604 nm shows a drop in intensity as PIP2 was added to the membranes. We interpret this as the quenching of the Alexa568 fluorescence as the JM peptides aggregate on the membrane surface. Similar results were observed for the positively charged JM peptides corresponding to the Neu receptor.[11]

We next performed similar experiments for TM−JM peptides reconstituted into large unilamellar vesicles composed of POPC and POPS in a 10:3 molar ratio. In the Supporting Information, we show using solid-state NMR that the TM helix breaks at the TM−JM boundary. The JM region is unstructured. Figure 1, panels B−D present the fluorescence spectra of the TM−JM (WT), TM−JM (G380R), and TM−JM (A391E) peptides. Figure 1B shows that the fluorescence intensity of the TM−JM (WT) peptide decreased with the addition of PIP2, similar to the JM sequence alone. In contrast, no significant changes were observed in the fluorescence intensity of the TM−JM (G380R) and TM−JM (A391E) peptides with the addition of PIP2, as shown in Figure 1C,D, respectively. We attribute the lack of fluorescence changes in the TM−JM (G380R) and TM−JM (A391E) sequences to a lack of JM−JM association, which suggests that the JM sequence had partitioned off of the membrane.

We further confirmed the interaction of the JM region with the acidic membrane by tryptophan fluorescence. Binding and insertion of tryptophan into the hydrophobic membrane results in a blue-shift of the fluorescence emission band. We chemically

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synthesized FGFR3 peptides (residues 367−422) that corresponded to the wild-type sequence, the G380R and A391E mutants, and having a tryptophan residue at the C-terminus. The peptides were reconstituted into lipid bilayers composed of POPC/POPS (10:3). Here we set the peptide/lipid ratio to 1:100 (or even up to 1:50) as opposed to that of 1:1000 for observing the fluorescence from Alexa568. In our system, the high ratio was required to overcome the effect of light scattering from the lipid vesicles. We confirmed that the addition of PIP2 to the system reduces the fluorescence intensity for the wild-type sequence, whereas PIP2 addition has no effect on the intensity from mutant sequences (data not shown), which is in agreement with the results of the Alexa568 fluorescence at a 1:1000 molar ratio. Figure 2 shows the results

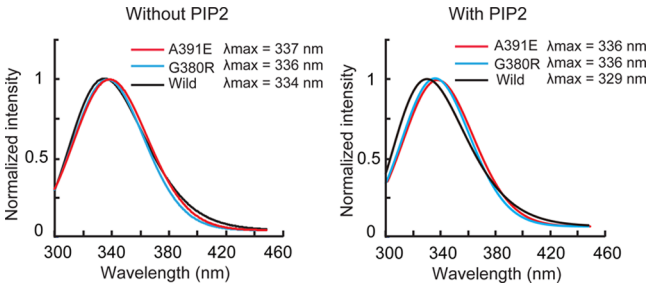

Figure 2. Comparison of tryptophan fluorescence spectra of the wild-type FGFR3 TM−JM peptide with the G380R and A391E mutants. Interaction between the JM region and the membrane was monitored. Spectra show the fluorescence from the peptides reconstituted into the membrane without PIP2 (on the left) and with PIP2 (on the right).

of the fluorescence measurements from tryptophan for each sequence. Intensities were normalized to the fluorescence intensity value measured at the wavelength of the emission maximum. In the system without PIP2, we observed only a small difference in the value of λ_{max}. The difference becomes distinct with the addition of PIP2 to vesicles. The spectrum from the wild-type sequence was blue-shifted 7 nm relative to that of the mutant sequences. The results agree with our interpretation of the data in Figure 1, that the JM region binds to the acidic membrane in the wild-type sequence and is released from the membrane in the G380R and A391E mutant sequences.

The G380R and A391E Mutations Induce a Change in Helix Orientation. The fluorescence experiments described above argue that the Alexa568 and tryptophan labels at the C-terminus of the JM region are in different environments in the G380R and A391E mutants than in the wild-type peptide. In this section, we address whether there is a change in the orientation of the TM portion of the peptide in these mutants using polarized FT-IR spectroscopy.

The TM−JM (WT), TM−JM (G380R), and TM−JM (A391E) peptides were reconstituted into POPC/POPS (10:3) or DMPC/DMPG (10:3) vesicles, and the vesicles were aligned on an ATR plate for polarized FT-IR measurements. Figure 3 shows the deconvoluted polarized FT-IR spectra of these sequences. The peak at 1657 cm^{-1} corresponds to the α-helical structure within the TM sequence. The dichroic ratio of each sequence is also shown in the figure. The values presented are the average results from multiple measurements (>3). The dichroic ratios of the TM−JM (G380R) and TM−JM (A391E) peptides were higher than that of the TM−JM (WT) peptide, suggesting that the TM helices with the

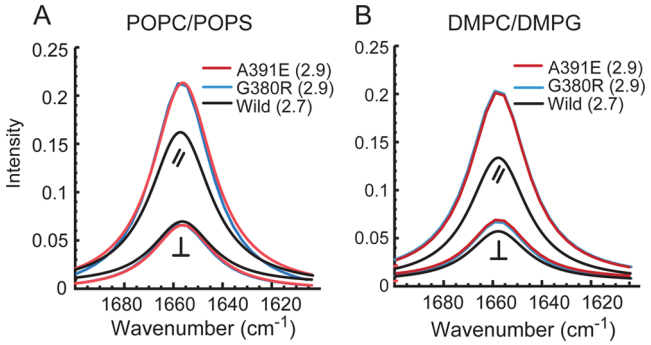

Figure 3. Comparison of polarized FT-IR spectra of the wild-type FGFR3 TM−JM peptide with the G380R and A391E mutants. The three TM−JM peptides were separately reconstituted into POPC/POPS (10:3) (A) or DMPC/DMPG (10:3) (B) membranes, and FT-IR spectra were obtained with light polarized parallel (||) or perpendicular (⊥) to the membrane normal. Only the amide I region of the IR spectrum is shown for clarity. The frequency of the amide I band at ∼1657 cm^{-1} is characteristic of α-helical secondary structure.

pathogenic mutations are less tilted than the TM helix of the wild-type peptide. This tendency was observed for measurements in both POPC/POPS and DMPC/DMPG membranes.

We then focused on the wild-type sequence and examined whether the orientation of the TM helix affected the release of the JM region from the membrane. The TM helix tilt was changed by reconstituting the TM−JM (WT) peptide into lipid bilayers with different thicknesses, which were varied by using lipids with different acyl chain lengths. It has been shown that the tilt angle of model TM peptides (WALP and KALP) decreases as membrane thickness increases due to the hydrophobic mismatch.[27,28] In addition, it has been shown that the tilt angle of the TM helix of the platelet-derived growth factor receptor, another RTK, decreases as the membrane thickness increases. For these experiments, the TM−JM (WT) peptide was reconstituted into different bilayers containing POPC, C18, or C19, respectively. In each case, POPS (23%) was added to maintain a negatively charged membrane surface. The dichroic ratio was observed to increase with an increase in the length of the acyl chain (C18, 2.8; C19, 3.3). The thicknesses of the lipid bilayers used were expected to be 27 Å for POPC,[29] 29.5 Å for C18, and 30.5 Å for C19.[30] A dichroic ratio of 3.3 corresponds to a helix tilt angle of ∼20° and a crossing angle between helices in a dimer of ∼40°, which is a typical value of helices in a left-handed coiled-coil geometry.

To examine the effect of the TM helix orientation on the interaction of the JM sequence with the membrane surface, we performed similar experiments as described above to determine whether PIP2 affected the fluorescence intensity of the Alexa probe incorporated at the C-terminus of the TM−JM (WT) peptide. The results are shown in Figure 4. For the POPC vesicles, the fluorescence intensity decreased upon the addition of PIP2 (Figure 4A); for the C18 vesicles, the intensity first increased with the initial addition of PIP2 and then decreased with additional PIP2 (Figure 4B). The decrease in the intensity of POPC and C18 vesicles can be attributed to the association of JM strands and the interactions of the vesicles with PIP2. However, for the C19 vesicles, the intensity was not responsive to the addition of PIP2 (Figure 4C), indicating that no interaction occurred between the JM region and PIP2 and further suggesting that the JM sequence was released from the membrane. A simple conclusion that can be drawn from the

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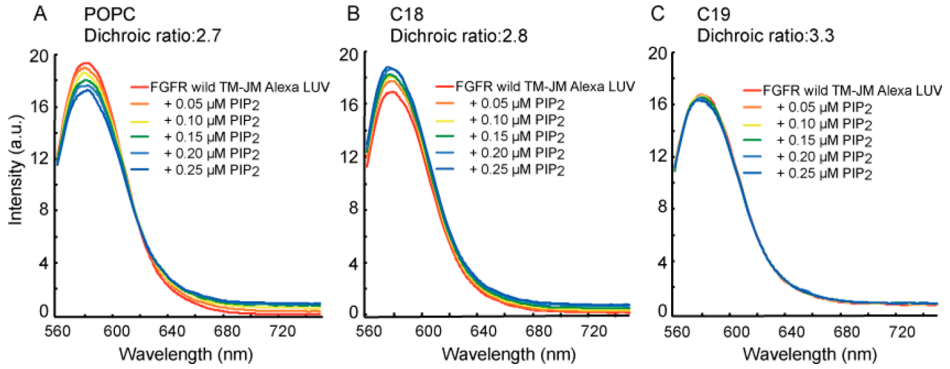

Figure 4. Dependence of JM–JM interactions in the TM–JM peptides on membrane thickness. The TM–JM peptide in the wild-type sequence was reconstituted into bilayers containing POPC (A), C18 (B), and C19 (C) along with 23% POPS. The influence of PIP2 on JM interactions was measured. The dichroic ratios of POPC, C18, and C19 samples obtained from polarized FT-IR experiments are also shown.

polarized FT-IR and fluorescence experiments is that for FGFR3, the JM region is released from the membrane as the TM helix orientation is shifted closer to the membrane normal.

Deuterium NMR Provides a Probe of Helix Dimerization, Orientation, and Motion. The FT-IR spectra reveal a distinct change in helix tilt between the TM–JM peptides corresponding to the inactive wild-type and active mutant sequences. Hristova and co-workers[14,17] have shown that the TM sequences of the wild-type, G380R, and A391E peptides of FGFR3 have the potential to dimerize in membrane bilayers. Comparison of dimerization between the wild-type and G380R peptides showed that the G380R mutation did not alter the monomer–dimer equilibrium in lipid bilayers.[17] In contrast, the comparison of the wild-type peptide and the A391E mutant peptide showed that the A391E mutation stabilizes the TM dimer.[14]

Deuterium MAS NMR spectroscopy provides a simple method to probe helix dimerization, orientation, and motion.[31] The intensities of the spinning side bands in the deuterium MAS spectrum are sensitive to molecular motion. Leucine, with a single deuterated methyl group at the end of its long side chain, is highly mobile in TM helices when facing the surrounding lipids and is relatively more constrained when packed within a dimer interface, suggesting its use as a probe of the interface for interacting TM helices. We used deuterium NMR spectroscopy here to probe whether the wild-type peptide sequence exhibits differences compared to the G380R and A391E peptides, but that G380R and A391E behave in a similar fashion, as observed by fluorescence and FT-IR spectroscopy.

Deuterated leucine was introduced to positions 385 and 388 of the TM–JM sequences of the wild-type, G380R, and A391E peptides. These leucines are in the middle of the TM domain. The spectra are shown in Figure 5. Distinct differences can be observed between the spectra of Leu385 and Leu388 in the TM–JM (WT) peptides. The line shape of Leu385 is broader than that of Leu388, which corresponds to a decrease in mobility of the Leu385 side chain. (Differences in the breadth of the line shape are reflected, for example, in the intensity of the ±2 spinning side bands.) The line shape of Leu385 is typical of that observed when the labeled leucine is in a dimer interface.[11,32] In contrast, the ±2 sidebands in the spectrum of Leu388 are much less intense than the ±1 side bands, indicating that Leu388 is more mobile, which is consistent with an orientation facing the lipids. Together, these two spectra suggest that the wild-type TM–JM peptides dimerize with an

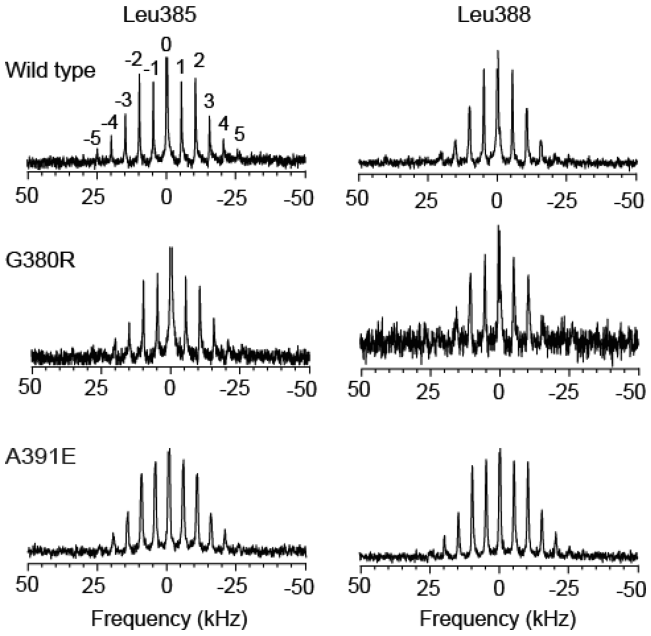

Figure 5. Deuterium MAS spectra of 5,5,5–2H Leu385 and Leu388 incorporated to the wild-type, G380R, and A391E FGFR3 TM–JM peptides. Wild-type (top), G380R (middle), and A391E (bottom) peptides with deuterium labeling at Leu385 (left column) and Leu388 (right column) were reconstituted into DMPC:DMPG vesicles. The spectra were obtained at 25 °C with a MAS frequency of 5 kHz. The MAS side bands are labeled as ±1, ± 2, ± 3, etc. from the center band at a frequency of 0 kHz. The spacing between side bands corresponds to the MAS frequency of 5 kHz.

interface mediated by S378-G382-L385. The S378-G382 interface is consistent with the small amino acid motif originally identified in the N-termini of RTKs by Sternberg and Gullick.[33] The dimer interface that we predict here agrees with the one obtained from molecular dynamics simulations by Sansom and co-workers on FGFR3.[34]

For A391E, little difference was observed between the spectra from Leu385 and Leu388. However, a comparison of the spectrum of Leu388 with that of the wild-type shows that the difference in intensities between ±1 and ±2 spinning sidebands is smaller for the A391E peptide. The line shape of Leu388 from the A391E peptide is broader than the one from the wild-type peptide. Even considering that TM helices may obtain some mobility from rotational diffusion due to the JM release, this suggests that the mobility of the side chain of

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Leu388 on the A391E peptide is relatively more restricted than the one on the wild-type. In contrast, the side chain of Leu385 exhibits increased mobility in the A391E mutant. These results imply that the A391E mutation induces a change in the dimer interface.

For the G380R peptide, the line shapes from Leu385 and Leu388 are similar, suggesting that both side chains are mobile. The increased mobility can be caused either by increased rotational diffusion induced by the JM release from the membrane or by the reorientation of TM helices or both.

DISCUSSION

The FGFR3 is similar to the ErbB2 or Neu receptor in that the mutation of specific hydrophobic residues in the TM sequence to glutamate or arginine can result in receptor activation. In the case of the Neu receptor, the V664E mutation shifts the monomer−dimer equilibrium toward the dimeric state, and the specific orientation of the helices in the receptor dimer facilitates the partitioning of the positively charged JM sequence from the membrane surface.[11] In the FGFR3, there are two clinically important mutations (G380R and A391E) that lead to receptor activation. The extensive studies of Hristova and co-workers have indicated that these mutations influence TM helix interactions in FGFR3. In fact, the A391E mutation in FGFR3 appears analogous to the V664E mutation in the Neu receptor in that both strengthen the association of the helices in a specific active orientation. The major finding in our current studies is that these mutations lead to similar changes in the orientation of the TM helices and to the spectral changes on probes attached to the JM domain. We interpret these changes as being due to the partitioning of the JM region from the membrane surface.

The intracellular JM region has long been recognized as an important regulatory site in RTKs.[35] Intracellular JM sequences are often highly positively charged. The positive charge not only serves as the stop sequence for the insertion of TM helices into cell membranes but it also terminates the TM helix. McLaughlin and others[32,36,37] have shown that such highly positively charged sequences can bind to negatively charged membrane surfaces and serve to sequester the highly negatively charged phosphoinositide, PIP2. While PIP2 can act as a second messenger in signal transduction, and has been associated with EGFR activity,[38] we are simply using PIP2 in the current studies as a way to measure membrane association of the JM region of the FGFR3. Another regulatory mechanism involving the JM region of RTKs is the interaction with calcium calmodulin, which is known to bind to the JM region of the EGFR.[39] Calcium calmodulin interacts with the JM region of the EGFR and facilitates its dissociation from the membrane, which in turn increases the transphosphorylation of its intracellular domain.[36] Phosphorylation typically occurs within the activation loop or the C-terminus of the kinase domain; however, it can also occur within the intracellular JM region itself. The studies presented here and previously[11] provide insight into how the JM region can function in a regulatory role.

For the FGFR subfamily, there is only limited structural information regarding how the extracellular domain is coupled to the intracellular kinase domain. Here, we have characterized the intracellular JM region of FGFR3 in order to see if there are parallels with the ErbB receptor subfamily. The JM region from FGFR3 binds to the acidic membrane containing PIP2. Importantly, this region is released from the membrane with an activating mutation, G380R or A391E, in the TM sequence.

The positively charged sequence of the JM region suggests that electrostatic interactions are essential for both membrane binding and release. In the ErbB system, we modulated the helix orientation with the V664E mutation and with engineered dimers.[11] We assumed that in the G380R and A391E peptides, the TM and JM regions are in active configurations. As mentioned above, the dimerization propensity differs between these mutant sequences,[14,17] and consequently, the mechanism for activity enhancement without ligand binding cannot be fully explained on the basis of dimerization alone. It is possible that there are two different changes in the TM region (induced by the two different activating mutations) that lead to similar changes on the intracellular side of membrane. Here we observed a decrease in the helix tilt angle relative to the membrane normal (i.e., the helices "stand up" in the membrane) as a common feature for these two mutant sequences. Furthermore, for the wild-type sequence, we found that the decrease of the TM helix tilt allows the release of the JM region from the membrane. On the basis of similar observations regarding the TM helix tilt and the JM release in the two FGFR3 mutant peptides, we propose that the orientation of the TM helices in active full-length receptor dimers contributes to the release of the JM region, allowing asymmetric interactions to occur between intracellular kinase domains.

The mechanism for release of the JM region from the membrane has still not been elucidated. One important consideration is whether the JM sequence is simply a helical extension of the TM helix or the secondary structure breaks at the TM−JM boundary and the JM sequence is unstructured. Preliminary solid-state NMR experiments on peptides containing 1-^{13}C labeled amino acids show that the TM helix breaks at the TM−JM boundary and suggest that the JM region is unstructured (Supporting Information). A similar observation was made for the Neu receptor peptides. In the wild-type peptides (and for the isolated JM peptides), the unstructured, positively charged JM sequence interacts electrostatically with the negatively charged membrane surface.[11,32] We propose that as the TM helices become less tilted in the membrane, or as dimerization propensity is increased with the A391E mutation,[14] the TM helices are brought together on the C-terminal side of the membrane. This change would bring the positively charged ends of the helices into closer proximity and result in repulsion between the JM positive charges. We suggest that this might facilitate the release of the JM region from the membrane.

The deuterium NMR results suggest that Leu385 lies in the dimer interface of the wild-type receptor. This orientation is consistent with the presence of the S378-G382 sequence at the N-terminus of the TM helix (Figure 5). An SxxxA motif has been shown to mediate the dimerization of the TM region of ErbB2,[40] while both GxxxG[41] and SxxxS[42] sequences have been implicated in TM dimerization. The dimer interface that we predicted agrees with the one obtained for FGFR3 by Sansom and co-workers using molecular dynamics.[34] However, the result does not agree with the interface observed in the high-resolution structure reported by Bocharov et al.,[43] rather, it corresponds to the activated configuration that they proposed. The difference from our structure may arise from their use of detergent micelles as a membrane-mimicking media. Micelles and bilayers may impose different constraints on the tilt angles of the interacting helices and hence the lowest energy interface.

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On the basis of our findings, together with the discussion above, Figure 6 presents a schematic model for the activation of

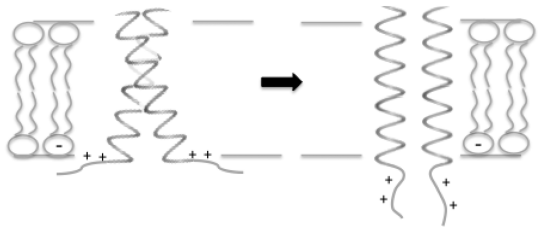

Figure 6. Schematic of TM−JM coupling in FGFR3. The isolated TM−JM peptides corresponding to FGFR3 have a propensity to form dimers in membrane bilayers.[45] The tilt angle of the helices in the wild-type receptor is estimated from the polarized IR experiments to be ∼40°. The positively charged JM regions associate with the membrane surface. The incorporation of PIP2 leads to the aggregation of the JM domains and the quenching of the fluorescence of the Alexa label attached to the C-terminus of the JM sequence. The incorporation of the activating G380R and A391E mutations changes the helix orientation and leads to a loss of quenching. We propose that the loss of quenching results from a partitioning of the JM sequence from the membrane surface. The A391E mutation on the C-terminal side of the TM domain increases dimerization[14] and may bring the helices together on the C-terminal side of the membrane, which in turn brings the positively charged ends into closer proximity.

FGFR3. Focusing on the TM−JM region, we propose that a change in orientation or tilt angle of the TM helices in the dimer results in the release of the JM region from the membrane surface. A similar mechanism has been shown for the thrombopoietin receptor, where a five residue sequence at the intracellular JM boundary modulates the helix angle and receptor dimerization.[44] This suggests that generally the structure and interactions of TM helices and their adjacent intracellular JM sequences may be coupled and that this coupling may be a target of regulation of receptor activity.

■ ASSOCIATED CONTENT

Supporting Information

Solid-state NMR ^{13}C spectra for the secondary structure of the FGFR3 TM−JM region. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

H.T. performed experiments, interpreted data, and contributed to writing the paper; Y.F. performed experiments and interpreted data; R.Y. and H.H. synthesized peptides; S.A. and S.O.S. analyzed data and helped write the manuscript; and T.S. is the project leader, designed experiments, performed experiments, and wrote the manuscript.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS:

ATR, attenuated total reflection; C18, 1,2-dioctadecanoyl-*sn*-glycero-3-phosphocholine; C19, 1,2-dinonadecanoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)]; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; FT-IR, Fourier transform−infrared spectroscopy; JM, juxtamembrane; MALDI, matrix-assisted laser desorption ionization; MAS, magic angle spinning; NMR, nuclear magnetic resonance; PIP2, phosphatidylinositol 4,5-bisphosphate; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; PS, phospho-L-serine; RP-HPLC, reversed-phase high-performance liquid chromatography; RTK, receptor tyrosine kinase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TM, transmembrane

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