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Rotational Coupling of the Transmembrane and Kinase Domains of the Neu Receptor Tyrosine Kinase

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Ligand binding to receptor tyrosine kinases (RTKs) regulates receptor dimerization and activation of the kinase domain. To examine the role of the transmembrane domain in regulation of RTK activation, we have exploited a simplified transmembrane motif, $[VVVEVVV]_n$, previously shown to activate the Neu receptor. Here we demonstrate rotational linkage of the transmembrane domain with the kinase domain, as evidenced by a periodic activation of Neu as the dimerization motif is shifted across the transmembrane domain. These results indicate that activation requires a specific orientation of the kinase domains with respect to each other. Results obtained with platelet-derived growth factor receptor-β suggest that this rotational linkage of the transmembrane domain to the kinase domain may be a general feature of RTKs. These observations suggest that activating mutations in RTK transmembrane and juxtamembrane domains will be limited to those residues that position the kinase domains in an allowed rotational conformation.

INTRODUCTION

Numerous cellular responses are mediated by growth factors that bind to and activate specific receptors with tyrosine kinase activity. These receptor tyrosine kinases (RTKs) share a common structure containing an extracellular ligand-binding domain, a single transmembrane domain, and an intracellular tyrosine kinase domain. Ligand binding results in receptor dimerization followed by transphosphorylation and activation of the kinase domain. Various models have been proposed as to how ligand binding regulates receptor activity (Williams, 1989; Ullrich and Schlessinger, 1990; Fantl *et al.*, 1993; Heldin, 1995, 1996; Weiss *et al.*, 1997; Weiss and Schlessinger, 1998). Although required for receptor activation, dimerization alone is not sufficient to activate RTKs. Recent reports have shown that dimerization of the RTK Neu can be induced by substitution of the unrelated transmembrane domain from glycophorin A (Burke *et al.*, 1997). However, although the receptor is dimerized, it lacks biological activity, demonstrating that additional requirements beyond dimerization are required for RTK activation.

The transmembrane domains of RTKs have been shown to play a critical role in the regulation of receptor dimerization and activation. Point mutations have been identified in the transmembrane domains of at least two RTKs, Neu and fibroblast growth factor receptor 3 (FGFR3), which lead to constitutive activation in the absence of ligand. Neu was isolated from an ethylnitrosourea-induced rat neuro/glioblastoma and shown to be activated by the mutation $Val^{664} \rightarrow Glu$ in the transmembrane domain (Bargmann *et al.*, 1986a,b; 1988b). Subsequently, it was postulated that this mutation facilitates hydrogen bonding between receptor monomers to yield a dimerized and activated receptor complex (Sternberg and Gullick, 1989, 1990; Weiner *et al.*, 1989). The mutation $Gly^{380} \rightarrow Arg$ in human FGFR3 is found in individuals with achondroplasia, the most common form of dwarfism (Rousseau *et al.*, 1994; Shiang *et al.*, 1994), and results in constitutive FGFR3 activation (Naski *et al.*, 1996; Webster and Donoghue, 1996, 1997). Importantly, these two mutations occur at the same relative position in their transmembrane domains, suggesting a common mechanism for receptor activation.

Transmembrane domain sequence requirements for activation of Neu have been well-characterized, and Neu serves as a valuable paradigm for studying the role of RTK transmembrane domains in signal transduction. Previous experiments have demonstrated that the mutant Glu residue in the transmembrane domain alone is sufficient for ligand-independent dimerization and activation of Neu (Bargmann and Weinberg, 1988b; Cao *et al.*, 1992). Receptors with a simplified transmembrane domain, containing Val residues interspersed with two Glu residues spaced seven residues apart (SPVVVVV.VVVE^{664}VVV.VVVEVVV.VVVVVVKR),

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Abbreviations used: ΔEC, deleted extracellular domain; EPOR, erythropoietin receptor; FGFR, fibroblast growth factor receptor; PDGFR-β, platelet-derived growth factor receptor-β; TM-shift, transmembrane-shift.

undergo receptor dimerization and activation, whereas an all-Val transmembrane domain does not induce receptor dimerization and activation (Chen *et al.*, 1997). These results demonstrate that the Glu residues in this transmembrane domain create a motif that is capable of mediating dimerization and activation of Neu.

To characterize the regulatory role that RTK transmembrane domains have in activation of the receptor, we designed a series of transmembrane domain mutants (termed "TM-shift" mutants) that sequentially move two Glu residues, within a simplified transmembrane domain, across the entire transmembrane domain. These mutant transmembrane domains were introduced into the Neu RTK where the movement of this dimerization motif is expected to rotate the kinase domains ~103° per residue. We show that rotation of this interface does not affect the ability of Neu to homodimerize, but leads to a periodic oscillation in kinase activation, as reflected by phosphotyrosine incorporation, as well as induction of immediate-early genes and morphological transformation. A similar periodic activation was observed with platelet-derived growth factor receptor-β (PDGFR-β), suggesting that this mechanism of receptor activation may be universal among different families of RTKs.

We therefore propose a model for the regulation of RTK activation that is dependent upon optimal rotational positioning of monomer subunits with respect to each other within a dimer. In wild-type receptors, this rotational positioning may be mediated by ligand binding to the extracellular domain of RTKs, but may also result from point mutations at specific positions within the transmembrane domain sequence or extracellular domain that lead to constitutive signaling in the absence of ligand.

MATERIALS AND METHODS

Construction of TM-shift Mutants, "Deleted Extracellular Domain" Mutants, and PDGFR-β Derivatives

Previously, we described a derivative of pSV2NeuN (Bargmann *et al.*, 1986b) in which silent restriction sites were introduced upstream (*Nhe*I) or downstream (*Sac*I) of the transmembrane domain-encoding sequence (Webster and Donoghue, 1997). The *Nhe*I site corresponds to bases 1973–1978, and the *Sac*I site corresponds to bases 2114–2119, in the published nucleotide sequence encoding $p185^{c\text{-}Neu}$ (Bargmann *et al.*, 1986a). The parental clone for the TM-shift mutants was generated by subcloning a pair of complementary synthetic oligonucleotides (D1371/D1372), with *Nhe*I/*Sac*I cohesive ends, into this pSV2NeuN derivative. These oligonucleotides also introduced a silent *Sal*I site immediately after the transmembrane domain, at nucleotides 2061–2066 (changing GAAGGA → GTCGAC). The *Nhe*I site and the new *Sal*I site were then used to generate the TM-shift mutants by ligating complementary pairs of oligonucleotides between the *Nhe*I and *Sal*I sites. For example, to construct the −7 shift shown in Figure 1D, the following pair of complementary oligonucleotides was synthesized: D1377 (sense strand): 5′-C T. A G C. C C G. G T T. G A A. G T C. G T A. G T C. G T C. G T T. G T A. G A G. G T C. G T A. G T C. G T G. G T C. G T A. G T A. G T G. G T C. G T A. G T A. G T C. G T C. G T T. G T G. G T C. A A A. C G-3′, and D1378 (compliment): 5′-T C G A C G T T T G A C C A C A A C G A C G A C T A C T A C G A C C A C T A C T A C T A C G A C C A C G A C T A C G A C C T C T A C A A C G A C G A C T A C G A C T T C A A C C G G G-3′. These oligonucleotides were synthesized, purified by PAGE, and recovered as described previously (Chen *et al.*, 1997).

Neu TM-shift mutants with a deletion of amino acids 30–628 in the extracellular domain (referred to as ΔEC-shift mutants) were based on the 5′Tx mutant created by Bargmann and Weinberg (1988a). They were constructed by synthesizing oligonucleotides (D1919/D1920) with *Hin*dIII-*Nhe*I overhangs encoding amino acids 1–29 of Neu, which includes the signal peptide, plus amino acids 629–653. Importantly, the ΔEC mutants also contain Cys → Ala mutations of the five remaining Cys residues in the extracellular domain (residues 29, 631, 635, 639, and 647), to eliminate the possibility of disulfide-bond–mediated dimerization as reported for several RTKs, including Ret (Santoro *et al.*, 1995), epidermal growth factor receptor (Sorokin *et al.*, 1994), Neu (Burke *et al.*, 1997), FGFR2 (Galvin *et al.*, 1996), and FGFR3 (D'Avis *et al.*, 1998). This insert was initially ligated into pSV2NeuN, confirmed by sequencing, and then the *Hin*dIII-*Nhe*I fragment was subcloned into the TM-shift mutant plasmids.

The PDGFR-β TM-shift mutants were constructed from the murine PDGFR-β/neu chimera (pNNTM-9) (Petti *et al.*, 1998), kindly provided by Dr. Daniel DiMaio. The PDGFR-β/neu gene was subcloned into pcDNA3, after which *Nhe*I and *Sal*I sites were created flanking the transmembrane domain by using polymerase chain reaction-based site-directed mutagenesis (Quik-Change; Stratagene, La Jolla, CA), creating a derivative PDGFR-β/neu(*Nhe*I/*Sal*I) vector. This new *Nhe*I site was introduced at nucleotides 1723–1728 (changing TTCAAA → GCTAGC), and the *Sal*I site was introduced at nucleotides 1811–1816 (changing AGCCAC → GTCGAC), in the DNA sequence of mPDGFR-β (Yarden *et al.*, 1986). The introduction of these sites also changed several residues in PDGFR-β to match

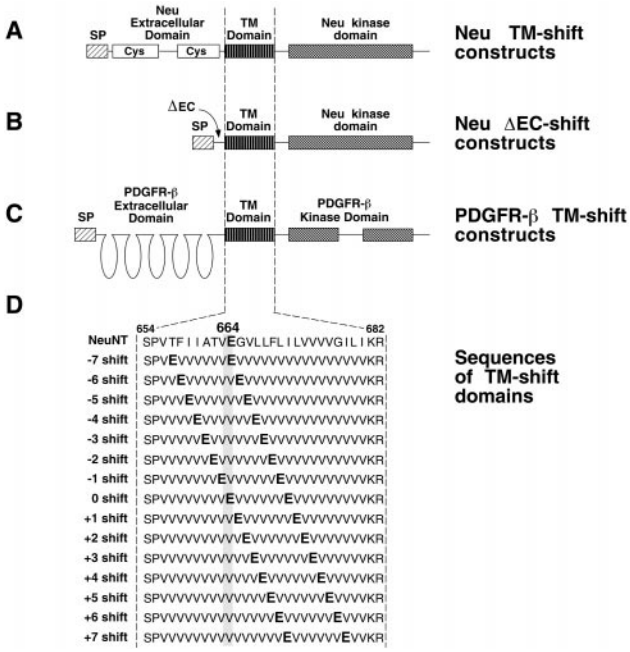

Figure 1. Structure of the TM-shift mutants. (A) Neu TM-shift constructs containing simplified transmembrane domains with a dimerization motif. (B) Neu ΔEC-shift constructs containing simplified transmembrane domains with a dimerization motif. In these constructs, nearly the entire extracellular domain (amino acids 30–628) was deleted (ΔEC). (C) PDGFR-β constructs containing TM-shift domains with a dimerization motif. (D) The amino acid sequence is shown for each of the 15 transmembrane domains of the TM-shift mutants. The cysteine-rich regions (Cys) of the extracellular domain of Neu are shown. All constructs contain a signal peptide (SP) for membrane translocation and a mutated transmembrane (TM) domain that replaces residues 654–682 of Neu and residues 499–527 of murine PDGFR-β.

the amino acid sequence of Neu; these changes were $F^{498} \rightarrow A$, $K^{499} \rightarrow S$, $K^{527} \rightarrow R$, and $P^{528} \rightarrow R$. *Nhe*I-*Sal*I DNA fragments containing the TM-shift domains from the constructs described above were isolated by electrophoresis through an 8% nondenaturing polyacrylamide gel, followed by elution and precipitation. These fragments were then ligated into the PDGFR-β/neu(*Nhe*I/*Sal*I) vector. To use the pLXSN retrovirus vector (Petti *et al.*, 1998), BsiWI-*Apa*I fragments were subcloned back into pNTMRV-14 (PDGFR-β/neu*) (Petti *et al.*, 1998). All constructs derived from oligonucleotides were confirmed by dideoxynucleotide sequencing.

Focus Assays

NIH3T3 cells were plated at a density of 2×10^5 cells/60-mm plate 24 h before transfection. Cells were transfected by using a modified calcium phosphate transfection protocol (Chen and Okayama, 1987) with 10 μg of DNA for the Neu TM-shift and Neu ΔEC-shift clones or 5 μg of DNA for the PDGFR-β TM-shift clones. Between 18 and 24 h after transfection, cells were refed with Dulbecco's modified Eagle medium (DME) containing 10% calf serum. Cells were split 1:12 onto 100-mm plates 24 h later. Foci were counted after 12–14 days. For the PDGFR-β TM-shift constructs, 24 h after splitting onto 100-mm plates, the DME was switched to DME containing 4% calf serum. Transfection efficiencies were determined by G418-resistant colonies on parallel plates. The PDGFR-β derivatives were expressed in the pLXSN vector that carries the neo gene, whereas the Neu derivatives were cotransfected with 50 ng of pRSVneo. Mock transfected cells yielded an average of 0.2 ± 0.1 foci/plate. The positive control NeuNT yielded an average of 251 ± 20 foci/plate. The negative control PDGFR-β/neu, which contains the wild-type transmembrane domain of Neu, yielded an average of 1 ± 0.7 foci/plate, whereas the positive control PDGFR-β/neu* had 176 ± 20 foci/plate.

Immunoprecipitations

COS-1 cells were split at a density of 2×10^5 cells/60-mm plate and transfected with 10 μg of DNA the next day (Chen and Okayama, 1987). Two days after transfection, the cells were rinsed with phosphate-buffered saline containing 10 mM iodoacetamide and lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (10 mM $NaPO_4$, pH 7.0, 1% Triton X-100, 0.1% SDS, 1% deoxycholic acid, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, 100 μM phenylmethylsulfonyl fluoride, and 10 mM iodoacetamide). Neu immunoprecipitations were prepared from RIPA lysates by using monoclonal antibody 7.16.4 (Ab-4; Oncogene Research, Cambridge, MA). For phosphotyrosine content, RIPA lysates were immunoprecipitated with 4G10 mAb (Upstate Biotechnology, Lake Placid, NY). After incubation, immune complexes were bound to protein A-Sepharose beads and washed four times in lysis buffer.

Immunoblotting

Whole-cell lysates or immunoprecipitations were resolved on 4–12% gradient SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked with 1× Tris-buffered saline, 3% milk, 0.02% Tween 20, and 0.02% sodium azide for either 1 h at room temperature or overnight at 4°C. The membranes were incubated with a rabbit polyclonal c-Neu antiserum (C-18; Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer for either 3 h at room temperature or overnight at 4°C. Following washes with 1× Tris-buffered saline, 0.02% Tween 20, membranes were incubated with anti-rabbit IgG-horseradish peroxidase (Amersham, Indianapolis, IN) (1:3000) in blocking buffer lacking sodium azide. Membranes were washed extensively and immunoreactive proteins were detected by enhanced chemiluminescence (ECL) (Amersham).

Dimerization Assays

To examine receptor dimerization as described by others (Weiner *et al.*, 1989; Burke *et al.*, 1997), Neu immunoprecipitates prepared as described above were boiled in 2× nonreducing sample buffer (4% SDS, 10 mM sodium phosphate, pH 7.0, 20% glycerol, 0.08% bromophenol blue). For reduced samples, an aliquot of nonreduced samples was removed, 2-mercaptoethanol added to 10%, and then reboiled. The samples were separated on 4–12% SDS-PAGE gradient gels, transferred to nitrocellulose, and immunoblotted as described above.

Transcription Assays

COS-1 cells were transfected with 2 μg of the pFL700 reporter (Hu *et al.*, 1995), together with 8 μg of each Neu TM-shift construct. Cells were refed the following day, and then 24 h later, starved in medium containing 0.1% fetal bovine serum for 48 h. Luciferase assays were performed with the luciferase assay system (Promega, Madison, WI). Mutants were assayed in at least three independent experiments.

RESULTS

Description of Neu TM-shift Domains

The $Val^{664} \rightarrow Glu$ mutation of rat Neu (designated NeuNT) is responsible for the oncogenicity of this mutant. This mutation has been shown to result in constitutive activation of Neu by facilitating receptor dimerization, postulated to occur by hydrogen bonding between the hydrophilic Glu residues in the hydrophobic transmembrane domain (Sternberg and Gullick, 1989, 1990; Weiner *et al.*, 1989). The Neu transmembrane domain, typical of other RTK transmembrane domains, consists of a stretch of aliphatic amino acid residues in an α-helical conformation (Brandt-Rauf *et al.*, 1990; Gullick *et al.*, 1992; Smith *et al.*, 1996). Each turn of the helix contains 3.5 amino acids so that amino acids 7 residues apart lie on the same face of the helix. Neighboring residues are positioned at 103° (360°/3.5) relative to each other.

We have previously demonstrated that activation of wild-type Neu (designated as NeuN) can be achieved by replacing the native transmembrane domain with one containing a dimerization motif embedded within a highly simplified sequence (Chen *et al.*, 1997). In the present study, we have further exploited this dimerization motif within a simplified transmembrane domain, defined by the hydrogen bonds of two Glu residues spaced 7 residues apart, to define a fixed point of interaction between subunits in a receptor dimer. By systematically moving this dimerization motif across the transmembrane domain, we were able to rotate the kinase domain with respect to this fixed point. The addition (or subtraction) of a single residue that lies between the dimerization motif and the end of the transmembrane domain effectively leads to a clockwise (or counterclockwise) rotation of 103°/residue. Changes in the extent of kinase activation within this series of mutants, referred to as TM-shift mutants, were then examined by transformation assays, phosphotyrosine incorporation, or activation of transcriptional reporters.

These TM-shift mutants are described in Figure 1, and are based on the 0 shift mutant with Glu residues at positions 664 and 671 (SPVVVVV.VVVE^{664}VVV.VVVEVVV.VVVVVVKR), previously described as $CONS.C^{2xE}$ (Chen *et al.*, 1997). If the transmembrane and kinase domains are rotationally coupled, then using the

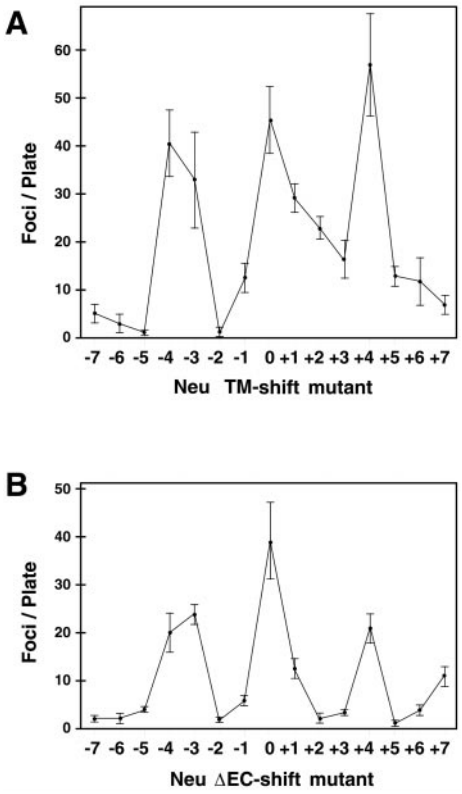

Figure 2. Transforming activity is presented for Neu TM-shift mutants (A) and ΔEC-shift mutants (B). The number of foci/10-cm plate/2.5 μg of DNA is presented. Four to ten plates were counted for each mutant. The SE of the mean is shown. The mock and the positive NeuNT controls are described in MATERIALS AND METHODS.

0 shift as a starting point, the −7 shift and +7 shift mutants will add or subtract two full turns of the α-helix, resulting in a rotation of the kinase domain back to the same relative position as the 0 shift. The −4 shift/+4 shift derivatives (±412°), and the −3 shift/+3 shift derivatives (±309°), will result in rotation of the kinase domain to a position relatively close to 360°, equivalent to the starting position defined by the 0 shift. In contrast, the other shift mutants only rotate the kinase domain a portion of the way around the α-helix, causing the kinase domain of each subunit to be rotated to the opposite face of the dimer with respect to the 0 shift. These rotations are depicted in the summary diagram of Figure 7.

Periodicity of Transforming Activity of Neu TM-shift Mutants

To examine whether rotation of the simplified transmembrane domain affected transforming activity of Neu, focus-forming assays were performed by using NIH3T3 cells. The −4 shift, −3 shift, and +4 shift mutants have the kinase domains of these constructs in similar positions to that of the 0 shift mutant, as explained above. These mutants displayed transforming activity similar to that of the 0 shift (Figure 2A). None of the other TM-shift mutants was significantly transforming. These constructs have their kinase domains in an opposite orientation from the 0 shift mutant, apparently rendering the receptors inactive.

Given the activity observed for the −3 shift mutant, one might expect the +3 shift mutant to be transforming, based on the position of the kinase domains. However, focus assays revealed the lack of transformation (Figure 2A). At the present time, we have no explanation for this surprising observation except to note that each of the 7 positions within a complete heptad are in fact unique, and structural requirements in both the receptor and associated downstream signaling molecules likely impose additional constraints beyond the predicted rotational angle.

In addition, the absence of strong transforming activity for the −7 shift and +7 shift mutants might be explained by the location of one of the Glu residues in the dimerization motif very near one of the ends of the transmembrane domain. This may distort the α-helical structure by allowing the Glu residue to become charged, or by shifting the α-helix up or down so that the Glu is no longer within the lipid environment. This is also consistent with the failure of these mutant proteins to exhibit dimerization (see below).

Extracellular Domain of Neu Does Not Inhibit Receptor Activity

A recent study demonstrated the existence of a dimer interface in the extracellular juxtamembrane region of Neu (Burke and Stern, 1998). One possible explanation of the periodic transforming activity exhibited by the Neu TM-shift mutants would be that the large extracellular domain, if rigidly coupled to the transmembrane domain through the juxtamembrane interface, could sterically hinder receptor interactions. To examine this possibility, we created a series of ΔEC-shift mutants, with the majority of the extracellular domain of Neu deleted. If the periodic transforming activity exhibited by the Neu TM-shift mutants was mediated by the extracellular domain, then all of the ΔEC-shift mutants should be active in the focus-forming assay. However, as shown in Figure 2B, the same periodic oscillation was observed for the ΔEC-shift mutants as with full-length receptors. These results demonstrate that the observed periodicity of TM-shift mutants is not due to extracellular domain interactions. However, it is still possible that interactions at the juxtamembrane interface have a stabilizing effect on the conformation of activated receptors.

Comparison of the graphs in Figure 2 suggests that the extracellular domain may exert some effect on transformation because the +1 and +2 TM-shifts still exhibit some activity in Figure 2A. When the extracellular domain is deleted, the +1 and +2 ΔEC-shift mutants no longer exhibit significant activity as shown in Figure 2B, and the rotational linkage of the transmembrane and kinase domains is more sharply defined. Similarly, some activity is observed for the +5 and +6 TM-shifts (Figure 2A), which is eliminated by removal of the extracellular domain (Figure 2B). We believe these differences may reflect steric hindrance between the extracellular domains, impeding rotation within the dimeric complex in response to transmembrane deletions/insertions. In this case, removal of the extracellular domain would eliminate this steric hindrance, yielding the data presented in Figure 2B.

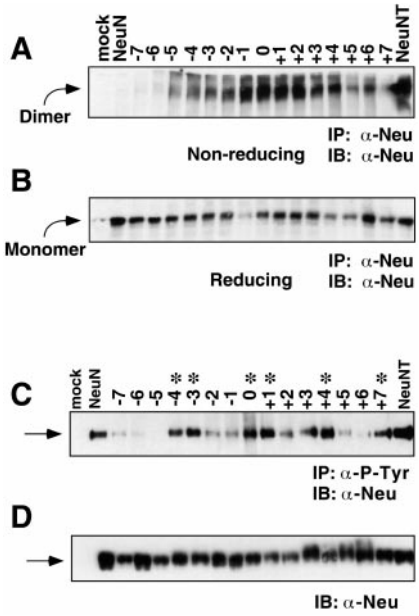

Figure 3. (A) Dimerization of Neu TM-shift mutants assayed by SDS-PAGE electrophoresis under nonreducing conditions, or reducing conditions (B). Receptors were immunoprecipitated with mAB 7.16.4, run on a 4–12% gradient gel, transferred to nitrocellulose, immunoblotted with C-18 Neu antisera and visualized by ECL. With the exception of the −7 shift, −6 shift, and +7 shift mutants, all other mutants exhibit a significant percentage of the receptor present as a dimer. Note that in the immunoblot shown, recovery of the −1 sample was anomalously low for unknown reasons. This was not observed for the −1 sample in other repeats of this experiment. (C) Tyrosine phosphorylation of Neu TM-shift mutants. RIPA lysates immunoprecipitated with 4G10 anti-phosphotyrosine mAb were electrophoresed on a 4–12% gradient SDS-PAGE gel, transferred to nitrocellulose, immunoblotted for Neu by using C-18 Neu antisera, and detected by ECL. *, mutants that exhibit an increase in P-tyr incorporation, which coincides with transformation. (D) Lysates from C were immunoblotted with C-18 Neu antisera to demonstrate approximately equivalent expression of Neu in all samples. Arrows in C and D indicate monomeric Neu receptors.

Dimerization of Neu TM-shift Mutants Does Not Correlate with Activity

Because dimerization of receptor monomers precedes their subsequent activation, we examined whether the Neu TM-shift mutants retained the ability to dimerize efficiently. Receptors expressed in COS-1 cells were immunoprecipitated with antisera to Neu, and separated by SDS-PAGE under reducing or nonreducing conditions. Immunoblot analysis of receptor monomers and dimers demonstrated that, with the exception of −7 shift, −6 shift, and +7 shift mutants, all of the Neu TM-shift mutants were able to dimerize to an extent comparable to NeuNT (Figure 3, A and B). Several of the mutants, notably −7, −6, and + 7, fail to exhibit significant dimerization. These mutants are predicted to have one of the glutamic acid residues at the edge of the transmembrane domain where the hydrophobic environment may differ considerably, potentially weakening the dimerization contacts. Because both nontransforming and transforming derivatives of Neu dimerized equally well, the lack of activity in transformation assays exhibited by some of the TM-shift mutants did not result from the absence of dimerization. Instead, these results imply that the orientation of monomers within the dimer is critical for activity.

Periodicity of Phosphotyrosine Incorporation in Neu TM-shift Mutants

Upon receptor dimerization, transphosphorylation of intracellular tyrosine residues generates sites that allow effector molecules to recognize and bind an activated receptor. Neu contains five intracellular tyrosine residues that are phosphorylated upon receptor activation, and that allow signaling through Shc, GRB2, phospholipase C-γ, and other effector molecules (Fazioli *et al.*, 1991; Qian *et al.*, 1995; Xie *et al.*, 1995; Pinkas-Kramarski *et al.*, 1996; Dankort *et al.*, 1997). We therefore examined the phosphorylation state of tyrosine residues in the Neu TM-shift mutants by immunoprecipitation with antisera against phosphotyrosine, and subsequent immunoblotting with antisera against Neu. As shown in Figure 3C, ~4- to 5-fold more NeuNT was recovered in phosphotyrosine immunoprecipitates compared with NeuN. Examination of the Neu TM-shift mutants demonstrated that the phosphotyrosine content of Neu receptors is coincident with the transforming activity of the TM-shift mutants. Mutants that were most active in focus-forming assays (−4 shift, −3 shift, 0 shift, and +4 shift) contained ~3-fold more phosphotyrosine compared with NeuN and were clearly phosphorylated to a higher degree than nontransforming TM-shift mutants (Figure 3C). Control immunoblotting with anti-Neu sera demonstrated approximately equivalent expression levels for each mutant (Figure 3D). These results demonstrate that only specific rotational conformations result in phosphorylation of dimerized Neu receptors.

Induction of Immediate-Early Genes

Ligand-induced activation of RTKs results in activation of the mitogen-activated protein kinase cascade leading to transcription of c-fos, an immediate-early gene (Deng and Karin, 1994). To assess the ability of the Neu TM-shift mutants to induce c-fos transcription, a luciferase reporter construct containing the c-fos promoter was cotransfected with the TM-shift mutants into COS-1 cells and luciferase activity was measured. The Neu TM-shift mutants exhibited a similar periodicity in luciferase activity (Figure 4) as previously observed for P-tyr incorporation and cellular transformation. These results further confirm that correct rotational positioning of kinase domains within receptor dimers is required for downstream signal transduction.

Restoration of Activity to the Inactive −2 ΔEC-shift Mutant

If the transmembrane domain and kinase domain are indeed rotationally linked, then it should be possible to "restore" activity to an inactive mutant by rotating the kinase domain to the correct or activating orientation, through the insertion of additional residues at the end of the transmembrane domain. Derivatives of the −2 ΔEC-shift mutant were made

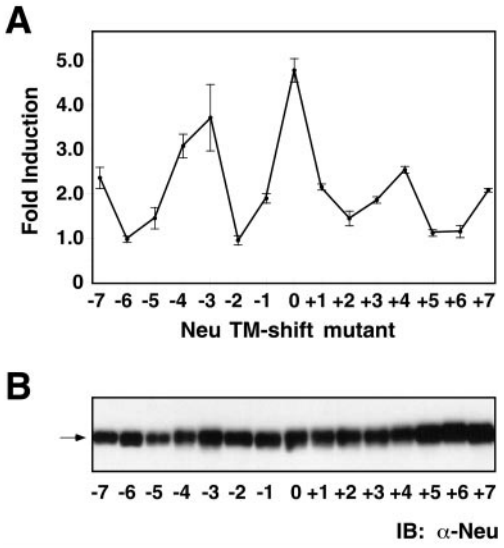

Figure 4. Fos-luciferase reporter assay. (A) Luciferase activity corresponds to transforming activity in cells transfected with Neu TM-shift mutants. The fold induction relative to mock transfected cells is set at 1. The positive control NeuNT exhibited a 5.4-fold induction. (B) Neu immunoblot of cell lysates demonstrates equivalent expression of the receptor in each sample. The arrow indicates the mobility of receptor monomers.

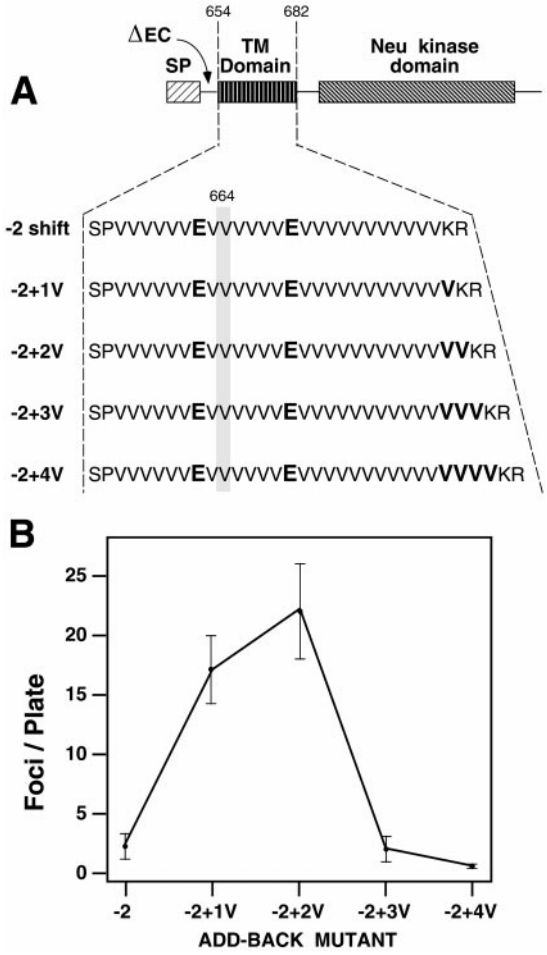

Figure 5. Restoration of activity to the inactive −2 ΔEC-shift mutant by insertion of additional Val residues in the add-back series. (A) The sequence of each transmembrane domain in this series of mutants is presented. (B) Transforming activity presented as the number of foci/10-cm plate/2.5 μg of DNA. Six plates from three independent experiments were counted. The SE of the mean is shown. The mock and positive NeuNT controls are described in MATERIALS AND METHODS.

that extended the artificial transmembrane domain by one, two, three, or four Val residues (Figure 5A). The extension of the α-helical transmembrane domain by each additional Val residue is predicted to result in a corresponding rotation of the kinase domain. When tested in focus forming assays, these "add-back" mutants displayed the transforming activity shown in Figure 5B. The −2 + 1V shift and −2 + 2V shift mutants were able to cause significant transformation compared with the −2 shift mutant, suggesting that the additional residues within the transmembrane domain rotated the kinase domains into an activated conformation.

In these add-back mutants, if one counts the number of residues that intervene between the kinase domain and the dimerization motif, represented by the Glu residues of the transmembrane domain, then it will be seen that −2 + 1V shift corresponds to the −3 shift mutant, and the −2 + 2V shift corresponds to the −4 shift mutant (Figure 1D). When the data of Figure 5B are compared with the data obtained from the TM-shift mutants presented in Figure 2, they are seen to represent nearly identical peaks of receptor activation.

Periodic Activation of PDGFR-β by TM-shift Mutations

To examine whether the rotational linkage of the transmembrane domain to the kinase domain characterized in Neu is a general feature of RTKs, we created additional derivatives containing the TM-shift domains in place of the PDGFR-β transmembrane domain (Figure 1C). These constructs were assayed for transformation of NIH3T3 fibroblasts, to determine whether the rotational orientation of the PDGFR-β kinase domain regulates activation. As shown in Figure 6A, these receptors also display periodic oscillation in their transformation activity, suggesting that rotational positioning of the monomer in the dimer may play an important role in regulating PDGFR-β activation.

It is noteworthy that the peaks of receptor activation displayed in this system do not coincide exactly with the peaks of activation observed for Neu, presented in Figure 2. The peaks in the periodicity of this system occur at −4, −1, +3, and +5. This difference could be a result of the transmembrane domain being distorted in the membrane, or differences in the length and composition of the transmembrane domain in the PDGFR-β and Neu. Although the transmembrane domains serve the same fundamental purpose of membrane anchoring and superficially resemble one another in being composed of largely hydrophobic residues, nonetheless these transmembrane domains differ consider-

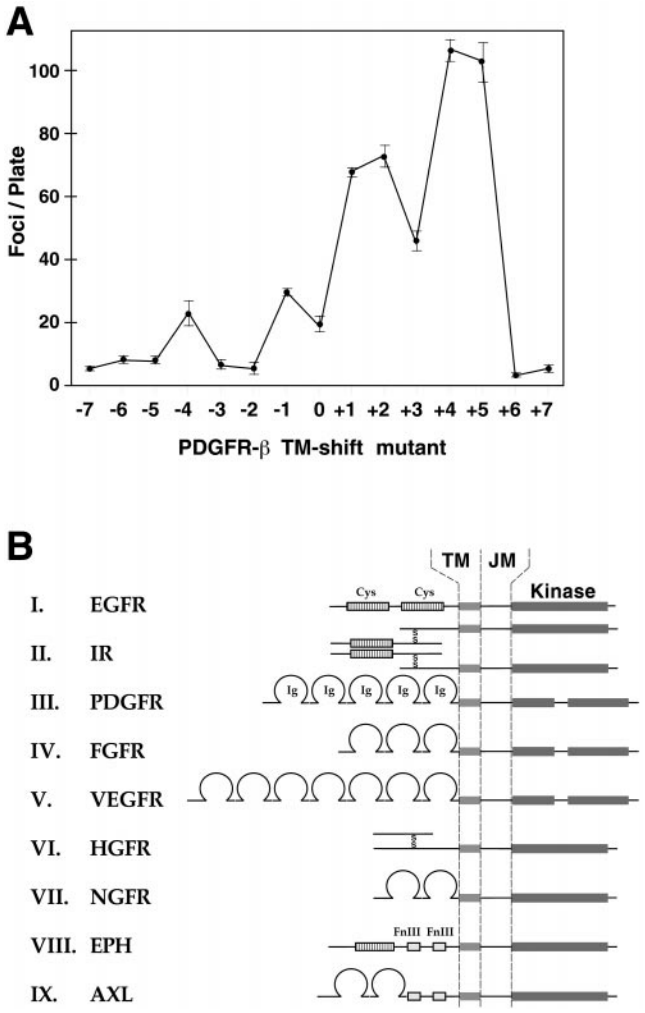

Figure 6. (A) Transformation assay of PDGFR-β constructs containing the TM-shift domains. Transforming activity is presented as the number of foci/10-cm plate/1.25 μg of DNA. Four plates were counted for each mutant and the SE of the mean is shown. The negative and positive controls, PDGFR-β/neu and PDGFR-β/neu*, respectively, are described in MATERIALS AND METHODS. (B) Partial summary of RTK families is presented, indicating the transmembrane (TM) and juxtamembrane domains (JM). Separation between the transmembrane domain and the kinase domain varies from ~35 to 75 residues, depending upon the specific RTK.

ably in many details. Therefore, we would not expect that the transmembrane domain would necessarily adopt the exact same helical conformation in the two systems. This could lead to differences or a shift in the periodicity when the transmembrane domain is put in the PDGFR-β system. However, the fact that periodicity is observed for the PDGFR-β TM-shift mutants suggests that the underlying phenomenon may be similar to that observed earlier for Neu, although further experiments will be required to establish the validity of this model. Possibly, the much larger extracellular domain may provide greater steric restrictions on rotational movements induced by deletions/insertions within the transmembrane domain.

As shown in Figure 6B, various families of RTKs exhibit a short cytoplasmic domain that connects the transmembrane domain with the kinase domain, ranging from ~35 to 75 residues. We would predict, therefore, that the results described here for Neu and for PDGFR-β may hold generally true for other RTKs as well.

DISCUSSION

It is well-established that RTKs are activated upon ligand binding. However, the mechanism by which this extracellular signal is transmitted to the intracellular kinase domain has remained elusive. The results presented here demonstrate that optimal rotational positioning of individual receptor kinase domains within the dimer is important in the regulation of RTK signaling. By using a simplified transmembrane domain sequence, we show that activation of RTKs occurs only when hydrophilic residues, responsible for dimerization in this system, are placed in specific positions within the transmembrane domain of the receptor. Given an α-helical transmembrane domain in Neu of 3.5 residues/turn (Gullick *et al.*, 1992; Smith *et al.*, 1996), deletions or insertions of approximately either 3–4 residues are expected to rotate the kinase domain ~360°, back to its original position with respect to its dimeric partner (Figure 7). Similarly, deletions or insertions of 7 residues are expected to rotate the kinase domain of each subunit by two full revolutions. Consistent with this model, experimental data obtained with Neu (Figure 2) indicate peaks of activity corresponding to TM-shift deletion/insertion positions of $-3/-4$, 0, and $+4$ residues. The observed periodicity in activation parameters cannot be accounted for by steric hindrance of the extracellular domain because removal of this region does not change the periodicity of activation. The extension of these observations to a member of a different family of RTKs, PDGFR-β, suggests that the rotational position of the kinase domain may represent an important aspect of regulation of RTKs in general. Rotational coupling potentially allows an additional level of fine-tuning the magnitude of RTK activation superimposed upon the requirement for dimerization.

Although data presented here are consistent with a model in which different deletion/insertion mutations lead to relative rotation of the kinase domains within the dimer, we cannot exclude the possibility that some of the mutations assayed may be affecting parameters other than relative orientation with unknown results on their biological activity. This reinforces the importance of future biophysical approaches to confirm the model presented here.

RTK Transmembrane Domain Mutations in Clinical Disease

Although activation of RTKs is usually mediated by ligand binding, several examples of transmembrane domain mutations have been identified that result in constitutive receptor activation and significant developmental dysmorphology. The NeuNT mutation ($Val^{664} \rightarrow Glu$) found in rat neuroglioblastoma represents the first discovery of an activating mutation in the transmembrane domain sequence of an RTK

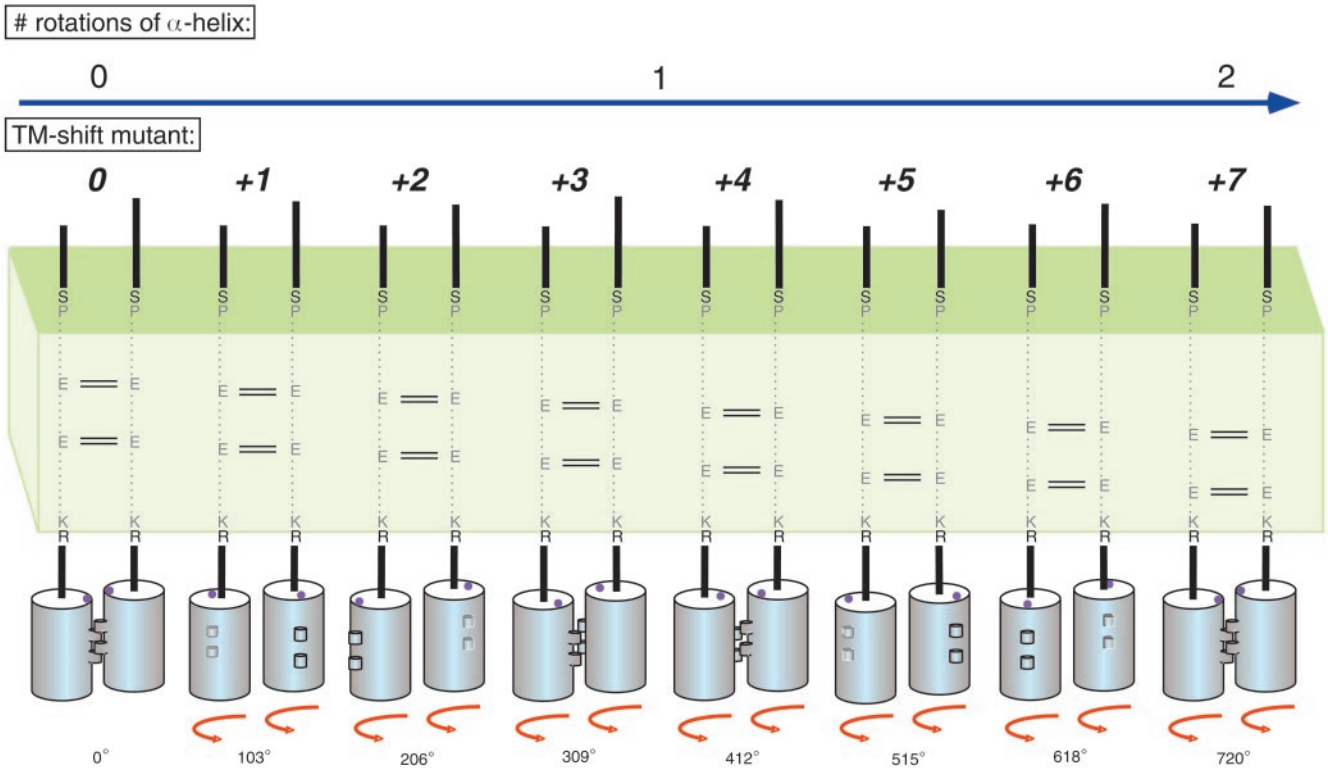

Figure 7. Model showing the role of rotational positioning in RTK activation. Moving the dimerization motif across the transmembrane domain rotates the Neu kinase domain monomers in the dimeric complex, leading to periodic activation of the kinase domains.

(Bargmann *et al.*, 1986b). Other transmembrane domain mutations have since been identified in a different family of RTKs, FGFR3, which lead to developmental defects in humans that differ in their nature and severity. For example, the mutation $Gly^{380} \rightarrow Arg$, which inserts a very polar residue into the FGFR3 transmembrane domain, accounts for the vast majority of cases of human achondroplasia (Rousseau *et al.*, 1994; Shiang *et al.*, 1994). However, the mutation $Ala^{391} \rightarrow Glu$, which also inserts a polar residue, results in a different clinical syndrome, Crouzon syndrome with acanthosis nigricans (Meyers *et al.*, 1995). Although both mutations likely induce receptor dimerization, altered positioning of the catalytic domains of FGFR3 in the dimer leading to differences in the extent of kinase activation and/or substrate accessibility may account for the variable severity of these different syndromes.

Interestingly, a variety of activating mutations have been described in the juxtamembrane domains of several human RTKs. These include a variety of small deletions arising in human c-erbB2 in a transgenic model of human breast cancer (Siegel *et al.*, 1994), and various mutations in the juxtamembrane of c-kit that are responsible for some human gastrointestinal stromal tumors (Kitayama *et al.*, 1995; Hirota *et al.*, 1998; Moskaluk *et al.*, 1999). Future experiments may reveal that these mutations lead to activation by facilitating a favorable rotational positioning of receptor subunits within the activated dimeric complex.

Modulation of Receptor Interactions by Rotational Positioning

The phosphotyrosine content of Neu TM-shift receptors clearly oscillates, correlating with receptor transforming activity. Because autophosphorylation of receptors in a dimeric complex occurs in trans, it seems likely that the active site of the kinase domains are rotated away from each other in dimeric complexes of the inactive derivatives. This concept provides insight for understanding why overexpression of receptors, such as seen with Neu in some breast cancer patients, could mimic ligand-induced activation. The high concentration of receptors within a confined area would increase the likelihood that some receptors would be positioned in a rotationally favorable conformation. Furthermore, a model of receptor activation requiring proper rotational orientation of the kinase domains provides a mechanism whereby various ligands could activate receptors to differing degrees.

Role of Ligand-Binding Domain in Rotational Positioning of Receptor Kinase Domains

We examined the role of the ligand-binding domain on the activity of the TM-shift mutants by deleting the extracellular domain of Neu. In these receptors, a similar pattern of periodic activation was observed as for the full-length receptors. Because dimerization of receptor monomers is nor-

mally mediated by ligand-binding interactions, the ligand-binding domain may play an important role in the rotational positioning of the intracellular kinase domains as well as dimerization of receptor monomers. The observation that only specific rotational orientations of interacting kinase domains allow for functional signaling reflects a regulatory mechanism that prevents inappropriate signaling from random interactions. The results presented here expand and complement recent results demonstrating first that dimerization is necessary but not sufficient for activation of Neu, and second that there exists a dimerization interface between the extracellular juxtamembrane and intramembrane α-helices of Neu (Burke *et al.*, 1997; Burke and Stern, 1998). The most recent of these studies show that only two extracellular juxtamembrane mutants of Neu, containing V656C and T657C mutations, exhibit significant transforming activity, whereas mutation to Cys of other residues within this region does not activate the receptor. Interestingly, these activating mutants are also predicted to lie on the same face of the α-helix as the V664E mutation.

Based on our results with Neu and PDGFR-β, we predict that ligand binding to RTK molecules first induces dimerization, and second, as a result, the intracellular kinase domains of the complex are rotationally positioned by a dimerization interface through the extracellular juxtamembrane, transmembrane α-helices, and intracellular juxtamembrane regions, leading to optimal activation of the kinase domain.

This model of RTK activation is consistent with structural studies of the erythropoietin receptor (EPOR), a member of the cytokine superfamily. Use of synthetic peptide agonists and antagonists has revealed detailed mechanisms of activation for EPOR. In one study, peptide agonist-induced activation and dimerization of EPOR revealed an altered dimerization structure compared with the structure of normal erythropoietin:EPOR complexes (Livnah *et al.*, 1996; Johnson *et al.*, 1998). Peptide antagonist binding to EPOR also induced dimerization of EPOR, but the conformation of this complex is altered in such a way as to inactivate the receptor complex (Livnah *et al.*, 1998). These studies suggest the possibility that EPOR dimers assume multiple conformations due to extracellular stimuli, of which only some may allow receptor activation. Subsequent structural studies of EPOR have shown that extracellular ligand binding to preformed dimers causes a large conformational change within the intracellular domain (Livnah *et al.*, 1999). Further structural studies of RTKs to examine transmembrane and intracellular conformational changes, as a result of mutation or ligand stimulation, should further our understanding of mechanistic requirements for RTK activation.

Evolutionary Development of RTKs

Interestingly, although the extracellular domains of RTKs are divergent, the intracellular domain structure remains largely conserved. RTKs in general contain only a short stretch of amino acids between the transmembrane and kinase domains, of ~35–75 residues (Ullrich and Schlessinger, 1990; Fantl *et al.*, 1993; Heldin, 1995) (Figure 6B). Our results suggest an evolutionary rationale for this compact structure is to maintain the rotational linkage of the transmembrane domains with the kinase domain. Many nonreceptor tyrosine kinases, such as src family members or the Janus family of kinases (JAKs), contain modular domains (SH2, SH3, PTB) that allow binding to effectors and/or substrates (Feller *et al.*, 1994; Feng and Pawson, 1994; Shokat, 1995; Kuriyan and Cowburn, 1997). Although these kinases are recruited to the plasma membrane, or localized there via lipid modifications, they do not require a direct signal from the extracellular environment to be activated. The presence of additional domains between the transmembrane and kinase domains would possibly allow the kinase domain to rotate more freely, destroying the rotational linkage and interfering with ligand-mediated regulation of RTK activation.

In this study, we have demonstrated variations in the extent of RTK activation within the dimeric complex, mediated by structural constraints imposed by the transmembrane domain. These results also provide an explanation for human developmental disorders and neoplasias, which may arise from mutations located at specific residues within the transmembrane or juxtamembrane domains of RTKs.

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