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STUDIES OF RECEPTOR TYROSINE KINASE TRANSMEMBRANE DOMAIN INTERACTIONS: THE EXCITATION-EMISSION FRET METHOD

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Abstract

The energetics of transmembrane (TM) helix dimerization in membranes, and the thermodynamic principles behind receptor tyrosine kinase (RTK) TM domain interactions during signal transduction, can be studied using Forster resonance energy transfer (FRET). For instance, FRET studies have yielded the stabilities of wild-type fibroblast growth factor receptor 3 (FGFR3) TM domains and two FGFR3 pathogenic mutants, Ala391Glu and Gly380Arg, in the native bilayer environment. To further our understanding of the molecular mechanisms of deregulated FGFR3 signaling underlying different pathologies, here we determine the effect of the Gly382Asp FGFR3 mutation, identified in a multiple myeloma cell line, on the energetics of FGFR3 TM domain dimerization. We measure dimerization energetics using a novel FRET acquisition and processing method, termed “excitation-emission FRET (EE-FRET)”, which improves the accuracy of thermodynamic measurements of TM helix association. The EE-FRET method, verified here by analyzing previously published data for wild-type FGFR3 TM domain, should have broad utility in studies of protein interactions, particularly in cases when fluorophore concentrations cannot be controlled.

INTRODUCTION

The receptor tyrosine kinase (RTK) family comprises a large group of single-pass membrane proteins that conduct biochemical signals upon dimerization in the plasma membrane (1). The dimerization process brings the catalytic domains in close proximity, and results in their cross-phosphorylation and activation (2;3). RTKs regulate cell growth, differentiation, and metabolism, and their deregulation leads to pathologies (4). Functional defects can arise due to chromosomal translocations, protein overexpression, or the occurrence of missense gain-of-function mutations. Often, such missense mutations occur in the transmembrane (TM) domains of RTKs. Two mechanisms have been proposed to contribute to pathogenesis due to RTK TM mutations (5): (1) direct dimer stabilization of the TM domain dimers, mediated by hydrogen bonds, disulfide bonds, cation- π interactions, or (2) defects in the downregulation of the activated receptor dimers in the cell. The relative importance of these two mechanisms in pathogenesis has not been explored in detail. Furthermore, it is not known if a specific mutation can affect both dimer stability and downregulation efficiency.

Previously, we have characterized the stability of wild-type fibroblast growth factor receptor 3 (FGFR3) TM domain (6), as well as two pathogenic mutants. FGFR3 is an RTK that plays an important role in human skeletal development (7), and the studied mutations were associated with skeletal dysplasias and cancer. The first one, Ala391Glu, is linked to Crouzon syndrome with acanthosis nigricans, an autosomal dominant disorder characterized by premature

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ossification of the skull (craniosynostosis) (8), accompanied by skin hyperpigmentation and thickening (hyperkeratosis), as well as to bladder cancer (9). The second one, Gly380Arg, is linked to achondroplasia (10;11), the most common form of human dwarfism, characterized by short stature (12), and is also found in some cancers (9). We have shown that while the Ala391Glu mutation stabilizes the FGFR3 TM domain dimer by -1.3 kcal/mole (13;14), the achondroplasia mutation, Gly380Arg, does not alter the stability of the FGFR3 TM domain dimer (15).

Such studies of dimerization propensities of isolated RTK TM domains and their pathogenic mutants can provide insights into pathogenesis mechanisms. If the isolated RTK TM dimer is stabilized due to a particular mutation, it could be expected that this mutation stabilizes the active dimeric state of the whole receptor. If, however, the mutation has no effect on TM dimer stability, other mechanisms of pathogenesis, such as slow downregulation of the activated receptors, are likely at play. Therefore, comparative studies of TM dimer stabilities can help us uncover the relative importance of direct stabilization of RTK dimers in various pathologies.

Here we investigate a Gly382Asp mutant identified in a multiple myeloma cell line (16). We find that the Gly382Asp mutation does not stabilize the FGFR3 TM dimer. This finding is consistent with a published structural model of the FGFR3 TM domain dimer (13), in which Gly382 does not participate in the helix-helix contacts. Therefore, pathogenesis associated with the Gly382Asp mutation is likely not due to dimer stabilization, and the exact mechanism is yet to be identified.

In this paper, we also present a robust FRET acquisition and processing method that gives the free energy of dimerization of TM helices with high precision. The method that we describe, termed “excitation-emission FRET”, i.e. EE-FRET, requires the acquisition of both emission and excitation spectra. These two spectra are combined with standard donor-only and acceptor-only emission and excitation spectra to yield the FRET efficiency, the actual donor and acceptor concentration, and the free energy of dimerization. We have verified the EE-FRET method using previously published data for wild-type FGFR3 TM domain (13), and we have used it to determine the dimerization energetics of the Gly382Asp mutant.

The presented EE-FRET method provides a tool to efficiently probe the stability of TM helix dimers, including RTK TM domain dimers and their pathogenic mutants. In addition, this method can have broad utility when the donor and the acceptor concentration cannot be controlled, such as in cellular studies.

MATERIALS AND METHODS

Materials

1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was purchased from Avanti. The 33 amino acid long transmembrane (TM) domain of fibroblast growth factor receptor 3 (FGFR3), (TM_{WT}, sequence RRAGSVYAGILSYGVGFFLFILVVAAVTLCRLR and the pathogenic mutant TM_{382Asp}, sequence RRAGSVYAGILSYGVDFFLFILVVAAVTLCRLR) were synthesized using solid phase peptide synthesis, as described previously (17). The peptides were purified using reverse-phase HPLC (Varian Analytical Instruments, Walnut Creek, CA) using a water/acetonitrile gradient. MALDI-TOF mass spectrometry confirmed the correct molecular weight of the peptides. The single Cys residue in the peptide was labeled with fluorescein-maleimide (donor) and rhodamine-maleimide (acceptor) (Molecular Probe) and purified as previously described (6; 18).

Vesicle preparation and FRET measurements

Lipids in chloroform and peptides in hexafluoro-2-propanol were mixed, as described (19). The organic solvents were removed under a steam of nitrogen gas; the mixture was lyophilized and then redissolved in 10 mM phosphate buffer, 500 mM NaCl, pH 7 to a concentrations of 0.25 mg/ml of lipids. Samples were freeze-thawed several times before the FRET experiments, as discussed in detail elsewhere (19).

Emission and excitation spectra of labeled peptides in vesicles were measured using a Fluorolog 3-22 fluorometer (Jobin Yvon). The vesicle solution was placed in a 10×2 mm quartz cuvette (Starna Cells, Inc.). For the emission scans, the excitation wavelength was set at 439 nm and the emission intensity was collected from 450 to 700 nm. In the excitation scans, the emission intensity was collected at 595 nm while the excitation wavelength was scanned from 400 to 570 nm.

The EE-FRET method

Overview—The method that we introduce, EE-FRET, requires the acquisition of both excitation and emission spectra for “the FRET sample”, i.e. the liposomal solution containing both donor-labeled and acceptor-labeled TM helices. Furthermore, both excitation and emission spectra need to be acquired for a donor-only and an acceptor-only “standards” of well known donor and acceptor concentration. In the EE-FRET method, we rely on these standard spectra to calculate not only the FRET efficiency, but also the actual donor and acceptor concentration, and therefore the equilibrium constants and the free energy of lateral dimerization with high experimental precision. As discussed below, uncertainties in final donor and acceptor concentrations, due to sample-to-sample variations as a result of low peptide solubility, can be resolved and just a few spectra are sufficient to reliably determine the energetics of interactions.

Theory—The efficiency of transfer E , from an excited donor to an acceptor, is defined as (20):

$$E = 1 - \frac{F_{DA}(\lambda_{em}^D)}{F_D(\lambda_{em}^D)} \quad (1)$$

where F_{DA} and F_D are the donor emissions in the presence and in the absence of an acceptor, respectively, and λ_{em}^D is the wavelength of the maximum donor fluorescence (see Figure 1B). Similarly, F_{AD} and F_A will be used to denote the acceptor emissions in the presence and in the absence of a donor. Note: F_{DA} (just as F_D , F_A , F_{AD} , etc.) denote the complete emission spectra, while $F_{DA}(\lambda_{em}^D)$ refers to the fluorescence intensity value at λ_{em}^D .

When both the donor and the acceptor are present, we can write:

$$F_{DA}(\lambda_{em}^D) = S_D \left[\varepsilon_D(\lambda_{ex}^D) \cdot c_D - \varepsilon_D(\lambda_{ex}^D) \cdot c_D \cdot E \right] \quad (2)$$

$$F_{AD}(\lambda_{em}^A) = S_A \left[\varepsilon_A(\lambda_{ex}^D) \cdot c_A + \varepsilon_D(\lambda_{ex}^D) \cdot c_D \cdot E \right] \quad (3)$$

where c_D and c_A are the donor and acceptor concentrations in the sample, $\varepsilon_A(\lambda_{ex}^D)$ and $\varepsilon_D(\lambda_{ex}^D)$ are the extinction coefficients of the acceptor and the donor, S_D is a scaling factor which depends on the quantum yield of the donor and the photodetector efficiency at λ_{em}^D , and S_A is a scaling factor which depends on the quantum yield of the acceptor and the photodetector efficiency at λ_{em}^A . At zero FRET efficiency ($E = 0$), the donor and the acceptor emit due to direct excitation only, such that $F_{DA} = F_D$ and $F_{AD} = F_A$. At non-zero FRET efficiency ($E \neq 0$), part of the energy, absorbed by the donor, is transferred to the acceptor. Other possible mechanisms of energy dissipation, resulting in donor quenching, are similar with or without FRET and can be included in the scaling factor S_D .

When FRET occurs, the donor fluorescence is quenched ($F_{DA} < F_D$) and the acceptor fluorescence is enhanced ($F_{AD} > F_A$). The acceptor enhancement, termed “sensitized fluorescence” F_{sen} , can be calculated as:

$$\begin{aligned} F_{sen} &= F_{AD} - F_A \\ F_{sen}(\lambda_{em}^A) &= S_A \cdot \varepsilon_D(\lambda_{ex}^D) \cdot c_D \cdot E \end{aligned} \quad (4)$$

The donor quenching is given by:

$$\begin{aligned} \Delta F_D &= F_D - F_{DA} \\ \Delta F_D(\lambda_{em}^D) &= S_D \cdot \varepsilon_D(\lambda_{ex}^D) \cdot c_D \cdot E = F_{sen}(\lambda_{em}^A) \cdot \frac{S_D}{S_A} \end{aligned} \quad (5)$$

Therefore, the efficiency of donor quenching at λ_{em}^D is proportional to the sensitized acceptor fluorescence at λ_{em}^A , with S_D/S_A being the scaling factor.

The scaling factor S_D/S_A depends on the dyes used and on the characteristics of the instrument, not on the particular dye concentration. S_D/S_A can be calculated from the characteristics of the FRET spectrum in a system of well-defined dye concentrations and known FRET efficiency according to:

$$\frac{S_D}{S_A} = \frac{\Delta F_D(\lambda_{em}^D)}{F_{sen}(\lambda_{em}^A)} \quad (6)$$

Alternatively, if the photodetector efficiency at λ_{em}^D and λ_{em}^A is the same (or if the fluorescence signal is normalized by the photodetector efficiency), then S_D/S_A equals the ratio of donor and acceptor quantum yields, ϕ_D/ϕ_A , which can be calculated from measured absorbance and emission spectra.

Once S_D/S_A is determined, it can be used to calculate the donor fluorescence in the absence of the acceptor (i.e. the donor control, F_D) from the measured donor fluorescence in the presence of the acceptor F_{DA} according to:

$$F_D(\lambda_{em}^D) = F_{DA}(\lambda_{em}^D) + F_{sen}(\lambda_{em}^A) \cdot \frac{S_D}{S_A} \quad (7)$$

The donor and acceptor standards—Figure 1 shows the excitation (A) and the emission (B) spectra of the fluorescein/rhodamine FRET pair, conjugated to the wild-type FGFR3 TM domain. The blue lines were recorded for 0.1mole % Fl-TM_{WT}, while the red lines are for 0.1mole % Rhod-TM_{WT}. We see that excitation at λ_{ex}^A , the wavelength of maximum acceptor emission, does not lead to donor excitation (Figure 1A). Thus, at λ_{ex}^A , the fluorescence of the acceptor in the presence and absence of the donor are the same (i.e., the black and the blue spectra coincide at λ_{ex}^A , see Figure 1B). This spectral feature is crucial for the applicability of the EE-FRET method.

The excitation and emission spectra of the donor only (blue lines) and the acceptor only (red lines) in Figure 1 are used as “standard” spectra in the EE-FRET method, and are referred to as “excitation and emission standards”. It is desirable that the amplitudes of the standard spectra are known with high accuracy. Therefore, multiple measurements at different dye concentrations are recommended to generate these standards. Once the standard spectra are available, the spectrum of every sample containing both donor and acceptor (i.e. every “FRET sample”) can be used to calculate the FRET efficiency and the free energy of interactions using the EE-FRET protocol described below.

We note that the amplitudes of the spectra in Figure 1 depend on the total fluorophore 3-dimensional (3D) concentrations (i.e. moles of labeled peptides per unit volume). The FRET efficiencies and the dimerization energetics in liposomes, however, are determined solely by the protein-to-lipid ratio (19). Therefore, experiments could be carried out at various protein and lipid 3D concentrations, and then interpreted with respect to the only relevant parameter, the protein-to-lipid ratio. Alternatively, all experiments can be preformed at fixed lipid 3D concentration, by varying the protein concentration (the protein-to-lipid ratio). The latter is the approach that we have taken in this work. In all experiments, the lipid concentration was fixed at 0.25 mg/ml (0.325 mmolar POPC solution). The protein concentration was varied, and reported in mole % (moles of protein per moles of lipid).

The EE-FRET Protocol

Step 1: A solution of liposomes containing donor-labeled and acceptor-labeled TM domains is prepared as previously described (19), and the excitation and emission spectra are acquired as discussed in Materials and Methods. Each FRET spectrum (i.e the spectrum of the FRET sample) has three contributions: direct donor emission in the presence of the acceptor F_{DA} , direct acceptor emission F_A , and sensitized acceptor emission F_{sen} . Furthermore, $F_{AD} = F_A + F_{sen}$.

Step 2: The acquired FRET excitation spectrum (Figure 2A, black line) is compared to the standard excitation spectrum of the acceptor (Figure 1A, red line). The standard spectrum is multiplied by a coefficient, ζ , to produce the red line in Figure 2A, such that the amplitude of the scaled standard (Figure 2A, red line) is identical with the FRET excitation spectrum (Figure 2A, black line) at λ_{ex}^A . This step gives the concentration of the acceptor in the sample as ζ times the standard acceptor concentration, in this case $\zeta \times 0.1\text{mole}\%$.

Step 3: The emission standard of the acceptor (Figure 1B, red line) is multiplied by ζ to obtain the direct emission contribution of the acceptor (Figure 2B, red line). The direct acceptor contribution (red line) is subtracted from the FRET emission spectrum (Figure 2B, black line), to reveal the sum of the direct donor emission and the sensitized acceptor emission, $F_{DA} + F_{sen}$. This sum is plotted in Figure 2C with the black dashed line.

Step 4: The emission standard spectrum of the donor (Figure 1B, blue line) is multiplied by a coefficient to produce the blue dashed line in Figure 2C, such that the amplitude of the scaled

standard (blue dashed line) is identical with the black dashed line in Figure 2C at λ_{em}^D . The difference between the black dashed and the blue dashed line is the sensitized acceptor emission, F_{sen} . The value of the sensitized emission at λ_{em}^A , $F_{sen}(\lambda_{em}^A)$, is related to the decrease in donor emission at λ_{em}^D , $\Delta F_D(\lambda_{em}^D)$, as shown in Equation (5).

Step 5: The value $\Delta F_D(\lambda_{em}^D)$, determined in Step 4, is added to $F_{DA}(\lambda_{em}^D)$ (Figure 2D) to give the value of the donor emission in the absence of acceptor at λ_{em}^D , i.e. $F_D(\lambda_{em}^D) = \Delta F_D(\lambda_{em}^D) + F_{DA}(\lambda_{em}^D)$. The donor emission standard (Figure 1B, blue line) is multiplied by a coefficient ζ , such that the amplitude of the standard spectrum at λ_{em}^D equals $F_D(\lambda_{em}^D)$. This step gives the concentration of the donor in the sample as ζ times the standard donor concentration, in this case $\zeta \times 0.1$ mole%. It also gives the complete emission spectrum of the donor in the absence of the acceptor F_D (Figure 2D, blue line) as the emission of the donor standard (Figure 1B, blue line), multiplied by ζ .

Further data analysis—Knowledge of the FRET efficiency and the donor and acceptor concentrations allows the calculation of equilibrium constants describing TM helix dimerization. As discussed previously (19), the measured FRET efficiency E has two contributions, one due to random colocalization of donors and acceptors, and one due to sequence-specific interactions,

$$E = E_{proximity} + E_{oligomer} \quad (8)$$

The FRET efficiency due to proximity effect $E_{proximity}$ is a function of acceptor concentration $[a]$, and can be determined as outlined in detail elsewhere (6;13). The FRET efficiency due to specific oligomerization $E_{oligomer}$ can be presented as:

$$E_{oligomer} = f_O \cdot p_D \cdot E_R \quad (9)$$

where f_O is the fraction of molecules in the oligomeric state, p_D is the probability for donor quenching in the oligomer, and E_R is the FRET efficiency in the oligomer. If the oligomers are smaller than the Forster radius, then $E_R = 1$. The probability of a donor-labeled molecule to dimerize with another donor-labeled or unlabeled molecule is $1 - x_A$, where x_A is the fraction

of the acceptor-labeled molecules, $x_A = \frac{[a]}{[d] / f_d + [a] / f_a}$, $[d]$ and $[a]$ are the donor and acceptor concentrations, and f_d and f_a are donor and acceptor labeling yields. The probability of a donor-labeled molecule to oligomerize only with donor-labeled or unlabeled molecules is $(1 - x_A)^{n-1}$, where n is the number of molecules in the oligomer. Any donor will be quenched if it oligomerizes with at least one acceptor, and therefore:

$$p_D = 1 - (1 - x_A)^{n-1}. \quad (10)$$

Since $[a]$ and $[d]$ can be determined using the EE-FRET method for each sample, $E_{proximity}$ and x_A can be calculated too, such that the fraction of molecules in the oligomeric state and the number of molecules in the oligomer can be calculated.

In the case of dimers, $n = 2$, $p_D = x_A$, and $f_O = 2[D]/[T]$, where $[D]$ is the dimer concentration and $[T]$ is the total peptide concentration. Note that all concentrations are in moles of peptide per moles of lipid (19).

RESULTS

EE-FRET Method Verification

To verify the EE-FRET method, we used previously published data for wild-type FGFR3 TM domain (13). The dimerization energetics for the wild-type is well characterized, based on an extensive study comprising multiple measurements for various peptide-to-lipid ratios and donor-to-acceptor ratios. We used these data in the following way: First, we determined the ratio S_D/S_A from averaged FRET spectra for 0.08, 0.2, 0.25 and 0.3 mole% peptide, donor-to-acceptor ratio=1 using Equation 6. Next, we used the calculated S_D/S_A and the EE-FRET method, as described in Materials and Methods, to analyze the FRET spectra acquired for 0.1 and 0.4 mole % peptide, and calculate the anticipated donor-only control (donor fluorescence in the absence of the acceptor, F_D). Finally, the calculated donor-only control was compared to the experimental donor-only control (see Figure 3).

S_D/S_A for the fluorescein-rhodamine FRET pair was determined from Eq. (6) as 1 ± 0.05 . As discussed in Materials and Methods, S_D/S_A can be determined, alternatively, by measuring the quantum yields of the donor and acceptor, ϕ_D and ϕ_A , at λ_{em}^D and λ_{em}^A , respectively. The quantum efficiency of the photodetector used at these wavelengths was practically the same, such that $S_D/S_A = \phi_D/\phi_A$. The ratio of the quantum yields ϕ_D/ϕ_A , determined by comparing UV absorbance and fluorescence spectra is $\phi_D/\phi_A = 1.06 \pm 0.1$, similar to a published value of $\phi_D/\phi_A = 1.02 \pm 0.04$ (21).

Based on all these measurements, a value of $S_D/S_A = 1.0$ was chosen to calculate the anticipated donor fluorescence in the absence of the acceptor from the experimental FRET spectra for 0.1 and 0.4 mole % wild-type. Figures 3A and B show the experimental F_{DA} spectra (solid lines), the calculated anticipated F_D spectra (dotted lines), and the experimental F_D control spectra (dashed lines) for 0.1 and 0.4 mole % wild-type, respectively. The comparison of the dashed and dotted lines shows that the calculated F_D spectra are within 2% of the experimental donor-only controls, validating the EE-FRET method.

We further show that the EE-FRET method allows accurate calculations of the free energy of dimerization, even from a single experiment. A FRET spectrum (solid line) and an experimental donor-only control (dashed line) are shown in Figure 4. The FRET spectrum was measured for a single sample, a liposomal solution with 0.15 mole% (intended) fluorescein-labeled TM_{WT} and 0.15 mole% (intended) rhodamine-labeled TM_{WT}. The calculations of the dimerization free energy based on donor quenching from the two experimental spectra in Figure 4, a method that we have used previously to calculate the free energy of dimerization (19), gives -1.8 kcal/mole (the detailed protocol for such calculations has been published elsewhere (6;13;19)). This value is different from the value obtained in an extended experimental study, based on multiple measurements at various peptide concentrations, -2.8 ± 0.1 kcal/mole (13). Using the FRET spectrum (solid line) and the EE-FRET method, we determined the “actual” concentrations of FL-TM_{WT} and Rhod-TM_{WT} as 0.172 mole% and 0.123 mole%, and we calculated the dimerization free energy as -2.9 kcal/mole, which is within experimental error of the published value of -2.8 ± 0.1 kcal/mole (13).

The EE-FRET analysis of the FRET spectrum in Figure 4 demonstrates that the fluorophore concentrations in the sample are different from the intended 0.15 mole%. As a result, the donor-to-acceptor ratio is 1.4 instead of 1, thus affecting the free energy calculations. Furthermore, the experimental florescein-only control (dashed line) is not the appropriate one, since the concentration of the donor in the “FRET sample” is higher than intended. The incorrect donor and acceptor concentrations and the improper florescein-only control lead to an inaccurate free energy calculation, -1.8 kcal/mole. The basis of the error is the limited experimental precision in the preparation of samples of well-defined donor and acceptor concentrations, due to sample-

to-sample variation and/or incomplete solubilization of the very hydrophobic peptides. However, the actual concentrations of donor- and acceptor-labeled peptides, and the appropriate donor-only control (dotted line) can be calculated using the EE-FRET method, thus yielding the correct dimerization free energy and increasing the accuracy of the experimental measurements.

Dimerization energetics of Gly382Asp FGFR3 TM domain

Next we investigated the effect of the Gly382Asp mutation, identified in the KSM-18 myeloma cell line (16), on the dimerization propensity of FGFR3 TM domain. In particular, we characterized the dimerization energetics of the mutant TM domain, TM_{382Asp}, and compared it to published data for the wild-type (13). While cellular studies have produced contradicting results about the effect of the mutation on receptor activation (22), biophysical studies of the mutant Gly382Asp TM domain have never been carried out.

POPC liposomes containing donor- and acceptor- labeled TM_{382Asp} were prepared as previously described in Materials and Methods. Experiments were set up with TM_{382Asp} concentrations varying between 0.05 and 0.5 mole %, a concentration range shown to be optimal for FRET measurements (6;19). Altogether, 6 different FRET samples were prepared at various peptide-to-lipid ratios, donor-to-acceptor ratio = 1, and both emission and excitation spectra were acquired for all of them. Fig. 5 shows the “raw” fluorescence spectra for two different TM_{382Asp} concentrations. From such spectra, the FRET efficiencies E and the actual donor and acceptor concentrations $[d]$ and $[a]$, were determined using the EE-FRET method; the actual concentrations were generally within 20% of the intended ones. For the EE-FRET calculations, we used $S_D/S_A=1$ and the donor and acceptor standards established for the wild-type FGFR3 TM domain. To obtain FRET that is due to sequence-specific dimerization, the contribution from random colocalization $E_{proximity}$ was calculated as described in detail elsewhere (19) and subtracted from the measured FRET efficiency to give FRET due to sequence-specific dimerization, E_{dimer} (Eq. (8)). We emphasize that the two contributions to the measured FRET efficiencies, one due to specific interactions and one due to random dye co-localization, can be separated only when the acceptor concentration is accurately known.

The dimer fraction $[D]/[T]$ for TM_{382Asp} was determined using Eq. (9) for $E_R = 1$, corresponding to 100% energy transfer efficiency from the donor to the acceptor in the dimer (19). The dimer fraction, as a function of total peptide concentration, is shown in Fig. 6. The free energy of dimerization, calculated according to $\Delta G = -RT \ln K$ is -2.78 ± 0.04 kcal/mol. This value is the same as the one determined for wild-type, -2.8 ± 0.1 kcal/mol (13), indicating that the Gly382Asp mutation does not stabilize the FGFR3 TM domain dimer.

We note that the symbols in Figure 6 show results from single experiments. Yet, one can have high confidence in the data, because the donor and the acceptor concentrations, and therefore the total peptide concentrations, have been verified using the EE-FRET method. Calculations of dimerization free energies from donor quenching (i.e. not using the EE-FRET method) gives -2.9 ± 0.7 kcal/mole. Thus, the application of the EE-FRET method reduces the uncertainty in the free energy calculation from ± 0.7 to ± 0.04 kcal/mole, and therefore increases the experimental precision.

DISCUSSION

The EE-FRET method

Equilibrium constants describing TM helix interactions have been previously measured by acquiring either emission (6;13-15) or excitation FRET spectra (23). Experimental challenges can arise because the concentration of donors and acceptors cannot be verified in the “FRET

samples” (dye:lipid ratios never exceed 1/500 and absorbance measurements lack the requisite sensitivity). Therefore, sample-to-sample variations in protein concentrations, most probably due to the very low solubility of the hydrophobic TM helices, can introduce uncertainties in the measured FRET efficiencies and the calculated dimerization free energies.

The presented EE-FRET method, which gives the free energies of interactions with high precision, involves the acquisition of both emission and excitation spectra. In this paper, the method was verified using previously published data for wild-type FGFR3 TM domain (13), and was used to calculate the free energy of dimerization for the Gly382Asp pathogenic FGFR3 mutant as $\Delta G = -2.78 \pm 0.04$ kcal/mole. This value was determined with high precision from single measurements at six peptide-to-lipid ratios. The actual donor and acceptor concentrations were determined for each sample, the dimer fraction was calculated using the actual donor and acceptor concentrations rather than the intended concentrations, and the calculated dimer fractions were plotted as a function of the actual total peptide concentration (Figure 6). Thus, several sources of statistical errors were eliminated, resulting in high experimental precision.

As discussed above, there should be a wavelength range for which the fluorescence of the acceptor in the presence and absence of the donor are the same (i.e., the black and the blue spectra coincide at λ_{ex}^A , see Figure 1B). This spectral feature is crucial for the applicability of the EE-FRET method. We point out that this is a feature of many FRET pairs, such as Cy3 and Cy5, YFP and Cherry, etc. Thus, the EE-FRET method that we introduce is not restricted to fluorescein and rhodamine, but has a broad utility.

We note that the presented EE-FRET analysis is valid only when the fluorescence intensity of the dyes (in the absence of FRET) is linear with dye concentrations. Otherwise, self-quenching or inner filter effects, which occur at high concentrations, impair the quantitative FRET analysis. We have found that self-quenching does not occur for donor concentrations that are lower than 0.3 mol% (19). Therefore, concentrations that exceed 0.3 mol% are not recommended for measurements of TM helix dimerization using FRET. We further note that the measured fluorescence intensities are proportional to the total amount of fluorescent dyes in the probed volume. Thus, variations in lipid concentration will also introduce uncertainties in the protein-to-lipid ratios. Although these errors cannot be corrected using the EE-FRET method, we have found that these variations are small (19).

The presented EE-FRET method can be useful in cases when the donor and acceptor concentration, and the total protein concentration, are not controlled or are unknown. Such a situation occurs in cellular studies, when the expression of the donor-tagged and the acceptor-tagged proteins cannot be controlled. Previously, unknown concentrations have been estimated by photobleaching of the acceptor (24) or of the donor, such that FRET is “turned off”. The resulting changes in F_{DA} and F_{AD} can be linked to the actual initial F_D and F_A values and to the initial FRET efficiency. However, there are problems with such approach: (i) the bleaching rate of the free dyes and of dyes in the donor-acceptor complexes could be different, (ii) donor and acceptor spectra are often not well separated, such that the dyes cannot be bleached independently, and (iii) photoconversion during bleaching may impair further calculations. The described EE-FRET method is free from such problems, and thus could have applications in cases when the bleaching approach is not optimal.

The FGFR3 Gly382Asp mutation

The Gly382Asp mutation in the TM domain of FGFR3 has been identified in the KSM-18 multiple myeloma cell line (16). Unlike other FGFR3 mutations, it has not been so far identified in skeletal disorders. The mutation does not affect FGFR3 activity in the KSM-18 cell line, and it is not capable of inducing foci formation in NIH 3T3 cells(22). These findings are

consistent with our results, which demonstrate that the mutation does not affect the dimerization energetics of FGFR3 TM domain. A previously published model of FGFR3 TM domain, created with the software CHI, identifies the residues that mediate helix-helix contact to be Leu377, Val381, Phe384, and Ile387 (13). In this model, Gly382 does not interact with the neighboring helix and is exposed to lipids. Based on this model, it can be expected that the substitution of Gly382 with any other amino acids does not affect dimer stability, consistent with our findings. We note that the Gly382Asp mutation differs from the Crouzon Ala391Glu mutation, which stabilizes the FGFR3 TM dimer by -1.3 kcal/mole. It has been proposed that Glu in position 391 forms a hydrogen bond with the neighboring helix in the FGFR3 dimer (13). However, Asp in position 382 does not appear to participate in stabilizing hydrogen bonding interactions.

Ronchetti et al (22) found that, in transfected 293T cells, the Asp382 mutant activates the MAP-kinase pathway in a ligand-independent manner, while the wild-type required ligands. This result demonstrates that the Gly382Asp mutation is capable of causing over-activation, despite the fact that it does not stabilize the dimer. It can be therefore hypothesized that other mechanism of pathogenesis are at play. In this respect, the Gly382Asp mutation is similar to the achondroplasia Gly380Arg mutation, which does not affect FGFR3 TM dimer stability, but causes deregulated signaling.

Some RTK mutations have been shown to induce slow downregulation of the activated receptor dimers. Examples include the achondroplasia mutation in the TM domain and the thanatophoric dysplasia II Lys650Glu mutation in the catalytic domain of FGFR3. These findings demonstrate that dimer stabilization is not necessarily required for pathogenesis. It could be expected that the Gly382Asp mutation also compromises the downregulation of the FGFR3 receptor. Indeed, immunofluorescence studies of the localization of the Gly382Asp receptor have revealed intense cytoplasmic staining (22).

In model bilayers, the achondroplasia mutation has been shown to induce a shift in the hydrocarbon core embedded segment of FGFR3 TM domain. Based on this result, we have previously hypothesized that a mutation-induced change in membrane topology may be the structural basis for the slow receptor downregulation in achondroplasia. Asp382 is not expected to participate in the dimer interface and is likely exposed to lipids, which is energetically unfavorable and may lead to changes in membrane topology. Future structural studies of the Gly382Asp mutant in bilayers, as well as cellular studies that address the fate of the Gly382Asp activated receptors, should shed further light on the role of the Gly382Asp mutation in multiple myeloma pathogenesis.

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Figure 1.

Emission and excitation spectra of fluorescein (FL) and rhodamine (Rhod), a common FRET pair, conjugated to TM_{WT}. (A) Excitation spectra, collected by recording emission at 595 nm while scanning the excitation from 400 to 570 nm. (B) Emission spectra. The excitation was fixed at 439 nm, and the emission was scanned from 450 to 700 nm. In (A) and (B), the blue lines correspond to 0.1mole % FL-TM_{WT}, while the red lines are for 0.1mole % Rhod-TM_{WT}. The blue and red spectra serve as standard excitation and emission spectra, to be used with the EE-FRET method. These spectra are averages, derived from measurements of multiple samples, prepared as described in Materials and Methods. Also shown are the excitation and emission spectra of 0.1mole % FL-TM_{WT} and 0.1mole % Rhod-TM_{WT} (the donor/acceptor sample, or “FRET sample”, black lines). The FRET spectrum (black) is the sum of three contributions: direct donor emission in the presence of the acceptor F_{DA} , direct acceptor emission F_A , and sensitized acceptor emission F_{sen} . Inspection of the excitation spectra in (A) reveals that at excitation wavelength λ_{ex}^A the acceptor excitation reaches its maximum, while the donor excitation is negligible. As a result, the excitation of the donor/acceptor sample at λ_{ex}^A is contributed by the acceptor only. In (B), the emission of the donor and the acceptor reach their maxima at λ_{em}^D and λ_{em}^A , respectively. Note that the emission of the acceptor is low, but not negligible, at λ_{em}^D . $\lambda_{ex}^D = 439$ nm is the excitation wavelength used in the acquisition of the emission spectra.

Figure 2.

The EE-FRET method.

(A). An acquired FRET excitation spectrum (black line) is compared to the excitation standard spectrum of the acceptor (Figure 1A, red line). This step gives the concentration of the acceptor in the sample.

(B). The emission standard of the acceptor (Figure 1B, red line) is scaled according to the acceptor concentration determined in (A) (red line). The difference between the red line and the FRET emission spectrum (black line) is the sum $F_{DA} + F_{sen}$.

(C). Dashed black line: $F_{DA} + F_{sen}$, sum of the direct donor emission in the presence of the acceptor, F_{DA} , and the sensitized acceptor emission, F_{sen} . The standard emission spectrum of the donor (Figure 1B, blue line) is scaled, such that the amplitude of the scaled standard (blue dashed line) is identical to the amplitude of the dashed black line at λ_{em}^D . The difference between the black dashed and the blue dashed line is the sensitized acceptor emission, F_{sen} . The value of the sensitized emission at λ_{em}^A , $F_{sen}(\lambda_{em}^A)$, is related to the decrease in donor emission at (λ_{em}^D) , $\Delta F_D(\lambda_{em}^D)$ (Equation (7)).

(D). The value $\Delta F_D(\lambda_{em}^D)$, determined in (C), is used to determine the donor emission in the absence of the acceptor F_D (blue line), and the donor concentration.

Figure 3.

Emission spectra of FI/Rhod-labeled TM_{WT} in POPC. All experimental spectra are from a previous extended study of this protein (13), and are based on multiple measurements. (A) Solid line: 0.04 mole % FI-TM_{WT} (intended) and 0.04 mole % Rhod-TM_{WT} (intended). The actual FI-TM_{WT} concentration is calculated as 0.0393%, and the calculated donor-only control spectrum is shown with the dotted line. (B) 0.1 mole % FI-TM_{WT} (intended) and 0.1 mole % Rhod-TM_{382Asp} (intended). FI-TM_{WT} concentration is calculated as 0.099%, the calculated donor-only control spectrum is shown with the dotted line. The experimental donor-only controls, shown with the dashed lines, are very similar to the calculated ones (dotted lines).

Figure 4.

A calculation of free energy of dimerization from a single FRET experiment. The intended molar concentration of both FL-TM_{wt} and Rhod-TM_{wt} is 0.15 mole % (0.15 peptides per 100 lipids; total lipid concentration is 0.25 mg/ml). The emission spectrum (black solid line) is acquired by exciting at 439 nm and scanning the emission from 450 to 750 nm. The fluorescein-only control (0.15 mole % FL-TM_{wt}, 0.25 mg/ml, dashed line) is the average of multiple sample measurements. Using the experimental fluorescein-only control (dashed line) and the single FRET spectrum (solid line), we determine, from the decrease in donor quenching, the dimerization free energy as -1.8 kcal/mole. Using the FRET spectrum and the EE-FRET method, we calculate the “actual” concentrations of FL-TM_{WT} and Rhod-TM_{WT} as 0.172 mole

% and 0.123 mole%, and the dimerization free energy as -2.9 kcal/mole. The value from an extended experimental study, based on multiple measurements at various peptide concentrations is -2.8 ± 0.1 kcal/mole.

Figure 5.

Fluorescence spectra of FI/Rhod-labeled TM_{382Asp} in POPC. (A) Excitation spectra. (B) Emission spectra. Solid lines: 0.025 mole % FI-TM_{382Asp} (intended) and 0.025 mole % Rhod-TM_{382Asp} (intended). The actual concentrations were 0.0212 mol% (FI-TM_{382Asp}) and 0.029 mol% (Rhod-TM_{382Asp}). Dashed lines: 0.15 mole % FI-TM_{382Asp} (intended) and 0.15 mole % Rhod-TM_{382Asp} (intended). The actual concentrations were 0.149 mole % (FI-TM_{382Asp}) and 0.135 mole % (Rhod-TM_{382Asp}). Samples were prepared as described in Materials and Methods.

Figure 6.

Dimer fraction $[D]/[T]$ vs peptide concentration $[T]$ for TM_{382Asp} . The symbols represent the dimer fractions calculated using the EE-FRET method, for different protein concentrations. The solid line is the theoretical equilibrium curve, obtained as described in details previously (6;13). The free energy of dimerization, as the average of the six experiments, is -2.78 ± 0.04 kcal/mole. For comparison, the average free energy value, obtained from donor quenching as previously described (19)(i.e. not using the EE-FRET method), is -2.9 ± 0.7 kcal/mole. Thus, the EE-FRET method increases the precision of TM helix dimerization energetics measurements.