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Cooperative interactions between VEGFR2 extracellular Ig-like subdomains ensure VEGFR2 dimerization

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Abstract

Background—Prior studies have suggested that the interactions occurring between VEGFR2 extracellular domains in the absence of ligand are complex. Here we seek novel insights into these interactions, and into the role of the different Ig-like domains (D1 through D7) in VEGFR2 dimerization.

Methods—We study the dimerization of a single amino acid mutant and of three deletion mutants in the plasma membrane using two photon microscopy and fully quantified spectral imaging.

Results—We demonstrate that a set of cooperative interactions between the different Ig-like domains ensure that VEGFR2 dimerizes with a specific affinity instead of forming oligomers.

Conclusions—The contributions of subunits D7 and D4 seem to be the most critical, as they appear essential for strong lateral interactions and for the formation of dimers, respectively.

General Significance—This study provides new insights into the mechanism of VEGFR2 dimerization and activation.

Graphical abstract

The authors declare no conflict of interest

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INTRODUCTION

There are three VEGF receptors belonging to the large family of receptor tyrosine kinases (RTKs). They conduct biochemical signals via lateral dimerization in the plasma membrane, a process that is regulated by several VEGF ligands (1–5). Of these three receptors, VEGFR2 is the primary regulator of endothelial cell proliferation and migration (6). It consists of an extracellular (EC) domain composed of 7 immunoglobulin-like (Ig-like) subdomains D1 through D7, a single-pass alpha helical transmembrane (TM) domain, and an intracellular (IC) portion containing a kinase domain. VEGFR2 binds to, and is activated by VEGF-A and processed forms of VEGF-C and VEGF-D (7). The VEGF binding site on the receptor encompasses regions of Ig-like domains D2 and D3, and the D2/D3 linker (7–9). X-ray crystallography, small angle X-ray scattering, and electron microscopy of isolated VEGFR2 EC domains, as well as biochemical studies of full-length VEGFR2, have demonstrated that in addition to receptor-ligand contacts, there are also receptor-receptor contacts in the ligand-bound state (9–13). In particular, there are inter-receptor contacts between Ig-like domains D4, D5, and D7, and these contacts are required for receptor phosphorylation and activation (10,11) upon ligand binding.

While VEGFR2 has long been believed to undergo dimerization and activation only upon ligand binding, recent work has demonstrated that VEGFR2 forms dimers also in the absence of ligand (14). A substantial fraction of full length VEGFR2 (30 to 70 %) is dimeric for receptor expression levels reported for endothelial cells. Furthermore, these unliganded dimeric receptors are phosphorylated on several tyrosines (15), leading to phosphorylation of downstream kinases even in the absence of ligand (16,17).

Published work involving mutagenesis, domain deletions and domain substitutions has provided some insights into the interactions that stabilize VEGFR-2 dimers in the plasma membrane in the absence of ligand (14). The deletion of the entire VEGFR-2 EC domain increased VEGFR2 dimerization, demonstrating that VEGFR-2 EC domain, as a whole, inhibits VEGFR2 dimerization in the absence of ligand (14). On the other hand, VEGFR2 TM domains and IC domains contribute favorably to dimerization. Thus, VEGFR2 unliganded dimers are stabilized by contacts between the TM and IC domains, while the EC domains oppose these favorable interactions, likely through steric repulsion effects that occur in the absence of bound ligand (14).

The above view of VEGFR2 unliganded dimerization needs to be reconciled with a crystal structure of the isolated D7 VEGFR2 EC domains (PDB entry 3KVQ), which forms dimers that exhibit a prominent inter-molecular salt bridge between residues D731 and R726 (11). Such specific interactions between the two D7 domains could stabilize VEGFR2 unliganded dimers. In our previous work, we therefore asked if the D731-R726 salt bridge plays a role in dimer stabilization, in the absence of ligand (18). We reasoned that if this salt bridge forms in the absence of ligand and if it stabilizes the dimer, its elimination will reduce, or even eliminate, VEGFR2 dimerization. If, however, the salt bridge does not form and plays no role in unliganded dimerization, its elimination will have no effect on VEGFR2 dimerization. To investigate which of these possibilities occur, we introduced a D731A mutation in a VEGFR2 construct in which the intracellular domain was substituted with a

fluorescent protein to allow for FRET detection, and we assessed the effect of the D731A mutation on its dimerization in the absence of ligand (18). To our surprise, none of the two hypothesized scenarios occurred in these experiments. Instead, we observed that the introduced mutation converted the VEGFR2 dimer into an oligomer, i.e., altered the association state of the receptor. This suggested that the D731-R726 salt bridge likely plays a role in dimer stabilization, but in an unexpected way. In particular, its role seemed to be to prevent interactions that lead to higher oligomers, thus ensuring proper VEGFR2 dimerization in the absence of ligand.

These prior limited studies of VEGFR2 unliganded dimerization suggest that the interactions occurring in the absence of ligand are complex. Here we seek novel insights into these interactions, and into the role of the different Ig-like domains in VEGFR2 dimerization. The approach that we take is to utilize serial deletions of specific Ig-like domains of VEGFR2 EC-TM constructs. The interactions between several truncated versions of the VEGFR2 receptor are studied using two photon microscopy and the recently introduced Fully Quantified Spectral Imaging (FSI) method (18). This method yields approximation-free measurements of the apparent FRET efficiency as well as receptor concentrations in the plasma membrane. We find that the addition of different Ig-like subunits drastically changes the oligomerization state of VEGFR2, with positive and negative cooperativity ultimately working to ensure VEGFR2 dimerization instead of higher-order oligomerization.

MATERIALS AND METHODS

Fluorescent Proteins

Monomeric yellow fluorescent protein (YFP) and mTurquoise (mTurq) with an N-terminal 6x His tag were expressed and purified as described previously (19). Fluorescent protein stocks were buffer exchanged into PBS buffer with a 10kDa MWCO concentrator (Pierce #88527) and filtered with a 0.2 μ m syringe filter. For each imaging session, the stocks were then diluted in PBS buffer to micromolar concentrations to produce 100%, 75%, and 50% fluorescent protein solution standards. The mTurquoise and YFP solution standard concentrations were measured in a 1 cm path length quartz cuvette using NanoDrop 2000C (Thermo Scientific). Molar absorption coefficients of 83,400 Mol*cm⁻¹ and 30,000 Mol*cm⁻¹ were used to calculate the concentrations of the solution standards from the YFP and mTurquoise absorption maxima of 514 nm and 434 nm, respectively. Images of the 100%, 75%, 50% fluorescent protein solution standards and a PBS buffer-only control were acquired at both excitation wavelengths and were used for the calculation of the pixel-level intensity versus concentration slope values utilized with the FSI method, as described previously (18).

Plasmid Constructs

pBE plasmids encoding for the VEGFR2 signal sequence, extracellular domain, transmembrane domain, a 15 amino acid GGS linker, and a fluorescent protein (either mTurq or YFP), described previously (18), were the starting products for this work. Truncated constructs were designed based on the sequence locations of the VEGFR2 Ig-like subdomains in UniProt. D4:D7-TM, D5:D7-TM, and D7-TM connected to GGS₅-mTurquoise

or YFP were cloned using the NEBuilder® HiFi DNA Assembly Master Mix (E2621L) cloning kit, according to the manufacturer's protocol. Briefly, the pcDNA3.1+ was linearized with PCR. Using the online NEBuilder Assembly Tool, primers for the truncated EC-TM-FP inserts were designed with 18 bp complementary overhangs to the pcDNA vector, which was produced via PCR using the pcDNA 3.1+ VEGFR2 ECTM-FP plasmid as a template. Two-piece assembly reactions were then performed with a 2:1 insert to vector ratio. All plasmids were completely sequenced and then verified with the NCBI BLAST tool.

Cell Culture and Transient Transfection

HEK293T cells, used for the expression of the VEGFR2-EC-TM-FP plasmid constructs, were a kind gift from Dr. D. Wirtz, Johns Hopkins University. The cells were cultured in DMEM supplemented with 10% FBS and 20mM glucose, at 37° C in a 5% CO₂ environment.

Twenty-four hours prior to transfection, HEK293T cells were seeded in collagen-coated, glass bottom 35mm petri dishes (MatTek, P35GCOL-1.5-14-C) at a density of 2.5e5 cells per dish to achieve 60–70 percent confluency at the time of transfection, 24 hours later. Single transfections were performed with a total of 3 µg of plasmid DNA and co-transfections were performed with a total of 4–9 µg of plasmid DNA, using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. Six hours after transfection, 10mM sodium butyrate was added to the cells to enhance protein expression. Twelve hours after transfection, the cells were rinsed twice with phenol-red free, serum free DMEM (Sigma, D2902) to remove all traces of phenol red and the cells were serum starved for at least 12 hours in the presence of 10 mM sodium butyrate, prior to the application of osmotic stress and imaging.

Cells Under Reversible Osmotic Stress

Hypotonic swelling media was composed of serum-free media, diluted 1:9 with diH₂O, buffered with 25mM HEPES, and 0.2µm sterile filtered. Just prior to imaging, the starvation media was aspirated from the petri dishes and was gently replaced with 1mL of 37°C hypotonic swelling media. The cells in each dish were allowed to stabilize for ~10 minutes before imaging, and swollen cell images were acquired for up to two hours per dish post swelling.

Fully Quantified Spectral Imaging (FSI)

Here we briefly review the primary equations that are the foundation of the FSI methodology (18). These equations are shown below:

$$E_{app} = F^D_{RET, \lambda 1} / F^D_{\lambda 1} = 1 - F^{DA}_{\lambda 1} / F^D_{\lambda 1} \quad (1)$$

$$[D] = \frac{F_{D,\lambda_1}^D}{i_{D,\lambda_1}} = \frac{1}{i_{D,\lambda_1}} (F_{\lambda_1}^{DA} + \frac{Q^D}{Q^A} (F_{\lambda_1}^{AD} - \frac{i_{A,\lambda_1}}{i_{A,\lambda_2}} F_{\lambda_2}^A)) \quad (2)$$

$$[A] = \frac{F_{A,\lambda_2}^A}{i_{A,\lambda_2}} = \frac{1}{i_{A,\lambda_2}} (F_{\lambda_2}^{AD} - \frac{i_{D,\lambda_2}}{i_{D,\lambda_1}} F_{\lambda_1}^{AD}) \cdot (1 - \frac{i_{A,\lambda_1}}{i_{A,\lambda_2}} \frac{i_{D,\lambda_2}}{i_{D,\lambda_1}})^{-1} \quad (3)$$

In these equations, E_{app} is the measured total apparent FRET efficiency, F_{RET,λ_1}^D is the theoretical fluorescence of the donor that is transferred to an acceptor instead of being emitted, upon excitation in the FRET scan at λ_1 . $F_{\lambda_1,2}^{D,A}$ is the total fluorescence of the donor or acceptor in the absence of FRET for excitation at λ_1 or λ_2 . $F_{\lambda_1}^{DA}$ is the measured fluorescence of the donor in the presence of acceptors, and $F_{\lambda_2}^{AD}$ is the measured fluorescence of the acceptor, enhanced due to FRET. i_{D,λ_1} and i_{A,λ_2} are the slopes of the solution standard intensity versus micromolar concentration curves determined by imaging solution standards of known concentration.

In order to perform surface density measurements, the apparent pixel-level fluorescence values (donor and acceptor) calculated during the image analysis are integrated across the diffraction limited segment of the membrane in order to properly determine the 2D surface density from the fluorescence and the calibration curves (18). To do so, F^D , F^A , and F^{AD} are integrated (summed) over every pixel selected in the region,

$$F_{\lambda_1,Reg}^{D,A} = \int F_{region}^{D or A} dA = \sum F_{i,j}^{D or A}. \text{ The apparent FRET efficiency of the region is then}$$

$$\text{calculated as } E_{app} = 1 - \frac{F_{\lambda_1,Reg}^{DA}}{F_{\lambda_1,Reg}^D}.$$

Thermodynamic Analysis of Receptor Dimerization and Oligomerization

The total apparent FRET efficiencies of oligomers of donor-labeled and acceptor-labeled proteins are calculated using the kinetic theory of FRET formalism (20–22):

$$E_{app} = \frac{E_{prox} + E_{oligo} - 2 E_{prox} E_{oligo}}{1 - E_{oligo} E_{prox}} \quad (4)$$

Equation 4 describes the total FRET efficiency that will be measured for two-dimensional distributions of proteins in terms of the intra-oligomeric FRET, E_{oligo} , occurring due to donor- and acceptor-labeled proteins, and the non-negligible inter-oligomeric proximity FRET, E_{prox} , that occurs when fluorophores are confined to two dimensions. The intra-oligomeric FRET is modeled based on Raicu's kinetic theory formalism (20):

$$E_{oligo}^{Dq} = \frac{\mu_{oligo}}{[D]_T} \sum_{k=1}^{n-1} \frac{k(n-k)\bar{E}}{1+(n-k-1)\bar{E}} \binom{n}{k} P_D^k P_A^{n-k} \quad (5)$$

In equation 5, n represents the oligomer order. μ_{oligo} is the concentration of oligomers. P_D and P_A are the fractions of donors and acceptors in the oligomer. For large numbers of molecules, these are equal to the fraction of donor and acceptors, respectively: x_D and x_A . $x_A = \frac{[A]}{[D] + [A]}$, with $[D]$ and $[A]$ representing the donor and acceptor concentrations, and $x_D + x_A = 1$. $\bar{E}$ is the “Intrinsic FRET” or “pair-wise FRET efficiency” which primarily depends on the distance between the fluorescent proteins in the oligomer (22,23).

Equations (4) and (5) provide the theoretical apparent donor-quenched energy transfer efficiency for mixtures of monomers and oligomers, assuming an equal donor to acceptor distance for all D-A pairs in the oligomer. For the case of $n = 2$, a dimer, this is always correct as there is only one donor and one acceptor in the dimer pair. For trimers and above, this is an approximation which minimizes the number of adjustable parameters in the theoretical model for FRET.

The theoretical FRET efficiency due to specific interactions in the oligomer is given by Equation 6.

$$E_{oligo}^{Dq} = \frac{f_{oligo}}{n \cdot x_D} \cdot E \quad (6)$$

In this equation, $E = \sum_{k=1}^{n-1} \frac{k(n-k)\bar{E}}{1+(n-k-1)\bar{E}} \binom{n}{k} P_D^k P_A^{n-k} \cdot f_{oligo}$ is the fraction of proteins in the oligomeric state, which depends of the association constant K according to equation (7)

$$f_{oligo} = \frac{n\mu_{oligo}}{[T]} = \frac{nK[m]^n}{[T]} \quad (7)$$

We use equation (7) to fit models for $n = 1$ to 6, corresponding to the cases of monomer, monomer-dimer, monomer-trimer, monomer-tetramer, monomer-pentamer, and monomer-hexamer thermodynamic equilibria, to the experimental data as described in detail previously (18). We calculate the overall mean squared error (MSE) for all oligomer models, and the model that yields the lowest overall MSE is chosen as the model that best represents the experimental FRET data. During the data fitting procedure, the theoretical total apparent FRET efficiencies, E_{app} , are calculated with a numerically-estimated proximity FRET contribution (E_{prox} in equation (5)). This proximity FRET contribution is well understood (24–26) and the procedure for data analysis has been recently described (22).

RESULTS

The D731R VEGFR2 Mutant

In previous work, we introduced a D731A mutation in a VEGFR2 construct in which the intracellular domain has been substituted with a fluorescent protein to allow for FRET

detection of protein-protein interactions. We then showed that the effect of the mutation was to convert the VEGFR2 EC-TM dimer into an oligomer, i.e., to alter the association state of the receptor (18). This mutation was introduced to destroy a salt bridge observed between two isolated D7 VEGFR2 domains in crystals (PDB entry 3KVQ (11)). Given the surprising outcome of these prior experiments (18), here we started our investigations by creating an alternate mutation to eliminate the same salt bridge. By mutating D731 to R, we completely reversed the polarity of the side chain. The D731R substitution was engineered in the previously used EC-TM VEGFR2 construct which contained the EC domain, the TM domain, and a fluorescent protein attached to the C-terminus of the TM domain via a flexible (GGG)₅ linker. The schematic of this construct is shown in Figure 1A.

Measurements of dimer and oligomer stabilities (free energies of oligomerization) in membranes require knowledge of the 2D concentration of the proteins in the membrane and thus, detailed knowledge of membrane topologies and membrane areas (18). The topology of the plasma membrane of cells, however, is very complex because cells possess 2 to 3 times the membrane needed to sustain their shape (27,28). It has been shown, however, that the plasma membrane topology can be controlled in a completely reversible and non-lethal manner by subjecting cells to reversible osmotic stress (29). As a result of the osmotic stress, cells flatten their caveolae (60–80 nm cup-shaped invaginations) and “un-wrinkle” their membranes (29). Hypoosmotic swelling results in a simple vesicle-like membrane topology (18,30,31), allowing for the determination of two-dimensional receptor concentrations in the membrane (18). Here we use such live cells under reversible osmotic stress to characterize the interactions between the EC-TM VEGFR2 constructs in FRET experiments. Previously, we have shown that the osmotic stress treatment does not alter the FRET efficiencies measured for this construct, and thus does not alter the nature of the protein-protein interactions (18).

Cells were co-transfected with plasmids encoding D371R EC-TM VEGFR2-mTurq and D371R EC-TM VEGFR2-YFP, in a 1:3 ratio. After receptor expression, the cells were subjected to reversible osmotic stress and imaged in accordance with the FSI methodology, in a two-photon microscope equipped with the OptiMiS spectral imaging system (20,32). Full emission spectra for each pixel were acquired in two scans: a “FRET scan” at λ_1 , in which the donor, mTurq, was primarily excited, and an “Acceptor scan” at λ_2 in which the acceptor, YFP, was maximally excited. Figure 1C shows the emission spectra of mTurq and YFP. The emission spectra acquired for each pixel of the image were then unmixed to yield the donor and acceptor fluorescence contributions, for each pixel (Figure 1D). The pixel-level emission spectra of VEGFR2 EC-TM-mTurq and VEGFR2 EC-TM-YFP, expressed in HEK 293T cells, were integrated to produce the cell image in Figure 1E. The cells under reversible osmotic stress exhibit large areas of stretched membrane with homogeneously distributed VEGFR2 fluorescence, such that VEGFR2 concentration in the plasma membrane can be calculated (18). For data analysis, regions of homogenous, diffraction-limited membrane fluorescence of approximately 3 μm in length were chosen and analyzed. Two such membrane regions are shown in Figure 1E. The FRET efficiency, the donor concentration, and the acceptor concentrations were calculated for each region using equations (1) through (3) in Materials and Methods, as described (18).

Figure 2 shows the results for the VEGFR2 EC-TM D731R construct. In all, 118 cells were imaged and one or two membrane areas per cell were selected for analysis, yielding 145 data points. The measured total apparent FRET efficiency is plotted in Figure 2A as a function of total VEGFR2 EC-TM D731R surface density, in purple. In Figure 2A, the total apparent FRET efficiency rapidly increases as a function of the total receptor concentration, suggesting a concentration dependent protein-protein association in the membrane. Figure 2B shows the measured mole-fraction of VEGFR2 EC-TM-YFP (the fraction acceptor-tagged receptors, x_A), for the selected membrane regions (purple bars). The average x_A values agree with those expected for the 1:3 donor-to-acceptor ratio used in transfection. There is a high variability in total receptor expression levels as well as acceptor fractions, due to transient transfection of the HEK293T cells. This natural variability is utilized in the FSI methodology to ultimately produce binding curves for the receptors through a sampling of many different total receptor concentrations.

The D371R VEGFR2 EC-TM total apparent FRET efficiencies, donor concentrations and acceptor concentrations in Figure 2A are analyzed with a theoretical model of the total apparent FRET efficiency occurring due to membrane protein association. The protocol has been described in detail in published work (22), and is overviewed in the Materials and Methods section (see equations (4) through (7)). Briefly, the association strength and oligomer sizes are varied to determine the type of oligomer in the membrane (dimer, trimer, tetramer, etc.) that best describes the measured data. Equations 4 and 5 are used to compute the theoretical apparent FRET efficiency as a function of total concentration for the different possible oligomeric association states (e.g., monomer-only, monomer-dimer, monomer-trimer, monomer-tetramer, etc.). These models, which include a contribution for proximity FRET (22,26), are fitted to the experimental FRET data using equation (7). The least squared error is minimized for each oligomeric model. The model which gives the lowest mean squared error (MSE) is considered the best overall model to represent the data. Dimer formation is identified through an MSE minimum at $n=2$, while higher-order oligomer formation yields a minimum MSE at $n>2$. While the exact order of the oligomer ($n=3, 4$, etc.) cannot be discerned from bulk, two-color static quenching FRET experiments (22), these experiments are able to correctly differentiate between dimers and oligomers, and correctly yield the fraction of receptors in an associated state, as well as the fraction of monomeric receptors (18,22).

In Figure 2C, we plot the best-fit MSE as a function of oligomer order (black stars). The minimum is at $n>2$, indicating oligomer formation. In Figure 2C, we also show the MSE's for the D731A mutant (grey stars) and the wild-type EC-TM construct (crosses). The behavior of the MSE as a function of oligomer order for the two mutants is the same, while for wild-type VEGFR2 EC-TM, the best-fit is achieved for the monomer-dimer equilibrium model, $n=2$. Thus, the mutation of D731 to either A or R alters the association of EC-TM VEGFR2 from dimer formation to oligomer formation.

In Figure 2A, we show the best-fit theoretical total apparent FRET efficiency for the D731R construct. Figure 2D shows the oligomeric fractions, calculated from the measured total apparent FRET efficiencies. They are binned and plotted as a function of the total concentration, along with the best-fit theoretical oligomeric fraction obtained through the

analysis. Figure 6B compares the oligomeric fractions measured for the D731A and D731R mutants. We see that the oligomeric fraction for the D731R mutant is higher than the oligomeric fraction for the D731A mutant.

The D7-TM VEGFR2 Mutant

Next, a series of truncations of the VEGFR2 EC domain were created to determine which subunits are essential for the dimerization of the EC-TM VEGFR2 construct. A cartoon schematic of the truncated constructs, with fluorescent proteins attached to VEGFR2 TM domains via (GGG)₅ flexible linkers, is shown in Figure 1B.

To begin with, the entire EC domain was removed except for the juxtamembrane subunit, D7. 666 cells expressing the VEGFR2 D7-TM truncated receptor were imaged, yielding 1,170 regions for analysis with the FSI method. Figure 3A shows the measured total apparent FRET efficiency as a function of total concentration in yellow. The D:A (mTurq/YFP) transfection ratios were purposely varied from 1:3 to 3:1 with this construct, as shown by the histogram of acceptor fraction values in Figure 3B. As a result, we see a wide range of measured total apparent FRET efficiency values in Figure 3A, as we should.

The data shown in Figure 3A were analyzed to determine the association state of the receptor using equations (4) through (7), and the results are shown in Figure 3C. The best-fit MSE is shown as a function of oligomer order. The minimum in the MSE occurs for $n > 4$, indicating that the D7-TM construct forms oligomers in the plasma membrane. The best-fit total apparent FRET efficiency is plotted in red in Figure 3A alongside the measured total apparent FRET efficiency. The best-fit total apparent FRET efficiency is in very good agreement with the measured data. In Figure 3D, the measured total apparent FRET efficiency values are algebraically transformed into the measured oligomeric fraction, binned, and plotted alongside the best-fit oligomeric fraction. In Figure 6B, we compare the oligomeric fraction of the D7-TM construct with that of the VEGFR2 EC-TM D731A and D731R oligomeric fractions. The D7-TM construct and the D731R EC-TM construct exhibit similar oligomeric fractions, higher than that observed for the D731A mutant.

The D5:D7-TM VEGFR2 Mutant

The D5:D7-TM construct lacks the distal domain D1, the ligand-binding domains D2 and D3, and the domain D4, which has been shown to participate in homotypic interactions in the ligand-bound VEGFR2 dimer (10). Images of 216 cells were acquired, and 347 regions of homogenous, diffraction-limited membrane fluorescence were chosen for FSI analysis. Figure 4 shows the results of these measurements and the analysis of the data. In Figure 4A, the total apparent FRET efficiency is plotted as a function of the measured D5:D7:TM surface density in magenta. Figure 4B shows histograms of the measured acceptor fractions, which agree with the 1:3 D:A ratio of plasmids utilized for the transient transfections.

Figure 4C shows the best-fit MSE as a function of the oligomer order. The lowest best-fit MSE occurs for $n > 2$, and thus the D5:D7-TM construct also forms oligomers. The best-fit theoretical total apparent FRET efficiency for oligomer formation is plotted in red in Figure 4A, and agrees well with the measured data. The measured oligomeric fraction is binned, and plotted as a function of the total D5:D7-TM surface density, along with the best-fit

oligomeric fraction in Figure 4D. The comparison of the oligomeric fraction of the D5:D7-TM construct and the D7-TM construct in Figure 6B shows that the presence of the D5 and D6 subunits significantly inhibits the interactions between the VEGFR2 EC-TM constructs.

The D4:D7-TM VEGFR2 Mutant

Next, we created the D4:D7-TM-GGS₅-mTur/YFP truncated VEGFR2 construct which contains D4, but lacks the distal domain, D1, and the ligand binding domains D2 and D3. 366 HEK293T cells, co-transfected with the VEGFR2 D4:D7-TM-mTurq/YFP constructs were imaged, yielding 549 data points for analysis. Figure 5 shows the measured data and the analysis results. The measured total apparent FRET efficiency is plotted as a function of the total VEGFR2 D4:D7-TM surface density in Figure 5A, in gray. Figure 5B shows the histogram of the measured acceptor fractions, which agree with the 1:3 donor-to-acceptor ratio used for the transfection. In Figure 5C, the best-fit MSE is plotted as a function of the oligomer order. The overall lowest MSE is found for the $n = 2$ model, indicating that this construct forms dimers instead of oligomers, just as wild-type EC-TM VEGFR2. The best-fit total apparent FRET efficiency for the monomer-dimer model is plotted in red in Figure 5A. Figure 5-D shows the measured dimeric fraction and the theoretical dimeric fraction as a function of total concentration.

In Figure 6A, we compare the dimeric fraction of D4:D7-TM with the previously measured dimeric fraction of wild-type EC-TM VEGFR2 (18). The Gibbs free energy of dimerization of D4:D7-TM is calculated as $\Delta G^\circ = -RT \ln \left(K_A / K_0 \right) = -3.0 \pm 0.1 \text{ kcal/mol}$, with the standard state defined as $K_0 = 1^{rec} / nm^2$. The Gibbs free energy of association of the VEGFR2 EC-TM construct, characterized previously (18), is 0.5 kcal/mol more favorable than that of the truncated D4:D7-TM construct. Thus, the truncated construct exhibits slightly reduced dimerization, compared to the VEGFR2 EC-TM construct.

DISCUSSION

Here we sought to investigate the interactions between VEGFR2 EC domains by destabilizing an inter-molecular salt bridge observed in crystal structures of isolated D7 dimers, and by creating versions of VEGFR2 with various truncations in the extracellular domain. First, we confirmed prior observations of perturbed interactions when D731 in D7 is mutated (18), re-affirming the idea that the inter-molecular Asp731-Arg726 salt bridge contributes to the fact that VEGFR2 forms a dimer and not a higher-order oligomer in the absence of ligand.

Second, here we investigated the role of the different Ig-like domains in VEGFR2 unliganded dimerization, by studying the association states of three truncated versions of the EC domain. We found that an EC-TM VEGFR2 construct that lacks all but the D7 domain in its EC portion (the D7-TM construct) has a strong propensity to form oligomers. This is a surprising result, as this construct should be capable of forming the Asp731-Arg729 salt bridge, and thus dimers. Based on the fact that oligomers are formed instead of dimers, it

appears that the presence of amino acids Asp731 and Arg726 alone are not sufficient to ensure stable VEGFR2 dimer formation.

The addition of domains D5 and D6 to the D7-TM construct (D5:D7-TM) did not change the association state of the receptor. Indeed, the D5:D7-TM truncated construct also formed oligomers, but with significantly reduced oligomerization affinity. Thus, it appears that the role of the D5 and D6 domains is to reduce the propensity for strong lateral associations exhibited by the D7-TM construct, without affecting the oligomerization state.

The addition of subunit D4 in the VEGFR2 D4:D7-TM construct nearly fully restored the dimerization that we observed for wild-type EC-TM VEGFR2 in prior work (18). Thus, it appears that specific D7-D7 homotypic interactions, likely involving the Asp731-Arg726 salt bridge, work together with the homotypic contacts between the D4 subunits to ensure the formation of VEGFR2 dimers instead of higher-order oligomers.

The D4:D7-TM construct has slightly weaker dimerization affinity than the VEGFR2 EC-TM construct containing the entire EC domain. This reveals a slightly favorable combined contribution of domains D1, D2, and D3, which are not present in the D4:D7-TM construct, to VEGFR2 dimerization in the absence of ligand.

Our results show that the different Ig-like subunits of the VEGFR2 extracellular domain play diverse roles to ensure that VEGFR2 dimerizes with a specific affinity instead of forming oligomers. The positively-cooperative contributions of D7 and D4 seem to be the most critical, as they appear necessary for strong lateral interactions and for the formation of dimers, respectively. The same Ig-like subunits (D4 and D7) have been shown to be important for VEGFR2 activation in response to VEGF ligands (10). For instance, mutant forms of VEGFR2 in which the salt-bridge between the D7 domains is broken exhibit drastically reduced tyrosine phosphorylation in the presence of VEGF (11). These results were interpreted previously to mean that D731A VEGFR2 mutant is predominantly monomeric, but the association state was not measured experimentally. Here we show that perturbations of interactions through single amino acid mutagenesis or domain deletions do not necessarily render the receptor monomeric as expected, but instead induce oligomerization. It is also interesting that the effect of adding the D5 and D6 subunits to the D7-TM construct was to destabilize the oligomer without altering the oligomeric state. Thus, these subunits introduce negative cooperativity into the EC-TM system, and reduce the interactions in the VEGFR2 EC domain.

In the ligand bound state, the stabilizing contacts between VEGFR2 extracellular domains have been mapped to D4, D5, and D7 using electron microscopy (12). A very recent structure of the entire extracellular domain of VEGFR1 in complex with VEGF-A also reveals contacts between D4, D5, and D7 (10). Our investigations of the unliganded VEGFR2 dimer suggest that contacts between D4 and D7 are also important for VEGFR2 dimerization in the absence of ligand. However, our data do not point to specific interactions between the D5 domains in the unliganded VEGFR2 dimer. Thus, the conformations of the ligand-bound and unliganded VEGFR2 EC domain dimers are likely different. The conformations of the TM portion of the VEGFR2 dimer in the absence and presence of

ligand have also been shown to be different (14). It can be thus hypothesized that ligand binding leads to a conformational change that engages D5 in lateral interactions. The structural changes in the EC domains are then transmitted to the TM helices, triggering a switch to an alternate dimer configuration. From the TM domains, the conformational change is propagated to the kinase domains, to fully activate VEGFR2.

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Highlights

We investigate the role of the Ig-like domains in unliganded VEGFR2 dimerization

Deletion of Ig domains D1 through D6 leads to VEGFR2 oligomerization

Addition of subunits D5 and D6 significantly reduces the oligomerization affinity

The addition of D4 restores the dimerization observed for the wild-type

The contributions of subunits D7 and D4 are the most critical for dimerization

Figure 1.

Experimental design and methods. **(A)** A cartoon of the VEGFR2 receptor and the EC-TM VEGFR2 construct used in the FRET experiments. VEGFR2 has three primary domains: Extracellular (EC), Transmembrane (TM), and intracellular kinase domain. The EC domain consists of seven Ig-like subunits known as subunits D1 through D7, with D1 at the N-terminus and D7 attached to the TM domain. The subunits D2 and D3, colored in yellow, are the ligand binding domains. D4 and D7, colored in blue, have been shown to be important in stabilizing the VEGFR2 ligand-bound dimer (10), but their role in the absence of ligand has not been investigated in detail. The location of the D731/R726 salt bridge is depicted with a gold star on subunit D7. **(B)** The different truncations of VEGFR2 EC-TM used in this work.

The D7-TM construct lacks domains D1 through D6, and contains only the D7 subunit and the TM domain. The D4:D7-TM construct lacks the D1, D2 and D3 domains. The D5:D7-TM construct lacks domains D1 through D4. **(C)** Emission spectra of mTurq (blue) and YFP (green), a FRET pair. **(D)** A single pixel's fluorescence signal (black) is decomposed as a linear sum (red line) of the donor (blue line), acceptor (green line), and background (gray line) contributions (see (18)). **(E)** A HEK293T cell under reversible osmotic stress displays large stretches of homogenous “unwrinkled” membrane. Scale bar is 10 μm . Membrane regions, $\sim 3\mu\text{m}$ in length, are selected for FSI analysis as described (18). Two such regions are shown in yellow.

Figure 2.

Results for the D731R EC-TM VEGFR2 mutant construct. **A.** The measured total apparent FRET efficiency (purple) and the best-fit theoretical total apparent FRET efficiency (red) as a function of the total VEGFR2 EC-TM expression. **B.** A histogram of measured acceptor fractions. **C.** The best-fit MSE as a function of oligomer order for D731R EC-TM (black stars). The best-fit model to the D731R EC-TM data is a monomer-oligomer equilibrium ($n > 2$). Also shown are the MSEs for D731A EC-TM (grey stars) and wild-type EC-TM (crosses), determined in prior work (18). **D.** The best-fit oligomeric fraction for D731R EC-TM is plotted as a function of the total EC-TM VEGFR2 concentration.

Figure 3.

Results for the D7-TM VEGFR2 truncated construct. **A.** The measured total apparent FRET efficiency (yellow) and the best-fit theoretical total apparent FRET efficiency (red) as a function of the total D7-TM VEGFR2 concentration. **B.** A histogram of measured acceptor fractions. **C.** The best-fit MSE as a function of oligomer order. The minimum occurs when $n > 2$, indicative of oligomerization. **D.** The best-fit oligomeric fraction is plotted as a function of the total D7-TM VEGFR2 concentration, along with the measured oligomeric fraction of receptors.

Figure 4.

Results for the D5:D7-TM VEGFR2 truncated construct. **A.** The measured total apparent FRET efficiency (magenta) and the best-fit theoretical total apparent FRET efficiency (red) as a function of total D5:D7-TM VEGFR2 concentration. **B.** A histogram of measured acceptor fractions. **C.** The best-fit MSE as a function of oligomer order. The best-fit model is a monomer-oligomer equilibrium model, $n > 2$. **D.** The best-fit oligomeric fraction is plotted as a function of the total D5:D7-TM VEGFR2 concentration, along with the measured oligomeric fraction of receptors.

Figure 5.

Results for the D4:D7-TM VEGFR2 truncated construct. **A.** The measured total apparent FRET efficiency (gray) and the best-fit theoretical total apparent FRET efficiency (red) as a function of the total D4:D7-TM VEGFR2 concentration. **B.** A histogram of measured acceptor fractions. **C.** The best-fit MSE as a function of oligomer order. The best-fit model is a monomer-dimer equilibrium model, $n = 2$. **D.** The best-fit dimeric fraction is plotted as a function of the total D4:D7-TM VEGFR2 concentration along with the measured and binned dimeric receptor fraction.

Figure 6.

Predicted dimeric, oligomeric, and monomeric fractions. **(A)** The best-fit dimeric fraction for the EC-TM and the D4:D7-TM VEGFR2 constructs. **(B)** The best-fit oligomeric fraction for the D7-TM, D5:D7-TM, EC-TM D731A, and EC-TM D731R constructs as a function of total receptor concentration. **(C)** Best-fit monomeric fractions for the truncated constructs.