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Physical-chemical principles underlying RTK activation, and their implications for human disease

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Abstract

RTKs, the second largest family of membrane receptors, exert control over cell proliferation, differentiation and migration. In recent years, our understanding of RTK structure and activation in health and disease has skyrocketed. Here we describe experimental approaches used to interrogate RTKs, and we review the quantitative biophysical frameworks and structural considerations that shape our understanding of RTK function. We discuss current knowledge about RTK interactions, focusing on the role of different domains in RTK homodimerization, and on the importance and challenges in RTK heterodimerization studies. We also review our understanding of pathogenic RTK mutations, and the underlying physical-chemical causes for the pathologies.

Introduction

RTKs, the second largest family of membrane receptors, transduce biochemical signals upon lateral dimerization in the membrane plane (1–3). RTKs are single-pass membrane proteins, shown schematically in Figure 1. The N-terminal extracellular (EC) domains, usually several hundred amino acids long, vary between families and contain characteristic arrays of structural motifs. The single transmembrane TM domain is followed by a 40 to 80 amino acid juxtamembrane (JM) region and a ~250 amino acid kinase domain, homologous to soluble tyrosine kinases (1;4;5).

RTK dimerization controls and ensures the close contact of the two kinase domains in the dimer. Upon dimerization each kinase domain catalyzes the phosphorylation of critical tyrosine residues in the activation loop of the neighboring kinase (6). This is followed by the phosphorylation of additional tyrosine residues in the juxtamembrane and catalytic domains, which serve as binding sites for docking proteins. Upon recruitment and/or phosphorylation, these docking proteins initiate intracellular signaling cascades that control vital cellular processes (7–9).

RTKs, with a single exception (ErbB2), are ligand-binding proteins. The ligands play intricate roles in the activation process (10), but are not always essential for RTK dimerization and activation. Their contribution to RTK activation is believed to be both

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thermodynamic (stabilizing RTK dimers) and structural (inducing RTK structural changes which enhance RTK activity).

There are two requirements for successful activation of an RTK. The first requirement is the close approach of the two kinase domains in the dimer. This is accomplished via dimerization. The dimerization efficiency, and therefore the time the kinase domains spend in close proximity of each other, is controlled by the dimerization constant. Dimerization is modulated by ligand binding, and thus the proximity of the kinase domains is also regulated by the ligand concentration and the ligand binding constant. The second requirement for successful activation of an RTK is that the structure of the kinase domain dimer is suited for the transfer of a PO_4 group from a bound ATP molecule to a particular tyrosine (9;11). The phosphorylation-competent dimer structure is established via specific receptor-receptor and receptor-ligand interactions. Structural constraints in the extracellular and TM domains, imposed upon dimerization and ligand binding, propagate into the kinase domain and control RTK phosphorylation and activity.

Physical - chemical models of RTK activation

We and others have proposed that RTK activation can be described with physical-chemical models which account for dimerization, ligand binding and phosphorylation (12–16). Such models have been shown to give an adequate description of RTK phosphorylation data, despite their simplicity. Usually the models do not take explicitly into account all interactions that regulate RTK activation in cellular membranes. Rather, all these events contribute to apparent constants describing different steps in RTK activation. Here we provide a brief overview of such models.

A. Dimerization

Ligand-independent lateral dimerization, i. e., the lateral association of two RTK monomers into an unliganded dimer, is the first critical step in RTK activation. It is controlled by the dimerization constant K_1 and is given by the following reaction scheme:

$$M+M \overset{K_1}{\longleftrightarrow} d \tag{1}$$

The dimerization constant K_1 is defined as $K_1 = [d]/[M]^2$, with $[M]$ and $[d]$ being the monomer and dimer concentrations, respectively. The total receptor concentration is $[T] = 2[d] + [M]$. The free energy of dimerization is calculated as $\Delta G = -RT \ln K_1$.

Unliganded RTK dimers can be phosphorylated or not. If the unliganded dimers are phosphorylated, equation (1) accounts for the so-called "*basal or constitutive activation*". If the unliganded RTK dimers are not phosphorylated (inactive), equation (1) accounts for the so-called "*pre-dimerization*". These two terms are vaguely defined in biology, but they have the same physical-chemical basis, i.e. equation (1).

B. Ligand binding

The next step in RTK activation is ligand binding, which is believed to stabilize RTK dimers and likely alter their structure (discussed below). The processes of dimerization and ligand binding are coupled, and can be described by the following reaction scheme:

$$\begin{array}{c} M+M \overset{K_1}{\longleftrightarrow} d \\ +L \updownarrow K_M \updownarrow K_D \updownarrow +2L, \\ ML+ML \underset{K_2}{\longleftrightarrow} D \end{array} \quad (2)$$

where *D* denotes the liganded dimer (see also Figure 2).

For the sake of simplicity, sometimes it is assumed that the ligand binds preferentially to the unliganded dimers, rather than the monomers (14). In this case, scheme (2) is reduced to the following two coupled reactions:

$$\begin{array}{c} M+M \overset{K_1}{\longleftrightarrow} d \\ d+L_2 \overset{K_D}{\longleftrightarrow} D \end{array} \quad (3)$$

In scheme (3), the dimerization and the ligand binding constants are defined as $K_1=[d]/[M]^2$ and $K_D=[D]/[d][L]^2$, respectively. The dimerization reaction is driven, in part, by ligand binding: Ligand binding depletes the unliganded dimers by converting them to liganded dimers, thus promoting the dimerization of the monomers.

All three schemes, (1) through (3), as well as more complex models accounting for negative cooperativity in ligand binding, have been used in the literature to rationalize experimental data and to gain insights into how dimerization and ligand binding regulate RTK activation (12–16). While these models are simple and do not capture the full complexity of RTK signaling, they are very useful in comparative studies, such as studies of the effects of sequence variations and pathogenic mutations on different steps in RTK activation, as discussed below.

C. Phosphorylation

In the dimer, the two receptors autophosphorylate each other, with each kinase domain acting as an enzyme which facilitates the transfer of a phosphate group to the neighboring kinase. One way to account for the efficiency of phosphorylation is to define receptor phosphorylation probabilities, Φ_d and Φ_D, within the unliganded and liganded dimers, respectively (17). The concentration of phosphorylated receptors is then calculated according to:

$$[P]=2\Phi_d[d]+2\Phi_D[D]. \quad (4)$$

Note that $\Phi_d = 0$ corresponds to inactive unliganded dimers and describes predimerization, while $\Phi_d \neq 0$ describes active unliganded dimers and accounts for basal or constitutive activation.

Experimental characterization of RTKs

A. Measurements of RTK dimerization

To characterize RTK dimerization in the context of equation (1), one needs to measure directly two of the following three parameters over a range of RTK concentrations: (i) concentrations of RTK dimers, (ii) concentrations of RTK monomers, and (iii) total RTK concentrations. Quantitative measurements of these concentrations, and thus calculations of dimerization constants for full-length RTKs is a challenge. However, some widely used biochemical methods allow us to estimate and compare dimerization propensities.

One such method is chemical cross-linking (18–21). In this method the cells are incubated with a cross-linker, lysed, and analyzed using Western blotting. Since only RTKs in very close proximity can be cross-linked, the presence of cross-linked bands on Western blots suggests that dimers exist on the cell surface. Furthermore, higher cross-linking correlates with higher dimerization. While the method is useful and well established, it should be kept in mind that the cross-linker is non-specific, such that it cross-links all proteins in close proximity. As a result, gels of cross-linked proteins are usually smeared, and very difficult to accurately quantify. Furthermore, a limitation of this experimental approach is that the cross-linking propensities depend not only on close proximity, but also on structure. Thus, a structural change can alter the probability for RTK cross-linking, despite the fact that RTK dimerization is not affected.

Another method used in the literature for assessment of RTK dimerization is immunoprecipitation, followed by SDS-PAGE (22). Disulfide-linked RTK dimers have been observed using this technique under oxidizing conditions without cross-linking. In this case, however, the assay reports on interactions occurring within the immunoprecipitates. These might be different from interactions in the native plasma membrane, which imposes structural constraints on RTK dimers. In particular, the presence of unpaired cysteines in the receptors may lead to the formation of non-native disulfide bonds in the immunoprecipitate and give a false positive for dimer formation.

A powerful method used to study dimerization is Förster resonance energy transfer (FRET) (23–27). FRET involves the non-radiative transfer from a fluorescent donor to a fluorescent acceptor (28–32), and is manifested in a decrease in donor fluorescence and an increase in acceptor fluorescence (30–32) upon dimerization. The efficiency of energy transfer *E* is inversely proportional to the sixth power of the distance, *r*, between the donor and acceptor. The transfer efficiency *E* is a function of *r* and *Ro*, the characteristic Förster radius for the donor and acceptor pair:

$$E=1/[1+(r/R_0)^6] \quad (5)$$

Typical donor/acceptor pairs, such as the widely used fluorescent proteins, have R_o of 50–60 Å. Thus, if the two fluorophores are closer than 50 Å in a dimer, FRET will occur.

FRET can be measured in the native cellular environment. In these experiments the genes encoding the RTKs are modified by attaching sequences encoding fluorescent proteins, usually at RTK's C-termini. While most in-cell FRET experiments measure the sensitized acceptor emission, sophisticated FRET techniques have been developed to assess donor quenching (33–38). When measuring interactions in membranes using FRET, however, special care needs to be exercised since FRET occurs even if there are no specific interactions, due to random co-localization of donors and acceptors (39–42). Furthermore, challenges in data interpretation arise because the cellular environment is highly heterogeneous, and supramolecular organizations in clusters/domains introduce additional heterogeneities affecting the measured FRET efficiency and complicating data analysis.

To overcome the above challenges, we have established plasma membrane-derived vesicles (43–48) as a model system for studies of RTK interactions in mammalian membranes via FRET (Figure 3). Plasma membrane-derived vesicles are produced using either a mechanical method, by breaking the plasma membrane, or using chemical methods, by disrupting the cytoskeleton in a direct or indirect (apoptotic) way (49–52). Plasma membrane-derived vesicles are a simplified model of the cell membrane because there is no cytoskeleton and no TM potential (43). Yet, plasma membrane-derived vesicles possess

complex features that are characteristic of native cellular membranes (43–48). Their lipid composition is similar to the one in the native membrane (43), and they maintain the plasma membrane asymmetry (44–46). The membranes of the vesicles contain various membrane proteins and mimic the natural crowded membrane environment.

We have demonstrated that quantitative measurements of RTK dimerization in single vesicles are feasible with the QI-FRET method (53). The QI-FRET method, discussed in (53–55), yields the unknown donor and acceptor concentrations, and the FRET efficiency (i.e. the three parameters that are sufficient to calculate free energies of dimerization) (38). Because the experiments utilize transient transfection, expression levels vary from cell to cell, and vesicles with a wide range of receptor concentrations (i.e., number of receptors per unit membrane area) can be produced in a single transfection experiment. Thus, a wide protein concentration range is sampled, and the dimerization energetics are measured for different receptor concentrations, to obtain dimerization curves.

Using this methodology, we have measured the energetics of FGFR3 TM domain dimerization in the plasma membrane of CHO cells, as well as the dimerization energetics of a construct containing both FGFR3 EC and TM domains (55). The two dimerization free energies were determined as $\Delta G(\mathrm{TM}) = -RT\ln K_D(\mathrm{TM}) = -4.2 \pm 0.2$ kcal/mole and $\Delta G(\mathrm{EC+TM}) = -3.3 \pm 0.1$ kcal/mole, respectively.

B. Characterization of ligand binding

The binding of ligands to full-length receptors on the surface of living cells is usually measured by quantifying bound radiolabeled or fluorescently-labeled ligands. Binding of ligands to isolated RTK extracellular domains can be characterized using isothermal titration calorimetry, ultracentrifugation, and surface plasmon resonance (SPR), as described below.

The most popular method to probe interactions between ligands and cell surface receptors involves quantification of bound radiolabeled ligands. In these experiments, cells are incubated with radiolabled ligands either on ice or at ambient temperature (to minimize receptor cell uptake). The free ligands are removed, a process which usually requires extensive washing with concentrated salt solutions. Then the cells are lysed and the radioactivity of the receptor-bound ligands is measured. Binding curves can be generated by performing the measurements at different concentrations of ligands. This method has been used to investigate EGF binding affinity to EGFR (56;57), and the interactions between FGF1 and FGF receptors (58;59). Furthermore, the bound ligands can be crosslinked to the receptors, such that the ligand-receptor complexes remain intact when the cell lysates are run on SDS-PAGE gels (59).

Fluorescence-based methods are an alternative to the traditional radiolabeling approach since the fluorescence intensity of the bound ligand is easily measured in a standard spectrofluorometer (60). The receptor–ligand interactions on the cell surface can be further investigated using single molecule fluorescence techniques. These techniques have allowed the visualization of single bound EGF molecules, and have provided insight into the binding kinetics (61;62). Fluorescence correlation spectroscopy (FCS*)* with single-molecule sensitivity is also used to quantitatively analyze ligand-receptor interaction on surface of living cell. This technique has yielded the dissociation constant for EGF-EGFR interactions, via the analysis of fluorescence intensity fluctuations in a small confocal volume (63).

Ligand binding to the isolated RTK extracellular domains has also been characterized. For instance, isothermal titration calorimetry, ultracentrifugation and molecular modeling (64) has been used to characterize the energetics and kinetics of the interactions between FGF1, FGFR1 extracellular domain, and heparan sulfate in solution. Alternatively, the ligands or

the extracellular domains can be attached to a surface, and the interactions can be analyzed using SPR. Such studies have provided valuable insights about the specificity in FGF-FGFR interactions (65–67).

C. RTK phosphorylation measurements

Methods to measure RTK phosphorylation are well established. Phosphorylation levels are monitored by either (1) in-vitro kinase assays, (2) quantification of bound antibodies that specifically recognize phosphorylated tyrosines, or (3) mass spectrometry identifying attached phosphate groups.

In vitro kinase assays often utilize radioactive phosphorous. In these assays cell lysates containing RTKs of interest are incubated with kinase buffer with ATP containing radiolabeled phosphates. The radioactivity of the receptors is quantified upon the transfer of the radioactive phosphate groups to the kinase domains (68). Alternatively, RTK phosphorylation can be quantified without the use of radioactivity by coupling it with the oxidation of NADH, and then following NADH oxidation using spectrophotometric assays (69).

RTK phosphorylation can be measured with the use of antibodies that recognize phosphorylated tyrosines. The amounts of such bound antibodies can be determined using traditional Western blots, ELISA, or in-cell Western blot techniques. These experiments usually involve secondary antibodies which interact with the primary ones, and are conjugated to reporter moieties, detectable using either chemiluminescence or fluorescence. Two different types of primary antibodies can be used: (1) antibodies recognizing all phospho-tyrosines, such as the 4G10 anti-phosphotyrosine antibody, capable of detecting over 30 tyrosine kinases, including ErbB2 (70), PDGFR (71), and FGFR3 (72), and (2) antibodies generated against specific phosphorylated sites, recognizing a particular phosphotyrosine together with the sequence around it (14;15). The latter are useful because RTK kinase domains normally contain more than one tyrosine residue, and the phosphorylation of the different tyrosines occurs sequentially (73;74).

In recent years, mass-spectrometry has become popular in studies of RTK phosphorylation (75–77), and we foresee that its usage will continue to increase. In this method, the phosphorylated RTKs are purified via immunoprecipitation and are digested with trypsin. The products of digestion (peptides) are analyzed by either Matrix-associated laser desorption ionisation time-of-flight Mass Spectrometry (MALDI-TOF MS) or Electrospray Ionization Mass Spectrometry (ESI-MS) (78). By comparing the masses of the peptides with a “theoretical digest”, the presence of phosphate groups can be detected. This method has been used to identify the phosphorylation sites of PDGFR (79) and EGFR (80).

Structural requirements for RTK activation

As discussed above, RTK activation requires precise orientation and positioning of the catalytic domains with respect to each other, such that the phosphate group can be successfully transferred from ATP to the neighboring receptor. For EGFR, this entails the formation of an asymmetric kinase dimer in which the C-lobe of one kinase contacts the N-lobe of the second kinase and positions the activation loop of the second kinase to catalyze phosphate group transfer (9). The existence of asymmetric dimers have been proposed for FGF receptors, too (81). It is not yet clear, however, whether such asymmetric dimers form for all RTKs, and whether the two kinases alternate over time and act both as catalysts and substrates.

The order of the phosphorylation of the different tyrosines is also controlled by the structure of the kinase domain. For instance, the order of tyrosine phosphorylation in FGFR1 kinase domain is strict, and kinetically controlled. It is limited by the rate of transfer of the phosphate group from ATP to the tyrosines (73), and strongly influenced by the kinase tertiary structure (73;74).

The structure and orientation of the kinase domains, on the other hand, are controlled by specific interactions between the TM domains, as well as interactions between the EC domains and the ligands, as discussed below.

Structural constraints imposed by the TM domain dimers

The TM domain structure has been shown to control the orientation of the kinase domains in the dimer, such that successful phosphorylation can occur (11). In particular, rotation of the dimer interface has been shown to induce periodic oscillations in kinase activity (11). Therefore, the structure of the TM domain dimer is an important determinant in RTK signal transduction. About 20 years ago, a five-residue sequence motif (the so-called P0–P4 motif) was proposed to mediate dimerization of RTK TM domains (82). The characteristics of the P0-P4 motif are: P0 exhibits a small side chain, such as Gly, Ala, Ser, Thr, or Pro; P3 requires an aliphatic side chain, i.e. Ala, Val, Leu, or Ile; and P4 must be a Gly or Ala residue. The P0-P4 motif is similar to the GxxxG motif (83;84), shown to be important for Glycophorin A (GpA) dimerization. Later, the TM sequences of ErbB1, ErbB2, and ErbB4 were noticed to have at least two such GxxxG-like motifs (85), and the different GxxxG-like motifs were proposed to mediate either homo or hetero-dimerization (90;91). Alternatively, based on calculations of dimerization free energies of alternate ErbB2 TM domain structures, it was proposed that some RTK TM domains may form two alternative homodimer structures utilizing different GxxxG-like motifs, a phosphorylation-competent structure and an inactive structure (17;86;87).

The solved crystal structure of the ErbB2 TM dimer provided demonstration that dimerization occurs via the N terminal GxxxG dimerization motif, while the C terminal motif does not participate in the dimer interface (88). A similar GxxxG-like motif is involved in the dimerization of a different RTK, EphA1 (89). Yet, the GxxxG motif is not required for the dimerization of all RTKs. For instance, the dimer interfaces of ErbB3, EphA2, PDGFR and FGFR3 TM domains do not involve GxxxG-like motifs, despite the fact that their sequences contain several such motifs (92–96).

Structures of ligand-bound extracellular domain dimers

The solved crystal structures of isolated RTK extracellular domains reveal tight contacts between the two EC domains, and between the EC domains and ligands. The contacts between EGFR EC domains are mediated via a “dimerization arm”, exposed only upon ligand binding (97–99). A recent crystal structure of an EGFR drosophila variant reveals very strong interactions between the two EC domains when only one ligand is bound to dimer (100).

In the FGF2 structure found to FGFR1, there are many contacts between each ligand and the two receptors, as well as direct receptor-receptor interactions (101–103). The structure further reveals a positively-charged lysine-rich “canyon” where heparin oligosaccharides bind, interacting with both the extracellular domains and the ligands, further stabilizing the dimer. Growth factors for families other than EGFRs and FGFRs, (such as KIT and VEGFR), are covalently linked homodimers that bring the extracellular domains together, which promotes additional interactions between the extracellular domains (104;105).

Structural changes mediated by ligands

The ligands of some receptors are believed to not only introduce a structural change in the extracellular domain, but also affect the conformation of the kinase domain, thus increasing RTK activation. For instance, the EGFR unliganded dimer is believed to be inactive because the asymmetric kinase dimer, required for activity (9), cannot form in the absence of ligand. Ligand binding likely induces a rotation in the EGFR dimer and ensures correct positioning of the two kinase domains for phosphorylation (106). The dimeric ligand of KIT, believed to stabilize the dimer by cross-linking the two monomers (104), also induces a structural change which enables receptor-receptor interactions. These interactions are weak as compared to ligand-mediated dimer stabilization and thus they do not drive dimerization by themselves. However, these interactions define the relative orientations of the two receptors, which likely helps to position the kinase domains in the correct orientation for productive phosphorylation.

RTK involvement in human disease

A. Pathogenic mutations in RTKs

Since RTKs play a key role in the regulation of cellular processes that are critical for cell growth, differentiation, and motility, defects in their activation lead to human pathologies. There are many pathogenic single amino acid mutations in RTKs that are usually (but not always) gain-of-function mutations. Because RTK activity is determined by RTK phosphorylation, studies of pathogenic mutations invariably involve experiments which assess the effect of these mutations on phosphorylation. The simplest way to address this question is to compare wild-type and mutant expression and phosphorylation, side-by-side on Western blots. If the expression of the wild-type and the mutant are the same, then the phosphorylation levels can be directly compared. Such direct comparisons have shed light on the molecular basis behind many pathologies (68;107–109).

Direct comparisons of phosphorylation on Western blots have also shed light on how different mutations in a particular RTK can give rise to different phenotypes. One such study compared the phosphorylation of two FGFR3 mutants, linked to achondroplasia (ACH) and thanatophoric dysplasia (TD) (109). ACH, the most common form of human dwarfism(110;111), is a relatively mild phenotype characterized by short stature (112).

On the other hand, TD is much more severe and always lethal in the neonatal period, characterized by severe shortening of the limbs, macrocephaly, and a narrow thorax with small ribs (112). The phosphorylation of the R248C and K650E mutants, associated with TD, has been shown to be higher than the phosphorylation of the G380R mutant linked to ACH (109). Thus, there is a link between higher phosphorylation and more severe phenotypes.

The effect of pathogenic mutations may be diverse, and may include processing defects, such as impeded trafficking and defective downregulation (111;113;114). In many cases, the expression of the mutant is different from the expression of the wild-type in cell lines that are widely used in biomedical research. If the expressions are different, side-by-side comparison of phosphorylation on Western blots is not very informative. To be able to carry out such a comparison, we need to obtain an absolute measure of the phosphorylation at a particular expression level. We have shown that measurements of “phosphorylated fractions” as a function of expression levels can serve this purpose (15;16). In these experiments we treat RTKs with their ligands and we measure phosphorylation over a very wide range of ligand concentrations, including very high ligand concentrations (14): At high ligand concentration all receptors that are exposed to ligand and capable of binding ligand are driven to their liganded dimeric state. At these levels, phosphorylation is saturated and is

not further increased when more ligand is added, providing a measure of the maximum possible phosphorylation (14;16). Phosphorylated fractions are then determined as the ratio of measured phosphorylation at a particular ligand concentration over the maximum possible phosphorylation (14;16), and are independent of the specific conditions used in the Western blot experiments. Using this technique, we have compared the phosphorylation of wild-type FGFR3 and the pathogenic A391E mutant linked to Crouzon syndrome in the absence of ligand, demonstrating an increase in FGFR3 phosphorylation due to the A391E mutation, despite the fact that the expression of the wild-type and the mutant were different (15).

B. Physical-chemical causes for RTK-linked pathologies

Altered dimerization—Some pathogenic mutations stabilize RTK dimers, by promoting more intimate contacts between the two mutant receptors in the dimer (see Table 1 for examples). As a result, the dimerization propensity (i.e. K_I in schemes (1) through (3)) increases. The distribution between inactive monomers and active dimers shifts towards the active dimeric state, increasing the over-all RTK activity.

To test for enhanced dimerization due to pathogenic mutations, one needs to use a direct dimerization assay such as the one shown in Figure 3. In our lab we have used this assay to investigate the effect of two pathogenic FGFR3 mutations, A391E and G380R. While the A391E mutation linked to Crouzon syndrome increases FGFR3 dimerization, the G380R mutation linked to ACH does not (paper in preparation). This behavior correlates with the mutation-induced stabilization of the isolated FGFR3 TM domain dimers in lipid bilayers (95;115).

Receptor overactivation due to increased dimerization may occur even for wild-type receptors if the receptor is overexpressed. This mechanism can be understood using equation (1). As the total concentration of receptors increases, the equilibrium is shifted towards the active dimeric state.

Altered ligand binding—There are pathogenic RTK mutations that increase ligand binding (K_M or K_D in schemes (2) and (3)). Increased ligand binding leads to dimer overstabilization and enhanced phosphorylation. Techniques that assess ligand binding strengths are well established (and reviewed above), and can be used to compare the binding of ligands to wild-type and mutant receptors. Such direct binding measurements, for example, have demonstrated aberrant ligand binding to FGFR mutants implicated in human growth disorders (Table 1).

Structural changes in the kinase domains—Some pathogenic mutations induce structural changes in RTK dimers (Table 1), which affect the receptor phosphorylation probabilities, Φ_d and Φ_D in equation (4). In this case the number of dimers is not increased, but the mutant dimers are more active than the wild-type dimers, leading to an over-all increase in activity. Presumably, this occurs because it is easier to phosphorylate critical tyrosines in the mutant dimers, as compared to the wild-type dimers (116). Such structural changes may originate outside the kinase domain and propagate throughout the whole structure (106).

High resolution structures of full-length RTKs are not available yet, due to experimental challenges in the expression of full-length RTKs in large quantities (117). Since the determination of full-length RTK dimer structures is challenging, studies of the effect of pathogenic mutations on dimerization, ligand binding, and phosphorylation may help us deduce possible structural changes in mutant RTK dimers. For example, the G380R mutation increases FGFR3 phosphorylation, but does not increase FGFR3 cross-linking or ligand binding (14). We have interpreted these findings as an indication for a structural

change. In support of this view, a molecular model of the FGFR3 TM dimer structure suggests that the mutation induces a rotation in the TM dimer interface (14). On the other hand, the changes in the phosphorylation and dimerization propensities due to the A391E mutation linked to Crouzon syndrome are very similar, suggesting that the mutation likely affects FGFR3 dimerization propensity, but does not induce a significant structural change (15;95).

Multiple effects—Some mutations may affect multiple steps in the RTK activation process. An approach to understand the effect of pathogenic mutations on dimerization, ligand binding, or receptor phosphorylation probabilities is to model RTK activation using physical-chemical models such as the ones given by equations (1) through (4). By fitting the models to experimental data, we can determine if a mutation affects dimerization (i.e K_1 or K_2), ligand binding, (K_L or K_M) or phosphorylation (Φ_d or Φ_D). In one example, Pike and colleagues measured EGF binding to EGFR while varying both the receptor and ligand concentrations (12;13). By fitting a model similar to the one given by equation (2) to their data, they were able to determine that an engineered mutation in the extracellular domain of EGFR, Y246D, decreases ligand binding, while an L680N mutation in the kinase domain of EGFR affects both EGFR dimerization and ligand binding. While quantitative measurements of RTK phosphorylation, dimerization, and ligand binding may be very tedious, their pay-off could be significant: The understanding of how a mutation affects RTK activity is the first step towards the development of new therapeutic strategies with high efficiencies and low toxicities.

Thermodynamics of RTK dimerization

In this last section, we overview recent findings that pertain to the thermodynamics of RTK dimerization as a regulator of RTK activity.

A. Contributions of RTK domains to RTK dimerization thermodynamics

Transmembrane (TM) domains—Biophysical studies of the isolated RTK TM domains have provided a direct assessment of the contribution of RTK TM domains to RTK dimerization energetics. All isolated RTK TM domains have been shown to dimerize in bacterial membranes, with the dimerization strength varying between families and within the families (118). Quantitative studies of dimerization in lipid vesicles have shown that the contribution of FGFR3, ErbB1 and EphA TM domains to dimerization is about −3 kcal/mole (119–123). FGFR3 TM domains has been further shown to drive ligand-independent dimerization in plasma membrane-derived vesicles in the absence of ligand in the presence of the EC domain (54). As a result, sequence changes in RTK TM domains affect RTK dimerization at zero ligand and at low ligand concentrations (124;125). At high ligand concentrations, however, the contribution of the TM domains to dimerization is overshadowed by the large contributions of the EC domains and ligands. Thus, TM domain sequence alterations do not affect RTK dimerization and activation significantly at high ligand concentrations (124;126).

Extracellular domains and ligands—Based on the solved dimeric structures of isolated extracellular domains bound to ligand, it can be expected that ligand binding stabilizes RTK dimers. Indeed, in all such structures, despite a great deal of structural diversity, we see tight contacts between the two EC domains in the dimer and between the EC domains and ligands (discussed above). However, this view was questioned recently for EGFR in a study of EGFR diffusion, as ligand binding *per se* did not appear to stabilize EGFR dimers (127).

The crystal structure of the EC domain dimer of a drosophila EGFR variant shows very intimate contacts between the two EC domains when only one ligand is bound (100). The

comparison of this structure to the symmetric human EGFR structure in the presence of two bound ligands (97;128) suggests that the binding of the first ligand is strongly stabilizing, while the second ligand binding event decreases dimer stability. This new finding suggests that the mechanism of ligand-induced RTK dimer stabilization is unexpectedly complex.

In the absence of ligand, the EC domains have been shown to inhibit dimerization. The contribution of FGFR3 extracellular domain to dimerization has been demonstrated to be inhibitory in plasma membrane derived vesicles, and its magnitude has been measured as $\Delta\Delta G = 0.9 \pm 0.2$ kcal/mole (55). Thus, the EC domains play a dual thermodynamic role in RTK dimerization, inhibiting dimerization in the absence of ligand and stabilizing the dimers in the presence of ligand.

Catalytic domains—The cross-phosphorylation of the kinase domains implies the occurrence of contacts between them (7–9). These contacts are likely be stabilizing. Yet, the contribution of the kinase domains to RTK dimerization energetics has not been measured experimentally thus far. It is possible that this contribution depends on the phosphorylation state. The JM domain, a 40 to 80 amino acid-long sequence between the TM domain and the catalytic domain may be further contributing to the interactions, by stabilizing the active conformation of the kinase domain, and mediating direct receptor-receptor contacts (129;130).

B. RTK heterodimerization

Heterodimerization between RTKs is a means of signal amplification, as well as diversification (131). RTK heterodimers have been shown to enhance receptor activation and downsteam signaling, as compared to homodimers (131–133). For instance, the ErbB2/ErbB3 heterodimer is believed to be the most biologically active and the most pro-tumorigenic of all ErbB homo and heterodimers (134;135). Multiple studies, focusing primarily on the ErbB family of receptors, have demonstrated the importance of heterodimerization in normal function and in disease (136–140). For example, ErbB1/ErbB2, ErbB1/ErbB4, and ErbB2/ErbB4 heterodimers have been shown to play a role in cell transformation. Furthermore, ErbB3 is overexpressed in many tumors that overexpress ErbB2, including breast, bladder, and melanomas (131;140). Tumors that overexpress ErbB2 also exhibit elevated ErbB3 phosphorylation levels (134). Yet, our understanding of RTK heterodimerization is only rudimentary, in part due to a paucity of methods that provide quantitative information about RTK heterodimerization. Thus far, heterodimerization between the isolated ErbB TM domains has been studied in detergents using FRET (141) and in bacterial membranes using the genetic assay GALLEX (91). These studies have suggested that ErbB TM domains form both homodimers and heterodimers with various stabilities.

Germ-line mutations in FGFR receptors cause heterozygous disorders of bone development (112;142;143), as the homozygous conditions are lethal. Thus both wild-type and mutant receptors are co-expressed in cells, and questions arise if wild-type/mutant heterodimers form and what their activity is. The interpretation of heterodimerization studies, however, is very challenging because three different dimeric species usually exist: (1) wild-type homodimers, (2) mutant homodimers, and (3) wild-type/mutant heterodimers. These dimers are indistinguishable in many biochemical experiments, as wild-type and mutants are reactive to the same antibodies. One way to characterize heterodimerization is to use FRET, with the wild-type labeled with a donor and the mutant with an acceptor (or vice versa). In this case, the FRET efficiency can be measured easily. However, the heterodimerization propensities are challenging to quantify because RTKs also form homodimers. Figure 4A illustrates the challenge in such measurements, arising due to coupling between the homo

and heterodimerization equilibria. The association constant of heterodimerization, K_{xy}, depends on the monomer concentrations of the two receptors, [X] and [Y]. The same monomer concentrations, however, appear in the equations describing the homodimerization constants K_x and K_y. Thus, homodimer stabilities need to be determined first, and then these are used to determine heterodimer stabilities (141;144).

We have demonstrated the feasibility of this approach by determining the propensity for heterodimer formation between the wild-type FGFR3 TM domain and its A391E mutant (144), linked to Crouzon syndrome. The free energy of heterodimerization was determined as −3.37 ± 0.25 kcal/mol (144). Comparison of this value to the homodimerization free energies for the wild-type, −2.8 ± 0.2 kcal/mol, and the mutant, −4.1 ± 0.2 kcal/mol, demonstrates that the heterodimer stability is the average of the two homodimer stabilities (144).

Another approach to investigate the formation of RTK heterodimers is to design an RTK construct that lacks the kinase domain, and then monitor the formation of heterodimers between this construct and full length RTKs (Figure 4B). The full-length/truncated heterodimers are inactive. They deplete the pool of full-length receptors capable of forming homodimers and ultimately reduce the concentration of active homodimers. Thus, the presence of the truncated receptors leads to a decrease in the phosphorylation of the full-length receptors if the full-length and the truncated receptors dimerize. This decrease in phosphorylation can be measured experimentally. Because the contributions of the kinase and the juxtamembrane domains to RTK dimerization may be non-negligible and different for different RTKs, this approach can only be used to assess the effect of pathogenic mutations in the extracellular and TM domains on heterodimerization (124). This is because the contributions of the intracellular domains are expected to be the same for the wild-type and mutant homodimers and heterodimers, but not for heterodimers of different RTKs. This approach has revealed that heterodimers of wild-type FGFR3 and the G380R mutant linked to ACH form with lower probability than wild-type FGFR3 homodimers in cellular membranes (124).

Final remark

As discussed in this review, quantitative biophysical frameworks, as well as sophisticated biochemical and biophysical experimental methods are now being used to unravel the physical-chemical principles behind RTK activation and their involvement in disease. The recent progress in the field has been impressive, and we look forward to future studies that will further deepen our understanding of RTK structure and function and pave the way for the development of novel targeted therapies for RTK-linked pathologies.

Highlights

Physical-chemical models provide an adequate description of RTK activation.

Quantitative experimental methods shed new light on RTK function.

Methods are available to characterize RTK homo and heterodimerization.

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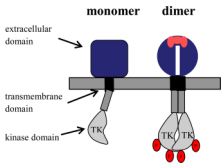


Figure 1.

A schematic of RTK architecture, showing the extracellular (EC) domain, the transmembrane (TM) domain and the tyrosine kinase domain. The kinase domain is linked to the TM domain via a 40–80 amino acid juxtamembrane (JM) domain. RTKs transduce biochemical signals via lateral dimerization in the membrane. While the monomers (left) are inactive, the dimers (right) are phosphorylated and active.

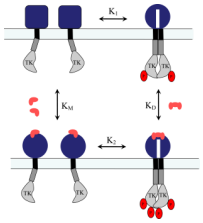


Figure 2.
A schematic representation of the activation model given by equation (2). Two monomers interact laterally to form dimers, both in the absence and presence of bound ligand (dimerization constants K_1 and K_2, respectively). The ligand binds to both monomers (association constant K_M) and dimers (association constant K_D). The receptors in the unliganded and liganded dimeric states are phosphorylated, but the probabilities for phosphorylation may be different (Φ_d for the unliganded state and Φ_D for the liganded state).

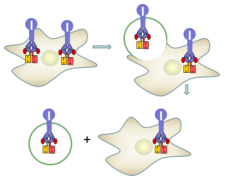
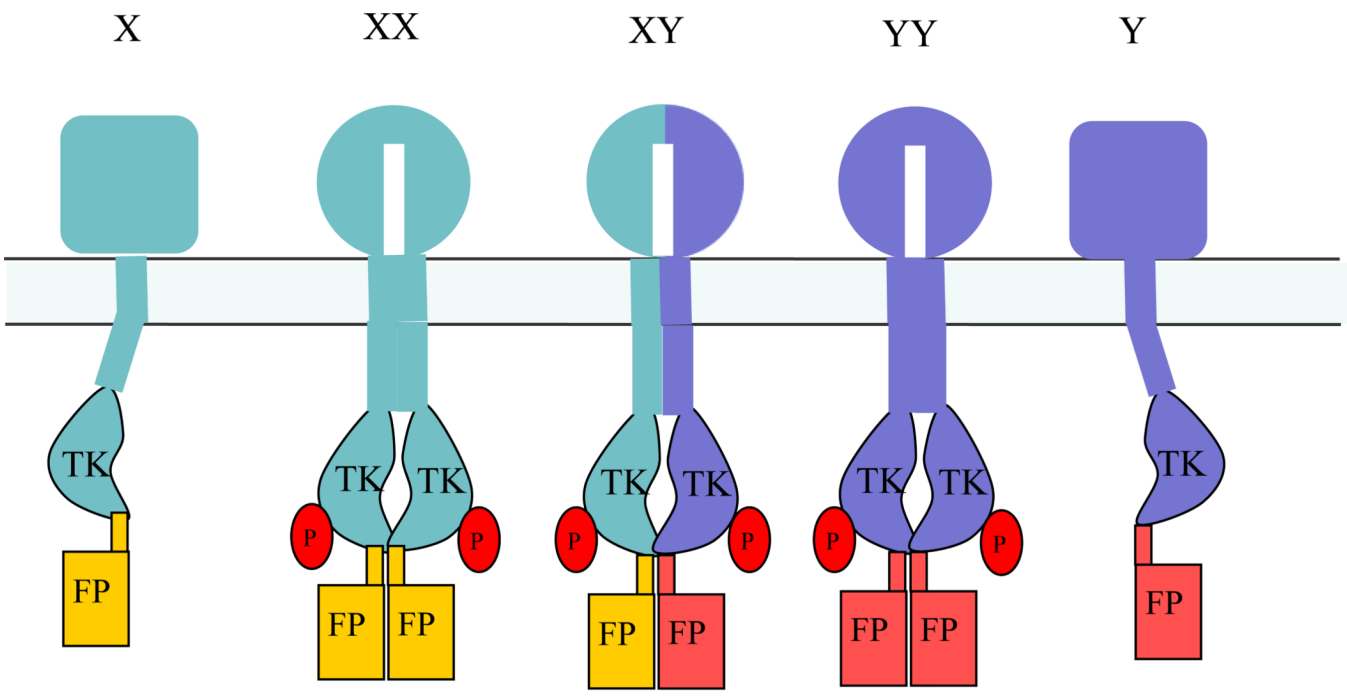


Figure 3.
Overview of RTK dimerization measurements in plasma membrane-derived vesicles. Cells are transfected with genes encoding RTKs fused to fluorescent proteins, and then vesiculated using established protocols. The vesicles are imaged in a confocal microscope, acquiring donor, acceptor and FRET images for each vesicle. The QI-FRET method, described in (53–55), is used to determine the donor and acceptor concentration, and the FRET efficiency in each vesicle. This information is then used to determine the concentrations of monomers and dimers in each vesicle, the dimerization constant K_1 and the free energy of dimerization.

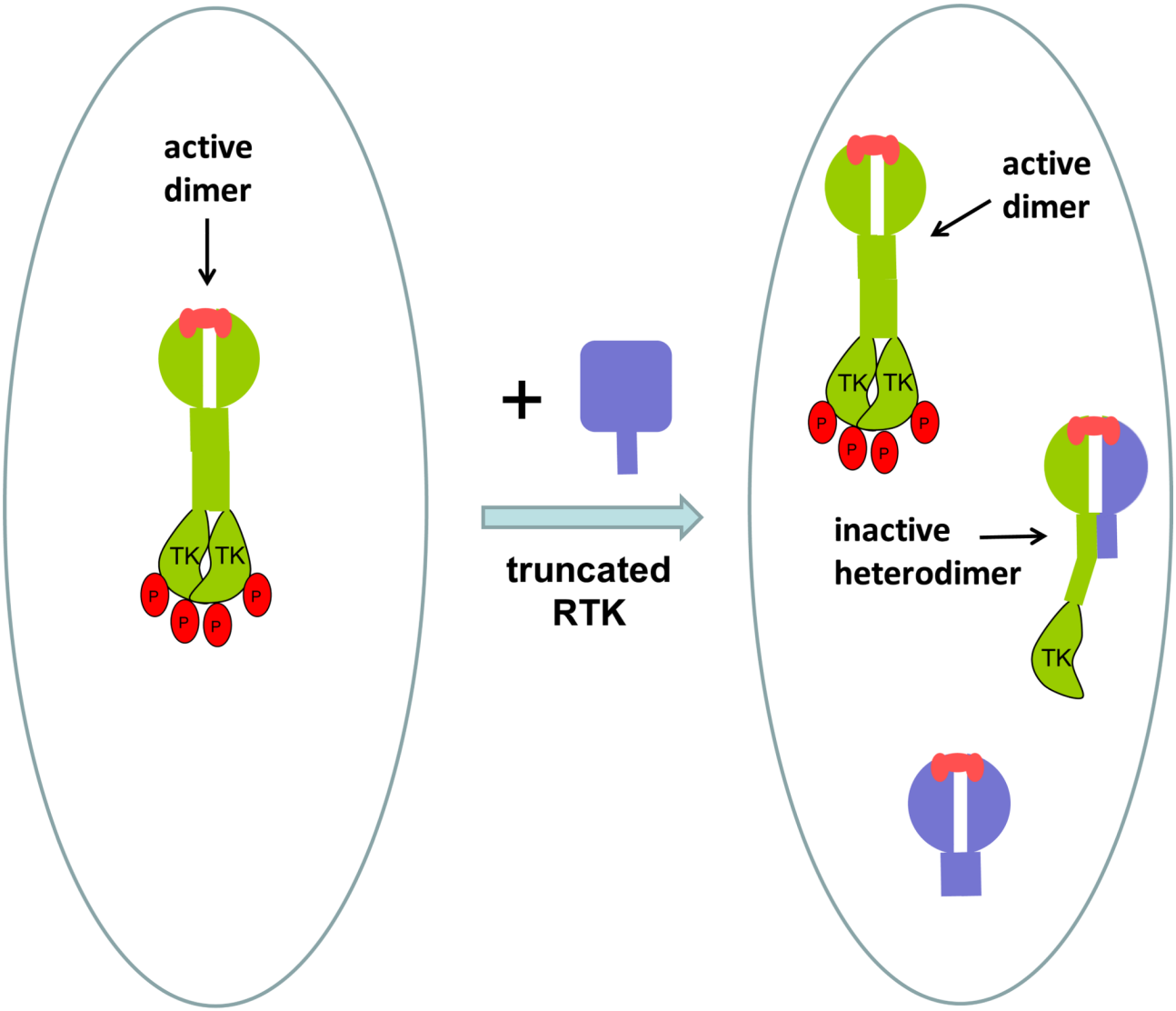


$$X + X \xleftrightarrow{K_X} XX \qquad K_X = \frac{[XX]}{[X]^2} \qquad \Delta G_{XX} = -RT \ln K_X$$

$$X + Y \xleftrightarrow{K_{XY}} XY \qquad K_{XY} = \frac{[XY]}{[X]\cdot[Y]} \qquad \Delta G_{XY} = -RT \ln K_{XY}$$

$$Y + Y \xleftrightarrow{K_Y} YY \qquad K_Y = \frac{[YY]}{[Y]^2} \qquad \Delta G_{YY} = -RT \ln K_Y$$

4A.



4B

Figure 4.

Characterization of RTK heterodimerization.

(A). FRET measurements of heterodimerization propensities between RTKs. One RTK is labeled with a donor, and the second RTK is labeled with the acceptor, such that the FRET efficiency can be measured. However, the calculation of the heterodimerization free energy is not trivial because the homo and heterodimerization equilibria are coupled. A detailed protocol for characterization of heterodimerization energetics using FRET is given in (144).

(B). An overview of an assay for RTK heterodimerization that does not require modification with fluorescent proteins. A stable cell line expressing full-length RTKs (green) is transiently transfected with a truncated version of a second RTK (blue) lacking the

intracellular domain. The inactive full-length/truncated heterodimers deplete the pool of full-length receptors capable of forming homodimers and reduce their phosphorylation. The decrease in the phosphorylation of the full-length receptors is measured experimentally. This decrease is a reporter of heterodimerization strength between the RTKs that are stably and transiently expressed. Detailed description of the assay is given in (124).

Table 1

A list of characterized pathogenic RTK mutations, and the underlying physical-chemical cause for the pathologies. All RTKs are human, unless stated otherwise.

RTK	Mutations	Location	Phenotype	Cause for pathology
FGFR1	P252R	Extracellular domain	Pfeiffer syndrome	Increased ligand binding (145).
FGFR1	Y372C	Extracellular domain	Osteoglophonic dysplasia	Increased dimerization (103).
FGFR2	S252W, P253R	Extracellular domain	Apert syndrome	Increased ligand binding (146).
FGFR2	D321A	Extracellular domain	Pfeiffer syndrome	Increased ligand binding (146).
FGFR2	C278F, C342Y,	Extracellular domain	Crouzon syndrome and Pfeiffer Syndrome	Increased dimerization (22).
FGFR2	W290G, S354C, Y340H C342Y	Extracellular domain	Crouzon syndrome	Increased dimerization (22;147).
FGFR2	T341P	Extracellular domain	Pfeiffer syndrome	Increased dimerization (22).
FGFR3	R248C, S249C	Extracellular domain	Thanatophoric dysplasia type I	Increased dimerization (148).
FGFR3	P250R	Extracellular domain	Muenke syndrome	Increased ligand binding (145).
FGFR3	G370C, S371C, Y373C	Transmembrane domain	Thanatophoric dysplasia type I	Increased dimerization(148;149).
FGFR3	G375C	Transmembrane domain	Achondroplasia	Increased dimerization (149).
FGFR3	G380R	Transmembrane domain	Achondroplasia	Structural change (14).
FGFR3	A391E	Transmembrane domain	Crouzon syndrome with acanthosis nigricans	Increased dimerization (15).
FGFR3	K650E	Kinase domain	Thanatophoric dysplasia type 2	Structural change in the kinase domain (150).
FGFR4	Y367C	Extracellular domain	Breast cancer	Increased dimerization (151).
EGFR	EGFRvIII. 6-273 deletion	Extracellular domain	Glioblastoma	Structural change (152).
EGFR	T263P, A289V, P596L, G598V	Extracellular domain	Glioblastoma	Increased dimerization (153).
EGFR	G719S	Kinase domain	Non-small-cell lung carcinoma	Structural change in the kinase domain (154).
EGFR	L858R	Kinase domain	Non-small-cell lung carcinoma	Structural change in the kinase domain and increased ligand binding (9;155).
EGFR	752–759 deletion and L861Q	Kinase domain	Non-small-cell lung carcinoma	Structural change in the kinase domain (155).
EGFR	959–1030 deletion	C-terminal tail	Glioblastoma	Structural change in the kinase domain (156).
ErbB2 (Rat)	V664E	Transmembrane domain	Oncogenic in rat	Increased dimerization (16).
KIT	417–419 deletion and T417I	Extracellular domain	Acute myeloid leukemia (AML)	Increased dimerization (157).
KIT	V559D	Juxtamembrane domain	Gastrointestinal stromal tumor	Structural change in the juxtamembrane domain (158) (159).
KIT	V560G	Juxtamembrane domain	Mastocytosis	Increased dimerization (158;160).
KIT (Murine)	KΔ27.547–555 deletion and D560K	Juxtamembrane domain	Mastocytoma	Increased dimerization (161).
KIT	D816V	Kinase domain	Mastocytosis	Structural change and increased dimerization (under debate) (162–164).

RTK	Mutations	Location	Phenotype	Cause for pathology
PDGFRA	V561D	Juxtamembrane domain	Gastrointestinal stromal tumors	Increased dimerization (165).
PDGFRA	D842V	Kinase domain	Gastrointestinal stromal tumors	Increased dimerization (165).