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FGFR3 unliganded dimer stabilization by the juxtamembrane domain

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Abstract

Receptor Tyrosine Kinases (RTKs) conduct biochemical signals upon dimerization in the membrane plane. While RTKs are generally known to be activated in response to ligand binding, many of these receptors are capable of forming unliganded dimers that are likely important intermediates in the signaling process. All 58 RTKs consist of an extracellular domain, a transmembrane (TM) domain, and an intracellular domain which includes a juxtamembrane (JM) sequence and a kinase domain. Here we investigate directly the effect of the JM domain on unliganded dimer stability of FGFR3, a receptor that is critically important for skeletal development. The data suggest that FGFR3 unliganded dimers are stabilized by receptor-receptor contacts that involve the JM domains. The contribution is significant, as it is similar in magnitude to the stabilizing contribution of a pathogenic mutation and the repulsive contribution of the extracellular domain. Furthermore, we show that the effects of the JM domain and a TM pathogenic mutation on unliganded FGFR3 dimer stability are additive. We observe that the JM-mediated dimer stabilization occurs when the JM domain is linked to FGFR3 TM domain and not simply anchored to the plasma membrane. These results point to a coordinated stabilization of the unliganded dimeric state of FGFR3 by its JM and TM domains via a mechanism that is distinctly different from the case of another well studied receptor, EGFR.

INTRODUCTION

Receptor tyrosine kinases (RTKs) are membrane proteins that control cell proliferation, differentiation, survival and migration^{1,2}. They are thus implicated in many pathologies, including tumorigenesis, cancer progression, and developmental abnormalities^{1,3}. The basic architecture of all RTKs consists of a ligand-binding extracellular (EC) domain, a single transmembrane domain and an intracellular portion composed of a juxtamembrane (JM) sequence, and a kinase domain^{4,5}. The activation of RTKs requires that they dimerize in the plasma membrane, an event that brings the two kinase domains in close proximity and leads to their phosphorylation⁶. This is followed by the phosphorylation of additional tyrosine

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residues in the intracellular domains, which triggers the recruitment of adaptor proteins, and the initiation of intracellular signaling cascades^{7–9}.

While the general principles of RTK activation are now well established, we still lack comprehensive mechanistic understanding of this process, despite very active research in the field. In the “canonical” model of RTK activation⁴, RTKs are monomers in the absence of ligand, but dimerize and cross-phosphorylate/activate each other upon ligand binding. However, recent work has identified unliganded dimers for many RTKs^{10–14}, and thus an alternative “pre-formed dimer” model of RTK activation was proposed in which the RTKs are dimeric in the absence of ligand, and ligand binding induces a structural change in the dimer that reorients the catalytic domains for efficient activation^{15–17}. Activation may be therefore viewed as a thermodynamic change of state, i.e. a transition from an unliganded dimeric state which lacks full activity, to the fully active liganded dimeric state¹⁶.

Recent studies of EGFR, the most widely researched RTK, has suggested that its JM domain is critical for establishing the distinct structural features of the liganded and unliganded states, and for the transition between the two states^{11,18,19}. In particular, while the liganded state is stabilized by direct N-terminal JM-JM contacts, in the unliganded state the JM domain interacts with membrane lipids and does not engage in stabilizing contacts^{11,19}. Intriguingly, the behavior of EGFR JM domain appears to be impacted by the lateral interactions between the TM domains, via a mechanism that is not completely understood¹¹.

Here we investigate the role of the JM domain of another RTK, FGFR3, in stabilizing the unliganded state. We also study the coordination of FGFR3 JM and TM domains in the absence of ligand. Many developmental abnormalities have been linked to dysregulated unliganded FGFR3 dimerization^{20–26}, but the physical interactions that stabilize the unliganded FGFR3 dimer are not completely known. Here we make direct measurements of the effect of the JM domain on dimer stabilization, within and out of the context of the FGFR3 dimer. The results show that the FGFR3 JM domain, unlike the EGFR JM domain, plays an active role in stabilizing the unliganded FGFR3 dimers, in coordination with FGFR3 TM domain.

RESULTS

The substitution of an unstructured linker with the FGFR3 JM domain stabilizes FGFR3 dimers in the absence of ligand

Previously, we have shown that the FGFR3 construct EC-TM-(GGS)₅-YFP/mCherry (Table 1), in which the intracellular domain has been substituted with a fluorescent protein attached via a (GGS)₅ linker, forms dimers in mammalian plasma membrane derived vesicles in the absence of ligand²⁷. The (GGS)₅ linker has been shown to be unstructured and to behave like a random coil²⁸. Here we substituted this 15-residue flexible linker with the 72 amino acid long JM domain of FGFR3 (see Figure 1) and we asked how this substitution affects dimerization. To answer this question we measured and compared the dimerization of EC-TM-(GGS)₅-YFP/mCherry and EC-TM-JM-YFP/mCherry in plasma membrane derived vesicles from CHO cells.

In these experiments, CHO cells were first co-transfected with plasmids encoding either EC-TM-JM-YFP and EC-TM-JM-mCherry or EC-TM-(GGS)₅-YFP and EC-TM-(GGS)₅-mCherry. After the receptors were produced and trafficked to the plasma membrane, the cells were induced to form plasma membrane derived vesicles using an osmotic stress method described recently²⁹. The dimerization of the two FGFR3 constructs was characterized with a FRET-based method termed QI-FRET as described previously³⁰. The FRET experiments were performed with a laser-scanning confocal microscope, imaging a thin slice through the equator of each vesicle (see Figure 2). For each receptor, 600 to 1000 individual plasma membrane-derived vesicles were imaged and analyzed with the QI-FRET method which yields (i) the donor concentration, (ii) the acceptor concentration, and (iii) the FRET efficiencies in each vesicle. The total receptor concentration and the dimeric receptor fraction were calculated in each vesicle, and data from many vesicles were combined to yield dimerization curves (Figure 3A).

The QI-FRET methodology has been published as a detailed step-by-step protocol³⁰, and the reader is referred to this protocol for details. In addition, basic concepts behind the QI-FRET method are briefly outlined in Supplemental Material to this manuscript (see also Figure 2 for an illustration of vesicle image processing).

Unique aspects of the methodology are: (i) we design our experiments such that receptor concentrations are varied over a wide range, and (ii) we independently measure donor and acceptor concentrations in the plasma membranes, along with FRET efficiencies. Thus, we can assess if the data is described by a dimerization model, calculate dimeric fractions, and predict dimeric fractions for receptor concentrations that are not experimentally accessible. From the data, we determine (i) the dimerization constant, **K**, and the dimer stability, or the dimerization free energy $G = -RT \ln K$ and (ii) the purely structural parameter “Intrinsic FRET”, $\tilde{E}$ ³⁰. The measured Intrinsic FRET value depends on the dimer structure, in particular on the distance and orientation of the fluorescent proteins in the dimer. Most importantly, this is a parameter that affects the measured FRET efficiencies, and it needs to be determined and accounted for in order to correctly measure **K** and the dimer stability G . The Intrinsic FRET value $\tilde{E}$ is a means to compare structures and follow large-scale structural perturbations.

The FRET efficiencies measured for the two FGFR3 constructs are shown in Figure S1, with each data point representing a single vesicle. The donor concentration in each vesicle is also shown in Figure S1 versus the acceptor concentration in the same vesicle. Thus, each vesicle contains different concentrations of donors and acceptors, and different total receptor concentrations. From the FRET efficiencies and the donor to acceptor ratio in Figure S1 and following the step-by step QI-FRET protocol³⁰, we obtain the dimerization curves shown in Figure 3A, as well as the dimerization constant **K** and $\tilde{E}$ shown in Table 1.

In Figure S1, the FRET efficiencies appear higher in the presence of the JM domain, suggesting that the dimerization is higher when the JM domain is present. After accounting for the effect of $\tilde{E}$, in Figure 3A we show that the dimeric fraction is higher in the presence of the JM domain. The dimerization free energy changes from -3.4 kcal/mole to -5.4 kcal/

mole upon the substitution of the unstructured linker with the JM domain. Thus, this substitution increases the stability of the FGFR3 dimer by $G_{JM} = -2$ kcal/mole.

The interplay of FGFR3 domain interactions

To understand the interplay of the different domains in FGFR3 unliganded dimerization, next we studied how the dimerization of the FGFR3 construct studied above changes when the EC domains were deleted. Specifically, we worked with (a) a FGFR3 construct containing the TM domain, the JM domain, and fluorescent proteins (TM-JM-YFP/mCherry) and (b) a FGFR3 construct containing the TM domain, a 15 amino acid flexible (GGS)₅ linker, and fluorescent proteins (TM-(GGS)₅-YFP/mCherry), see Figure 1. The dimerization results for the two proteins are shown in Figure S2 and Figure 3B.

For TM-(GGS)₅-YFP/mCherry, we measure unliganded dimer stability of -5.2 kcal/mole (Figure 3B), which is higher than the stability in the presence of FGFR3 EC domain. Comparison of the stabilities of the EC+TM and TM dimers (-3.4 and -5.2 kcal/mole, respectively), demonstrates that the deletion of the EC domain stabilizes the dimer by 1.8 kcal/mole, with the positive sign indicating that the contribution is inhibitory. This result is similar to our previous measurements in vesicles produced via chemical vesiculation using formaldehyde and DTT^{31–33}, which yielded ~ 1 kcal/mole for the inhibitory EC domain contribution²⁷.

In Figure S2, the FRET efficiency for TM-JM-YFP/mCherry construct does not depend on the concentrations, which suggests that the construct is 100% dimer over the concentration range we study. In this case, the exact values of K and G_{JM} cannot be determined. However, the data demonstrate that the stability of the FGFR3 TM dimer is increased by at least -1.5 kcal/mol in the presence of the JM domain.

The data in Figure 3 suggest that the substitution of the unstructured linker with the JM domain stabilizes the FGFR3 dimer by -2 kcal/mole, while the EC domain inhibits dimerization by 1.8 kcal/mole. In other words, the stabilizing effect of the JM domain cancels the inhibitory contribution of the EC domain in the FGFR3 unliganded dimer.

The (GGS)₅ linker does not contribute significantly to dimerization

The (GGS)₅ linker has been previously assumed to have a negligible effect on protein interactions as it is thought to be long enough to allow for flexibility and to prevent significant steric overlap between the fluorescent proteins in the dimer^{28,30}. Here, we sought experimental support for this assumption.

We reasoned that if the linker affects dimerization, for instance due to the overlap of the random coil radii of the two linkers, or due to the steric clash of the fluorescent proteins, its contribution will depend on its length. We therefore compared the interactions between TM helices when the fluorescent proteins were attached to 6, 9, and 15 amino acid long linkers. To address this question in a most general context, we performed the experiments with the TM helix of glycophorin A (GpA), a well-characterized sequence that is traditionally used as a model in transmembrane helix dimerization studies^{34,35}. In particular, we characterized the dimerization of GpA TM domain when the fluorescent proteins are connected via

(GGS)₂, (GGS)₃, and (GGS)₅ linkers (see Figure 1). Dimerization results for the three constructs are shown in Figure S7, Figure 4A, and in Table 1. The length of the linker had no effect on GpA dimer stability. The dimerization curves in Figure 4A are essentially identical, as also shown by the resulting dimerization free energies in Table 1. Furthermore, the value of the Intrinsic FRET, $\tilde{E}$, is the same for GpA-(GGS)₃-YFP/mCherry and GpA-(GGS)₅-YFP/mCherry (0.52). The value of $\tilde{E}$ is somewhat lower for GpA-(GGS)₂-YFP/mCherry (0.41), which may be indicative of restrictions in mobility of the fluorescent proteins when attached to the shortest linker.

Overall, these data support the idea that the (GGS)₅ linker does not contribute measurably to the stabilization of TM dimers. Thus, the G_{JM} contribution that we measure above represents the stabilizing contribution of the JM domain.

Tethering the JM domain to the membrane does not lead to stabilizing interactions

The behavior of the JM domain of EGFR has been shown to depend on the TM domain^{11,19}. To test if the FGFR3 JM domain has inherent stabilizing interactions that arise simply from membrane tethering, we attached the JM domain to the unrelated GpA TM domain via a short, flexible (GGS)₂ linker (GpA-(GGS)₂-JM-YFP/mCherry, see Figure 1). The role of GpA in this experiment was to anchor the protein to the plasma membrane and bring the two JM domains into close proximity upon dimerization. The role of the (GGS)₂ linker was to decouple the JM domain from the GpA helices, since the GpA and FGFR3 dimer structures are different, and the cytoplasmic ends of the dimeric helices have different separations.

We compared the dimerization of the GpA-(GGS)₂-JM-YFP/mCherry and GpA-(GGS)₅-YFP/mCherry constructs. The results for these two constructs are shown in Figure S4, Figure 4B and in Table 1. There is no difference in the dimerization of the two constructs. Thus, the effect of the substitution of the (GGS)₅ linker with (GGS)₂-JM was negligible. The JM domains therefore engaged in stabilizing contacts only when attached to the TM domain of FGFR3 in the appropriate context, but not when closely tethered to the membrane via a short, flexible (GGS)₂ linker on a different TM domain. Thus, the behavior of FGFR3 JM domain in our experiments depends on the details of the TM domain dimer. In FGFR3, the two domains seem to act in synergy to stabilize the dimeric form.

The interactions between the JM domains occur within the N-terminal part of the JM sequence

In EGFR, direct JM-JM contacts in the liganded state occur in the N-terminal portion of the JM domain^{18,19}. Here we asked whether, similarly, the N-terminal end of the FGFR3 JM domain might be responsible for the JM-mediated stabilization of the FGFR3 unliganded dimers. In particular, we investigated if the removal of a C-terminal segment from the JM segment will decrease FGFR3 dimer stability in the absence of ligand. We thus shortened the JM domain by 12 residues, using a convenient restriction site in the DNA sequence encoding the JM domain. We then measured the dimerization of the construct EC-TM-JM₆₀-YFP/mCherry containing the shorter 60 amino acid JM sequence. Results are shown in Figure S5, Figure 5 and in Table 1. The dimer stabilities for EC-TM-JM₆₀-FP and EC-TM-JM-FP are the same, demonstrating that the C-terminal 12 amino acid segment of the

JM domain does not participate in JM-JM stabilizing interactions. Furthermore, the value of the intrinsic FRET, $\tilde{E}$, is somewhat higher in the case of the shorter linker (0.7 versus 0.57, see Table 1). This suggests that the distance between the fluorescent proteins may be shorter in the dimer when the JM domain is shorter (46 Å versus 51 Å, assuming free fluorophore rotation), a finding which is consistent with the idea of JM-JM interactions occurring within the N-terminal part of the JM domain, close to the membrane.

FGFR3 JM domain stabilizes the A391E FGFR3 dimer

Previously, we have characterized the dimerization of FGFR3 in the presence of the A391E mutation which causes a craniosynostosis, Crouzon syndrome with acanthosis nigricans²². We have shown that the mutation stabilizes the unliganded FGFR3 dimer by -1.4 kcal/mole in plasma membrane derived vesicles produced with the DTT/formaldehyde vesiculation buffer³⁶. Here we asked if the substitution of the (GGS)₅ linker with the JM domain will alter the stability of the pathogenic A391E dimer. We therefore engineered the A391E mutation into the ECTM-JM₆₀-YFP/mCherry sequences and then we measured and compared the dimerization of EC-TM-A391E-(GGS)₅-YFP/mCherry and EC-TM-A391E-JM₆₀-YFP/mCherry in vesicles produced with the osmotic stress method. Results are shown in Figure S6, Figure 6 and in Table 1. The mutation stabilized the FGFR3 dimer by -1.4 kcal/mole, consistent with previous results³⁶, and the JM domain stabilized the A391E mutant dimer by an additional -1.8 kcal/mole. The latter is within experimental error of the contribution of the JM domain in the wild-type protein. Thus, the contributions of the A391E pathogenic mutation in the TM domain and of the JM domain to dimer stability are additive.

DISCUSSION

Goals and findings of this study

Our long term goal is to understand the roles of the different RTK domains in RTK unliganded dimerization. Here we focus on FGFR3 JM domain, and we find that it stabilizes the unliganded FGFR3 dimer. The contribution is significant, when compared to the contribution of other FGFR3 domains. For instance, the JM contribution completely cancels the inhibitory contribution of FGFR3 EC domain. Furthermore, the magnitude of the stabilizing effect is similar to the effect of a pathogenic FGFR3 mutation, and the two effects are additive. These comparisons suggest that the stabilizing effect that we measure here is likely important for FGFR3 biological function.

Interestingly, the JM domain stabilizes the dimer only when it is attached to FGFR3 TM domain (Figure 3). On the other hand, simply anchoring of the JM domain to the membrane does not potentiate JM-JM interactions (Figure 4B). These findings suggest that the TM and the JM domains in FGFR3 work synergistically to stabilize the unliganded FGFR3 dimer.

Experimental approach

Studies of the effect of different RTK domains on RTK dimer stability have been challenging, as the interactions of interest occur within the two dimensional plasma membrane. Soluble isolated domains are sometimes produced and studied in solution, but

results acquired for the three dimensional case cannot be extrapolated to two dimensions^{37,38}. This is why, here we used a methodology that allowed us to directly explore the thermodynamics of RTK dimerization in plasma membrane derived vesicles.

The QI-FRET method that we use here has been described previously³⁰. Yet, this work required significant improvements in experimental protocols as in this case we compared the dimerization of RTK constructs that utilized different attachments of the fluorescent proteins. Thus, no *a priori* assumptions could be made about changes in dimer structure and the Intrinsic FRET, $\tilde{E}$, upon the incorporation of the JM domain. Instead, all the data were subjected to two parameter fits which yielded the values of $\tilde{E}$ along with the values of the dimerization constant K and the dimer stability G . These fits were possible because the RTK concentrations in our experiments spanned about two orders of magnitude, an improvement of an order of magnitude over our previous work. In particular, we were able to decrease the lowest accessible receptor concentration by a factor of ~ 5 , while increasing the maximum expression levels by a factor of ~ 2 . Additionally, for this project we used vesicles that are produced using an osmotic stress method²⁹, and thus were not exposed to DTT and formaldehyde which may introduce perturbations in RTK interactions.

Comparison with EGFR

Previously, we have shown that the substitution of EGFR JM domain with an unstructured (GGG)₁₀ sequence has no effect on unliganded EGFR dimerization³⁹. Thus, in the EGFR case the JM domain does not stabilize the dimer in the absence of ligand, a behavior that is very different from the behavior that we observe here. This prompted us to seek differences in sequence characteristics of the FGFR3 and EGFR JM domains. We used the web-based program JPRED to evaluate the propensity of FGFR3 JM domain for folding into secondary structure (see Supplemental data). The FGFR3 sequence was predicted to be mainly unstructured, with low beta sheet content but no helical content. On the other hand, EGFR JM domain has a short alpha-helical segment close to its N-terminus which has been implicated in the interactions of EGFR JM domain with lipids in the absence of ligand¹⁹. Thus, the differences in JM domain secondary structures may underlie the difference in behavior.

The activity of all RTKs is often modeled after the mode of activation of the extensively studied EGFR. Yet, it is not clear if the lessons learned about EGFR are directly transferable to other RTKs. For instance, there are distinct differences in the activation mode of the kinases from different RTK families⁵. Here we further show that the behavior of FGFR3 JM domain is distinctly different from EGFR JM domain, which engages in JM-JM stabilizing contacts only upon ligand binding. These results reinforce the idea that different families of RTKs have evolved different mechanisms of dimerization and activation, and thus all the 58 RTKs merit in-depth investigation.

Implications

There is evidence in the literature that unliganded FGFR dimers are important for FGFR biological function¹⁴. Unliganded FGR dimers are phosphorylated, and this basal phosphorylation has been suggested to “prime” the kinases for rapid activation upon ligand

binding^{14,15}. Furthermore, FGFR mutations have been shown to cause disease by specifically increasing unliganded dimerization^{40–42}. For instance, the G380R mutation in FGFR3, which causes the most common form of human dwarfism, increases ligand-independent FGFR3 dimerization and phosphorylation, without having a significant effect on FGFR3 phosphorylation in the presence of ligand³⁹.

Within the “pre-formed dimer” model of RTK activation, the transition from unliganded to liganded dimers can be considered as a thermodynamic change of state¹⁶. Thus, unliganded dimers are an intermediate in the activation process. When unliganded dimers are present, the response of the pre-formed dimers to ligand is not limited by the diffusion of the receptors in the plasma membrane and is thus faster. As a result, there is interest in understanding the unliganded dimer state, with the long-term goal of understanding RTK activation.

This work yields new knowledge about the unliganded FGFR3 state by demonstrating that FGFR3 JM domain plays an important role in unliganded dimer stabilization. The biological significance of our observations comes from the fact that the JM contribution is similar in magnitude to the effect of pathogenic mutations that stabilize the FGFR3 dimers, and is additive. Furthermore, the effect opposes and cancels the inhibitory contribution of the EC domain.

Since RTKs are implicated in many human cancers and developmental abnormalities, new strategies are being sought to inhibit their dimerization and activation. And because the unliganded FGFR3 dimer is an activation intermediate, its destabilization could be a viable therapeutic strategy. The significant stabilization of FGFR3 unliganded dimers by the JM domains suggests that JM-mediated interactions could be a novel target.

EXPERIMENTAL METHODS

The YFP plasmid was a gift from Dr. M. Betenbaugh (Johns Hopkins University, Baltimore, MD) and pRSET-mCherry was obtained from Dr. R. Tsien (University of California, San Diego). The plasmid encoding human wild-type FGFR3 was a gift from Dr. D. J. Donoghue (University of California, San Diego). All primers were purchased from Invitrogen.

22 different gene constructs, inserted into the multiple cloning site of pcDNA3.1(+) between HindIII and XbaI (Table 1) were used in this study. The cloning procedures for FGFR3 TM-(GGG)₅-YFP, FGFR3 TM-(GGG)₅-mCherry, FGFR3 EC-TM-(GGG)₅-YFP, FGFR3 EC-TM-(GGG)₅-mCherry, GpA-(GGG)₅-YFP and GpA-(GGG)₅-mCherry, FGFR3 EC-TM-A391E-(GGG)₅-YFP, and FGFR3 EC-TM-A391E-(GGG)₅-mCherry have been described previously^{27,30,36}. All constructs included the signal peptide of FGFR3 (MGAPACALALCVAVAIAGASS) at the N-terminus.

To create the FGFR3 EC-TM-JM-YFP and FGFR3 EC-TM-JM-mCherry plasmids, the AsiSI site before (GGG)₅ in FGFR3 EC-TM-(GGG)₅-YFP and FGFR3 EC-TM-(GGG)₅-mCherry was mutated to a BsrGI site. The FGFR3 EC-TM-(GGG)₅-YFP and FGFR3 EC-TM-(GGG)₅-mCherry plasmid constructs containing the BsrGI site were then double digested using BsrGI and AgeI restriction enzymes. The complementary DNA (cDNA)

encoding the JM domain of FGFR3 was amplified using Polymerase Chain Reaction (PCR), double digested with BsrGI and AgeI and ligated with FGFR3 EC-TM-(GGG)₅-YFP and FGFR3 EC-TM-(GGG)₅-mCherry that have been digested with the BsrGI and AgeI restriction enzymes.

To create the FGFR3 EC-TM-JM₆₀-YFP and FGFR3 EC-TM-JM₆₀-mCherry plasmid constructs, a XhoI site in the JM sequence of FGFR3 was utilized to double digest EC-TM-JM₆₀ using Hind III and XhoI. This cDNA was then inserted into the multiple cloning sites in the pcDNA3.1(+) vector plasmids encoding YFP and mCherry between the HindIII and XhoI sites.

To generate GpA-(GGG)₂-JM-YFP and GpA-(GGG)₂-JM-mCherry, a BsrGI site was first created at the 7th Glycine position in the (GGG)₅ linker sequence in the GpA-(GGG)₅-YFP and GpA-(GGG)₅-mCherry sequences. The cDNA encoding the JM domain of FGFR3 together with YFP or mCherry was then amplified from FGFR3 EC-TM-JM-YFP and FGFR3 EC-TM-JM-mCherry using PCR, and double digested with the BsrGI and XbaI restriction enzymes. The JM-YFP/mCherry digested cDNA was then ligated into the pCDNA-GpA-TM-(GGG)₅ vector which has been double digested with BsrGI and XbaI.

The A391E mutation was generated in the FGFR3 EC-TM-JM₆₀-YFP and FGFR3 EC-TM-JM₆₀-mCherry plasmid sequences using a QuickChange ® II XL Site-Directed Mutagenesis Kit (Stratagene, CA).

The GpA-(GGG)₂-YFP/mCherry and GpA-(GGG)₃-YFP/mCherry plasmid constructs were created from GpA-(GGG)₅-YFP/mCherry. The GpA-(GGG)₂ and GpA-(GGG)₃ cDNAs were amplified using PCR. The GpA-(GGG)₅-YFP/mCherry plasmids were double digested with HindIII and AsiSI restriction enzymes. The amplified GpA-(GGG)₂ and GpA-(GGG)₃ cDNAs were then double digested with HindIII and AsiSI and ligated with the double digested GpA-(GGG)₅-YFP/mCherry cDNAs.

Cell culture and transfection

Chinese Hamster Ovary (CHO) cells were a kind gift of Dr. M. Betenbaugh (Johns Hopkins University, Baltimore, MD). The cells were cultured at 37 °C with 5% CO₂ for 24h. Transfection was carried out using Fugene HD transfection reagent (Roche Applied Science), following the manufacturer's protocol. Cells were co-transfected with total of 4-5 µg total DNA encoding YFP and mCherry tagged constructs.

Production of mammalian plasma membrane vesicles

Vesiculation was performed as described previously²⁹. CHO cells were rinsed twice with 30% PBS (pH 7.4), and incubated with 1 mL of chloride salt vesiculation buffer overnight at 37 °C. The vesiculation buffer consisted of 200 mM NaCl, 5 mM KCl, 0.5 mM MgSO₄, 0.75 mM CaCl₂, 100 mM bicine and protease inhibitor cocktail (Complete mini EDTA-free tabs, Roche Applied Science) adjusted to PH of 8.5. A large number of vesicles were produced after 1.5 h, and the vesicles were transferred into 4-well Nunc Lab-Tek II chambered coverslips for imaging.

Fluorescence Image Acquisition—Vesicles were imaged using a Nikon Eclipse confocal laser scanning microscope using a 60× water immersion objective. All the images were collected and stored at a 512 × 512 resolution. Three different scans were performed for each vesicle: (1) excitation at 488 nm, with a 500-530 nm emission filter (donor scan); (2) excitation at 488 nm, with a 565-615 nm emission filter (FRET scan); and (3) excitation at 543 nm, with a 650 nm longpass filter (acceptor scan). Gains of 8.0 and pixel dwell time of 1.68 μ s were used for the three scans.

The imaged vesicles exhibited uniform fluorescence intensities (see Figure 2), which allowed us to determine the concentrations of the fluorescent proteins in the membrane using solutions of purified YFP and mCherry solutions of known concentration as described in³⁰. The fluorescent protein solutions were prepared as described in⁴³. They were imaged in the microscope using the same settings used for vesicle imaging, to allow direct comparison of solution and vesicle intensities.

Each vesicle was analyzed using a Matlab® program to determine the fluorescence intensity across the membrane^{27,44}, which was fitted to a Gaussian function and the background intensity was approximated as an error function (see Figure 2). The donor, acceptor, and FRET intensities for each vesicle were used to determine (i) the donor concentration, (ii) the acceptor concentration, and (iii) the FRET efficiency in each vesicle as described in detail elsewhere^{27,44} and in Supplementary Material.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- FGFR3 juxtamembrane domain (JMD) stabilizes FGFR3 unliganded dimers
- The JMD contribution to dimer stability is similar to that of a pathogenic mutation
- The contributions of the JMD and a pathogenic mutation to stability are additive
- The JMD contribution cancels the repulsive contribution of the extracellular domain

Figure 1.

The plasmid constructs used in the FRET experiments. SP: FGFR3 signal peptide (MGAPACALALCVAVAIVAGASS), EC: extracellular domain, FGFR3 TM domain: (DEAGSVYAG ILSYGVGFLLFILVVAAVTLCRLR), GpA TM domain: (LIIFGVMAGVIGTILLISYGIRRL), FGFR3 JM domain: (S PPKKGLGSPT VHKISRFPLK RQVSLESNAS MSSNTPLVRI ARLSSGEGPT LANVSELELP ADPKWELSRAR), FGFR3 JM₆₀ (S PPKKGLGSPT VHKISRFPLK RQVSLESNAS MSSNTPLVRI ARLSSGEGPT LANVSELEL), FP: Fluorescent protein, either YFP or mCherry (a FRET pair).

Figure 2.

A vesicle, imaged and analyzed in the FRET, acceptor, and donor channels. Images were acquired with a Nikon laser scanning confocal microscope. The images are analyzed with a Matlab code that has been discussed in detail in a previous publication³⁰. The intensity across the membrane (open blue symbols) is fit to a Gaussian (solid line) after background correction. The green dotted line is the residual from the fit.

EC-TM-(GGS)₅-YFP/mCherry

EC-TM-JM-YFP/mCherry

TM-(GGS)₅-YFP/mCherry

TM-JM-YFP/mCherry

Figure 3.

Dimerization curves for (A) EC-TM-(GGS)₅-YFP/mCherry and EC-TM-JM-YFP/mCherry and (B) TM-(GGS)₅-YFP/mCherry and TM-JM-YFP/mCherry. The dimers that contain the JM domains are more stable.

Figure 4.

Dimerization curves for (A) three Glycophorin A TM helix constructs in which the fluorescent proteins are attached to the TM helix via different length linkers and for (B) GpA-(GGS)₅-YFP/mCherry and GpA-(GGS)₂-JM-YFP/mCherry. The linker length and the JM domain do not affect GpA dimer stability.

Figure 5.
Dimerization curves for EC-TM-(GGS)₅-YFP/mCherry and EC-TM-JM₆₀-YFP/mCherry.
The shorter JM domain stabilizes the dimer to the same extent as the full-length JM domain
(see Figure 3A and Table 1).

EC-TM-A391E-(GGS)₅-YFP/mCherry
 EC-TM-A391E-JM₆₀-YFP/mCherry

Figure 6.

Dimerization curves for and EC-TM-A391E-(GGS)₅-YFP/mCherry and EC-TM-A391E-JM₆₀-YFP/mCherry. The A391E mutation is the genetic cause for Crouzon syndrome with acanthosis nigricans. The JM domain stabilizes the mutant dimer.

Table 1

Dimer stabilities and Intrinsic FRET for the proteins studies here. Shown are optimal values and 95% confidence intervals. $\tilde{E}$ is the value of the Intrinsic FRET, which depends on the distance between the fluorescent proteins and on their mobility within the dimer. Average distance between the fluorescent proteins (D) is calculated under the assumption of free fluorescent protein rotation, which may not be correct in all cases.

	G (kcal/mol)	G _{JM} (kcal/mol)	Intrinsic FRET, $\tilde{E}$	D(Å)
FGFR3 EC-TM-(GGG) ₅ -FP	-3.4 (-3.2 to -3.6)		0.52(0.46 to 0.57)	52.42 (50.7 to 54.6)
FGFR3 EC-TM-JM-FP	-5.4 (-5.0 to -6.0)	-2	0.57(0.53 to 0.61)	50.7 (49.3 to 52.1)
FGFR3 EC-TM-JM ₆₀ -FP	-5.3 (-5.0 to -5.9)	-1.9	0.7(0.66 to 0.74)	46.1 (44.6 to 47.6)
FGFR3 TM-(GGG) ₅ -FP	-5.2 (-5.0 to -5.4)		0.65(0.64 to 0.67)	47.9 (47.2 to 48.2)
FGFR3 TM-JM-FP	100% dimer-	> -1.5	0.38±0.01	57.6 (56.6 to 58.6)
GpA-(GGG) ₅ -FP	-5.3 (-5.0 to -5.4)		0.52(0.5 to 0.54)	52.4 (51.7 to 53.1)
GpA-(GGG) ₃ -FP	-5.2 (-5.0 to -5.4)		0.53(0.52 to 0.55)	52.0 (51.4 to 52.4)
GpA-(GGG) ₂ -FP	-5.4 (-5.2 to -5.6)		0.41(0.39 to 0.43)	56.5 (55.7 to 57.2)
GpA-(GGG) ₂ -JM-FP	-5.3 (-5.0 to -5.9)	0	0.54(0.52 to 0.57)	51.7 (50.7 to 52.4)
FGFR3 EC-TM-A391E-(GGG) ₅ -FP	-4.8 (-4.5 to -5.0)		0.72(0.68 to 0.74)	45.4 (44.6 to 46.85)
FGFR3 EC-TM-A391E-JM ₆₀ -FP	-6.6 (-6.2 to -8.9)	-1.8	0.83(0.8 to 0.86)	40.8 (42.2 to 39.2)