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The Transmembrane Domains of ErbB Receptors do not Dimerize Strongly in Micelles

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The epidermal growth factor receptors (erbB) constitute an important class of single pass transmembrane receptors involved in the transduction of signals important for cell proliferation and differentiation. Receptor association is a key event in the signal transduction process, but the molecular basis of this interaction is not fully understood. Previous biochemical and genetic studies have suggested that the single transmembrane helices of these receptor proteins might play a role in stabilizing the receptor complexes. To determine if the erbB transmembrane domains could provide a driving force to stabilize the receptor dimers, we carried out a thermodynamic study of these domains expressed as C-terminal fusion proteins with staphylococcal nuclease. Similar fusion constructs have been used successfully to investigate the oligomerization and association thermodynamics of a number of transmembrane sequences, including that of glycophorin A. Using SDS-PAGE analysis and sedimentation equilibrium analytical ultracentrifugation, we do not find strong, specific homo or hetero-interactions between the transmembrane domains of the erbB receptors in micellar solutions. Our results indicate that any preferential interactions between these domains in micellar solutions are extremely modest, of the order of 1 kcal mol^{-1} or less. We applied a thermodynamic formalism to assess the effect of weakly interacting TM segments on the behavior of a covalently attached soluble domain. In the case of the ligand-bound EGFR ectodomain, we find that restriction of the ectodomain to the micellar phase by a hydrophobic TM, even in the absence of strong specific interactions, is largely sufficient to account for the previously reported increase in dimerization affinity.

Keywords: transmembrane dimer; membrane protein interactions; thermodynamics; erbB receptor; EGF receptor

Introduction

Transmission of molecular signals across biological membranes is essential for normal cellular function. The epidermal growth factor receptors represent an essential class of receptor tyrosine kinases (RTKs) involved in the transduction of signals important for cell proliferation and differentiation. In humans, this family includes four receptor proteins: the epidermal growth factor receptor (EGFR, erbB1, HER1), erbB2 (HER2), erbB3 (HER3), and erbB4 (HER4). The generally accepted paradigm for signaling by these receptors is a ligand-induced rearrangement of the extracellular domains followed by receptor association. The subsequent trans-phosphorylation of the intracellular kinase domains results in downstream signaling.[1,2] The nature of the signal is determined by the specific identity of the complex; these receptors are able interact with a number of hormone ligands to form a variety of homo and hetero-oligomeric complexes.[3] This diversity of interactions yields a vast signaling network, and

Abbreviations used: CAT, chloramphenicol acetyltransferase; C_8E_5, pentaethylene glycol monooctyl ether; EGFR, epidermal growth factor receptor; HER, human epidermal growth factor receptor; GpA, glycophorin A; RTK, receptor tyrosine kinase; SB-C14, 3-(*N*,*N*-dimethylmyristyl-ammonio)propanesulfonate; SDS, sodium dodecyl sulfate; SN, staphylococcal nuclease; SNGpA, staphylococcal nuclease/glycophorin A fusion protein; SNerbB, staphylococcal nuclease/erbB fusion protein; SRV, square-root of the variance; Strimer, separate trimer; TM, transmembrane; WT, wild-type.

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unregulated signaling within the network is implicated in a number of human cancers.[4]

Discovery of the molecular basis behind the protein association reaction in the erbB receptor complexes is critical to understanding the signal transduction process. Recent structures of the ligand-binding domains of the erbB receptors have begun to provide insight into the mechanisms of signaling. With the exception of erbB2, it is now clear that the extracellular domains (ECD) of these receptors exist in an auto-inhibited conformation in the absence of ligand. Instead of bridging two receptors, ligand-binding induces a conformational change in the ECD that reveals a cryptic protein–protein interface.[5] In contrast, the ECD of ErbB2, for which there is no known ligand, exists in an already extended conformation reminiscent of the structure induced in the ECD of the other erbBs by ligand-binding. Thus, the recent structures revealed that an interface between any two erbB proteins must be entirely receptor-mediated.[5]

While the crystal structures of the extracellular portions of the erbB proteins revealed a receptor–receptor interface, the extracellular ligand-binding domains alone are not capable of recapitulating all of the erbB interactions that are known to exist.[6,7] In some cases, the ligand-binding domain is not even necessary to form an active complex. The v-*erbB* oncogene product is a truncated homolog of erbB1 that lacks most of its ligand-binding domain, but is capable of dimerizing and transforming cells in a ligand-independent manner.[8,9] Additionally, a version of erbB2 with a truncated N-terminal domain also leads to more efficient transformation than its full-length counterpart, even though the ECD is presumably not normally auto-inhibited.[10,11] Interestingly, the extracellular domain of erbB2-(HER2) is proteolytically cleaved in tumor cells to yield a soluble fragment, detectable in the serum of breast cancer patients, and an active membrane fragment. The domain structure of the membrane fragment, with just a transmembrane segment and a tyrosine kinase domain, is reminiscent of the v-*erbB* oncogene product.[12,13] Together, these results suggest that other regions of the protein are important for stabilizing and/or regulating receptor–receptor interactions, and it raises questions about the relative contribution of the single transmembrane helix and the intracellular kinase domain to the receptor association.

A number of studies have suggested that specific interactions between the transmembrane helices are in fact important for stabilizing the receptor complexes. Attention first focused on the transmembrane domain as a potentially important site of interaction when the *neu* oncogene was identified as a point mutation of valine to glutamic acid within the transmembrane (TM) of a rat ortholog of erbB2.[14] Molecular modeling and solid-state NMR experiments suggest the glutamic acid might stabilize receptor dimers by forming inter-helical hydrogen bonds.[15,16] The transforming effect of the neu glutamic acid substitution was found to depend upon the surrounding sequence context,[17] as well as absolute position in the TM.[18] The surrounding sequence incorporates a five amino acid residue motif, Small-x-x-Large-G/A, consisting of a small residue (Gly, Ala, Ser, Thr, or Pro) in the zero position, a large aliphatic residue (Ala, Val, Leu, or Ile) in position 3, followed by Gly or Ala in position four.[15] This motif was identified in a large number of receptor tyrosine kinases, suggesting the motif could also be important for normal receptor function.[19] Recently, it was postulated that the two copies of the sequence motif in erbB1 have distinct functional roles, one being important for homo-interactions and the other important for hetero-interactions with erbB2.[20]

Additional evidence for a role for the transmembrane segment in receptor association resulted from a study of the ligand-induced dimerization of the extracellular domain of EGFR (erbB1). Extending this fragment to include the membrane-spanning segment enhanced the level of observed dimerization upon addition of the EGF ligand.[21] In other studies *in vivo*, peptides corresponding to the TM regions of the protein are capable of specifically inhibiting their cognate receptor.[22,23] Interfering with the transmembrane domain can also affect hetero-dimerization. Replacement of the transmembrane domain of erbB3 with the TM sequence from the fibroblast growth factor receptor or with a minimal lipid-anchor impairs hetero-dimerization with erbB2 relative to the wild-type erbB3, suggesting the TM segment functions as something more than a membrane tether.[6]

More recently, it was found that all four of the erbB TM segments gave strong positive responses in TOXCAT, a genetic assay for helix–helix association.[24] The erbB TM domains gave TOXCAT signals that were about half the strength of wild-type glycophorin A (GpA), a well-characterized TM model system known to have strong helix–helix interactions.[23,24] All four erbB TOXCAT responses were significantly stronger than that of the disruptive GpA mutant G83I.[24] This response level in TOXCAT can be interpreted as an indication of a high propensity for the transmembrane sequence to self-associate in bacterial membranes. Moreover, mutational analysis showed that changing residues in the Sternberg & Gullick motif[15,19] and the closely related GG_4 motifs[25] affected the response levels observed in TOXCAT.[24] Surprisingly, a valine to glutamic acid mutation in erbB2, analogous to that found in rat *neu*, which is generally believed to stabilize receptor dimers, resulted in a reduction in the TOXCAT signal.[24]

Whereas TOXCAT appeared to be reporting on a sequence-specific interaction, several studies indicate active receptors are actually quite promiscuous in the transmembrane sequences they can tolerate. EGFR can accommodate a number of proline substitutions in the TM and remain responsive to EGF.[26] Several erbB1 constructs with extended or truncated transmembrane domains will still bind ligand and retain their kinase activity.[26,27]

Additionally, the transmembrane domain of the rat neu receptor can be reduced to a simplified sequence of polyvaline with two appropriately placed glutamic acid residues without a loss of receptor dimerization or activation.[28,29] Thus, whether the transmembrane domains of the erbB receptors provide a strong and specific driving force for receptor interaction remains an open question.

In this study, we address the intrinsic propensity of the erbB transmembrane sequences to self-associate using a well-characterized fusion protein expression system coupled with sedimentation equilibrium analytical ultracentrifugation. We employed a construct in which the erbB transmembrane domains were fused *via* a 14 amino acid residue linker region to the C terminus of staphylococcal nuclease (SN). Similar SN-TM fusion constructs have been used to successfully probe the sequence-dependent oligomerization of several transmembrane domains, including glycophorin A, bNIP3, phospholamban, and several designed sequences.[30–33] Compared to these helix–helix oligomers, we find that the transmembrane domains of the erbB receptors have only a minimal propensity for interaction in micellar solutions, despite giving strong signals in TOXCAT.

Results

Table 1 shows the amino acid sequences for the TM segments of the four wild-type human erbB receptors that were used in this study. SNerbB2 was originally cloned without C-terminal charges, but subsequent inclusion of these charges in the SNerbB2KRR clone did not significantly impact the results. A construct in which the rat neu valine to glutamic acid mutation was mapped onto the human erbB2 sequence and another construct with a valine to isoleucine mutation that has been associated with a lower risk of cancer[34,35] were also cloned and expressed.

Strong dimerization cannot be detected in SDS micelles

The association of the SNerbB fusion constructs was first analyzed by SDS-PAGE. This method can detect stable oligomeric complexes of membrane proteins, and it has been used previously to map the interfaces of several TM helix oligomers.[30–32] Since SDS is a detergent micelle environment, membrane proteins may retain their structure, and some non-covalent interactions can be preserved. Indeed, the isolated transmembrane domain from the rat homolog of erbB2, protoneu, has been shown to retain a helical secondary structure in SDS micelles.[36]

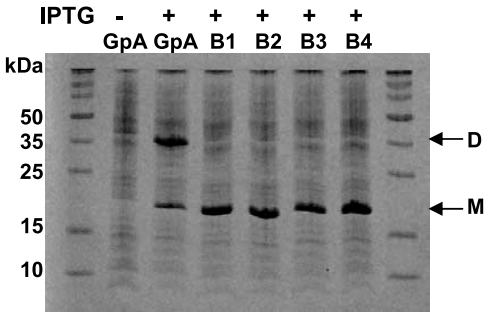


Figure 1. SDS-PAGE analysis of whole cell lysates for proteins expressing SN fusion proteins using a 20% acrylamide Phast gel. Arrows indicate the position to which monomers and dimers migrate. Lane 1, markers; lane 2, −IPTG SNGpA; lane 3, +IPTG SNGpA; lane 4, +IPTG SNerbB1; lane 5, +IPTG SNerbB2, lane 6, +IPTG SNerbB3; lane 7, +IPTG SNerbB4, lane 8, markers.

Figure 1 shows the SDS-PAGE of whole cell lysates, which revealed that the SNerbB fusion proteins migrated as monomers. For comparison, lysates from cells expressing SNGpA, which migrates predominantly as a dimer on SDS-PAGE, are also shown. Using purified protein, much higher concentrations (>100 μM) could be loaded onto the gel, but the protein still migrated as monomer (data not shown). Both SNerbB2 mutants, SNerbB2neu and SNerbB2VI, expressed as monomers, but the proteins were ill-behaved and neither could be stably purified in sufficient quantities for thermodynamic studies.

SNerbB proteins are predominantly monomeric in C_8E_5

In the case of the glycophorin A transmembrane domain, the free energy of the helix–helix interactions is known to be weaker in SDS relative to

Table 1. A list of the transmembrane sequences considered in this study, given by single-letter amino acid codes

Clone name	TM AA sequence
SNerbB1	SIATGMVGALLLLLVVALGIGLFMRRR
SNerbB2	LTSIVSAVVGILLVVVLGVVFGILI
SNerbB2KRR	LTSIVSAVVGILLVVVLGVVFGILIKRR
SNerbB2neu	LTSIVSAV**E**GILLVVVLGVVFGILIKRR
SNerbB2VI	LTSI**I**VSAV**V**GILLVVVLGVVFGILIKRR
SNerbB3	LTM**A**LTVIAGLVVIFMMLGGTFLYWRGRR
SNerbB4	LIAAGVIGGLFILVIVGLTFAVYVRRK

These sequences were fused to the C-terminal end of the staphylococcal nuclease *via* a linker region. Mutations are in bold and underlined.

other detergent environments.[37,38] Previously, sedimentation equilibrium analytical ultracentrifugation has been used to detect significant dimer populations for mutants of GpA that were monomeric by the SDS-PAGE assay.[39] In contrast to SDS-PAGE, analytical ultracentrifugation also allows for equilibrium measurements of the species in solution over a wide concentration range. This approach has been used to determine the thermodynamics of association of wild-type glycophorin A and more than 50 sequence variants.[39,40] Therefore, to more rigorously investigate the association of the erbB transmembrane segments, we carried out sedimentation equilibrium experiments in the neutrally buoyant detergent C_8E_5.

The SNGpA construct is greater than 90% dimeric in 23 mM C_8E_5 at the concentrations used in the centrifuge, and even the very disruptive GpA mutant G83I is greater than 20% dimeric under these conditions.[39] Therefore, we expected that we would be able to detect moderately stable interactions of the erbB TMs at this detergent concentration. Representative data sets for each SNerbB protein are shown in Figure 2. Using a non-linear, least-squares global analysis procedure, the data for each of SNerbB1, SNerbB2, and SNerbB3 were well described by a model for a single ideal species with the molecular mass of a monomer. Only SNerbB4 could not be described by a simple monomer model at 23 mM C_8E_5, as evidenced by the non-random residuals in Figure 2. However, if the concentration was raised to 50 mM C_8E_5, SNerbB4 was monomeric, suggesting the interactions in C_8E_5 are reversible. In contrast, most GpA mutants have a detectable population of dimer under these experimental conditions. These initial experiments suggest interactions between the erbB TMs are very weak, with SNerbB4 showing a slightly greater propensity for association relative to the other three.

At limiting protein to detergent ratios, SNerbB proteins self-associate

The apparent dissociation constant for dimerization of the GpA TM decreases as the detergent concentration is decreased, as a result of the reduced volume of the hydrophobic phase available to the protein.[41] Therefore, to populate the SNerbB4 oligomer(s), as well as possibly detect even weaker interactions for the other erbB TM domains, we decreased the detergent concentration in the sedimentation equilibrium experiments to 11 mM, a condition under which the disruptive glycophorin A mutant G83I would be greater than 50% dimeric.

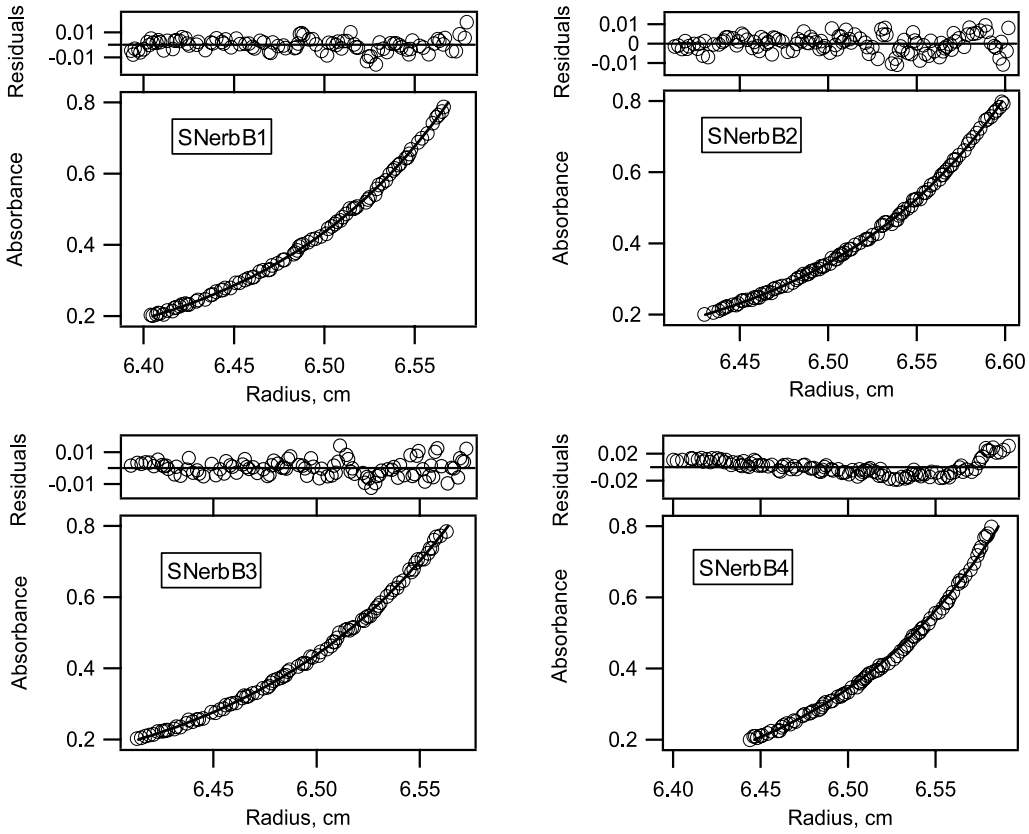


Figure 2. SNerbB proteins are monomeric at most detergent concentrations. Representative data sets from equilibrium AUC experiments are shown for the four erbB fusion constructs at 23 mM C_8E_5. The protein distribution was monitored by measuring absorbance at 280 nm. Raw data are shown as open circles; the continuous lines represent global fits for single ideal species with the molecular mass of a monomer. The upper panel for each data set shows the residuals for the fit. The residuals for the single ideal fit are small and random for SNerbB1, SNerbB2, and SNerbB3, indicating those data are well described by a monomer model. The residuals for SNerbB4, however, are larger and non-random.

A stochastic distribution model predicts some non-specific interactions at these high protein:detergent ratios used in the 11 mM C_8E_5 experiments,[42] but we sought to determine if we could drive the system to a preferential oligomer.

For all SNerbB constructs, the data at 11 mM were no longer described by a single monomeric species model and showed evidence for protein self-association. However, from these data alone, it was difficult to fit a specific association model (see discussion below), so we used the value for the reduced molecular mass, sigma (σ), as a model-independent parameter for expressing the extent of association, where σ is defined by Yphantis as:[43]

$$\sigma = \frac{M(1 - \bar{\upsilon}\rho)\omega^2}{RT} \quad (1)$$

where M is molecular mass, $\bar{\upsilon}$ is the partial specific volume of the protein (ml g^{-1}), ρ is the solvent density (g ml^{-1}), ω is the rotor speed (rad s^{-1}), R is the universal gas constant, and T is the temperature (K). For a single ideal species fit, the value of σ represents an approximate measure of the weight-average molecular mass of the species in solution. The expected value of σ for a given protein can be calculated from the amino acid sequence and the buffer components. If a protein is monomeric, then the experimental value for σ from a single ideal species model, σ_{exp}, would be equal to the calculated value, σ_{calc}. Thus, a ratio of $\sigma_{exp}/\sigma_{calc}$ greater than 1 indicates the presence of higher-order species, and larger ratios can indicate a greater extent of self-association. For SNerbB1, SNerbB2, and SNerbB3, the σ ratio is within error of unity at 23 mM C_8E_5, whereas the SNerbB4 ratio is significantly larger than 1 under the same conditions (Table 2). Even so, the experimental σ for SNerbB4 in 23 mM C_8E_5 is only 16% larger than the value calculated for monomer, indicating that monomer is still a significantly populated species. In contrast to the 23 mM data, Table 2 shows that the σ ratios for all four proteins are significantly greater than 1 when experiments are carried out in 11 mM C_8E_5. This result reflects the presence of higher-order species in all four proteins. The fact that the data at 11 mM C_8E_5 were no longer well described by a monomeric species alone is also reflected in the increase in the values of the square-root of the variance (SRV) for the single species fits as compared to the SRVs at 23 mM C_8E_5 (Table 2).

The absence of strong interactions was surprising, because the erbB TM domains show positive signals in the genetic assay TOXCAT, a result that is generally interpreted as an indication of strong helix–helix interactions.[23,24] In the TOXCAT assay, the activity of the erbB TMs was approximately half the activity of wild-type glycophorin A. Moreover, the erbB signals were several-fold greater than the disruptive glycophorin A mutant G83I. In contrast, in our experiments, the SNerbB proteins were monomeric under conditions where even G83I has a significant population of dimer.

Although the absolute strengths of the SNerbB interactions were much weaker than expected from the TOXCAT results by comparison to GpA, a consideration of just the relative signals of the erbBs suggests that the association trends in bacterial membranes as measured by TOXCAT are mirrored in the extent of association observed in the centrifuge in 11 mM C_8E_5. This comparison is shown in Figure 3, where it can be seen that in each assay the TM sequences of erbB1, erbB2, and erbB3 exhibit about the same levels of association, whereas there is evidence for a somewhat greater extent of association for erbB4. Although SNerbB4 exhibits a somewhat greater propensity for interaction than the other three sequences, the interactions of all four proteins in micellar solutions are very weak, especially by comparison to the GpA sequences.[39]

Specific association models for SNerbBs are difficult to define

We fit the sedimentation equilibrium data with a number of self-association models to determine the nature of species present in 11 mM C_8E_5. We tried a number of monomer–*N*mer models and found that for all four proteins a monomer–trimer model was the best description of the data from these simple two-species models. Table 3 shows the results of these fits for one of the experiments with SNerbB4, and results were similar for the other three proteins. The fact that a monomer–trimer equilibrium was the best fit to the data from the simple self-association models was surprising, since the

Table 2. Analysis of SNerbB AUC data using single ideal fits

		23 mM C_8E_5			11 mM C_8E_5		
SNerbB	σ_{calc}	SRV (10^{-3})	σ_{exp}	$\sigma_{calc}/\sigma_{exp}$	SRV (10^{-3})	σ_{exp}	$\sigma_{calc}/\sigma_{exp}$
1	0.9575	5.55	1.0036	1.0481	7.95	1.3657	1.4263
2	0.9287	6.71	0.9844	1.0600	8.18	1.2943	1.3937
3	0.9841	5.79	1.0581	1.0751	7.39	1.3593	1.3813
4	0.9533	7.79	1.1088	1.16311	9.06	1.6260	1.7057

At detergent concentrations of 23 mM or greater, the SNerbB proteins are monomeric in solution. When the detergent concentration is reduced to 11 mM, evidence of higher-order species can be seen. The values of σ for the four SNerbB fusion constructs were calculated using SEDNTERP for 20,000 rpm. σ_{exp} is the value returned by the NONLIN[47] fitting procedure for a single ideal species at 20,000 rpm. SRV is the square-root of the variance for these fits. The experimental numbers in the Table represent the average of at least two independent measurements.

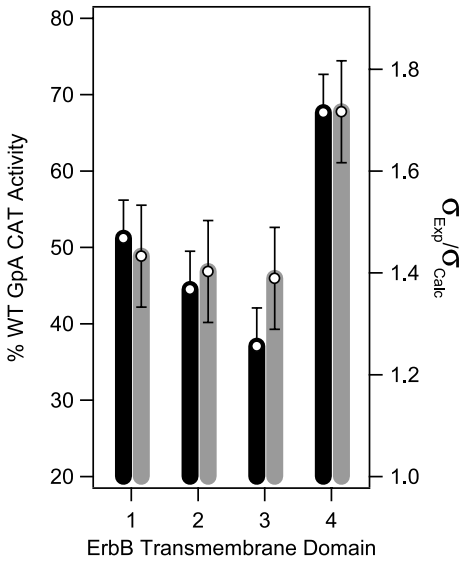
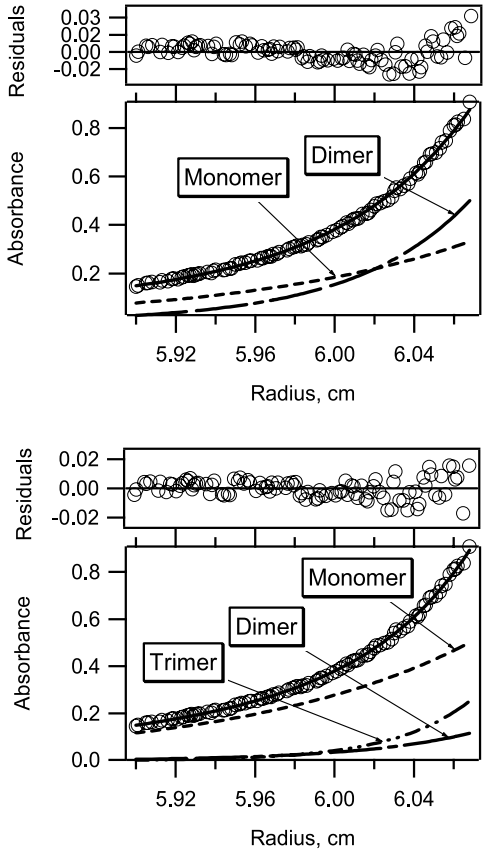


Figure 3. A comparison of the relative CAT activities of the erbB transmembrane sequences to the σ ratios from 11 mM C_8E_5 determined by sedimentation equilibrium using a single ideal species fit. It can be seen that in both assays, the transmembrane segment of erbB4 appears to be the most associated of the four transmembrane domains.

biology of the erbB receptors suggests that a monomer–dimer equilibrium would likely be the most appropriate model to describe the data.[44,45] Nevertheless, Figure 4 shows a comparison of the species plots for these fits, where it can easily be seen from the non-random residuals that a monomer–dimer equilibrium is not as good a description of the data as a monomer–trimer model.

Therefore, to independently identify the oligomers present in solution and to better discriminate between models for the sedimentation equilibrium data, we carried out glutaraldehyde cross-linking experiments. Cross-linking techniques can capture the oligomers that are preferentially populated in solution. The fusion proteins were cross-linked in 11 mM C_8E_5 at the highest protein concentrations used in the centrifugation experiments. Figure 5 shows that bands for both dimers and trimers could be seen, as well as traces of higher-order aggregates. At extended times, the SNerbB proteins all cross-link as large aggregates. The cross-linking results did confirm the presence of a dimer species, but they also revealed that it is likely at least three species are present in solution, which may be why a unique fit using the AUC data alone was difficult to define.

The cross-linking results and the biology of the



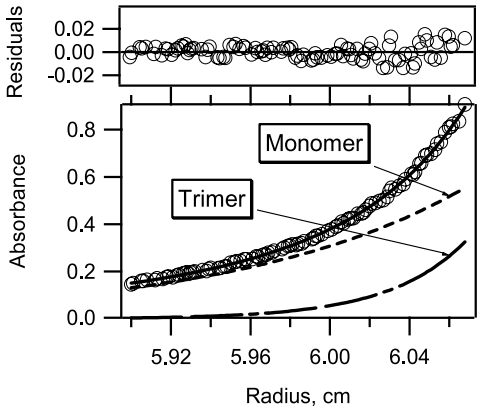


Table 3: Various association models fit to data for SNerbB4 in 11mmC_8E_5

Model	SRV
Single Ideal	8.56e-3
Monomer-Dimer	7.31e-3
Monomer-Trimer	6.49e-3
Monomer-Tetramer	7.92e-3
Monomer-Dimer-Trimer	6.42e-3
Monomer-DImer-Tetramer	6.45e-3

Figure 4. Species plots for various association models fit to data for SNerbB4 in 11 mM C_8E_5. Although only one data set is shown, a global fitting procedure using nine data sets, three concentrations at three rotor speeds was employed. It was expected that the equilibrium for SNerbB4 would be described by a monomer–dimer equilibrium, but higher order models appear to fit equally as well if not better, thus making it difficult to distinguish which is the appropriate description of the system.

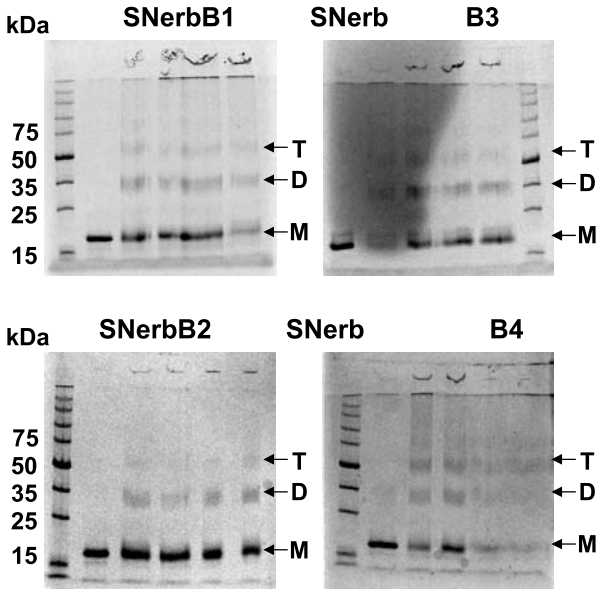


Figure 5. Higher-order SNerbB oligomers can be visualized by SDS-PAGE of glutaraldehyde cross-linked proteins. The proteins were cross-linked in 11 mM C_8E_5 at 43 μM with 0.1% glutaraldehyde and run on a 12.5% acrylamide Phast gel. Lane 1, molecular mass markers; lanes 2–6: 0, five, ten, 30, 90, and 120 seconds, respectively. Bands can be seen for both dimers and trimers; traces of higher-order aggregates can also be seen. At long times, all the proteins cross-link as large aggregates that crash out of solution.

erbBs justify the use of three species models that include dimers for the analysis of the sedimentation equilibrium data. Therefore, we fit the data with a monomer–dimer–trimer model, and the statistics of the fit and the shapes of the residuals indicated the model was a reasonable description of the data (Figure 4). Separation of the trimer terms, which can be interpreted as a non-equilibrating population for that species, led to an improvement in the fit. The inclusion of the additional parameters was supported by the Akaike information criterion[46] statistical test and analysis of the residuals by a runs test.[47]

Stochastic association largely accounts for the observed interactions

The observed species distributions for the SNerbB proteins in 11 mM C_8E_5 were compared to what is predicted by a purely stochastic distribution under these conditions. At these high protein to detergent ratios where the number of micelles approaches the number of protein molecules, the probability that more than one protein molecule can occupy a given micelle in the absence of a preferential interaction is non-negligible, and the distribution of proteins in micelles is given by a Poisson distribution as described in Materials and Methods.[42] Figure 6 shows a comparison of the experimentally observed species plots for SNerbB4 to those predicted by a stochastic model. The comparison shows that the fraction of monomer for SNerbB4 decreases more quickly than one would expect from purely stochastic interactions. It also demonstrates that the fraction of dimers expected from a random distribution is non-negligible at the concentrations used in the sedimentation equilibrium experiments, highlighted by the thickened portion of the line. The presence of yet another distinct species, namely the stochastic dimer, may also have contributed to the difficulty in defining a model for the AUC data at 11 mM C_8E_5. Attempts were made to fit the data with models that included a parameter to approximate the stochastic dimer, but this did not improve the fits. SNerbB4 exhibits the greatest propensity of the erbBs to associate, so the curves for SNerbB1, SnerbB2, and SNerbB3 were shifted even closer to the random distribution curves. They do remain to the left of the simulated data, indicating that they are slightly more associated than one would expect from a purely random distribution. However, we estimate that any preferential interactions between the erbB TMs are extremely modest, certainly less than 1 kcal mol^{-1}, and we conclude that stochastic interactions account for a large portion of the SNerbB association seen in 11 mM C_8E_5. It is worth noting though that at 23 mM C_8E_5, the stochastic model predicts a negligible amount of association but SNerbB4 is not monomeric under these conditions.

Heterodimers of the SNerbB proteins could not be detected

Since interactions in the transmembrane domains have been implicated in the heterodimerization of the erbB receptor tyrosine kinases,[6,20] we carried out experiments to detect hetero-interactions of the SNerbB fusions using SDS-PAGE. For each of the six pairwise interactions, equal amounts of both proteins were combined, but only monomer was visible on the gel when the samples were analyzed by SDS-PAGE (data not shown). We also attempted to detect modest interactions by sedimentation equilibrium between SNerbB2 and SNerbB3. We chose this pair because the erbB2/erbB3 heterodimer is the most potent signaling complex[48,49] and only weak hetero-interactions can be detected between the soluble ligand-binding domains,[7] so we hypothesized that if TM–TM interactions were important for stabilizing any of the hetero-complexes it would be the erbB2/erbB3 complex. Our results, however, showed that an equimolar amount of SNerbB2 and SNerbB3 at 23 mM C_8E_5 yielded a molecular mass consistent with protein monomers in solution (Figure 7).

Discussion

ErbB TM interactions are difficult to detect in micellar solutions

Transmembrane helix association as a driving

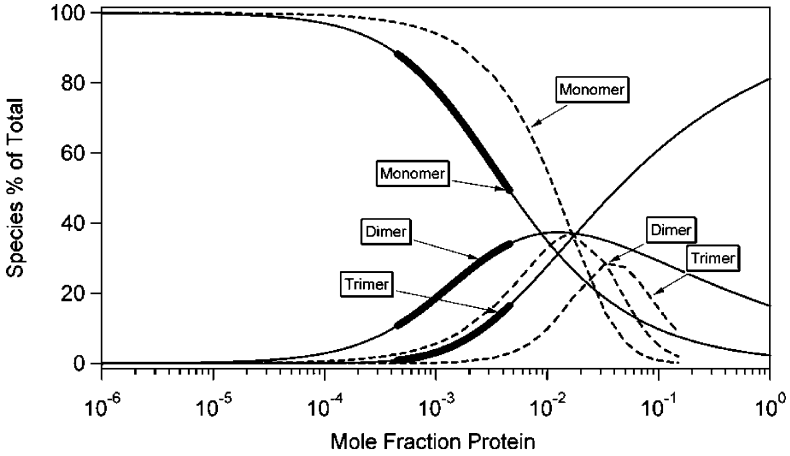


Figure 6. An overlay of a stochastic distribution and the distribution calculated from a monomer–dimer–separate trimer fit to one of the SNerbB4 data sets. The dotted lines represent the monomer, dimer, and trimer populations simulated using a random distribution of proteins into micelles for an aggregation number of 60 and a maximum occupancy of $k=5$. The continuous lines represent the distributions calculated using the global K_{12} constant and the K_{13} from the data set for the middle concentration at 24,500 rpm for 11 mM C_8E_5. The thickened black portion of the line represents the concentration region where the data were actually collected. It can be seen that the association of SNerbB4 is only very moderately shifted from what one would expect from a stochastic distribution.

force for protein association is not unprecedented, and several TM segments have been identified that are capable of driving oligomerization.[30–32] Much consideration has been given as to whether this is the role of the erbB transmembrane domains. Some studies have indicated that active EGF receptors can tolerate a variety of transmembrane sequences,[26–29] but others studies have suggested a specific role for the transmembrane domain in dimerization, with perhaps the most suggestive evidence for a role for TM helix interaction coming from the genetic assay TOXCAT.[15,21,24]

Contrary to what was expected from the TOXCAT,[24] we found that the transmembrane domains of the erbB receptors were not sufficient to drive the strong dimerization of an SN fusion partner in detergent micelles. This distinguishes the erbB TM sequences from the transmembrane domains of GpA and bNIP3, which are capable of driving robust dimerization of chimeric proteins in a number of environments, including detergent micelles and bacterial membranes.[30–32,37,39,50–52] It was also initially surprising that the erbB TMs did not interact, given that the sequences contain GxxxG motifs, which can be an important sequence motif in strong helix–helix interactions.[25,31] However, it has been subsequently shown that a GxxxG motif is not sufficient to drive TM helix dimerization.[39,42] Our results are also consistent with an earlier report of a similar SN/erbB1 TM fusion construct migrating as a monomer on SDS-PAGE.[53]

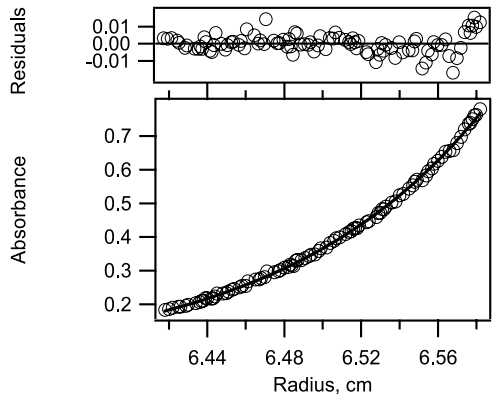


Figure 7. A representative sedimentation equilibrium data set for a mixture of equimolar amounts of SNerbB2 and SNerbB3 fit to a single ideal species. The raw data are represented as open circles and the continuous line represents the fit and the upper panel shows the residuals for the fit. The data are well described by a single ideal species with a molecular mass of 21,273 kDa, which is equal to the average of the two monomers, SNerbB2 (20,876 kDa) and SNerbB3 (21,669 kDa).

It is possible that the lack of interaction is a consequence of the SN fusion construct that was used, but we believe this is unlikely. Our linker region is 14 amino acid residues long and is comparable to what has been used in the past to successfully measure the energetics of glycophorin A, a number of GpA mutants, and other TM helices.[31,33,37,39,40] In all of these studies, the SN fusion did not appear to interfere, and the linker length was sufficient to allow the TMs to interact. Additionally, extending the linker between the SN domain and the TM sequence had no effect on the interaction behavior of the CCK-4 TM domain.[42] Furthermore, it has been reported that a peptide corresponding to the TM of the EGF receptor (erbB1) migrated as a monomer in SDS-PAGE,[54] and isolated erbB2 TM peptides also migrate predominantly as monomers.[55] These results with isolated TM peptides are consistent with the SDS-PAGE analysis of our SN-fusion constructs. Some reports do indicate, however, peptides corresponding to the TM of the proto-oncogenic neu receptor from rats, a homolog of erbB2, migrate as something larger than monomers on SDS-PAGE.[36,55,56]

While specific protein–protein interactions determined by the amino acid sequence are important for TM helix interactions, the environment can also be a critical factor in the investigation of membrane proteins. Successful solution studies can depend on the choice of an appropriate detergent. However, our inability to detect strong interactions does not

seem to be simply the effect of the selected experimental environment, as the protein did not interact in a number of detergents. The SNerbB proteins are principally monomeric in the non-ionic detergent C_8E_5, as well as in the ionic detergent SDS. Preliminary sedimentation equilibrium experiments in the zwitterionic detergent SB-C14 also failed to detect strong SNerbB interactions (data not shown).

Detergent micelles have proven to be powerful and useful tools in many membrane protein studies, but detergent micelles are sometimes criticized as being poor substitutes for the two-dimensional fluid of a lipid bilayer. In the case of the erbB proteins, it may be that the bilayer imposes structural constraints that favor dimerization-competent conformations of the erbB transmembrane domains but these constraints would be lacking in a micellar environment. The results from the TOXCAT assay, which is carried out in bacterial membranes, are certainly consistent with this idea.[24] However, the populations in solid-state NMR experiments in model bilayers seem to be in agreement with the level of association we observe. While the solid-state NMR experiments are not a direct measure of association and can be difficult to interpret, these studies suggest that the erbB1 TM is largely monomeric in bilayers, with the only signs of interactions being seen at very high concentrations of protein.[57–61] The erbB2 TM appears to exhibit a somewhat greater tendency to associate in the solid-state NMR experiments, but there is still a significant population of molecules that behave as one would expect for an unrestricted monomer.[61] These NMR experiments suggest that the erbB TM peptides are not constitutively associated in bilayers, and thus our inability to detect strong interactions between the erbB TM domains may not be the consequence of the micellar environment required for the sedimentation equilibrium studies. We cannot, however, eliminate the possibility that interactions we detect as extremely weak in solution are enhanced by the environment of the native bilayer and are biologically relevant.

Another possible explanation for the apparent discrepancy between TOXCAT and the other assays for helix–helix interactions is the difference in the domains flanking the transmembrane segment. In the full-length receptors, the transmembrane helix connects a ligand-binding ectodomain and an intracellular kinase domain. It may be that the TM segments of the erbB receptors cannot function as independent interaction domains isolated from the context of the full-length protein. This deficiency may somehow be compensated for by the nature of the soluble domains in the TOXCAT chimera. Of the studies considered here, the TOXCAT construct is the only construct that has a soluble domain with a dimerization interface, in the form of the ToxR DNA-binding domain. The TOXCAT construct is also the only construct with a C-terminal soluble domain. It may be that the erbB TMs require interactions in the soluble domains to pre-arrange the TM helices and that the TOXCAT fusion protein has an architecture to accomplish this. It could also be that the presence of a structured C-terminal domain is important for stabilizing interactions at the end of the transmembrane segment necessary for the receptor association. Previously, it has been suggested that the erbB transmembrane helix and the intracellular kinase domain are rotationally coupled, implying some degree of rigidity in the linker between the two.[28] It is also worth noting that this C-terminal juxtamembrane region of EGFR contains an important phosphorylation site for protein kinase C[62,63] and that the addition of peptides corresponding to this juxtamembrane region leads to increased receptor autophosphorylation.[64]

Consequences of including a TM lacking strong specific self-associations

In studies of SNGpA fusion proteins, it has been shown that it is appropriate to treat the micelles as a distinct thermodynamic phase and to consider the transmembrane segments confined to this phase.[41,65,66] Such a consideration leads to the expression:[41]

$$\Delta G_{\text{app}} = \Delta G_x^0 + RT \ln[\text{Micellar Det}]_{\text{w}} \qquad (2)$$

where ΔG_{app} is the observed apparent free energy in bulk aqueous concentrations, ΔG_x^0 is the standard state mole fraction free energy of association, R is the universal gas constant, T is the temperature (K), and $[\text{Micellar Det}]_{\text{w}}$ is the aqueous concentration of the detergent in micelles. The first term represents the association free energy of the intrinsic chemical components of the association reaction, and the second term represents a statistical energy of mixing, the magnitude of which varies with the micellar detergent concentration. In the case of GpA, we believe the contribution of the soluble SN domain to the standard state free energy ΔG_x^0 is negligible, and we interpret ΔG_x^0 as the contribution from interactions between the transmembrane domains.

However, the soluble domain of EGFR, when ligand-bound, does interact. Equation (2) predicts that extending these fragments to include a hydrophobic transmembrane segment should enhance the apparent dimerization affinity of the ECDs even in the absence of specific TM interactions. The TM domain would be restricted to the volume of the micellar phase, and thus the apparent free energy of association of the ECDs would now contain a contribution from the statistical energy term described above as well as the intrinsic free energy of dimerization of the soluble domains, ΔG_{int}. Applying the above formalism, we can substitute ΔG_{int} for ΔG_x^0 and rearrange equation (2) to write the apparent association equilibrium constant for an ECD-TM construct in micelles, K_{app}, as:

$$K_{\text{app}} = \frac{K_{\text{int}}}{[\text{Micellar Det}]_{\text{w}}} \qquad (3)$$

The equilibrium constants in equation (3) are in bulk molar concentration units. From equation (3), one would expect that the association of the receptor would depend on detergent concentration, with a tighter apparent association expected at lower detergent concentrations, and it has in fact been shown that the full-length EGFR, as well as a truncated fragment resembling v-erbB, interact more strongly at low detergent concentrations.[67,68]

A study by Tanner & Kyte, which compared the ligand-induced dimerization of the ECD of erbB1 to the dimerization of a fragment extended to include the TM, is also consistent with this formalism. This study found the longer fragment did indeed associate more readily, although complete dimerization was never observed.[21] The dimerization of the longer fragment was measured in 1% Triton X-100 (15 mM), and equation (3) predicts that restriction of the soluble fragment to the micellar phase would lead to an apparent 67-fold enhancement in the association. By comparing the dimerization of the ECD alone, which had been shown to be complete at 40 μM protein,[69] with the dimerization of the ECD-TM fragment in Triton micelles that they observed (2 nM), the authors estimated an observed enhancement of approximately 10,000-fold.[21] However, a subsequent study reports complete ligand-induced dimerization of the extracellular domain of erbB1 at concentrations as low as 4 μM protein;[7] therefore, the enhancement of ligand-induced dimerization of the ECD by the addition of the TM may be less than originally suggested and be more of the order of 1000-fold. Equation (3) suggests a large portion of this is simply the result of restricting the longer fragment to be stably inserted in the micelles. If approximately 65-fold arises from limiting the protein to the micellar phase, only a 15–30-fold enhancement arises from other features of the ECD-TM construct. This additional enhancement is modest, and it could arise from stabilization in the longer construct of structures in the juxtamembrane region important for dimerization or from restriction of the relative orientation of two ECDs on the surface of a micelle. The role of the transmembrane domain in the differences observed in the Tanner & Kyte study is still unclear, but our results suggest that it may not be necessary to invoke strong specific interactions between the TM domains to account for the observed enhancement.

A sterically permissible hydrophobic transmembrane segment may be sufficient

Some studies have indicated active receptors tolerate a good deal of sequence promiscuity in the transmembrane segment,[26,29] but others have implicated sequence specificity in the TM is necessary to maintain proper receptor interactions in the EGFR family.[17,18,23] If there are not strong interactions between the transmembrane domains, then why do some TM sequence variants affect receptor function? This dual behavior might be reconciled in a case where proper orientation of the soluble domains puts the transmembrane domains in close proximity without the transmembrane segments necessarily providing significant energy to stabilize the dimer. In other words, it may only be required that the transmembrane region dimers be sterically permissible. Examination of the crystal structures of the extended ligand-bound conformation of the erbB extracellular domains and structure-based models of the receptor dimers reveal that the C termini of domain IV point toward one another in way that could juxtapose the transmembrane domains of two receptors.[70–73] The need for a close-approach of the transmembrane domains might explain the occurrence of motifs that place small residues on the same face of the helix, such as the Stenberg & Gullick and GG_4 motifs.

The receptors could be destabilized by mutations in the TM that introduce large steric clashes in regions of the transmembrane segments positioned close in space by the orientation of the soluble domains. For mutations to have this effect, there would have to be some degree of rigidity in the structure so that the soluble domains and the mutated transmembrane sequence could not simultaneously adopt their optimal conformations. A previous study that explored the rotational coupling between the transmembrane segment and the kinase domain of the neu receptor has in fact suggested that the linker between the two domains is rigid.[28] Conversely, sequence variants that could be juxtaposed when the soluble domains of the protein adopt their preferred conformation in the dimer would be tolerated.

Such a situation would be consistent with what is observed for the neu mutation. The wild-type valine residues may be in close proximity in the transmembrane region, but not interacting to any significant extent. Placing a glutamic acid residue in an already optimal position introduces a hydrogen-bonding interaction, in an otherwise non-interacting domain, which stabilizes the dimer. The subsequent introduction of an unfavorable interaction, such as one might expect if glycine residues in close proximity are mutated to larger residues, would then hinder the receptor association.[17] When placed in a non-optimal position elsewhere in the transmembrane domain, no effect is observed.[18] It may be the case that the sterics of the soluble domains prevent the glutamic acid residues in the transmembrane domains from interacting. Alternatively, it may be an effect of the sequence context of the TM itself.[74] Since a number of studies have shown that the introduction of a single appropriately placed polar residue in a TM can lead to association of an otherwise non-interacting TM domain,[33,75,76] we do not believe that it is a prerequisite for the wild-type erbB transmembrane domains to have significant interactions in order for the neu mutation to effect cellular transformation by stabilization of receptor dimers.

Conclusions

Contrary to what was expected, given the behavior of the erbB transmembrane segments in TOXCAT, we do not detect strong specific interactions between the TM segments of the erbB proteins in detergent micelles using an SN-TM fusion system. This is in contrast to the transmembrane domains of GpA and bNIP3, which give strong TOXCAT signals and are capable of driving stable protein oligomerization in a number of environments. Our study suggests that one interpretation of the results is the transmembrane segments of the erbB proteins isolated from the full-length protein are not independent association domains and cannot drive oligomerization, at least in a micellar environment. It may be that interactions we detect in solution as extremely modest are significantly enhanced in a bilayer environment. However, given that the biology of the receptor dictates that protein exists in a monomer–dimer equilibrium, it may not be surprising that TM interactions are distinctly weaker than for those of glycophorin A. Even a non-interacting hydrophobic transmembrane domain, however, can increase the apparent affinity of the covalently attached soluble domain by restricting the protein to the hydrophobic phase of the solution. The role of the transmembrane domain in the context of the full-length protein remains unclear, but it may be sufficient for the transmembrane sequences of the erbB proteins to be sterically permissible. In this scenario, the transmembrane domains are passive, in the sense that they do not contribute significant energy; however, not just any sequence will suffice because the packing of the TM segments must not interfere with the association of the other domains. The contribution of the individual domains of the receptors to the full-length complexes is unclear, but it seems likely the association of the erbB receptors will be the sum of many interactions, including numerous weak contacts, distributed throughout all the domains.

Materials and Methods

Cloning and expression

Quikchange mutagenesis was used to introduce a SmaI site into the pet11b-SNGpA plasmid N-terminal to the GpA TM domain. The GpA TM sequence was excised from the SN vector containing an open reading frame for a staphylococcal nuclease C-terminal GpA fusion by restriction digest with either SmaI/BamHI or XmaI/BamHI, leaving behind the reading frame for staphylococcal nuclease and a C-terminal linker. The erbB2 transmembrane sequence was generated by a PCR of a pccB2 plasmid template, a generous gift from Dr Mark Lemmon and Dr Jeanine Mendrola, using primers containing SmaI and BglII restriction sites. BglII creates an overhang compatible with BamHI and the erbB2 TM sequence contains an internal BamHI site. The PCR product was digested and ligated into the SN vector. The other erbB sequences were generated by ordering synthetic 5′ phosphorylated oligonucleotides with the appropriate overhangs from Invitrogen and annealing the oligonucleotides by heating to 95 °C with subsequent cooling. The annealed oligonucleotides were then ligated into the SN vector. SnerbB2 variants were made using the Stratagene Quikchange protocol, and the sequences for all constructs were verified by DNA sequencing. The SNerbB plasmids were transformed into HMS174(DE3) cells, and expression of the protein was induced with IPTG. The SNerbB proteins were purified using Thesit® detergent according to the protocol published for SNGpA.[30,37]

SDS-PAGE assay

Cells were grown to an A_{600} of 0.8, induced with 1 mM IPTG for four hours, and harvested by centrifugation. Cell pellets were resuspended in SDS-PAGE loading buffer and analyzed using a 20% (w/v) acrylamide gel using a Pharmacia Phast gel system. For hetero-dimer interactions, equal amounts of protein at 25 μM in 20 mM NaPi 7.0, 100 mM NaCl, and 2% (w/v) Thesit® were mixed and incubated for 30 minutes at 37 °C. The samples were then analyzed on a 20% acrylamide Phast gel.

Sedimentation equilibrium analytical ultracentrifugation

Purified proteins were exchanged into the neutrally buoyant C_8E_5 detergent by ion-exchange chromatography as described.[37] Sedimentation equilibrium experiments were performed in Beckman XL-A analytical ultracentrifuge in six-sector cells according to the previously published protocol for SNGpA, with the exception that the protein was observed at 280 nm.[37] Data were collected at three initial protein concentrations (A_{280}=0.9, 0.6, 0.3) and three rotor speeds (20,000 rpm, 24,500 rpm, and 30,000 rpm). WINMATCH† was used to check that equilibrium was reached at each speed. For the data analysis, protein molecular masses and partial specific volumes were calculated using the program SEDNTERP.[77] Since none of the transmembrane sequences of interest contained tryptophan, the absorbance of the TM was considered negligible relative to the SN part of the fusion and an extinction coefficient of A_{280}=1 mg/ml was used. The radial distribution profiles for the nine data sets were globally fit by a non-linear, least-squares curve-fitting procedure in the Windows version of NONLIN.[47] The Akaike information criterion (AIC) was calculated using the equation:[46]

$$\mathrm{AIC} = -N \ln \sigma^2 - 2P \quad (4)$$

where N is the number of degrees of freedom, σ is the square-root of the variance, and P is the number of parameters in the model. The runs test score was calculated for each data set from the residuals of the global fit using the equations described earlier.[78]

Glutaraldehyde cross-linking

Glutaraldehyde (1%, w/v) in buffer was added to 40 μM protein in 11 mM C_8E_5, 20 mM NaPi 7.0, and 200 mM NaCl to a final concentration of 0.1% glutaraldehyde. Aliquots were taken at five, ten, 30, 90, and 120 seconds and quenched with SDS-PAGE loading buffer.

† www.bbri.org/rasmb

Samples were boiled for five minutes and analyzed on 12.5% acrylamide Phast gels.

Stochastic distribution data simulation

When the number of micelles and the number of protein monomers far exceeds the occupancy of a micelle, the distribution of the protein into the micelles due to random chance can be described by a Poisson distribution:

$$\mathrm{Lim}(m, n >> k) : \mathrm{Prob}[X_i = k] = \frac{1}{k!}\left(\frac{m}{n}\right)^k \mathrm{e}^{-m/n} \quad (5)$$

where X_i is the random variable that counts the number of proteins in a micelle i, k is the number of proteins occupying a micelle, m is the number of protein molecules, and n is the number of micelles.[42] Given a stochastic distribution, the fraction of proteins that are in a particular oligomeric state, f_j where j is a particular value of the occupancy, can be calculated from the following partition function, which considers only occupied micelles:

$$f_j = \frac{j\,\mathrm{Prob}[X_i = j]}{\sum_{k>0}^{k=5} k\,\mathrm{Prob}[X_i = k]} \quad (6)$$

The series was truncated at an occupancy of $k=5$ because oligomers larger than this did not contribute significantly to the distribution at micelle to protein ratios smaller than 1:1. Additionally, oligomers larger than pentamer would spin out at the speeds used in the sedimentation equilibrium experiments and are unobservable in the centrifuge. To calculate the species distribution as a function of total micellar detergent instead of the concentration of micelles, the ratio m/n was divided by the aggregation number. An aggregation number of 60, which lies between the aggregation numbers reported for C_8E_4 and C_8E_6,[79] was used.

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