Energetics of ErbB1 Transmembrane Domain Dimerization in Lipid Bilayers

Lirong Chen,[†] Mikhail Merzlyakov,[†] Tomer Cohen,[‡] Yechiel Shai,[‡] and Kalina Hristova[†*]
[†]Department of Materials Science and Engineering Johns Hopkins University, Baltimore MD 21218; and [‡]Department of Biological Chemistry, The Weizmann Institute of Science, Rehobot, Israel

ABSTRACT One of the most extensively studied receptor tyrosine kinases is EGFR/ErbB1. Although our knowledge of the role of the extracellular domains and ligands in ErbB1 activation has increased dramatically based on solved domain structures, the exact mechanism of signal transduction across the membrane remains unknown. The transmembrane domains are expected to play an important role in the dimerization process, but the contribution of ErbB1 TM domain to dimer stability is not known, with published results contradicting one another. We address this controversy by showing that ErbB1 TM domain dimerizes in lipid bilayers and by calculating its contribution to stability as −2.5 kcal/mol. The stability calculations use two different methods based on Förster resonance energy transfer, which give the same result. The ErbB1 TM domain contribution to stability exceeds the change in receptor tyrosine kinases dimerization propensities that can convert normal signaling processes into pathogenic processes, and is thus likely important for biological function.

Receptor tyrosine kinases (RTKs) are the second largest family of membrane receptors. They are single-pass membrane proteins consisting of an extracellular ligand-binding domain, a transmembrane domain (TM), and a cytosolic catalytic domain (1). Unlike G-protein coupled receptors, a single monomeric RTK cannot effectively transduce biochemical signals across the plasma membrane. Thus, lateral interactions between RTKs are critical for their function (2,3). It is well established that the first step in RTK signaling is the formation of an RTK dimer in the plasma membrane, a process that leads to the activation of the dimerized receptors. The dimerization event regulates RTK activity by controlling the distribution between inactive monomers and active dimers, and ultimately exerts control over various cellular processes, such as cell proliferation, differentiation, survival, and migration. Defects in dimerization lead to disease (4), and inhibitors of the dimerization events are already recognized as possible therapeutics with applications in the clinic, and are in trials or are approved (such as Vectibix (Amgen, Thousand Oaks, CA) and Erbitux (ImClone Systems, Branchburg, NJ) for ErbB1 and Pertuzumab (Genentech, San Francisco, CA) for ErbB2 (5)).

Crystal structures of isolated extracellular (ligand binding) domains have been solved over the past decade, and they have provided new insights into ligand binding and dimerization (6). For instance, the high-resolution structures of ErbB and FGFR ligand binding domains have shown that for these receptors, the ligands control the equilibrium between the dimerization-competent and incompetent states of the extracellular domains, by stabilizing the competent state (7–11). Thus, the successes in structure determination have lead to a dramatic increase in the mechanistic understanding of the initial steps of RTK dimerization. However, the exact mechanism of signal transduction across the membrane (i.e., the mechanism through which the interactions between the extracellular domains lead to phosphorylation within the catalytic domains) remains unknown. Because crystal structures of full-length receptors are not available, the question of how the information is communicated from the ligand binding domains to the catalytic domains has been addressed primarily in biochemical studies. In one such study, Bell et al. (12) observed that the rotation of the TM dimer interface leads to periodic oscillations in kinase activity. Furthermore, inserting residues in the C-terminal TM flanking region, which causes the kinase domain to rotate with respect to the TM domain, restores the kinase activity of the inactive receptors. These experiments show that 1), the optimal activation of an RTK occurs only for a specific TM dimer interface, and 2), the RTK TM dimer interface contains the critical structural information that positions the catalytic domains in such a way that they can phosphorylate each other. Consistent with this view, it has been proposed that at least some TM domains have two dimerization motifs, corresponding to active and inactive dimer structures (13,14). Within this view of RTK activation, the TM domains play an important structural role during the dimerization events, and are thus critical for the dimerization process and for RTK activation (15). In addition, the TM domains may be contributing to the stability of the full-length RTK dimers (16). In particular, if the TM domains form sequence-specific dimers, the RTK dimer interface would include contacts between the TM domains, and these contacts will contribute to dimer stabilization. A question remains, however, as to how large the TM domain contribution to dimer stability is.

One of the most extensively studied receptors is EGFR/ErbB. In one study, aimed at elucidating the relative contributions of the extracellular domain and the TM domain for this receptor, Tanner and Kyte showed that the extracellular EGFR domains dimerize strongly only in the presence of the TM domains (17). They estimated that the dissociation constant of the extracellular domains, when attached to the

Submitted November 25, 2008, and accepted for publication March 11, 2009.

*Correspondence: kh@jhu.edu

Editor: Mark Girvin.

0006-3495/09/06/4622/9 $2.00

doi: 10.1016/j.bpj.2009.03.004

TM domains, is at least 10,000-fold smaller, as compared with the dissociation constant of the isolated extracellular domains.

The dimerization of ErbB1 TM domains has been further investigated using the genetic TOXCAT or ToxR assays (13,14). These assays couple transmembrane segment association with the expression of chloramphenicol acetyltransferase or β-galactosidase, which is measured. These studies have shown that the TM domains of ErbB1 dimerize in bacterial membranes, consistent with the results of Tanner and Kyte (17). However, the TOXCAT/ToxR assays do not readily yield information about the oligomer size and the free energy of dimerization, a thermodynamic characteristic that allows to predict the occurrence of active dimeric species at any concentration (expression level), and to compare the contribution of the different domains in the dimerization process.

Techniques that can provide such quantitative thermodynamic data are Förster resonance energy transfer (FRET) and analytical ultracentrifugation, the latter being applicable only for detergent environments. Interactions between ErbB1 TM domains were detected in the detergent dimethyldodecylamine *N*-oxide using FRET (18). Surprisingly, no interactions were observed between ErbB1 TM domains in C_5E_8 detergent using analytical ultracentrifugation (19), in contradiction with the results of Tanner and Kyte (17), and with the TOXCAT/ToxR results. This contradiction raises two possibilities. The first is that the results of Tanner and Kyte (17) and the bacterial assay results do not register sequence-specific dimerization, but excluded volume or proximity effects, and the ErbB1 TM domains do not really interact (19). The second is that the TM domains dimerize in the native bilayer environment, but the interactions are abolished in detergent micelles, suggesting that detergents are not an appropriate system for studies of RTK TM domains, as proposed by Engelman and colleagues (20).

One way to resolve this controversy is to characterize the thermodynamics of ErbB1 dimerization in lipid bilayers. Quite remarkably, despite all the work with ErbB TM domains, such a measurement remains lacking for ErbB1. We present experimental results showing that ErbB1 TM domain dimerizes in a lipid bilayer environment. The free energy of dimerization is calculated using two different FRET-based methods, which give very similar results. These results reinforce the idea that RTK TM domains are not passive anchors during the dimerization process, and their interactions contribute a few kcal/mol to the overall stability of the RTK dimers. We show sequence-specific dimerization of ErbB1 TM domain in lipid bilayers, consistent with the proposed important structural role for RTK TM domains in signal transduction.

MATERIALS AND METHODS

Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) were purchased from Avanti Polar Lipids (Alabaster, AL). The ErbB1 TM domain, amino acid sequence KKKSIATGVVGALLLLVVALGIGLFMKKK, was synthesized using solid phase peptide synthesis, and labeled with the FRET pair of fluorescein (Fl) and rhodamine (Rhod). The labeled and unlabeled peptides were purified (>98% homogeneity) by reverse phase HPLC on a C_4 column using a linear gradient of 30%–80% acetonitrile in water containing 0.1% TFA, for 40 min. The peptides were subjected to amino acid analysis and mass spectrometry to confirm their composition and mass.

SDS-PAGE

Between 4–8 nmol of the peptide were subjected to SDS-PAGE using NuPAGE Novex Tris-Acetate Mini Gels (Invitrogen, Carlsbad, CA), following the manufacturer's protocol. The peptides were visualized with Coomassie blue.

Preparation of vesicles for circular dichroism and FRET measurements

Vesicles were prepared as described previously (21–23). Briefly, donor-labeled peptides, acceptor-labeled peptides, unlabeled peptides (as appropriate) and lipids were first mixed in TFE/chloroform, the organic solvents were removed, and the protein/lipid mixture was redissolved in 10 mM phosphate buffer, 500 mM NaCl, pH 7. Samples were then freeze-thawed several times, which substantially decreased the turbidity of the samples. We have shown that both circular dichroism (CD) and FRET spectra can be measured in such vesicles (23).

CD

The secondary structure of ErbB1 TM domain was recorded in TFE, as well as in POPC and DLPC vesicles in 10 mM phosphate with 500 mM NaCl, at pH = 7. The vesicles were prepared as described above. The CD spectra were collected using a Jasco spectropolarimeter (Oklahoma City, OK).

Oriented CD

Oriented CD measurements were carried out in DLPC multilayers as reported previously (24–26). Dropwise, a solution of peptides and lipids in TFE/chloroform was deposited on a quartz slide and the solvent was removed under a stream of nitrogen to form a multilamellar sample containing the peptides. The quartz slide with the deposited multilamellar sample was placed in a custom designed chamber, and hydrated through the vapor phase as described (27,28). CD spectra were recorded while the sample was rotated around the beam axis in increments of 45°. The eight recorded spectra were averaged and corrected for lipid background.

FRET

FRET experiments in DLPC vesicles were carried out in a Fluorolog fluorometer (Jobin Yvon, Edison, NJ) using Fl and Rhod as a donor/acceptor pair. The Förster radius, R_0, for this pair is 56 Å (29). FRET was measured in liposomes containing donor- and acceptor-labeled peptides, by fixing the excitation wavelength at 439 nm, and collecting emission spectra from 455 nm to 700 nm. The FRET efficiency, E, was calculated from the decrease in donor fluorescence at 519 nm, while using the EmEx-FRET method to eliminate uncertainties in peptide concentrations due to aliquoting (30). The measured FRET efficiency was used to calculate the free energy of dimerization as shown below.

Free energy calculations

The association constant K describing the monomer-dimer equilibrium in liposomes depends only on the protein/lipid ratio (23) and is given by:

$$K = \frac{[D]}{[M]^2}, \quad (1)$$

where $[D]$ is the dimer molar concentration in the liposomes (dimers per lipid) and $[M]$ is the monomer molar concentration in the liposomes (monomers per lipid).

Following the protocol of You et al. (23), E was measured as a function of peptide/lipid ratio. In these experiments, the measured FRET efficiency has two contributions: one due to sequence-specific dimerization and one due to random colocalization (proximity) of donors and acceptors. The proximity contribution, $E_{\text{proximity}}$, is modeled computationally as described in (21,23) and subtracted from the measured FRET efficiency E to obtain the contribution due to sequence-specific dimerization.

$$E_{\text{dimer}} = E - E_{\text{proximity}}. \tag{2}$$

On the other hand,

$$E_{\text{dimer}} = f_{\text{D}} \times x_{\text{a}}, \tag{3}$$

where $f_{\text{D}} = \dfrac{2[D]}{[T]}$ is the dimeric fraction, $x_{\text{a}} = \dfrac{[a]}{[d]+[a]}$ is the acceptor fraction, and T is the total peptide concentration, $[T] = [d]+[a]$. Here $[d]$ and $[a]$ denote the donor and acceptor concentration, respectively.

Thus, the dimer concentration can be calculated as:

$$[D] = \frac{E_{\text{dimer}} \times [T]}{2x_{\text{a}}}. \tag{4}$$

The monomer concentration is given by $[M] = [T]-2[D]$, and the equilibrium constant K is determined using Eq. 1.

Finally, the free energy of dimerization is calculated as:

$$\Delta G = -RT\ln K. \tag{5}$$

The major shortcoming of the above method is the fact that FRET due to sequence-specific dimerization is obtained by subtracting a calculated contribution from the experimentally measured FRET efficiency. Whereas multiple lines of evidence have shown that the proximity contribution model is correct (21,29,31), a second independent method to characterize the dimerization energetics that does not depend on the exact value of $E_{\text{proximity}}$ is highly desirable.

Occurrence of sequence-specific dimerization can be shown directly by measuring the change in the FRET efficiency upon the addition of unlabeled peptides (32). It has been shown that if dimerization occurs, the addition of unlabeled peptides to donor- and acceptor-labeled dimers will decrease the FRET efficiency (21,32). This control experiment has shown that glycophorin A and FGFR3 form sequence-specific dimers in SDS and in POPC vesicles, respectively (21,32). We show that this method can be extended to yield equilibrium constants, independently from the calculations described by Eqs. 2–4 above. The advantage of this new alternative method is that $E_{\text{proximity}}$ does not change in the presence of the unlabeled peptide (because it depends only on the acceptor concentration). Thus, the measured decrease in the FRET efficiency is only due to changes in the sequence-specific FRET, E_{dimer}. The change in the measured FRET efficiency, ΔE, from E_1 to E_2 after the addition of unlabeled peptide is:

$$\begin{aligned}\Delta E &= E_1 - E_2\\ &= \left(E_{\text{dimer},1} + E_{\text{proximity},1}\right) - \left(E_{\text{dimer},2} + E_{\text{proximity},2}\right)\\ &= E_{\text{dimer},1} - E_{\text{dimer},2}\\ &= f_{\text{D},1} \times x_{\text{a},1} - f_{\text{D},2} \times x_{\text{a},2}.\end{aligned} \tag{6}$$

Furthermore,

$$K = \frac{[D]}{[M]^2} = \frac{[D]}{([T]-2[D])^2}. \tag{7}$$

From Eq. 7, we obtain the following quadratic equation for $[D]$:

$$K([T]-2[D])^2 = [D].$$

The solution for this equation is:

$$[D] = \frac{4K[T] + 1 \pm \sqrt{8K[T]+1}}{8K},$$

and the dimetic fraction is:

$$f_{\text{D}} = \frac{2[D]}{[T]} = 1 + \frac{1}{4K[T]} \pm \frac{\sqrt{8K[T]+1}}{4K[T]}.$$

Because $f_{\text{D}} \le 1$,

$$f_{\text{D}} = 1 + \frac{1}{4K[T]} - \frac{\sqrt{8K[T]+1}}{4K[T]}.$$

By substituting f_{D} into Eq. 6, we obtain

$$\begin{aligned}\Delta E = &\left(1 + \frac{1}{4K[T]_1} - \frac{\sqrt{8K[T]_1+1}}{4K[T]_1}\right) \times \frac{[a]_1}{[a]_1+[d]_1}\\ &- \left(1 + \frac{1}{4K[T]_2} - \frac{\sqrt{8K[T]_2+1}}{4K[T]_2}\right)\\ &\times \frac{[a]_2}{[a]_2+[d]_2+[u]}.\end{aligned} \tag{8}$$

In Eq. 8, $[T]_1 = [d]_1 + [a]_1$, and $[T]_2 = [d]_2 + [a]_2 + [u]$, where $[d]$ and $[a]$ are the donor and acceptor concentrations defined above, and $[u]$ is the concentration of unlabeled peptide.

The only unknown in Eq. 8 is the equilibrium constant K, such that K can be easily determined. The free energy of dimerization is calculated according to Eq. 5.

A problem may arise, however, if the interactions between the TM domains are weak (such as the weak interactions measured for two other RTK TM domains, FGFR3 (29) and EphA1 (33)). Then ΔE would be small, and thus special precautions should be taken to reduce random errors. We thus used the following protocol for sample preparation. Lipids in chloroform were mixed with donor-labeled and acceptor-labeled peptides in TFE. Then, the sample was slit into two ''twin'' samples, and unlabeled peptides were added to only one of them. The organic solvents in the samples were removed and the samples were hydrated to produce liposomes. This procedure ensured that the labeled peptide and lipid concentrations were identical, and most importantly, the donor/acceptor molar ratio was exactly the same in both samples. Experimental uncertainties are limited to possible variations in the reconstitution of the peptides in the vesicles. Thus, the changes of the ratio of the sensitized acceptor emission I_{sen} to the emission of the donor in the presence of the acceptor I_{da} upon the addition of the unlabeled peptides was used to determine ΔE.

As discussed in detail in Merzlyakov et al. (30), the sensitized acceptor emission I_{sen} is equal to the decrease in donor emission in the presence of the acceptor ΔI_{d} for the Fl/Rhod pair, more precisely $I_{\text{sen}}/\Delta I_{\text{d}} = 1.00 \pm 0.05$ (30). Therefore:

$$E = \frac{\Delta I_{\text{d}}}{I_{\text{d}}} = \frac{\Delta I_{\text{d}}}{I_{\text{da}} + \Delta I_{\text{d}}} = \frac{I_{\text{sen}}}{I_{\text{da}} + I_{\text{sen}}} = \frac{I_{\text{sen}}/I_{\text{da}}}{I_{\text{sen}}/I_{\text{da}} + 1}. \tag{9}$$

The decrease in the FRET efficiency in the presence of unlabeled peptides was calculated as:

$$\Delta E = \left(\frac{I_{\text{sen}}/I_{\text{da}}}{I_{\text{sen}}/I_{\text{da}} + 1}\right)_{\text{no\_unlabeled}} - \left(\frac{I_{\text{sen}}/I_{\text{da}}}{I_{\text{sen}}/I_{\text{da}} + 1}\right)_{\text{with\_unlabeled}}. \tag{10}$$

RESULTS

SDS-PAGE

To probe the dimerization propensities of ErbB1 TM domains, we first subjected the TM domains to SDS-PAGE.

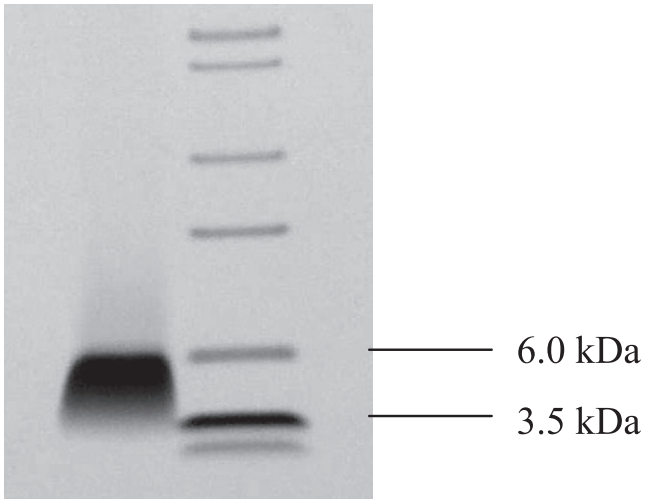

FIGURE 1 ErbB1 TM domain runs as a single band on an SDS-PAGE gel. In this experiment 8 nmol of the peptide were dissolved in 1× NuPAGE LDS sample buffer and heated at 70°C for 10 min before loading onto NuPAGE Novex Tris-Acetate Mini Gel. The gel was run at 150 V for 1 h. The peptide was visualized by staining with Coomassie brilliant blue.

As shown in Fig. 1, a single band was observed, corresponding to a molecular weight between the expected weights of the monomer (~3000) and the dimer (~6000). Because TM domains are known to sometimes run anomalously on a gel (34), it is not clear if this single band corresponds to monomeric or dimeric species. The observation of a single band is consistent with previous studies of a construct of ErbB1 TM domain linked to a soluble protein, which runs as a monomer (19). Thus, although our data cannot prove it, it can be argued that the band in Fig. 1 is monomeric.

The ErbB1 dimerization propensity in SDS can be compared to the propensity of other characterized RTK TM domains, such as FGFR3 TM domain and its pathogenic mutants (22,29,35), which exhibit both monomeric and dimeric bands on SDS gels. Thus, ErbB1 TM domain seems to have a lower dimerization propensity than FGFR3 TM domain in SDS.

Secondary structure of ErbB1 TM domain

The secondary structure of ErbB1 TM domains in different hydrophobic environments was characterized using CD. Fig. 2 *A* shows the CD spectrum in TFE, which is consistent with a helical structure, as is the spectrum in SDS (not shown). Next, we investigated the secondary structure in POPC vesicles. To our surprise, a single minimum was observed at ~220 nm (Fig. 2 *B*), which is characteristic of β-sheets, rather than α-helices. We hypothesized that the reason for this observation is that the ErbB1 TM helix with its flanking lysines may be too short to span the POPC bilayer. We thus measured the secondary structure of ErbB1 TM domain in thinner bilayers made of DLPC, having only 12 carbon acyl chains. As seen in Fig. 2 *C*, the CD signal in DLPC (*solid line*) seems consistent with α-helix formation, as evidenced by the presence of a minimum around 208 nm. The quality of this CD signal, however, is rather poor because of the high turbidity of the liposomal solutions.

Previous thorough investigations of the suitability of various liposomal systems for FRET studies have shown that both multilamellar vesicles that have been subjected to several freeze-thaw cycles and extruded large unilamellar vesicles are appropriate for FRET studies of TM helix

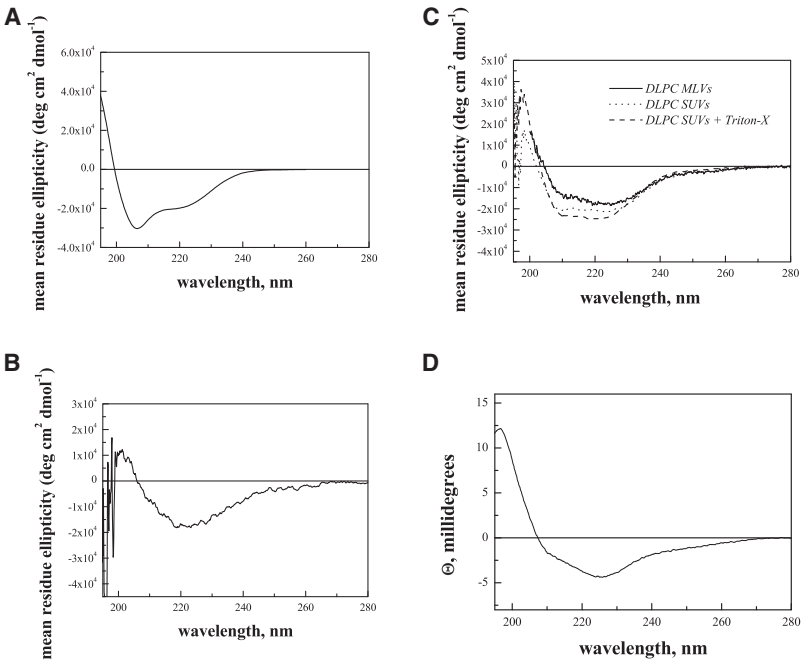

FIGURE 2 (*A*) CD spectrum of ErbB1 TM domain in TFE. (*B*) CD spectrum of ErbB1 TM domain in POPC vesicles. The spectrum suggests β-sheet secondary structure. (*C*) CD spectrum of ErbB1 TM domain in DLPC vesicles (*solid line*). Although the turbidity of the liposomal solution is high, this spectrum is indicative of α-helical secondary structure. The CD spectra after sonication (*dotted line*) and after the addition of Triton-X (2 detergent molecules per 100 lipids, *dashed line*) confirm that the secondary structure is α-helical (see text). (*D*) Oriented CD spectrum of ErbB1 TM domain in oriented DLPC multilayers. The spectrum is consistent with transmembrane orientation.

dimerization (23) and yield very similar results. Both of these systems, however, show very high turbidity that obscures the CD signal. Thus, to convince ourselves that the CD signal in Fig. 2 *C* (*solid line*) is indeed helical, we sonicated the liposomes and recorded the CD signal, which is shown with the dotted line in Fig. 2 *C*. We also added small amount of Triton-X (2 detergent molecules per 100 lipids). These two procedures reduced the turbidity and allowed us to record CD spectra of better quality, showing minima around 208 and 222 consistent with α-helical secondary structure. We thus concluded that DLPC, but not POPC, is an appropriate bilayer system for studies of ErbB1 dimerization.

Next we investigated if the helix is transmembrane in DLPC using oriented circular dichroism (OCD). TM helices exhibit characteristic OCD spectra with a single minimum around 220–230 nm and a maximum around 200 nm. In contrast, helices that are parallel to the membrane plane exhibit a much stronger OCD signal with minima at 208 and 222 nm (25,26). The samples used in the OCD experiments were DLPC multilayers containing 5 mol % ErbB1 TM domain, deposited on a quartz slide. The experimental OCD spectrum is shown in Fig. 2 *D*. The shape of the spectrum, with a single minimum around 230 nm, indicates that the ErbB1 TM domain is perpendicular to the DLPC bilayer normal.

In summary, CD and OCD measurements show that the ErbB1 peptide is helical and transmembrane in DLPC bilayers.

FRET and free energy calculations

For the FRET experiments, the ErbB1 TM domain was labeled with the FRET pair of Fl/Rhod. The labeled peptides were purified by HPLC, such that labeling yield was 100%. The FRET efficiencies for a series of samples containing Fl- and Rhod-labeled ErbB1 TM domains were measured as described in Materials and Methods. Fig. 3 shows typical emission spectra of liposomes containing the donor (Fl) only (*dashed line*) and liposomes containing the same concentration of donor in the presence of the acceptor (Fl and Rhod, *solid line*). In Fig. 3, FRET is obvious from the decrease in donor fluorescence and the appearance of sensitized acceptor fluorescence. FRET efficiency is calculated as $E = \Delta I_d/I_d$, where ΔI_d is the change in donor emission at 519 nm, and I_d is the donor emission in the absence of the acceptor at 519 nm.

First, FRET was measured for a constant peptide/lipid ratio, but varying donor/acceptor ratio. The linear dependence of the energy transfer on the acceptor mole ratio, shown in Fig. 4, is indicative of dimer formation (23,36–38). Therefore, ErbB1 TM domain exists in a monomer-dimer equilibrium and does not form higher order aggregates.

Next, FRET was measured for a constant donor/acceptor ratio, but varying peptide/lipid ratio. Fig. 5 shows the total

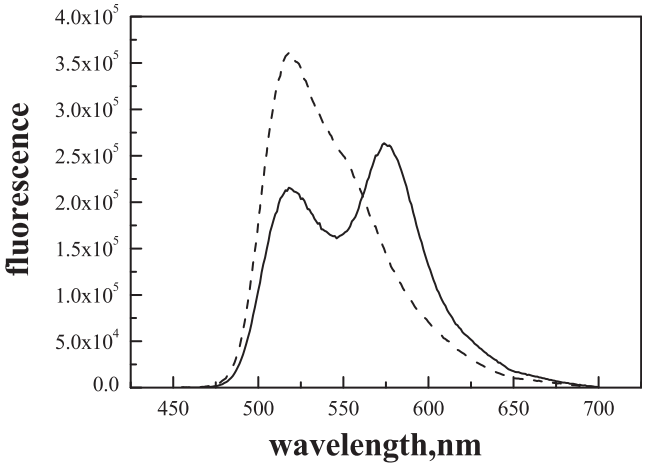

FIGURE 3 Fluorescence spectra of Fl- and Rhod-labeled peptide mixtures in liposomes. The spectra shown are for DLPC vesicles with 0.2 mol % Fl-labeled ErbB1 TM domain (*dotted line*) and for DLPC vesicles with 0.2 mol % Fl-labeled and 0.2 mol % Rhod-labeled ErbB1 TM domain (*solid line*) The excitation was fixed at 439 nm, such that only Fl was directly excited. The emission was scanned from 455 to 700 nm. FRET efficiency is calculated from the decrease of Fl emission in the presence of Rhod at 519 nm.

measured FRET efficiency E as a function of acceptor concentration (*squares*). The FRET efficiency that arises due to random proximity effects was modeled according to the analysis of Wolber and Hudson (39), and is shown with the dotted line for comparison. To calculate the FRET efficiency due to sequence-specific dimerization, E_{dimer}, the predicted FRET efficiency due to proximity was subtracted from the measured FRET signal (23). The equilibrium constant describing the dimerization process was calculated

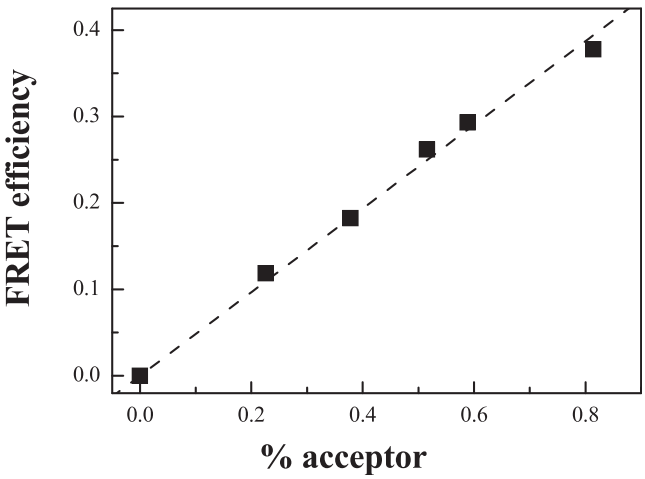

FIGURE 4 Measured FRET efficiency in DLPC vesicles as a function of acceptor mole ratio. The peptide/lipid ratio was kept constant at 1:500, whereas the ratio of donor-labeled/acceptor-labeled peptides was varied. The linear dependence of the energy transfer on the acceptor mole ratio is indicative of the formation of dimers and shows that there are no higher order aggregates.

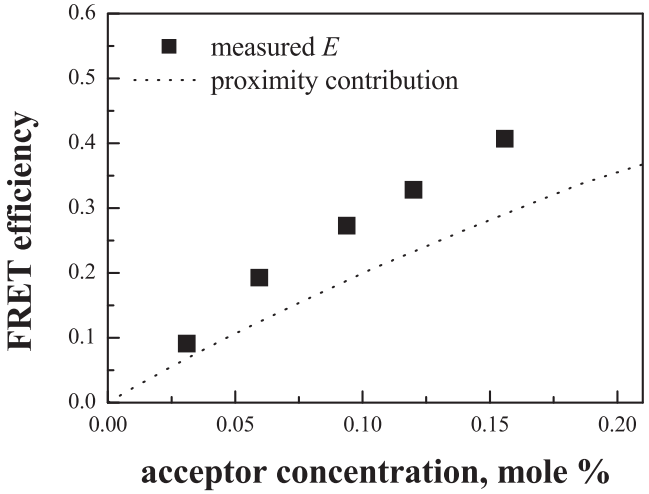

FIGURE 5 Measured FRET efficiencies for an equimolar mixture of Fl- and Rhod-labeled peptides in DLPC vesicles (*solid symbols*), as a function of Rhod (acceptor) concentration. Whereas the donor/acceptor ratio was held constant, the peptide/lipid concentration was varied. Also shown is the prediction for the proximity contribution to the FRET efficiency. For a quantitative description of dimerization, the measured FRET efficiency is corrected for this contribution. The free energies of sequence-specific dimerization are calculated using Eqs. 2–5 and are shown in Table 1.

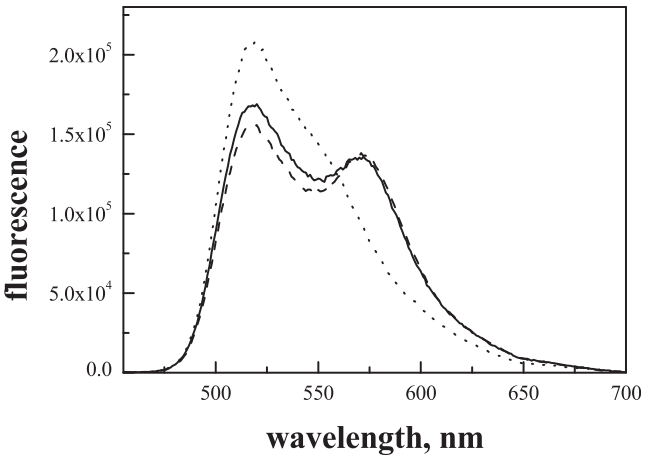

FIGURE 6 Fluorescence spectra of 0.1 mol % Fl-labeled and 0.1 mol % Rhod-labeled ErbB1 TM domain in DLPC vesicles (*dashed line*). The solid line is the FRET spectrum in the presence of 0.3 mol % unlabeled peptide. Thus, the FRET efficiency decreases in the presence of the unlabeled peptide. The free energy of dimerization is calculated from this decrease using Eqs. 6–10. Three different samples were prepared as described in the text, and the results for ΔG are shown in Table 2. The dotted line is the emission of Fl-labeled ErbB1 in the absence of Rhod-labeled peptides (i.e., the "no-FRET" control).

using Eqs 1 and 4. The free energy of dimerization of ErbB1 TM domain, ΔG, given by Eq. 5, was calculated as -2.5 ± 0.1 kcal/mol (Table 1).

As discussed in Materials and Methods, the major shortcoming of the above method is that the proximity contribution is predicted rather than measured. In particular, it depends on the value of the Förster's radius, R_0, and the predictions carry a certain degree of uncertainty (23). This uncertainly impacts the calculated values of the equilibrium constants and dimerization free energies, particularly for weakly dimerizing helices such as ErbB1. Previously, we have presented various arguments that the predicted proximity contribution is correct (21,29) and we have verified it using lifetime measurements (31). Nevertheless, in this study we developed and used a second independent method to characterize the dimerization energetics. In particular, we analyzed the decrease in FRET efficiency in the presence of unlabeled peptides. This experiment has been used previously to show sequence-specific dimerization (21,32), and now we show that this method can yield dimerization free energies.

TABLE 1 Calculations of the dimerization free energy of ErbB1 TM domain, ΔG, based on Eqs. 2–5 and the FRET efficiency measurements shown in Fig. 5

Protein/lipid ratio (%)	ΔG (kcal/mol)
0.05	−2.2
0.1	−2.6
0.2	−2.5
0.3	−2.5
0.4	−2.6
Average	-2.5 ± 0.1

Fig. 5 compares fluorescence spectra of Fl- and Rhod-labeled ErbB1 peptides in liposomes without and with unlabeled peptides. The unlabeled peptide is not expected to have an effect on the FRET signal if the FRET signal is solely due to random proximity of donors and acceptors. A decrease in FRET, however, is the hallmark of sequence specific dimerization (32), and it reflects the substitution of donors and acceptors in donor-acceptor dimers with unlabeled peptides. In Fig. 6 we see a decrease in the FRET efficiency in the presence of the unlabeled peptide, which seems rather modest. Such a modest effect is expected because the decrease is only in the portion of the measured FRET that is due to sequence specific dimerization. Although this modest decrease requires very careful measurements, it should be noted that because $E_{\text{proximity}}$ has no effect on the measured change in the FRET efficiency, this method yields the equilibrium constant K without the explicit knowledge of $E_{\text{proximity}}$ (see Eq. 8). Equations 5 and 8 were used to calculate K and ΔG for three different samples containing 0.1 mol % Fl, 0.1 mol % Rhod, and 0.3 mol % unlabeled peptide.

In three different vials, aliquots of lipids in chloroform, as well as donor-labeled and acceptor-labeled peptides in TFE were mixed. Then, the sample in each of the vials was slit into two "twin" samples, and unlabeled peptides were added to only one of them. Thus, the labeled peptide and lipid concentrations were identical within each twin set. A total of six samples, or three twin sets were examined, and three values for ΔG were calculated, one for each twin set, from the change in FRET according to Eq. 10. The results are shown in Table 2 and the average is $\Delta G = -2.4 \pm 0.4$ kcal/mol. The comparison of the values in Tables 1

TABLE 2 Calculations of the dimerization free energy of ErbB1 TM domain, ΔG, via dilution with unlabeled peptides, using Eqs. 6–10

	I_{sen}/I_{da}no unlabeled peptide	$I_{sen}/I_{da}$with unlabeled peptide	ΔE	ΔG (kcal/mol)
1	0.346	0.262	0.049	−2.9
2	0.367	0.348	0.010	−2.0
3	0.347	0.314	0.019	−2.3
Average				−2.4 ± 0.5

Calculations are based on FRET spectra for 0.1% Fl-labeled and 0.1% Rhod-labeled peptides in the presence and absence of 0.3% unlabeled peptides (Fig. 6).

and 2 shows that the two methods yield very similar results.

DISCUSSION

In this study, we assessed the dimerization propensity of ErbB1 TM domain in DLPC bilayers, and we calculated its dimerization free energy. This study addressed a controversy in the literature about the role of ErbB1 TM domains in the dimerization and activation of the full-length ErbB1 receptor. We showed that sequence-specific interactions between ErbB1 TM domains do occur in DLPC bilayers, and the free energy of dimerization is −2.5 kcal/mol. These results were obtained using FRET, and are based on two independent FRET-based calculations.

Measurements of TM helix dimerization based on FRET

As shown previously (23), FRET can yield information about TM helix dimerization in a lipid bilayer environment. FRET involves the nonradiative transfer of energy from the excited state of a donor to an acceptor, both coupled to the TM domains. If a donor-labeled peptide and an acceptor-labeled peptide dimerize, the donor and the acceptor will be brought in close contact in the dimer, such that FRET will occur. However, FRET can also arise due to proximity effects, i.e., random colocalization of the donor-labeled and the acceptor-labeled peptides. Therefore, the measured FRET efficiency E has two contributions, one due to sequence-specific FRET, E_{dimer}, and one due to proximity, $E_{proximity}$.

In this study, we used two methods based on FRET to calculate the dimerization free energy of ErbB1 TM domain. First, we measured the FRET efficiency for a series of liposomal samples containing donor- and acceptor-labeled ErbB1 TM, at different peptide/lipid ratios. The modeled FRET efficiency due to the proximity effect ($E_{proximity}$) was subtracted from the total efficiency (E) to obtain the FRET efficiency due to sequence specific dimerization (E_{dimer}). Knowledge of E_{dimer} allowed us to calculate the dimerization free energy, as described previously (23,35,40). The value is −2.5 ± 0.1 kcal/mol.

In this study, we also introduced a new method that is based on the fact that if sequence-specific dimerization occurs, the addition of unlabeled peptides to the vesicles containing donor- and acceptor-labeled peptides will decrease the measured FRET efficiency. This decrease is due to the formation of dimers between the unlabeled peptides and the donor- or acceptor-labeled peptides. The dimerization free energy was calculated from the measured decrease in the FRET efficiency as −2.4 ± 0.4 kcal/mol. Most importantly, this calculation did not require the explicit knowledge of the FRET efficiency that arises due to the random colocalization of donors and acceptors. The two methods give similar results, and we expect that both methods will have broad use in studies of TM helix dimerization in lipid environments.

Dimerization of RTK TM domains in lipid bilayers and in detergents

SDS-PAGE is a popular technique to asses whether TM domains have propensity for self-association. In this study, we observe that ErbB1 TM domain runs as a single band on a SDS gel, as reported previously (19). In DLPC bilayers, however, ErbB1 TM domains form dimers, and the free energy of dimerization in DLPC is −2.5 kcal/mol. This value is similar to the dimerization propensity of FGFR3 TM domain in POPC, −2.8 ± 0.1 kcal/mol.

One unexpected finding of this study is that although the strength of the interactions for ErbB1 and FGFR3 TM domains in lipid bilayers seems similar, ErbB1 runs as a single band on an SDS gel (most probably a monomer), whereas FGFR3 monomers and dimers coexist in SDS (21,22). These findings suggest that there is not always a strong correlation between TM helix dimerization behavior in detergents and in bilayers. This result has an important implication, because SDS-PAGE is often used for an initial assessment of interactions between TM helices.

RTK TM domains and signal transduction

We measured the dimerization free energy of ErbB1 TM domain in DLPC bilayers as −2.5 kcal/mol. A major finding of this study is that the strength of ErbB1 interactions is very similar to the measured dimerization strengths for two other RTK TM domains (FGFR3 (29) and EphA1 (33)). Thus, the interactions within these three RTK TM domain homodimers are rather weak, and in particular much weaker than the interactions within the well characterized stable glycophorin A dimer. This finding is not surprising because weak dimerization in RTK signaling should allow for a tight control over the monomer/dimer equilibrium (16). We find it remarkable, however, that the TM domains of ErbB1, FGFR3, and EphA1 exhibit very similar dimerization energetics, despite being members of three different RTK families. It is not known whether the dimerization propensities of all 59 RTK TM domains are similar, or whether there are variations

in the strength of their interactions. In one study (18), in the detergent dimethyldodecylamine *N*-oxide, a range of interaction strengths were observed for the TM domains of the ErbB family of receptors. A question remains whether such hierarchy exists in lipid bilayers and in cellular membranes.

One of the open questions in RTK signaling pertains to the relative contribution of the TM domain to full-length RTK dimer stability. This question is difficult to answer because association between TM domains is probed in 2D membrane systems, whereas the interactions between isolated soluble extracellular domains have been characterized in 3D only (i.e., in aqueous solution) (41). Thus, the measurements for the extracellular and TM domains are difficult to compare. However, we have shown previously that modest changes in receptor dimerization propensities, of the order of ~ −1 kcal/mol, may be enough to cause receptor overactivation, likely because the signals are amplified downstream in the signaling cascades (16,42). In this respect, it seems that the −2.5 to −3 kcal/mol that the TM domains are contributing to RTK dimer stability is important for their biological functions.

This study was supported by the Delores and Eugene M. Zemsky Johns Hopkins University-Weizmann Institute of Science Cooperative Program, and in part by National Institutes of Health GM068619 to K.H. Y.S. has the Harold S. and Harriet B. Brady Professorial Chair in Cancer Research.

REFERENCES

1. Fantl, W. J., D. E. Johnson, and L. T. Williams. 1993. Signaling by receptor tyrosine kinases. *Annu. Rev. Biochem.* 62:453–481.
2. Schlessinger, J. 2000. Cell signaling by receptor tyrosine kinases. *Cell.* 103:211–225.
3. Weiss, A., and J. Schlessinger. 1998. Switching signals on or off by receptor dimerization. *Cell.* 94:277–280.
4. Linggi, B., and G. Carpenter. 2006. ErbB receptors: new insights on mechanisms and biology. *Trends Cell Biol.* 16:649–656.
5. Sakai, K., H. Yokote, K. Murakami-Murofushi, T. Tamura, N. Saijo, et al. 2007. Pertuzumab, a novel HER dimerization inhibitor, inhibits the growth of human lung cancer cells mediated by the HER3 signaling pathway. *Cancer Sci.* 98:1498–1503.
6. Burgess, A. W., H. S. Cho, C. Eigenbrot, K. M. Ferguson, T. P. J. Garrett, et al. 2003. An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors. *Mol. Cell.* 12:541–552.
7. Cho, H. S., and D. J. Leahy. 2002. Structure of the extracellular region of HER3 reveals an interdomain tether. *Science.* 297:1330–1333.
8. Cho, H. S., K. Mason, K. X. Ramyar, A. M. Stanley, S. B. Gabelli, et al. 2003. Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. *Nature.* 421:756–760.
9. Bouyain, S., P. A. Longo, S. Q. Li, K. M. Ferguson, and D. J. Leahy. 2005. The extracellular region of ErbB4 adopts a tethered conformation in the absence of ligand. *Proc. Natl. Acad. Sci. USA.* 102:15024–15029.
10. Plotnikov, A. N., S. R. Hubbard, J. Schlessinger, and M. Mohammadi. 2000. Crystal structures of two FGF-FGFR complexes reveal the determinants of ligand-receptor specificity. *Cell.* 101:413–424.
11. Plotnikov, A. N., J. Schlessinger, S. R. Hubbard, and M. Mohammadi. 1999. Structural basis for FGF receptor dimerization and activation. *Cell.* 98:641–650.
12. Bell, C. A., J. A. Tynan, K. C. Hart, A. N. Meyer, S. C. Robertson, et al. 2000. Rotational coupling of the transmembrane and kinase domains of the Neu receptor tyrosine kinase. *Mol. Biol. Cell.* 11:3589–3599.
13. Mendrola, J. M., M. B. Berger, M. C. King, and M. A. Lemmon. 2002. The single transmembrane domains of ErbB receptors self-associate in cell membranes. *J. Biol. Chem.* 277:4704–4712.
14. Gerber, D., N. Sal-Man, and Y. Shai. 2004. Two motifs within a transmembrane domain, one for homodimerization and the other for heterodimerization. *J. Biol. Chem.* 279:21177–21182.
15. Fleishman, S. J., J. Schlessinger, and N. Ben-Tal. 2002. A putative molecular-activation switch in the transmembrane domain of erbB2. *Proc. Natl. Acad. Sci. USA.* 99:15937–15940.
16. Li, E., and K. Hristova. 2006. Role of receptor tyrosine kinase transmembrane domains in cell signaling and human pathologies. *Biochemistry.* 45:6241–6251.
17. Tanner, K. G., and J. Kyte. 1999. Dimerization of the extracellular domain of the receptor for epidermal growth factor containing the membrane-spanning segment in response to treatment with epidermal growth factor. *J. Biol. Chem.* 274:35985–35990.
18. Duneau, J. P., A. P. Vegh, and J. N. Sturgis. 2007. A dimerization hierarchy in the transmembrane domains of the HER receptor family. *Biochemistry.* 46:2010–2019.
19. Stanley, A. M., and K. G. Fleming. 2005. The transmembrane domains of ErbB receptors do not dimerize strongly in micelles. *J. Mol. Biol.* 347:759–772.
20. Matthews, E. E., M. Zoonens, and D. M. Engelman. 2006. Dynamic helix interactions in transmembrane signaling. *Cell.* 127:447–450.
21. Li, E., M. You, and K. Hristova. 2005. SDS-PAGE and FRET suggest weak interactions between FGFR3 TM domains in the absence of extracellular domains and ligands. *Biochemistry.* 44:352–360.
22. Iwamoto, T., M. You, E. Li, J. Spangler, J. M. Tomich, et al. 2005. Synthesis and initial characterization of FGFR3 transmembrane domain: Consequences of sequence modifications. *Biochim. Biophys. Acta.* 1668:240–247.
23. You, M., E. Li, W. C. Wimley, and K. Hristova. 2005. FRET in liposomes: measurements of TM helix dimerization in the native bilayer environment. *Anal. Biochem.* 340:154–164.
24. Vogel, H. 1987. Comparison of the conformation and orientation of alamethicin and melittin in lipid membranes. *Biochemistry.* 26:4562–4572.
25. Hristova, K., W. C. Wimley, V. K. Mishra, G. M. Anantharamaiah, J. P. Segrest, et al. 1999. An amphipathic α-helix at a membrane interface: a structural study using a novel x-ray diffraction method. *J. Mol. Biol.* 290:99–117.
26. Hristova, K., C. E. Dempsey, and S. H. White. 2001. Structure, location, and lipid perturbations of melittin at the membrane interface. *Biophys. J.* 80:801–811.
27. Han, X., M. Mihailescu, and K. Hristova. 2006. Neutron diffraction studies of fluid bilayers with transmembrane proteins: structural consequences of the achondroplasia mutation. *Biophys. J.* 91:3736–3747.
28. Han, X., K. Hristova, and W. C. Wimley. 2008. Protein folding in membranes: insights from neutron diffraction studies of a membrane beta-sheet oligomer. *Biophys. J.* 94:492–505.
29. Li, E., M. You, and K. Hristova. 2006. FGFR3 dimer stabilization due to a single amino acid pathogenic mutation. *J. Mol. Biol.* 356:600–612.
30. Merzlyakov, M., L. Chen, and K. Hristova. 2007. Studies of receptor tyrosine kinase transmembrane domain interactions: the EmEx-FRET method. *J. Membr. Biol.* 215:93–103.
31. Posokhov, Y. O., M. Merzlyakov, K. Hristova, and A. S. Ladokhin. 2008. A simple ''proximity'' correction for Förster resonance energy transfer efficiency determination in membranes using lifetime measurements. *Anal. Biochem.* 380:134–136.
32. Fisher, L. E., D. M. Engelman, and J. N. Sturgis. 1999. Detergents modulate dimerization, but not helicity, of the glycophorin A transmembrane domain. *J. Mol. Biol.* 293:639–651.
33. Artemenko, E. O., N. S. Egorova, A. S. Arseniev, and A. V. Feofanov. 2008. Transmembrane domain of EphA1 receptor forms dimers in membrane-like environment. *Biochim. Biophys. Acta.* 1778:2361–2367.

34. Therien, A. G., F. E. M. Grant, and C. M. Deber. 2001. Interhelical hydrogen bonds in the CFTR membrane domain. *Nat. Struct. Biol.* 8:597–601.

35. You, M., J. Spangler, E. Li, X. Han, P. Ghosh, et al. 2007. Effect of pathogenic cysteine mutations on FGFR3 transmembrane domain dimerization in detergents and lipid bilayers. *Biochemistry*. 46:11039–11046.

36. Li, E., and K. Hristova. 2004. Imaging FRET measurements of transmembrane helix interactions in lipid bilayers on a solid support. *Langmuir*. 20:9053–9060.

37. Adair, B. D., and D. M. Engelman. 1994. Glycophorin a helical transmembrane domains dimerize in phospholipid bilayers—a resonance energy transfer study. *Biochemistry*. 33:5539–5544.

38. Li, M., L. G. Reddy, R. Bennett, N. D. Silva, Jr., L. R. Jones, et al. 1999. A fluorescence energy transfer method for analyzing protein oligomeric structure: Application to phospholamban. *Biophys. J.* 76:2587–2599.

39. Wolber, P. K., and B. S. Hudson. 1979. An analytic solution to the Förster energy transfer problem in two dimensions. *Biophys. J.* 28: 197–210.

40. You, M., E. Li, and K. Hristova. 2006. The achondroplasia mutation does not alter the dimerization energetics of FGFR3 transmembrane domain. *Biochemistry*. 45:5551–5556.

41. Lemmon, M. A., Z. M. Bu, J. E. Ladbury, M. Zhou, D. Pinchasi, et al. 1997. Two EGF molecules contribute additively to stabilization of the EGFR dimer. *EMBO J.* 16:281–294.

42. He, L., and K. Hristova. 2008. Pathogenic activation of receptor tyrosine kinases in mammalian membranes. *J. Mol. Biol.* 384:1130–1142.