Point Mutations in Dimerization Motifs of the Transmembrane Domain Stabilize Active or Inactive State of the EphA2 Receptor Tyrosine Kinase*

Received for publication, February 16, 2014, and in revised form, April 11, 2014. Published, JBC Papers in Press, April 14, 2014, DOI 10.1074/jbc.M114.558783

George V. Sharonov,§ Edward V. Bocharov,§ Peter M. Kolosov, Maria V. Astapova, Alexander S. Arseniev,† and Alexey V. Feofanov‡

From the §Department of Structural Biology, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry of RAS, 117997 Moscow, Russia, the †Faculty of Medicine, Moscow State University, 119992 Moscow, Russia, and the ‡Department of Molecular Neurobiology, Institute of Higher Nervous Activity and Neurophysiology of RAS, 117485 Moscow, Russia, and the Biological Faculty, Moscow State University, 119992 Moscow, Russia

Background: Isolated Eph transmembrane domains (TMD) dimerize in membrane mimetics, but the functional significance of these interactions is unclear.

Results: Mutations introduced into the alternative dimerization motifs of the EphA2 TMD induced an opposite effect on receptor activity.

Conclusion: Alternative TMD interactions promote either the active or inactive EphA2 conformation.

Significance: The involvement of TMD interactions in Eph receptor activity is discovered.

The EphA2 receptor tyrosine kinase plays a central role in the regulation of cell adhesion and guidance in many human tissues. The activation of EphA2 occurs after proper dimerization/oligomerization in the plasma membrane, which occurs with the participation of extracellular and cytoplasmic domains. Our study revealed that the isolated transmembrane domain (TMD) of EphA2 embedded into the lipid bicine dimerized via the heptad repeat motif L535XG539X2A542X2V546X2L549 rather than through the alternative glycine zipper motif A536XG540X2G544 (typical for TMD dimerization in many proteins). To evaluate the significance of TMD interactions for full-length EphA2, we substituted key residues in the heptad repeat motif (HR variant: G539I, A542I, G553I) or in the glycine zipper motif (GZ variant: G540I, G544I) and expressed YFP-tagged EphA2 (WT, HR, and GZ variants) in HEK293T cells. Confocal microscopy revealed a similar distribution of all EphA2-YFP variants in cells. The expression of EphA2-YFP variants and their kinase activity (phosphorylation of Tyr residues in the cytoplasmic domain of EphA2) followed by dimerization of ephrin-EphA2 complexes and phosphorylation of Tyr residues in the cytoplasmic domain of EphA2 (6, 7). Accumulating evidence suggests that, even without ligands, EphA2 can form EphA2-EphA2 homodimers and oligomers (clusters) (7, 8) that facilitate the formation of signaling (ephrin-EphA2)2 heterotetramers (9). At a high local concentration of EphA2, the receptor oligomerization is accompanied by a ligand-independent receptor activation (7).

Receptor tyrosine kinases of the Eph family and their ephrin ligands are key regulators of cell-cell and cell-matrix adhesion, coordinating cell migration and positioning in various adult and embryonic tissues of human organisms (1, 2). The EphA2 receptor, a representative of the human 14-member Eph family, controls such diverse processes as capillary stabilization by pericytes (3), keratinocyte movement out of the basal layer (6, 7), blastocyst entry into the endometrial layer (4), and cardiac stem cell mobilization from the niche (5). Activation of EphA2 leads to cell detachment (mobilization), loss of intercellular contacts (an increase in cell layer permeability), or cell repulsion (guidance).

A classical model of EphA2 activation assumes the binding of a ligand (ephrin) situated at the membrane of a neighboring cell followed by dimerization of ephrin-EphA2 complexes and phosphorylation of Tyr residues in the cytoplasmic domain of EphA2 (6, 7). Accumulating evidence suggests that, even without ligands, EphA2 can form EphA2-EphA2 homodimers and oligomers (clusters) (7, 8) that facilitate the formation of signaling (ephrin-EphA2)2 heterotetramers (9). At a high local concentration of EphA2, the receptor oligomerization is accompanied by a ligand-independent receptor activation (7). Structural studies revealed several sites that are involved in ligand-dependent and ligand-independent EphA2 oligomerization. These sites were found along the extended extracellular domain (ECD), consisting of fibronectin III repeats (FN1 and FN2), ligand-binding domains (LBDs) and cysteine-rich domains, as well as in a cytoplasmic sterile α motif domain situated after the tyrosine kinase domain (TKD) (7, 9, 10). The functional importance of EphA2 oligomerization via the ECD...
Switching of EphA2 by Transmembrane Helix-Helix Interaction

FIGURE 1. Alternative dimer configurations of the EphA2 TMD. A, ribbon diagram of the left-handed TMD dimer of EphA2 formed via the heptad repeat motif according to NMR data (29). The heavy atom bonds are shown. The membrane is shown schematically by isolines. B, hydrophobicity map for the surface of a TMD helix (left blue helix from A) constructed as described previously (29). Contour isolines encircle regions with high values of molecular hydrophobicity potential. Spatial locations of two dimerization motifs, the heptad repeat motif L533XG539X2A542XV546X2L549 and the glycine zipper motif A536XG540XG544, are marked by red and green dashed ovals. The helix packing interface found in the NMR structure of the EphA2 TMD dimer is indicated by magenta dots. The amino acid substitutions in the HR (G539I, A542I, G553I) and GZ (G540I, G544I) variants of EphA2 are highlighted with red and green letters, respectively. C, ribbon diagram of the right-handed TMD dimer of EphA2 formed via the glycine zipper motif according to the molecular modeling performed with the PREDDIMER program (36).

was confirmed by using site-directed mutagenesis and cell-based signaling assays (7, 10). However, the participation of a single-span transmembrane domain (TMD) in EphA2 activation was not studied.

It should be mentioned that the view of the TMD as a mere membrane anchor of receptors has changed dramatically over the last decade. An advanced concept considers the TMD as a regulator of dynamic receptor assembly, conformational switching, and signal transduction (11–17). To a large degree, this concept is on the basis of the findings obtained for isolated TMDs that were either expressed in bacterial membranes or reconstituted in a membrane-like environment or simulated by computational modeling. Investigations of full-length receptors confirm this concept, but the list of studied proteins is rather limited: Neu/ErbB2 epidermal growth factor receptor 2 (18), fibroblast growth factor receptors (19, 20), platelet-derived growth factor receptor A (21), erythropoietin receptor (22), neuropilin 1 (23), p75 neurotrophin receptor (24), T cell receptor (25), class II major histocompatibility complex (26), integrins (27), syndecan 4 (28), E-cadherin (29), and amyloid precursor protein (30).

We have shown recently that isolated TMD helices of EphA2 dimerized in a membrane-mimicking environment (31). Being embedded into lipid bicelles, they formed a left-handed dimer via the heptad repeat motif L533XG539X2A542XV546X2L549 (Fig. 1A). The TMD of EphA2 contains another recognized dimerization sequence, A536XG540XG544 (Fig. 1B), the so-called glycine zipper motif (32). Interactions via the glycine zipper motif were not observed for the EphA2 TMD in the bicelles. At the same time, isolated TMDs of the EphA1 receptor formed two alternative dimers via a similar N-terminal glycine zipper and, presumably, via a C-terminal GG4-like dimerization motif that was, in fact, part of a heptad repeat sequence (33, 34). The appearance of an alternative dimerization mode was induced by the deprotonation of the Glu547 side chain, resulting in local structure perturbations near the N-terminal glycine zipper and a realignment of the helix-helix packing in the EphA1 TMD dimer (33). Moderate changes in the lipid composition of the bicelles caused an alteration in the observed conformational exchange (33). Thus, depending on the external and local membrane environment as well as ligand binding, the EphA1 TMD can be involved in different types of association. Accordingly, we have hypothesized about the dimerization of TMD in full-length EphA2 receptors, which could associate with the participation of either the heptad repeat or the glycine zipper motif (31).

Here we present experimental proof of the functional importance of TMD interactions for the EphA2 receptor. To characterize TMD participation in EphA2 receptor activation, we substituted key residues in the heptad repeat motif (HR variant) or in the glycine zipper motif (GZ variant) and expressed the WT, HR, and GZ variants of EphA2 tagged with YFP in HEK293T cells. We found a significant disturbance of ligand-dependent and ligand-independent activity (phosphorylation) of the HR and GZ variants compared with the WT receptor. It is the first time that involvement of the TMD in the activation of the receptor tyrosine kinase of the Eph family is discovered, supplementing available biophysical and biochemical data with useful insights into Eph functioning at the molecular level.

EXPERIMENTAL PROCEDURES

Cloning and Mutagenesis of Human EphA2—The full-length cdNA encoding EphA2 was cloned into the pTagYFP-N (neo) vector (Evrogen) under the control of the CMV RNA polymerase II promoter, as described previously (35). The PCR primers used for the generation of EphA2 mutants were as follows (5’ to 3’): G533I, AAGCAGAACATAGTGGCAGCAC; G544I, AGCAGGGCCAACAATGACAGCCAC; G540I, ACAGCCACGATGGCCACATCAC; G536I, GCCAATCACTGAGTTGCAG; and G539I and A542I, ACACCGACAATCAGCCGATGATCCACCC.

The mutagenesis procedure was carried out according to the PCR-based “megaprimer” method (36). Two rounds of PCR were performed with the same PCR mixture. To get megaprimer in the first PCR round, a direct primer was always 5’-GCACGAATTCGACGCTGTC-3’ (the EcoRI site is
underlined), and a reverse primer was one of the mutant primers 1–5 listed above. In the second PCR round, the megaprimer served as a direct primer, and a reverse primer was 5'-TTTG-TTCGACATGGGGATCCCCACAGTTTCA-3'(revpr_Sall, the Sall site is underlined). The first round was carried out in 25 μl of reaction mixture containing 20 ng of pTagYFP-EphA2 DNA, 0.48 μM direct primer, 0.6 μM reverse mutant primer, 0.2 mM dNTP, Pfu DNA polymerase reaction buffer (1×), and 0.75 units of Pfu Turbo DNA polymerase (Stratagene). The denaturation was carried out at 95°C (3 min) in the first cycle and at 94°C (30 s) in the next 20–25 cycles. The amplification included 30 s of primer annealing (60°C) and 40 s of elongation (72°C). The second round was performed after the addition of 12 pmol of the reverse primer (revpr_Sall) and 1.25 units of Pfu Turbo DNA polymerase to the PCR mixture. Amplification with the megaprimer was performed during 28 cycles (30 s at 94°C, 40 s at 65°C, and 140 s at 72°C). The resulting 1370-bp PCR product was purified in 1% agarose using a DNA extraction kit (Promega), hydrolyzed with EcoRI and Sall, and ligated with the pTagYFP-EphA2 plasmid pretreated with the same endonucleases. The resulting pTagYFP-EphA2 DNA, having point mutation, was further utilized to introduce the next mutation exactly as described above. The final ligated mixtures were utilized for the transformation of Escherichia coli strain T10. The cloned DNAs were sequenced, and characterized clones were used in experiments.

EphA2 Expression, Stimulation, and Fluorescent Staining—HEK293T cells were cultured in DMEM with low glucose and sodium pyruvate (HyClone) supplemented with 10% fetal bovine serum (HyClone). Cells were seeded in a 6-well plate, and, after 24 h, they were transfected with 1 μg of EphA2-YFP plasmid (WT, HR, or GZ) mixed with 2.5 μl of Lipofectamine 2000 (Invitrogen) per well. Two days after transfection, the cells were washed, placed in serum-free medium for 5 h, harvested with Versene solution (Paneco, Russia), and resuspended in PBS at a final concentration of (3 ± 1) × 10⁶ cells/ml, and kept at 37°C. Cells were activated either with 7 mM dNTP, a final concentration of (3

Switching of EphA2 by Transmembrane Helix-Helix Interaction

Confocal Microscopy—48 h after transfection, cells were seeded in the wells of a Lab-Tek chambered coverglass (Nunc) and analyzed the next day with a TCS SP5 confocal microscope (Leica) with a ×63 oil immersion objective and 514-nm excitation line of an argon laser.

Flow Cytometry and Data Analysis—Cells were analyzed with either FACS Canto II or LSR Fortessa (both from BD Biosciences). The fluorescence of EphA2-YFP, pEphA2-TRITC, and pEphA2-DyLight649 (or ephrin-A3/Cy5) was measured with 488-, 562-, and 633-nm excitation wavelengths and 530/30, 585/15, and 660/20 emission bandpass filters, respectively. No fluorescence spillover was observed between these channels, and, therefore, compensation was not applied.

Two-dimensional cytograms of pEphA2-DyLight649 versus EphA2-YFP (Fig. 2) were recalculated into dependences of EphA2-YFP activity on the receptor amount in cells in the following way. Data were processed with FloJo software (Treestar), where cells were gated on forward and side scatter and then with a specially written script. Briefly, the measured cells were subdivided into 15 segments in accordance with EphA2-YFP content (EphA2-YFP fluorescence intensity). For each segment, average EphA2-YFP activity (average pEphA2 fluorescence intensity) was calculated after correction for unresponsive cells. Note that JMD phosphorylation of EphA2-YFP was inhomogeneous over cells within a sample. Most cells responded to ephrin-A3 stimulation by increasing pEphA2, but some cells retained a background level of EphA2 activity for unknown reasons (Fig. 2). To discern such unresponsive cells, we performed fitting of frequency distribution of pEphA2 in cells (within each of 15 segments) with two Gaussian curves. The curve with a higher average pEphA2 value corresponded to responsive cells, and this average value was taken as a measure of EphA2 activation at a particular amount of EphA2-YFP in cells. Calculated dependences of EphA2-YFP activity on the receptor amount in cells were fitted with a sigmoidal curve with variable slope (Fig. 2), and simultaneously background levels of EphA2-YFP activity were defined.

To compare the abilities of WT, HR, and GZ variants of EphA2 to activation, we analyzed the dependence of pEphA2-DyLight649 on EphA2-YFP and introduced parameters called activating expression level (AEL), activation ability (AA), and integrated activity. The AEL is the amount of EphA2-YFP in cells that provides the pEphA2 increase of 500 fluorescence units over the background level. The AA is inversely proportional to the AEL and is calculated as 10⁶ / AEL. Integrated activity is an area under the fitted sigmoidal curve within the range of the observed EphA2-YFP intensity values. Six independent experiments were carried out, and their results were averaged. Statistical analysis was performed with paired t test in Prism software (GraphPad). Each time point for each experiment gave a total of n = 24 data point pairs for statistical analysis.

Molecular Modeling of the TMD Dimer—Molecular modeling of the self-association of EphA2 TMD (residues 532–562) was performed with the PREDDIMER program (38). The set of five dimer structures was predicted with the FSCOR values var-
ied from 1.593–2.426. The right-handed dimer formed via the glycine zipper motif (Fig. 1C) received the best rank ($F_{SCOR} = 2.426$). The second rank ($F_{SCOR} = 2.197$) was given to the left-handed dimer formed via the heptad repeat motif. Its helix packing interface was similar to the NMR-derived structure (Fig. 1A) (31).

**RESULTS**

The role of TMD interactions was studied using YFP-tagged EphA2 and two variants of EphA2-YFP with point mutations introduced into either the heptad repeat motif of the TMD (HR variant) or the glycine zipper motif of the TMD (GZ variant) (Fig. 1B). In the HR variant, three key weakly polar residues (Gly$^{539}$, Ala$^{542}$ and Gly$^{553}$), situated at the helix-helix packing interface (Fig. 1B) of the EphA2 TMD dimer (31), were substituted with bulky hydrophobic isoleucine residues. The GZ variant containing two mutations, G540I and G544I, was created to verify our hypothesis (31) that two dimerization motifs are involved in the stabilization of the alternative structures of the EphA2 dimers upon signal transduction. Similar amino acid substitutions strongly diminished the dimerization of isolated EphA1 TMD occurring via the homologous glycine zipper motif (34). In either case, the introduced mutation should disturb the EphA2 dimerization via the mutated motif and affect receptor functioning if the corresponding dimerization mode is realized for a full-length receptor.

We have demonstrated earlier that the EphA2 receptors tagged with YFP or cyan fluorescent protein on the C terminus and expressed in HEK293T cells bind ephrins, form dimers (oligomers), and participate in ligand-induced phosphorylation (35, 39). Accordingly, the WT, HR, and GZ variants of EphA2-
YFP were transiently expressed in HEK293T cells. Confocal microscopy analysis revealed a similar cellular localization and distribution of EphA2-YFP variants and the absence of novel features associated with the introduced mutations (Fig. 3A). The ligand-binding properties of the EphA2-YFP variants were compared with the aid of preclustered fluorescent ephrin-A3/Cy5 and flow cytometry. A linear relationship between ephrin-A3/Cy5 binding and EphA2-YFP expression was observed in two-dimensional cytograms (Fig. 3B) for most cells except for a fraction of cells with a high EphA2-YFP expression level. A partial decrease in ligand binding with such cells can point to an overexpression-related increase in the intracellular receptor pool that is inaccessible for ephrin-A3. A slope of the fitted line is indicated on each dot plot.

Receptor activity was analyzed for each individual cell by flow cytometry (Fig. 2) using specific antibodies that recognize phosphorylated tyrosines 588 and 594 in the JMD. Phosphorylation of these tyrosines in EphA2 was shown to be critical for signal transduction (40). In accordance with data published previously (7), receptors were found to be partially activated even without a ligand addition (Fig. 2). As shown earlier, such a type of activation is induced by ligand-independent dimerization (oligomerization) of receptors (7). In our experiments, ligand-independent phosphorylation grows as a function of the receptor content in cells. It occurs for all variants of EphA2-YFP, being greater for the HR variant and smaller for the GZ variant compared with the WT receptor (Fig. 2). The corresponding integrated activity values are (10 ± 1) × 10⁴, (7.2 ± 0.5) × 10⁴, and (5.0 ± 0.9) × 10⁴ arbitrary units (mean ± S.E.) for the HR, WT, and GZ receptors, respectively (Fig. 4A). It seems that mutations in the heptad repeat motif of the TMD enhance receptor activation, whereas mutations in the glycine zipper motif reduce it. This conclusion is consistent with the analysis of the ligand-independent AA, which shows that an equal level of activation is achieved at a lesser content of HR receptors and higher content of GZ receptors in cells (Figs. 2 and 4B). AA values averaged over six independent experiments are as follows: 1.27 ± 0.08, 0.93 ± 0.08, and 0.84 ± 0.1 (mean ± S.E.) for the HR, WT, and GZ receptors, respectively (Fig. 4B).

Ephrin-A3 induces the enhancement of phosphorylation for all variants of EphA2-YFP (Figs. 2 and 4). This enhancement is observed in most cells, including those with high a receptor

![FIGURE 3. Cellular distribution and ligand binding for the WT, HR, and GZ variants of EphA2-YFP. A, typical distribution of the WT, HR, and GZ variants of EphA2-YFP in live HEK293 cells recorded with a laser-scanning confocal microscopy. Representative individual cells are shown. B, comparison of ephrin-A3/Cy5 binding to the WT, HR, and GZ variants of EphA2-YFP in live HEK293 cells. Dot plots show ephrin-A3/Cy5 binding versus EphA2-YFP content for each analyzed cell. Data within the first half of the EphA2-YFP expression range were fitted with linear dependence (red). A slope of the fitted line is indicated on each dot plot.](image-url)
Switching of EphA2 by Transmembrane Helix-Helix Interaction

FIGURE 4. Activity comparison for the WT, HR, and GZ variants of EphA2-YFP. Comparative statistical analysis of data obtained for EphA2-YFP variants in six independent experiments. A, comparison of integrated activities (IA) of EphA2-YFP variants. B, comparison of the AA of EphA2-YFP variants. The abscissa is the time after addition of ephrinA3 to HEK293T cells. *, p < 0.05 (** or ***, p < 0.01; paired t test).

content, i.e. with an increased level of ligand-independent phosphorylation (Fig. 2). As shown by us earlier (35), activation of WT EphA2-YFP with ephrin A3 occurs in a transient (pulse-like) manner in cells. It achieves a maximum approximately 2 min after ligand addition and decays to the initial level after approximately 20 min. Similar behavior was observed for ligand-induced activation of the HR and GZ variants of EphA2-YFP (Fig. 4). The intensity of ligand-induced activation was found to decrease in the row of receptor variants, HR > WT > GZ. This behavior remains unchanged for cells with various contents of receptors in different periods after ligand addition (Fig. 2) and was reproduced in independent experiments (Fig. 4). A paired t test of all time points for six independent experiments gave a significant 1.15 ± 0.01-fold increase (p = 0.0015) of the AA for the HR variant and a 1.54 ± 0.22-fold decrease (p = 0.04) for the GZ mutant compared with the WT receptor. Similar differences were observed for integrated activity values that were 1.34 ± 0.02-fold higher (p = 0.008) for the HR variant and 1.30 ± 0.17-fold lower (p = 0.013) for the GZ variant compared with WT EphA2-YFP. There were no significant deviations from these values for particular time points immediately following the ligand addition. Thus, mutations in the heptad repeat motif of the TMD enhance ligand-induced receptor activation as opposed to the mutations in the glycine zipper motif that reduce it.

DISCUSSION

Using an approach on the basis of quantitative flow cytometry to measure EphA2-YFP phosphorylation in single cells, a set of data was obtained that clarified the role of the TMD in receptor functioning. Applying the original algorithm of data analysis, we were able to monitor and compare activation of the WT, HR, and GZ variants of EphA2-YFP in cells with various receptor content. It should be noted that a high expression level of EphA2 was reported in malignant states (41, 42), whereas most normal cells have low to moderate receptor content. In accordance with our suppositions, the mutations introduced in the TMD of EphA2 disturb receptor dimerization and, thus, affect receptor phosphorylation. The mutations to bulky nonpolar side chain residues used in our study do not completely undermine the functional bases of receptor signaling. Accordingly, the mutation effects are found to be moderate but reproducible and statistically significant. Apparently, the TMD of EphA2 simultaneously acts as a membrane anchor and as a structural element that participates in receptor functioning in a complex manner. Our findings indicate that both dimerization motifs in the TMD are involved in the regulation of EphA2 activity. They influence both ligand-dependent and ligand-independent EphA2 phosphorylation. According to the data of NMR spectroscopy and molecular modeling (Fig. 1), the heptad repeat and the glycine zipper motifs cannot participate in TMD dimerization simultaneously, and, therefore, they should promote the formation of two structurally different receptor dimers. This conclusion is supported by the fact that the heptad repeat and glycine zipper motifs have opposite effects on receptor activity. Disturbance of the TMD interactions through the heptad repeat motif increases the activation ability of EphA2, and, hence, the dimer formed via this motif corresponds to a configuration making activation unfavorable (contra-active configuration). The decrease in phosphorylation of EphA2 caused by glycine zipper motif disruption indicates that this motif is involved in the formation of a configuration favoring activation of the receptor dimer (proactive configuration).

The effects of the mutations in the TMD of EphA2 are clearly observed for cells with low and high receptor content, and their characteristic patterns remain unchanged (Fig. 2). It seems that both configurations of dimers coexist in cells with various EphA2 content. Enhancement of the receptor expression level does not lead to domination of a proactive configuration because disruption of the heptad repeat motif (responsible for contra-active configuration) still considerably increases ligand-independent EphA2 activation in cells with high EphA2 content (Fig. 2). Growth of ligand-independent activation of WT EphA2 with increasing receptor content is likely to occur because of an increase in the number of EphA2 involved in dimer formation, but only a part of newly formed dimers has a proactive TMD configuration. According to this model, ligand binding induces both the formation of dimers with a proactive TMD configuration and the transition of preformed dimers from a contra-active to a proactive configuration.

These findings allowed us to suggest that TMD dimerization switching is an essential mechanism underlying EphA2 signal transduction at the ligand-induced and spontaneous activation of the receptor. The TMD dimerization switching of unligated receptors can be modulated by local membrane properties (i.e. charge, thickness, curvature, lipid composition, and ordering). Thus, it has been demonstrated that cholesterol-rich membrane microdomains promote the ligand-independent clustering of Eph receptors and the formation of low-affinity homodimers (43).

EphA2 TMD interactions via the extended heptad repeat motif leads to left-handed dimerization of the TMD helixes with a small (approximately 15°) angle between helix axes (Fig. 1A). Molecular modeling shows that dimerization via the glycine zipper motif provides for the formation of the right-handed dimer, which has a scissor-like configuration with a large (approximately 45°) angle between the helix axes and
increased distance (~20 Å) between the TMD helix ends on the cytoplasmic side of the membrane (Fig. 1C). This model structure looks reliable because a similar structure was revealed with the NMR analysis for the isolated EphA1 TMD, which formed dimers via the N terminus glycine zipper motif (33). Moreover, right-handed dimerization is one of the conventional variants for the packing of interacting TMD helices of integral proteins, and the ~45° helix crossing angle is close to the frequently occurring angle for transmembrane helix-helix interactions (44).
Switching of EphA2 by Transmembrane Helix-Helix Interaction

Taking into account the dimer structures of the EphA2 TMD described above (Fig. 1), the transition from a contra-active configuration to a proactive one should be accompanied by the mutual rotation of TMDs around helix axes (approximately 160°) and considerable separation of their ends at the cytoplasmic side of the membrane (from approximately 10 to 20 Å). We surmise that such TMD realignments are a driving force for the conformational transition of the TKD into the active state. By analogy to other receptor tyrosine kinases (45–47), one can assume that the TKDs of adjacent EphA2 molecules form the dimer, which is transformed from a symmetric autoinhibited configuration to an active asymmetric configuration.

Recent crystal structure studies of the EphA2 ECD (7, 10) enable us to consider the tentative models of functional coordination between the ECD and TMD of EphA2 receptors. Experimental data indicate that EphA2 can participate in both dimeric and oligomeric interactions via the ECD (7, 10) but only in dimeric interactions via the TMD (31). Oligomeric interactions between well ordered ECDs can stabilize linear arrays of EphA2 (10) in which the LBD binds to the cysteine-rich domain of a neighboring molecule, forming the staggered, parallel packing of rigid rod-like ECDs (Fig. 5A). In such arrays, TMDs are supposed to form contra-active dimers, and TKDs adopt a symmetric autoinhibited configuration (Fig. 5A).

According to the crystal structure analysis (10), the ligand-bound ECDs form “in-register” arrays stabilized by LBD-LBD oligomeric and cysteine-rich domain-cysteine-rich domain dimeric interactions (Fig. 5, B and C). Pairs of ECDs cross in the region of FN1 domains. The FN1-FN2 linker has a hinge-like character, and the relative rotation of the FN2 domain (−70° in crystal) occurs, compared with the unliganded ECD conformation (10). Within in-register arrays of receptors, TMDs are supposed to form proactive dimers, and TKDs have an active asymmetric configuration (Fig. 5, B and C).

The transition from staggered packing to the ligand-bound (in-register) structure should be accompanied by the scaled reorientation of each second ECD in the array and the inevitable reorganization of TMD dimers.

A crystal structure study revealed that arrays of EphA2 ECDs can have the ligand-bound-like (in-register) conformation even without ligand (7). Such structures in EphA2 clusters should promote the ligand-independent formation of the proactive TMD dimer configuration and receptor activation. It is reasonable to suppose that the probability of the spontaneous formation of the unliganded in-register conformation in receptor clusters increases at a high receptor content in the membrane.

Because both ligand-bound and unliganded EphA2 can adopt the in-register conformation, the local ligand-induced reorganization of receptors from the staggered packing to the in-register conformation can provoke propagation of this reorganization and TMD-mediated receptor activation along the EphA2 cluster far from the place of ligand binding (Fig. 5, B and C), as it is observed in cells (43) and predicted by the so-called “seeding” mechanism (10). It seems that EphA2 clusters behave like a continuous excitable medium rather than a set of isolated dimers/oligomers.

Another question to be discussed is the stoichiometry of a minimal ephrin-EphA2 active complex in the context of the proposed model. The hypothetic structure of signaling (ephrin-EphA2)2 heterotetramers is shown in Fig. 5B. The distance between the C termini of FN2 domains in a such heterotetramer is approximately 12–17 nm, considering the possible rotation of the FN2 domain. It is larger than the length of two extracellular juxtamembrane segments (approximately 5–6 nm) connecting the FN2 domains with the corresponding N termini of the TMD helices. Obviously, these geometrical constraints cannot be resolved for the depicted signaling heterotetramers (Fig. 5B). At the same time, the available structures of ECD and TMD dimers can be united in signaling ephrin-EphA2 heterohexamers, for example, as shown in Fig. 5C. In this case, the distance between C-terminal ends of FN2 domains, which are linked to the TMD dimer, is 5–10 nm. So far, the formation of signaling ephrin-EphA2 heterohexamers was neither supported by experimental data nor discussed as a hypothesis. To maintain the classical concept of signaling (ephrin-EphA2)2 heterotetramers, the structure of some EphA2 domains should be re-examined.

In conclusion, we discovered the involvement of TMD interactions in Eph receptor activity. As discussed earlier (31), at least one dimerization motif can be found in the TMD sequence of any Eph receptor. For EphA1, the dimerization of the isolated TMD in the lipid environment have been tentatively confirmed, and two dimerization modes have been recognized (33, 34, 48). It seems that TMD participation in receptor activation via TMD dimerization can be a general property of Eph receptor tyrosine kinases.

The discovery of proactive and contra-active configurations of TMD dimers of EphA2 extends to a list of receptor tyrosine kinases, such as ErbB (45, 49) and FGFR3 (50, 51), in which the alternative dimerization of TMD is supposed to control receptor activation.

REFERENCES

1. Miao, H., and Wang, B. (2012) EphA receptor signaling: complexity and emerging themes. Semin. Cell Dev. Biol. 23, 16–25
2. Lin, S., Wang, B., and Getsios, S. (2012) Eph/ephrin signaling in epidermal differentiation and disease. Semin. Cell Dev. Biol. 23, 92–101
3. Okazaki, T., Ni, A., Baluk, P., Ayeni, O. A., Kearley, J., Coyle, A. J., Humb, A., and McDonald, D. M. (2009) Capillary defects and exaggerated inflammatory response in the airways of EphA2-deficient mice. Am. J. Pathol. 174, 2388–2399
4. Fuji, H., Fujiwara, H., Horie, A., Sugimana, K., Sato, Y., and Konishi, I. (2011) EpprinA1 stimulates cell attachment and inhibits cell aggregation through the EphA receptor pathway in human endometrial carcinoma-derived Ishihkahw cells. Hum. Reprod. 26, 1163–1170
5. Goichberg, P., Bai, Y., D’Amario, D., Ferreira-Martins, J., Fiorini, C., Zheng, H., Signore, S., del Monte, F., Ottolenghi, S., D’Alessandro, D. A., Michler, R. E., Hosoda, T., Anversa, P., Kajstura, J., Rota, M., and Leri, A. (2011) The ephrin A1-EphA2 system promotes cardiac stem cell migration after infarction. Circ. Res. 108, 1071–1083
6. Himanen, J. P. (2012) Ectodomain structures of Eph receptors. Semin. Cell Dev. Biol. 23, 35–42
7. Himanen, J. P., Vremekayva, L., Janes, P. W., Walker, J. R., Xu, K., Atapattu, L., Rajashankar, K. R., Mensinga, A., Lackmann, M., Nikolov, D. B., and Dhe-Paganon, S. (2010) Architecture of Eph receptor clusters. Proc. Natl. Acad. Sci. U.S.A. 107, 10860–10865
8. Himanen, J. P., Goldgur, Y., Miao, H., Myshkin, E., Guo, H., Buck, M., Nguyen, M., Rajashankar, K. R., Wang, B., and Nikolov, D. B. (2009) Ligand recognition by A-class Eph receptors: crystal structures of the EphA2 ligand-binding domain and the EphA2/ephrin-A1 complex. EMBO Rep.
ments in activation or autophosphorylation of receptor tyrosine kinases. 
Mol. Cells 29, 443–448
48. Artemenko, E. O., Egorova, N. S., Arseniev, A. S., and Feofanov, A. V. (2008) Transmembrane domain of EphA1 receptor forms dimers in membrane-like environment. Biochim. Biophys. Acta 1778, 2361–2367
49. Fleishman, S. J., Schlessinger, J., and Ben-Tal, N. (2002) A putative molecular-activation switch in the transmembrane domain of erbB2. Proc. Natl. Acad. Sci. U.S.A. 99, 15937–15940
50. Bocharov, E. V., Lesovoy, D. M., Goncharuk, S. A., Goncharuk, M. V., Hristova, K., and Arseniev, A. S. (2013) Structure of FGFR3 transmembrane domain dimer: implications for signaling and human pathologies. Structure 21, 2087–2093
51. Volynsky, P. E., Polyansky, A. A., Fakhruddinova, G. N., Bocharov, E. V., and Efremov, R. G. (2013) Role of dimerization efficiency of transmembrane domains in activation of fibroblast growth factor receptor 3. J. Am. Chem. Soc. 135, 8105–8108