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Characterization of Membrane Protein Interactions in Plasma Membrane Derived Vesicles with Quantitative Imaging FRET

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CONSPECTUS

Here we describe an experimental tool, termed Quantitative Imaging Förster Resonance Energy Transfer (QI-FRET), which enables the quantitative characterization of membrane protein interactions. The QI-FRET methodology allows us to acquire binding curves and calculate association constants for complex membrane proteins in the native plasma membrane environment. The method utilizes FRET detection, and thus requires that the proteins of interest are labeled with fluorescent proteins, either FRET donors or FRET acceptors. Since plasma membranes of cells have complex topologies precluding the acquisition of two-dimensional binding curves, the FRET measurements are performed in plasma membrane derived vesicles which bud off cells as a result of chemical or osmotic stress. The results overviewed here are acquired in vesicles produced with an osmotic vesiculation buffer developed in our laboratory, which does not utilize harsh chemicals. The concentrations of the donor-labeled and the acceptor-labeled proteins are determined, along with the FRET efficiencies, in each vesicle. The experiments utilize transient transfection, such that a wide variety of concentrations is sampled. Then, data from hundreds of vesicles are combined to yield dimerization curves. Here we discuss recent findings about the dimerization of receptor tyrosine kinases (RTKs), membrane proteins that control cell growth and differentiation via lateral dimerization in the plasma membrane. We focus on the dimerization of fibroblast growth factor receptor 3 (FGFR3), an RTK that plays a critically important role in skeletal development. We study the role of different FGFR3 domains in FGFR3 dimerization in the absence of ligand, and we show that FGFR3 extracellular domains inhibit unliganded dimerization, while contacts between the juxtamembrane domains, which connect the transmembrane domains to the kinase domains, stabilize the unliganded FGFR3 dimers. Since FGFR3 has been documented to harbor many pathogenic single amino acid mutations that cause skeletal and cranial dysplasias, as well as cancer, we also study the effects of these mutations on dimerization. First, we show that the A391E mutation, linked to Crouzon syndrome with acanthosis nigricans, and to bladder cancer, significantly enhances FGFR3 dimerization in the absence of ligand and thus induces aberrant receptor interactions. Second, we present results about the effect of three cysteine mutations that cause thanatophoric dysplasia, a lethal phenotype. Such cysteine mutations have been hypothesized previously to cause constitutive dimerization, but we find instead that they have a surprisingly modest effect on dimerization. Most of the studied pathogenic mutations also altered FGFR3 dimer structure, suggesting that both increases in dimerization propensities and changes in dimer structure contribute to the pathological

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phenotypes. The results acquired with the QI-FRET method further our understanding of the interactions between FGFR3 molecules, and RTK molecules in general. Since RTK dimerization regulates RTK signaling, our findings advance our knowledge of RTK activity in health and disease. The utility of the QI-FRET method is not restricted to RTKs, and we thus hope that in the future the QI-FRET method will be applied to other classes of membrane proteins, such as channels and G protein-coupled receptors.

Introduction

Interactions between proteins in cells occur in response to environmental stimuli and ultimately determine cell fate. Out of the many interactions that occur between biological macromolecules, the interactions between membrane proteins are particularly challenging to study experimentally (see^{1,2,3} for reviews) and thus remain the least characterized. Yet, membrane proteins play important roles in vital cellular processes such as signal transduction, nutrient uptake, and motility, and are thus critically important for normal cellular function. Therefore, there is an urgent need to better understand the physical-chemical determinants of their behavior.

Here we describe a tool, termed Quantitative Imaging FRET (QI-FRET), which enables the quantitative characterization of protein interactions in plasma membrane derived vesicles. The QI-FRET method yields binding curves and equilibrium constants in native membrane environments, for membrane proteins that have been labeled with donor and acceptor fluorescent proteins (a FRET pair)^{4,5,6,7,8}. Here we also overview recently acquired basic knowledge about the second largest class of membrane receptors, the receptor tyrosine kinases (RTKs), which has been gained through the use of the QI-FRET methodology.

RTKs are single-pass transmembrane proteins which transduce biochemical signals across the membrane plane via lateral dimerization. Contact between the two catalytic domains in the dimer triggers kinase activity and results in the cross-phosphorylation of the receptor subunits. This activates the catalytic domains of the receptors for phosphorylation of cytoplasmic substrates, which in turn initiates signaling cascades that control cell growth, differentiation, and motility^{9,10,11}. The dysregulation of RTK interactions in the plasma membrane has been linked to many human diseases and disorders, including a variety of cancers^{12,13,14}. The QI-FRET method was developed to study the interactions between RTK molecules in the plasma membrane, as these interactions regulate RTK activity in health and disease.

The QI-FRET method

While FRET is measured routinely in many laboratories, the QI-FRET methodology is powerful because it allows us to assess if the data is described by a dimerization model, to calculate dimeric fractions, and to predict dimeric fractions for receptor concentrations that are not experimentally accessible^{4,5,8}. To accomplish this, we design our experiments to cover a broad range of receptor concentrations (from $\sim 10^2$ to as high as 10^4 receptors per μm^2 , depending on protein geometry), such that binding curves can be obtained. The advancement over other FRET methods is that we not only measure the FRET efficiency in

each vesicle, but also the concentrations of donor-tagged and acceptor-tagged receptors in the vesicle. We perform measurements in hundreds of vesicles, each expressing different amounts of receptors, and we determine (i) the dimerization constant, K , and the dimer stability, or the dimerization free energy, $G = -RT \ln K$ and (ii) the structural parameter “Intrinsic FRET”, F^A . The Intrinsic FRET reports on the dimer structure, in particular on the distance between the fluorescent proteins in the dimer, but does not depend on the dimerization propensity. The value of the Intrinsic FRET affects the FRET efficiency that we measure in an experiment, and it needs to be determined and accounted for so that the dimerization constant can be calculated correctly. As discussed below, the Intrinsic FRET can also provide valuable structural information about the receptor dimers.

Plasma membrane derived vesicles as a model system for studies of RTK interactions

To collect dimerization curves and determine dimerization free energies, one needs to determine both the FRET efficiencies and the concentrations of donor-labeled and acceptor-labeled membrane proteins. The concentrations can be determined by comparing the fluorescence intensity of the proteins in the membrane, and the intensities of solutions of purified fluorescent proteins of known concentration. However, the plasma membranes of cells have very complex topologies and are highly “wrinkled”, possessing 2 to 3 times more surface area than is needed to maintain their shape^{15,16,17}. This complex topology precludes the calculation of two dimensional membrane protein concentrations⁴. To be able to perform quantitative measurements of membrane protein association thermodynamics, one needs to use a model system with a well defined and simple topology. Plasma membrane vesicles are one such model system, because these vesicles are perfectly spherical, and because the distribution of the RTKs in these vesicles is homogeneous⁴. When a vesicle is imaged through its equator, the two-dimensional membrane is parallel to the field of view, allowing the determination of membrane concentrations as described in detail previously⁴.

In the plasma membrane derived vesicles, RTK interactions can be studied with no requirements for RTK purification and reconstitution into model systems⁴. Vesicles are produced from live cells following treatments with so-called “vesiculation buffers,” which stress the cells into budding off many vesicles¹⁸. The RTKs are expressed in mammalian cells, and thus they are subject to all necessary post-translational modifications, including glycosylation. The lipid composition of the cell-derived vesicles is similar to the native membrane¹⁸, and the plasma membrane asymmetry is largely retained¹⁹. The vesicles contain various membrane proteins and thus they mimic the natural crowded membrane environment. Furthermore, they have heparan sulfate proteoglycans on their surfaces, which are known to recruit and sequester RTK ligands^{20,21}.

Plasma membrane derived vesicles can be produced from different cell lines using an established vesiculation buffer that contains small amounts of the active chemical formaldehyde, as well as dithiothreitol²². Recently, we developed a novel alternative method of vesicle production that uses osmotic stress to bud vesicles from cells²³, and thus eliminates the use of the harsh chemicals. DTT and formaldehyde are known to cross-link

and reduce proteins, and thus the osmotic vesiculation method is the method of choice in our laboratory. The two types of vesicle preparations differ in a key property: soluble proteins are retained within the DTT/formaldehyde vesicles, but are not found inside the osmotic stress vesicles¹⁸. Thus, the vesicles produced with the osmotic stress method allow us to focus on the physical-chemical interactions that occur in the membrane, devoid of the modulating effects of soluble proteins.

We have measured the association of membrane proteins with no cytoplasmic domains (and thus no expected association with soluble proteins), in the two types of vesicles. For some cases, such as the case of glycoporphin A (GpA) TM domain, the results were identical in the two types of vesicle preparations²⁴, while in others (such as FGFR3 TM domain) the results differed by as much as ~1 kcal/mole²⁵. Since the lipid composition is the same in the two types of vesicles¹⁸, the observed difference may be a direct consequence of the presence or absence of DTT and formaldehyde. Alternatively, it may be due to a different degree of molecular crowding in the two types of vesicles. Indeed, we have shown that some membrane proteins are not efficiently incorporated into the DTT/formaldehyde vesicles, which may also be a consequence of formaldehyde-induced cross-linking during vesiculation¹⁸. Based on these reasons, in this review we show only data that are acquired in vesicles produced with the osmotic stress method.

Overview of the QI-FRET experimental protocol

In the QI-FRET experiments, the RTKs are tagged with YFP and mCherry as the FRET pair^{4,5,6,8,24}. The fluorescent proteins are attached to the receptor via a (GGG)₅ linker. The attachment can be either to the C-terminus of the full-length receptor, or to the C-terminus of a truncated receptor construct. In most of the experiments overviewed below, the attachment is directly to the TM domain, ultimately yielding insights into the TM conformation and structure (see Figure 1). The (GGG)₅ linker has been shown to be unstructured, to behave as a random coil²⁶, and to not affect dimerization²⁵.

Figure 1 depicts the QI-FRET experimental protocol. Mammalian cells are co-transfected with plasmids encoding for donor- and acceptor-tagged receptors. After receptor expression, the cells are vesiculated, and the vesicles are imaged in donor, acceptor, and FRET channels using a laser scanning confocal microscope. Details about the image acquisition and subsequent data processing are given in references^{4,25,27}.

In each experiment, at least 300 individual plasma membrane-derived vesicles are imaged at room temperature, and the FRET efficiency, the donor concentration, and the acceptor concentration are calculated in each vesicle. Because transient expression levels vary from cell to cell, a wide range of receptor concentrations is sampled in a single transfection experiment. The FRET efficiencies are corrected for the so-called proximity or stochastic FRET, which occurs because the membrane proteins are confined to two-dimensional membranes²⁸. A fit of a dimerization model to the corrected FRET data yields the association constant **K** and the Intrinsic FRET value for the dimer^{4,5,6,8,24}.

Utility of the QI-FRET method, as applied to RTKs

While RTKs are generally known to be activated in response to ligand binding²⁹, it has been shown that at least some RTKs are capable of forming unliganded dimers that are likely important intermediates in the signaling process^{30,31,32}. We have used the QI-FRET method to study the unliganded dimerization of RTKs, and here we overview results for fibroblast growth factor receptor 3 (FGFR3), an RTK which is critically important for skeletal development and which harbors many mutations linked to skeletal disorders and cancers^{33,34,35,36,37,38,39}.

FGFR3, just like most RTKs, consists of an N-terminal extracellular (EC) ligand-binding domain, a single transmembrane (TM) domain, followed by a juxtamembrane (JM) domain, and a kinase domain^{34,40}. Below we focus on the role of these domains in FGFR3 unliganded dimerization and the effects of pathogenic FGFR3 mutations on dimerization, as revealed by QI-FRET measurements.

FGFR3 TM domain has a propensity for dimerization

For many years, RTK TM domains were believed to be passive membrane anchors^{41,42}. Later, a plethora of pathogenic TM domain mutations were identified, and some of them were shown to be activating^{43,44,45,46}. Isolated RTK TM domains were shown to dimerize in lipid bilayers and bacterial membranes^{47,48,49,50,51,52}, and they were proposed to play an important role in RTK dimerization^{53,54}. Using the QI-FRET method, we characterized the dimerization of the FGFR3 TM domain^{5,25}. Data acquired in vesicles produced with the osmotic stress method are shown in Figure 2 (solid red symbols). The FGFR3 TM construct contained the FGFR3 signal sequence, FGFR3 TM domain, a (GGG)₅ flexible linker, and the fluorescent proteins (these constructs are denoted as TM-(GGG)₅-YFP/mCherry). The dimerization curve is shown in Figure 2 as a red solid line, with dimeric fraction varying between ~60 and ~90% over the concentration range accessible in the experiments. The dimerization free energy was determined as -5.2 ± 0.2 kcal/mole. Thus, FGFR3 TM domains have a robust propensity for dimerization in mammalian plasma membranes, and could provide a driving force for receptor dimerization even in the absence of ligand.

FGFR3 EC domain inhibits unliganded dimerization

RTK EC domains are the ligand binding domains, and thus they are critical for ligand-induced dimerization and activation. Isolated EC domains in aqueous solutions do not form dimers in the absence of ligand⁵⁵. However, the confinement of proteins to two-dimensional membranes can have a significant effect on their interactions⁵⁶. Thus, questions arise as to what the contribution of the EC domains to RTK dimerization in membranes might be: inhibiting dimerization, promoting dimerization, or having no effect. To directly determine this contribution, we compared the dimer stability of the FGFR3 TM-(GGG)₅-YFP/mCherry construct discussed above, and an FGFR3 EC-TM-(GGG)₅-YFP/mCherry construct which also contained the EC domain^{5,25}. The results are shown in Figure 2. The measured dimer stabilities of -5.2 ± 0.2 kcal/mole and -3.4 ± 0.2 kcal/mole for the TM and EC+TM constructs, respectively, reveal that the deletion of the EC domain stabilizes the FGFR3 dimer by -1.8 ± 0.3 kcal/mole²⁵. This is a direct demonstration that FGFR3 EC domain

inhibits dimerization in the absence of ligand. This domain therefore plays a dual role in FGFR3 dimerization and activation, as it works to stabilize the dimer in the presence of ligand, but inhibits dimer formation in the absence of ligand.

FGFR3 JM domain stabilizes the FGFR3 unliganded dimer

The JM domain is the sequence between the TM domain and the kinase domain. The JM domains of different RTKs have been shown to play diverse roles in signaling, ranging from autoinhibitory to activating^{57,58}. We asked whether the JM domain contributes to the stability of the FGFR3 dimer in the absence of ligand, and we measured this contribution directly with the QI-FRET method²⁵. In particular, we compared the dimerization of two FGFR3 constructs: EC-TM-(GGS)₅-YFP/mCherry, and a construct in which the 15-residue flexible linker was substituted with the 72 amino acid long JM domain of FGFR3 (see Figure 3). Upon inclusion of the JM domain, the dimerization free energy increased from -3.4 ± 0.2 kcal/mole to -5.4 ± 0.5 kcal/mole. The (GGS)₅ linker does not affect dimerization²⁵, and thus the contribution of the JM domain to dimerization is favorable, -2.0 ± 0.5 kcal/mole. Interestingly, this favorable contribution cancels the inhibitory contribution of the EC domain.

We further found that the JM domain stabilizes the dimer only when it is attached to FGFR3 TM domain²⁵. On the other hand, simply anchoring the JM domain to the membrane does not promote JM-JM interactions. Thus, the TM and the JM domains in FGFR3 work synergistically to stabilize the unliganded FGFR3 dimer.

Pathogenic FGFR3 mutations affect the propensity for unliganded dimerization

Mutations in FGFR3 can lead to dominant disorders of bone development, including the common dwarfism phenotypes achondroplasia and hypochondroplasia^{33,59}. These mutations have also been found in cancers⁶⁰. We have therefore studied several pathogenic FGFR3 mutants using the QI-FRET method.

Crouzon syndrome with acanthosis nigricans—Crouzon syndrome with acanthosis nigricans arises due to the A391E mutation in the TM domain of FGFR3. Crouzon syndrome is a craniosynostosis disorder with an incidence of 1 in 25,000 live births, characterized by premature ossification of the skull⁶¹. Due to the obliterations of the sutures, the skull is unable to grow normally, the eye sockets are shallow, and the upper jaw is underdeveloped. Acanthosis nigricans is a skin disorder, characterized by hyperpigmentation and hyperkeratosis of the skin.

We have previously investigated the molecular basis behind this disorder, and we have found that the A391E mutation over-activates FGFR3^{62,63}. Using QI-FRET, we have also directly measured the effect of the A391E mutation on dimerization in plasma membrane derived vesicles, by comparing the dimer stabilities of the wild-type FGFR3 EC+TM construct and a similar construct harboring the mutation (Figure 4). The difference in dimer stability due to the A391E substitution was measured as -1.4 kcal/mole^{24,25}. Thus, the A391E mutation over-stabilizes the FGFR3 dimers⁵⁰, suggesting that the increased activity of the mutant receptor is due to its enhanced dimerization propensity. Since the measured effect of -1.4

kcal/mole is consistent with previous reports of hydrogen bond strengths in proteins^{64,65,66}, we hypothesize that the mutant dimer is over-stabilized by Glu391-mediated hydrogen bonds^{50,54}.

Notably, the A391E mutation not only leads to dimer over-stabilization, but also induces a change in Intrinsic FRET²⁵. In particular, the Intrinsic FRET of EC+TM FGFR3 changes from 0.52 to 0.72 due to the mutation. Thus, the A391E mutation leads to a decrease in the separation of the fluorescent proteins by about 7 Å. Since the fluorescent proteins are attached to the TM domains, this finding suggests that the C-termini of the TM domains come closer together due to the mutation. This structural change, along with the increased dimerization propensity, is likely contributing to the pathology in Crouzon syndrome with acanthosis nigricans.

Thanatophoric dysplasia type I (TDI)—Thanatophoric dysplasia type I (TDI) is known to arise due to five different mutations, all involving the introduction of a cysteine residue into FGFR3: R248C, S249C, G370C, S371C, and Y373C^{34,37,67}. The R248C and Y373C mutations are responsible for 60–80% of all cases of TDI. TDI is a lethal human skeletal growth disorder with a prevalence of 1 in 20,000 to 1 in 50,000 births. In the literature, TDI mutations have been linked to a variety of abnormal activities: increased phosphorylation and activation of the receptor in the absence of ligand^{68,69}, increased downstream ERK signaling⁷⁰, increased BaF3 cell proliferation⁷¹, compromised downregulation of activated FGFR3 dimers in the plasma membrane⁷², and increased retention of FGFR3 dimers in the endoplasmic reticulum⁷³.

More generally, cysteine mutations in RTKs have been proposed to induce constitutive dimerization in the absence of ligand, leading to receptor overactivation^{74,75,76,77}. To test the hypothesis that the TDI mutants are constitutive dimers, we used QI-FRET to characterize the dimerization of the R248C, S249C, and Y373C mutants in the absence of ligand (see Figure 5)²⁷. The dimer stabilities of the R248C, S249C, and Y373C mutant are -4.2 ± 0.1 , -3.7 ± 0.1 , and -3.8 ± 0.1 kcal/mol, respectively, which are only slightly more favorable than the wild-type value, -3.4 ± 0.1 kcal/mol. Thus, the R248C, S249C, and Y373C mutations stabilize the FGFR3 dimer by only -0.8 , -0.3 , and -0.4 kcal/mol, respectively. This modest effect does not support the idea of constitutive disulfide-bonded FGFR3 dimers.

The Intrinsic FRET for the R248C and S249C TDI mutants is different from the wild-type, implying that there are differences in the structures of the wild-type and mutant dimers. The fluorescent proteins are closer to each other in the mutant dimers, suggesting a decrease in the separation between the mutant TM domains²⁷. This structural change may be contributing to the diverse aberrant effects of the TDI mutants in cells, and to the very severe TDI phenotype.

Overall, these measurements provide mechanistic insights about the interactions between FGFR3 molecules in mammalian membranes. These interactions have never before been characterized in quantitative terms, due to lack of appropriate experimental methodologies. In some cases, as in the case of the TD mutations, the results are not in agreement with

current models in the field, highlighting the need for further research before we achieve a comprehensive understanding of FGFR3 signaling in health and disease.

Technical advantages of the QI-FRET method in RTK research

In RTK FRET experiments, the measured FRET efficiency depends on both the dimerization propensity (dimer stability) and the structure of the dimer (more specifically, the separation of the fluorescent proteins in the dimer structure). Unfortunately, the importance of both contributions is not always recognized during FRET data interpretation. Furthermore, other experimental techniques used in RTK research also produce results that can depend on both dimerization propensity and on structural factors. For example, Western blotting and anti-phospho-tyrosine antibodies have been used to assess receptor phosphorylation, but receptor kinase activity is dependent on both the dimeric state of the receptors and the relative positioning and orientation of the two catalytic domains. The technique has been used to show that many RTK mutations increase phosphorylation and activation, relative to the wild-type, but whether this observation is caused by an increase in dimerization propensity or a change in dimer structure remains unknown. Additionally, Western blotting and anti-receptor antibodies are frequently used to investigate cross-linking efficiencies. Cross-linking is contingent on both dimer formation and on the presence of suitable amine groups that are situated close enough to be cross-linked. Once again, the read-out of these experiments may indicate either a change in receptor interactions, a change in dimer structure, or both.

In the QI-FRET method, we overcome the limitations in data interpretation by separating dimerization effects from structural effects. This is accomplished by fitting experimental data to a dimerization model with two parameters, namely, the dimerization constant, K , and the structural parameter Intrinsic FRET, $\tilde{E}$. Thus, the QI-FRET method is uniquely suited to provide both structural and thermodynamic information about RTK interactions, leading to new insights into the mode of RTK signal transduction across the plasma membrane.

The QI-FRET method requires that the receptors are expressed over a broad concentration range. If the proteins exist in a monomer-dimer equilibrium, the broad range of concentrations is required such that the association model can be fitted to the data. In the case of constitutively dimeric receptors, data over a broad range is required so we can convince ourselves that the receptors are indeed 100% dimeric, by the lack of dependence of FRET on concentration⁷. In this case, the measured FRET depends only on the Intrinsic FRET and the acceptor fraction. Since the acceptor fraction is known, the Intrinsic FRET can be directly determined for constitutive dimers⁷. In this case, the QI-FRET method can be used as a structural assay. The QI-FRET method can be applied to any membrane protein, provided that it can be tagged with donors and acceptors, and that it can be expressed over a broad concentration range.

Conclusion

Here we described a method, QI-FRET, which allows us to characterize the association of membrane proteins in plasma membrane derived vesicles. We also discussed our recent

findings pertaining to the lateral interactions of FGFR3, acquired using the method. Work is underway in our laboratory to apply the QI-FRET method to full-length receptors. The biological significance of full-length RTK unliganded dimerization is under debate in the literature⁷⁸, and this work will provide new information about the abundance of unliganded dimers at physiological concentrations. The method can be used to study a variety of membrane proteins, not just RTKs, provided that they are labeled with fluorescent proteins and expressed over a broad concentration range. We are hopeful that the QI-FRET method will reveal new information about the interactions and functions of other important classes of membrane proteins, such as GPCRs, channels, transporters, and adhesion receptors.

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Figure 1. Overview of the QI-FRET experiments

Mammalian cells are co-transfected with plasmids encoding for membrane proteins that are labeled with fluorescent proteins (a FRET pair). The cells are vesiculated and the vesicles are imaged in a confocal microscope to determine the FRET efficiency, the donor concentration, and the acceptor concentration. Data for hundreds of vesicles are combined to obtain binding curves and association free energies.

Figure 2. Dimerization curves for FGFR3, in the presence and absence of the EC domain
The EC domain reduces FGFR3 dimer stability by 1.8 kcal/mole²⁵.

Figure 3. Dimerization curves for FGFR3, with and without its JM domain

The substitution of the flexible (GGS)₅ linker with the JM domain increases the stability of the FGFR3 dimer by -2 kcal/mole²⁵.

Figure 4. Dimerization curves for WT FGFR3 and the A391 mutant causing Crouzon Syndrome with acanthosis nigricans

The mutation increases FGFR3 dimerization propensity by -1.4 kcal/mole²⁵.

Figure 5. Dimerization curves for WT FGFR3 and three mutants linked to Thanatophoric Dysplasia I

A reduced χ^2 analysis demonstrates that the differences between the mutant and wild-type dimeric fractions are statistically significant, but very modest²⁷.