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Transmembrane Helix Dimerization: Beyond the Search for Sequence Motifs

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Abstract

Studies of the dimerization of transmembrane (TM) helices have been ongoing for many years now, and have provided clues to the fundamental principles behind membrane protein (MP) folding. Our understanding of TM helix dimerization has been dominated by the idea that sequence motifs, simple recognizable amino acid sequences that drive lateral interaction, can be used to explain and predict the lateral interactions between TM helices in membrane proteins. But as more and more unique interacting helices are characterized, it is becoming clear that the sequence motif paradigm is incomplete. Experimental evidence suggests that the search for sequence motifs, as mediators of TM helix dimerization, cannot solve the membrane protein folding problem alone. Here we review the current understanding in the field, as it has evolved from the paradigm of sequence motifs into a view in which the interactions between TM helices are much more complex.

1. Introduction

In every organism, about a quarter of all open reading frames code for integral membrane proteins (1;2). Yet, despite their abundance, important biological roles, and utility in medicine and biotechnology, the detailed principles of membrane protein structure and folding are not well understood. The prediction of membrane protein structure from sequence is not reliable and, in fact, lags far behind soluble protein structure prediction. Just as for water-soluble proteins, understanding, manipulating, designing or engineering the biological activity of membrane proteins will require a detailed understanding of how and why their sequences drive them to fold into their unique, native, three dimensional structures (3).

By analogy to the soluble protein folding paradigm (4), the folding of membrane proteins is believed to be dictated by the amino acid sequence. However, membrane proteins fold with different constraints imposed by the architecture of the lipid bilayer (5–8). These constraints change the nature of the folding problem, and it has been tempting to describe MP folding in terms of five distinct steps, as shown in Figure 1, consisting of binding, secondary structure formation, insertion, lateral dimerization and higher-order lateral self-assembly (6;9).

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Furthermore, within this multi-step conceptual framework for MP folding, it has been useful and enlightening to study the dimerization of TM helices as fundamentally important step in the folding process (9;10). A great deal of work has been done to understand the strength and specificity of TM helix dimerization. An important milestone in understanding membrane protein structure and folding has been the idea that sequence motifs, i.e. simple recognizable amino acid sequences that drive lateral interaction, can be used to understand and predict the dimerization of TM helices in membranes (10;11). After much research in the field, it has become clear however that the sequence motif concept, while powerful, cannot fully describe the interactions between TM helices, and cannot solve the membrane protein folding problem. Here we overview systems which are well described by the sequence motif paradigm, and systems which cannot be fully described by this paradigm. We discuss the current understanding in the field, as it has evolved from the paradigm of "sequence motifs" to a view in which the interactions are much more complex.

2. Folding in the lipid bilayer

The physical chemical context within which membrane proteins fold could hardly be more different from the context for soluble proteins. Thus, completely different theoretical frameworks must be considered to understand the structure and folding of each. Soluble proteins fold in the context of an isotropic, highly polar solvent which competes effectively for interactions between backbone polar groups, while also driving the collapse and exclusion of hydrophobic groups (4). Membrane proteins, on the other hand, by virtue of their propensity to partition into membranes, fold in an environment that is very different from water (6;12–14). The membrane is highly anisotropic, changing from bulk water to essentially pure hydrocarbon, back to bulk water over a distance that is equivalent to only 20 amino acid residues in a helical polypeptide-chain (5). The hydrophobic core of a bilayer exerts significant constraints on membrane protein structure and greatly impacts the membrane protein folding process. Specifically, the very low concentration of exogenous polar groups in the bilayer forces protein backbone groups into hydrogen bonds, such that the free energy cost of being in the bilayer are lower. Thus, the membrane strongly favors hydrogen bonded secondary structures, α-helices and β-sheets, that can span the entire bilayer(6).

The driving effect of bilayer hydrophobicity on the formation of TM helices and the fact that TM helices are roughly perpendicular to the bilayer plane has been well understood for some time (6;15;16). What concerns us most in this review, and what must be understood in molecular detail in order to solve the membrane protein folding problem, is how the lateral interactions between transmembrane (TM) α-helices depend on the sequence, and how the physical chemistry and architecture of the membrane modulates these interactions.

3. Physical chemistry of lateral helix interactions in membranes

A very simple, but useful, way to begin thinking about interactions between TM helices in membranes is to consider the bilayer containing TM helices as a two-dimensional co-solvent system. Such binary systems can be realistically modeled with Monte Carlo simulations, for example, in which the only parameters are the strength of the pair-wise, nearest neighbor interactions; lipid-lipid, helix-helix and helix-lipid (17). Due to the strict confinement of lipids and transmembrane helices within the bilayer, helices and lipids will interact at all times with a full set of nearest neighbors. Therefore, a "monomeric" helix is simply one which interacts at least slightly better with lipids than with other helices. Similarly, helical "self assembly" will result if helix-helix or lipid-lipid interactions are slightly favored over helix-lipid interactions. Such models of membrane organization are useful in thinking about the problem of helix dimerization in membranes. First, they provide a useful thermodynamic

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yardstick for understanding lateral interactions in membranes by showing that, because of the confinement to two-dimensions, differences in interaction energies as small a 1–2 times the thermal energy (k_BT) (i.e. less that 2 kcal/mol) can give rise to significant "demixing" of components, including TM helices, in a bilayer. Indeed, TM helix dimerization free energies of this magnitude have been measured in lipid bilayers (18–21). Second, these simple models remind us that helix dimerization does not take place in a vacuum. Just as the non-specific, unfavorable interaction of hydrophobic groups with water drives soluble proteins to fold into highly specific three dimensional structures, unfavorable helix-lipid interactions could play as important a role in determining the propensity of TM helices in membranes to self-associate. Next we discuss some of the physical chemical principles of lateral interactions between helices and lipids in membranes.

Nonpolar interactions and the "lipophobic effect"

What factors contribute to the sequence-specific interactions between TM helices? One factor may be a "surface tension"-like effect that arises from unfavorable interactions between the surface irregularities of TM helices and the linear alkyl chains of the bilayer lipids. From the perspective of helix-helix interactions, this effect has long been described as a "knobs into holes" or "ridges into grooves" interaction (22). However, by analogy to the hydrophobic effect in water, which is largely caused by unfavorable solvent interaction, hydrophobic helix dimerization in membranes should probably be considered predominantly to be a "lipophobic effect" arising, at least partially, from unfavorable peptide-lipid interactions (23;24). Again, by analogy to the hydrophobic effect, the specificity of a lipophobic interaction arises from the degree of surface complementarity. For example, a better fit between the surfaces gives rise to more favorable van der Waals interactions. However, the lipophobic effect is not well understood and yet not quantified in physical-chemical terms.

Polar and other interactions

Relative to a hydrophobic side-chain, a polar side-chain in a TM helix creates a thermodynamically unfavorable situation if it is exposed to the lipid hydrocarbon. Peptide and protein-based hydrophobicity scales (25–29) show that the cost of placing polar groups in a bilayer can be from 0.3–1.0 kcal/mol for a simple polar group (such as a hydroxyl) to 1–6 kcal/mol for a peptide bond or a charged side-chain (6;28). Salt bridges and hydrogen bonds between polar groups within the hydrophobic environment can reduce the energetic cost (30), thereby driving helix dimerization. It is sometimes assumed that TM helices with polar groups in bilayers will always be strongly driven to self-associate, making a TM polar residue the simplest of the "dimerization motifs". While this is sometimes the case, there are also examples of membrane-embedded polar groups that do not drive self association in membranes (31). In part this is because the energetic cost of inserting polar groups in the bilayer is smaller than previously believed (28;32) and in part it is because the helix/bilayer system can respond in ways that do not involve dimerization. As we discuss in detail below, polar group interactions in membranes can be important, but they are more context dependent, and less promiscuous, than frequently assumed.

Other types of interactions have also been proposed to be important contributors to helix dimerization, although direct evidence is generally lacking. For example, to explain the preponderance of glycine in helix-helix interfaces (e.g. GxxxG motifs), it has been proposed that polar dipole-dipole interactions between hydrogen-bonded peptide bonds brought into very close proximity by glycine-glycine contacts can contribute favorably to interactions (33). Cation-π interactions may help explain the occurrence of unpaired arginines in TM helices (34–36). Aromatic π-π (i.e. ring stacking) interactions (36) may occur between aromatics, especially Phe, in interacting TM helices (37;38).

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The contribution of bilayer properties to TM helix dimerization

The effect of lipid composition on TM helix dimerization is likely significant. Several measurements of GpA TM helix dimerization in bacterial and in mammalian membranes have suggested that only weak dimerization takes place in these complex environments (39–41), in contrast to the strong dimerization that takes place in detergents and in some synthetic membranes (42). But even in synthetic bilayers, dimerization of GpA is surprisingly sensitive to bilayer properties. Bowie and colleagues measured the dimerization of the GpA TM domain in synthetic membranes of varying composition and found very dramatic effects of membrane properties on dimerization (41). Specifically, they observed strong dimerization of GpA in pure phosphatidylcholine bilayers which decreased substantially when anionic lipids were added. They found that dimerization decreased even more when a model bacterial membrane protein was present, supporting the idea that GpA probably dimerizes weakly in bacterial and mammalian membranes. More studies like this one, which utilizes a direct measurement of dimerization, are needed before we really understand how much bilayer properties affect TM helix dimerization.

The contribution of the bilayer structural anisotropy to helix dimerization has barely been explored, but is probably significant. For example, the energetics of inserting polar groups in the bilayer depends sharply on their depth in the bilayer. The cost is highest in the exact bilayer midplane, and it decreases dramatically as the polar group moves away from the midplane (28;29). As a result, a TM helix with an unfavorable lipid-exposed polar group may be able to lower its free energy minimum by 1) dimerizing via salt bridge or hydrogen bond formation, 2) shifting its position vertically in the bilayer, or 3) a combination of both. Polar group-driven dimerization has been demonstrated for the pathogenic valine 664 to glutamate mutation in the rat ErbB2 TM domain (43–45). On the other hand, the pathogenic glycine to arginine mutation at position 380 in the TM sequence of human FGFR3 causes a vertical shift of the helix in the membrane with no change in dimerization propensity relative to the wild type sequence (31;46–48).

The contribution of “lipophobic effects”, which will depend on acyl chain flexibility, are also expected to change with bilayer depth. However, this contribution to dimerization should change in a direction opposite to that of polar effects. Because lipophobic effects are dependent on “surface tension” at the peptide-lipid interface, they should decrease toward the bilayer center because the acyl chain flexibility increases in the vicinity of the terminal methyl groups (49).

Juxtamembrane sequences and TM domain boundaries

In a native membrane protein or in a chimeric membrane protein used as a model system (e.g. ToxCAT or ToxR bacterial reporter systems), the transmembrane sequences are not isolated peptides, but rather are surrounded by the so called “juxtamembrane” regions. These interfacial or non-membrane inserted sequences are known to have significant effects on TM helix dimerization in at least some native systems (50) but are rarely considered in model system studies of TM domains. Juxtamembrane sequences can conceivably affect TM helix dimerization directly by specific interfacial interactions, or indirectly by influencing the local bilayer properties, the depth of TM domain membrane insertion or the allowed rotational angles of the TM domains. In model peptide systems, juxtamembrane sequences are usually absent or replaced by polycationic sequences (20;51–53), further complicating the interpretation of TM helix dimerization.

4. Membrane mimetic environments

The interactions between TM helices have been studied in a variety of “cell membrane mimetic” environments. The earliest experiments on helix dimerization were performed by

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SDS polyacrylamide gel electrophoresis (SDS PAGE) in which electrophoretic mobility is related to molecular weight (54;55). SDS PAGE is still frequently used, and can be informative (51;54;56;57): certain TM helices dimerize specifically and strongly in SDS PAGE, presumably because the SDS micellar environment mimics the hydrophobic core of the bilayer. On the other hand, there are TM helix systems that behave anomalously in SDS PAGE (58;59), so SDS PAGE results must be interpreted with caution. Other detergent micelles and detergent-lipid bicelle systems are also commonly used to study helix-helix interactions with techniques such as Förster resonance energy transfer (FRET) and analytical ultracentrifugation (60;61). While there are a few studies that address the effect of detergent species on helix dimerization propensities (62), the variety in the hydrophobic and hydrophilic moieties and the variation in the physical properties of detergent micelles suggest that the detergent-specific effects can be significant. It is also likely that certain types of helix-helix interactions are exaggerated (or diminished) in detergents, as compared to membranes.

Synthetic membranes have also been used to study helix-helix interactions, and while they present more technical challenges as experimental systems, they are closer to representing the “native” environment of a transmembrane helix. In bilayers, helix-helix interactions can be studied with FRET, thiol cross-linking, or the “steric trap” method (42;63–67), for example. In recent years, reporter systems in biological membranes have increasingly been used to probe TM helix dimerization. These include a variety of bacterial reporter systems (36–39;50;68–79) as well as mammalian systems (40;80). Combining biased genetic libraries with reporter systems that can also be used for selection has led to the discovery of hundreds of novel interacting TM helices (37;38;75;81).

Despite a large amount of data available, it is not known if the diversity of membranes and membrane mimetic environments that have been used to study helix-helix interactions has clarified or has clouded our understanding of the fundamental principles of membrane protein folding. The degree of overlap between the physical principles that guide membrane protein folding and TM helix interactions in these different environments remains to be determined. Furthermore, it remains to be determined if certain experimental systems favor specific types of interactions over others. In some sense, it might seem that we are as far from solving the membrane protein folding problem as ever because we cannot yet predict with accuracy whether or not a particular TM helix will dimerize in a particular hydrophobic environment.

5. Dimerization motifs

Glycophorin A and the GxxxG sequence motif

More than 30 years ago, it was shown that glycophorin A (GpA), one of the major sialoglycoproteins of human red blood cells, forms detergent-resistant dimers in SDS PAGE (82). Dimerization of GpA was also shown to occur in the membranes of intact cells (83). In the early 1990’s, experimental studies showed that GpA dimerization was driven by its single α-helical TM domain (54;84). The TM segment of GpA is a seemingly unremarkable, mostly hydrophobic sequence (I_{73}TLII$_{77}$FGVM$_{81}$AGVI$_{85}$GTIL$_{89}$LISY$_{93}$GI) with a hydrophobic composition that is typical of single span TM domains. Extensive deletion and site-directed mutagenesis studies revealed the sequence-dependence of GpA dimerization (55;84). Specifically, replacement of a particular set of residues (Leu75, Ile76, Gly79, Gly83, Val84, and Thr87) with other hydrophobic amino acids resulted in decreased dimerization as assessed by SDS PAGE. Thus, these residues were assumed to comprise the dimer interface (55). Other residues could be mutated with essentially no effects on dimerization, and were suggested to face lipids, away from the dimer interface (54;55;85). These results, combined with the model proposed by Treutlein et al (86) lead to the

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identification of a putative dimer interface: $L_{75}IxxG_{79}VxxG_{83}VxxT_{87}$. The pattern of interacting residues suggested that the GpA dimer formed a right-handed helical cross. The propensity of this sequence pattern to drive dimerization of TM helices was confirmed by showing that it can drive the dimerization of polyleucine membrane spanning helices (87).

The NMR structure of the GpA TM domain dimer in detergent micelles, and later in bilayers, confirmed the putative dimer interface and provided structural details (22;88). The structure showed that the two glycines (Gly79 and Gly83) form a “groove” that allows the helices to pack very closely against one another in a “ridges-into-grooves” manner. Since the “groove” glycines cannot be mutated without a significant disruption of dimerization, Engelman and colleagues concluded that the most important aspect of the GpA dimerization interface are the two glycines separated by three amino acids in the primary sequence (86). It was later shown that the GxxxG motif was highly overrepresented in the sequences of membrane proteins (11;89). The motif was also specifically selected in genetic screens for TM dimers (89). As a result, the GxxxG sequence was proposed to drive TM helix dimerization. Thus, the paradigm that a simple sequence motif is sufficient to drive association of TM helices, was born.

Although GxxxG is, statistically, the most over-represented simple sequence motif found in membrane proteins, similar motifs with other small side-chains separated by three residues were also found to be over-represented in membrane proteins (89). Thus, the SmxxxSm motif, where “Sm” is a small residue (Gly, Ala, Ser or Thr), often referred to as a “GxxxG-like motif”, was proposed as a more general interaction motif for TM helices. In the literature, SmxxxSm motifs are often suggested to be important in dimerization of TM helices in the absence of GxxxG motifs. Yet, such motifs are very abundant in membrane proteins (see Figure 2A), such that a dimerization interface or a protein fold cannot be predicted based on their occurrence.

In addition to glycophorin A, there are some additional recent examples of GxxxG or SmxxxSm motifs that seem to drive TM helix dimerization. A glycine-rich dimerization motif (GxxGxxxAxxG) was recently identified in the N-terminal transmembrane domain of scavenger receptor class B, type I (SRBI). The submotif GxxxAxxG was shown to play a significant role in receptor homodimerization and lipid uptake activity (90). The GxxxG motif in the first transmembrane segment of the Japanese encephalitis virus prM protein was shown to be involved in the heterodimerization with E proteins (91). Replacement of either Gly with Ala, Leu or Val showed that both glycines in the GxxxG motif are equally important for heterodimerization. Furthermore the crystal structures of the ErbB2 (92), EphA1 (93), and BNIP3 (94) (95) homodimers, and of the αIIb/β3 (96;97) and ErbB1/ErbB3 heterodimers (98) all reveal helical packing enabled by SmxxxSm motifs.

However, there are also many examples of GxxxG or SmxxxSm motifs that are not sufficient for strong TM helix dimerization. Schneider and colleagues studied the dimerization propensities of the 58 human receptor tyrosine kinase (RTK) TM domains using the ToxCAT dimerization assay (99), and found no obvious correlation between the measured dimerization propensity and the presence or absence of SmxxxSm motifs. All but seven of the 58 sequences contain at least one SmxxxSm motif and many contain two or more. Yet dimerization of these 58 sequences, relative to the glycophoprin A TM helix, ranged from very weak to very strong. As one particular example, the TM domains of the discoidin domain family of receptor tyrosine kinases (DDR1 and DDR2), two of the strongest dimerizing RTK TM domains in Schneider’s study, contain GxxxA motifs. However, mutations in the GxxxA motif did not change the dimerization propensity (70), suggesting that the GxxxA motif is not involved in dimerization, or that there is an alternate dimer structure. Similarly, the TM domain of the platelet-derived growth factor receptor

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beta (PDGFβ) contains a SxxxA motif, but mutation of the Ala residue to either Leu or Trp resulted in dimerization comparable to that of the wild-type sequence in bacterial membranes (50). Furthermore, significant changes in dimerization were observed when other residues in the sequence (which are not part of the SmxxxSm motif) were mutated. These studies, and earlier studies by Fleming and co-authors (100–102), have convincingly demonstrated that GxxxG and SmxxxSm motifs while sometimes dominant, are “neither necessary nor sufficient” (101) for TM helix dimerization. This view is corroborated by the solved structures of ErbB3 (103), EphA2 (104), ζζ (105), and DAP12 homodimers (106), and the Sx1A/Syb2 heterodimer (107). The interfaces of these dimers do not involve SmxxxSm motifs, despite the fact that such motifs are present in the sequences.

In a recent study that addresses the specificity of dimerization motifs (108), we used an SDS-PAGE based high throughput screen to select strongly homo-dimerizing sequences from a combinatorial library based on the rat *neu* (ErbB2) TM domain. In the 3,888-member peptide library there were a very large number of recognizable dimerization motifs. For example, every library member had at least one SmxxxSm motif, most library members had one or more polar groups, over 50% of the library members had at least one ionizable amino acid, and over 20% of the library (i.e over 800 members) contained GxxxG motifs. Yet, despite the high abundance of recognizable dimerization motifs we found only six dimerizing sequences in the entire library. Of these, only one contained a polar group other than threonine, and only three of the six dimerizing sequences contained GxxxG motifs (two of these three had contiguous GxxxGxxxG motifs). These results confirm that the specific structural context is a very important contributor to the dimerization propensities of peptides with known sequence motifs, and supports the idea that known motifs, while important, do not completely control the interactions between the TM helices.

Motifs with polar residues

The exposure of polar residues in a TM helix to the hydrocarbon core of a lipid bilayer membrane is energetically unfavorable (6). Thus, TM helices with polar residues may be expected to dimerize in a promiscuous way, so they can bury the polar groups in the dimer interface, away from the lipid environment. Consistent with this view, it has been shown that in some cases polar residues drive dimerization of natural TM domain sequences. For example, Shai and colleagues demonstrated that the two polar residues in the QxxS sequence motif of the bacterial aspartate receptor (Tar) transmembrane domain are essential for its dimerization (109). Interchanging the two polar residues had no effect on dimerization, while mutating them to nonpolar residues reduced dimerization (109). Furthermore, exchanging the QxxS motif with the GxxxG motif disrupted the dimer significantly.

Schneider and Engelman studied the effect of mutating Gly79 in GpA TM domain with hydrophobic and polar residues (77). Whereas most substitutions at Gly79 in GpA decreased dimerization substantially, serine caused only a slight disruption of dimer stability. The authors suggested that the small effect may arise because the substitution introduces a hydrogen bond between serine’s hydroxyl group and the backbone carbonyl of the adjacent helix. Furthermore, the incorporation of hydrophilic residues, such as Glu, Asp and Asn, into a hydrophobic TM domain can drive dimerization, but does not always do so (110;111). The incorporation of less polar residues such as Ser, Thr and Tyr frequently does not result in a significant change in dimerization (111).

In the absence of glycine, and thus in the absence of GxxxG motifs, Engelman and colleagues showed that SxxSSxxT and SxxxSSxxT motifs are the two most over represented motifs in a pseudo-random genetic library selected for TM helix dimerization in bacterial membranes (112). Mutagenesis and TOXCAT assays showed that the interaction between

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helices with these motifs is position-specific, and that the presence of multiple polar residues in the motif leads to a cooperative stabilization of the dimer, presumably through a network of interhelical hydrogen bonds (112).

Engelman and colleagues further showed that biological transmembrane domains with strongly polar residues are not always stabilized by the polar residue; only appropriately placed polar residues lead to a significant increase in dimerization (69). Thus hydrogen bonds are formed only if a dimer interface is already present. This is also likely the case in BNIP3, where histidines stabilize a dimer that also utilizes an AxxxG motif in the dimer interface (56). Consistent with this view, in a genetic screen of pseudo random sequences, histidines were shown to stabilize TM helix dimers more frequently when a GxxxG motif was also present in the sequence (75). Furthermore, in an asparagine scanning mutagenesis study, the erythropoietin TM dimer was shown to be stabilized by interhelical hydrogen bonds formed by Asn only when the mutated positions were already facing each other in the dimer (113). A similar effect can explain the effect of the pathogenic V664E mutation in the TM domain of Neu (rat ErbB2) receptor tyrosine kinase, as discussed below.

More recently, a set of strongly interacting, heterodimeric TM domains that contain the GxxxG motif in addition to ionizable residues of opposite charge were found from a combinatorial genetic screen (75). This finding prompted a genomic search which showed that this motif is significantly over-represented in putative TM domains. The dimerization assays showed that stabilization of heterodimers (from two TM domains with residues of opposite charges) is highly specific (e.g., exchanging Asp for Glu abolishes the dimer) and depends on the position of the ionizable residues (75).

The work reviewed above suggests that polar interactions (H-bonds and salt bridges) are relevant in dimerization of transmembrane helices. Polar residues can stabilize an existing dimer interface, but do not introduce promiscuous interactions and rarely create a novel dimerization interface.

6. Leucine zippers

In soluble proteins, a small fraction of interacting α-helices are known to interact through simple repeated sequence motifs comprised of hydrophobic residues that repeat every one or two helical turns. For example, the leucine zipper motif of coiled-coils is easily recognizable because it has hydrophobic residues at every fourth position in a seven residue (two helical turn) heptad repeat. The seven positions in the repeat are referenced as *abcdefg*. In a leucine zipper, the *d* residues are Leucine, Isoleucine, or Valine (114). These hydrophobic residues within a hydrophilic context form the contacts between the helices. In the study of dimerizing TM helices, “leucine zipper”-like motifs have been invoked when SmxxxSm motifs do not explain the available data (50;74;115). For example, Ruan and others (113) studied dimerization of the erythropoietin receptor TM domain using asparagine-scanning mutagenesis and bacterial membrane dimerization assays. The authors did not observe promiscuous dimerization caused by asparagine, but instead found that Asn residues had highly variable effects on dimerization. They reasoned that Asn mutations must stabilize the dimer only when the position of the Asn residue was already part of the dimer interface. Mutations in Leu234, Ser238, Leu241 and Ala245 resulted in the strongest dimerization. Because these positions (S_{231}xxLxxxS_{238}xxLxxxA_{245}) occur at the first and fourth residues of a putative heptad repeat, with leucines at every fourth position, the authors concluded that a leucine-zipper-like motif was involved in dimerization of this sequence in membranes. A leucine zipper motif has also been invoked to explain the strong self-association of the transmembrane domain of the discoidin domain family of receptor tyrosine kinases (DDR1 and DDR2) (70). The transmembrane domain contains a GxxxA motif, not important for

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dimerization, within a putative leucine zipper-like motif (I_{420}**G**xL_{423}x**A**xI_{427}xxL_{430}xxI_{433}I), and the mutation of the Leu-Leu and Ile-Ile pairs to Gly-Pro resulted in a significant reduction in the dimerization.

Unlike the case for soluble proteins, questions remain whether the leucine zipper can be considered a recognizable motif that can drive dimerization between TM helices. In the DDR1 and DDR2 study above, Gly-Pro substitutions are very dramatic mutations which may disrupt any helix-helix interaction motif. Also, the abundance of leucine, isoleucine and valine in TM domains overall is very high, and thus "leucine zipper" sequence motifs are very abundant (see Figure 2B). Multiple "leucine zipper" motifs can be recognized within almost every TM helix. These "motifs" are sometimes just parts of sequences consisting of many consecutive leucine and isoleucine residues, as they are in the erythropoietin receptor TM domain, which is inconsistent with the packing of true leucine zippers.

7. The sequence motifs paradigm as a surrogate tool for structure determination

In comparison to soluble proteins, high resolution structures of membrane proteins are rare because they are difficult to obtain. Major bottlenecks include difficulties of overexpression of membrane proteins in large quantities needed for such studies, misfolding during purification, low success rate in crystallization and difficulty with NMR structures because of the need to embed membrane proteins in micelles or bicelles. While the number of solved membrane protein structures is steadily increasing (116), for the foreseeable future, most novel membrane proteins under study will have no structural homologues in the databases. This makes the prediction of membrane protein structure and the solution of the membrane protein folding problem difficult to obtain.

TM helix dimers are inherently disordered, possibly heterogeneous in structure, and are not amenable to crystallography. NMR is the only direct route to structure determination, and thus solving TM helix dimer structures is particularly challenging. While recently we have witnessed a few successes, still there are only about ten structures of TM domain dimers available (22;92–98;104–107). These structures have not yet provided enough information that will allow us to predict the occurrence of dimerization based on sequence.

In the context of this lack of structural information, a surrogate tool for "structure determination" is based on the paradigm that sequence motifs completely control the interactions between TM helices. In this paradigm, the interaction will be abolished if the interacting sequence motif is perturbed. Following this line of thinking, the structure determination approach combines mutagenesis and dimerization propensity measurements, based on SDS PAGE or bacterial membrane reporter assays (50;56;70;117). If the paradigm is correct, a mutated residue participating in the dimer interface will cause destabilization, while a mutated residue that interacts with lipids will not change dimer stability. Thus, the contacts between the two helices can be identified. The underlying assumption is that the same TM dimer structure exists for the wild-type and for all mutants, and a destabilizing mutation affects monomer-dimer equilibrium, but does not alter the dimer structure. This approach has been successful in some cases. For example, it has been used to identify the critical residues that mediate helix-helix contacts in the wild-type GpA dimer (86;87) (reviewed above) and BNIP3 dimers (56). In these studies, the mutation of the motif residues inhibited dimerization. Yet, the effects of hydrophobic substitutions at the motif residues on dimer stabilities were varied, and could not be fully explained by the assumption that the mutants either adopt the wild-type structure or do not dimerize. For instance, the mutagenesis of some residues in BNIP3 that are not part of the dimer interface lead to modest dimer destabilization (see Figure 3).

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While the effects of mutations on stability are generally well rationalized within the context of the wild-type GpA and BNIP structures, in other cases mutagenesis does not produce meaningful results. One example pertains to FGFR3, which forms a unique dimer structure that was solved recently (118). Yet, mutagenesis of almost all amino acids in the sequence does not result in a significant disruption of the dimer (Li and Langosch, unpublished). Furthermore, multiple mutations in GpA have been shown to produce stable dimers with different, unknown structures (101). Thus, the utility of the mutagenesis-based structural approach is not universal. Even if there is a single wild-type dimer structure, sequence changes can affect both protein-lipid and protein-protein interactions, and alter the dimer structure.

8. The complete lack of mutant structures leaves us in a state of uncertainty

The effects of pathogenic mutations, or engineered changes in TM domains, are often rationalized within the context of the sequence motif paradigm. The default assumption in most cases is that the mutant structure is the same as the wild-type structure. This assumption is understandable, as no mutant structures are available. Yet, it is probably not always valid, as mutations can induce structural changes that cannot be predicted based on the wild-type structure. Thus, in the general case, the wild-type structure cannot be a guide or a reference state for the mutant structure. An example of a mutation inducing a structural change is the G380R mutation in FGFR3 TM domain. In the wild-type dimer structure, G380 is in the dimer interface (118), and thus it is tempting to speculate that the mutation is stabilizing the dimer, as Arg has hydrogen bonding capabilities. However, dimer stability measurements invariably show that the stabilities of the wild-type and mutant dimers are very similar, both for the isolated TM domain, and the full-length receptor (31;48). In synthetic membranes, TM domain studies show that the depth of the mutant and the wild-type helix in the bilayer are different and that the dimerization propensities are the same (34;48;119). Thus, the behavior of the mutant sequence cannot be predicted based on our knowledge of the wild-type sequence or structure.

9. The concept of a motif switch

As the importance of the GxxxG and SmxxxSm motifs in helix dimerization was becoming clear, some researchers noticed that there were many TM sequences with two or more distinct dimerization motifs. For instance, the TM domains of the human ErbB1, ErbB2, and ErbB4 receptors have at least two SmxxxSm motifs, one near the amino end and one near the carboxyl end of the TM domain, separated by roughly three turns of the α-helix. To explore which of these motifs is important for dimerization, Lemmon and colleagues mutated the critical glycines to valines in these motifs (117). They found that for ErbB1, only mutations in the C-terminal motif reduced dimerization, while for ErbB4, only mutations in an N-terminal motif reduced dimerization, suggesting that only one motif is used or each. For the ErbB2 TM domain, however, alteration of either motif reduced dimerization (117). Because it is unlikely that both motifs would be used at the same time, it was proposed that the ErbB2 TM domain has two alternative dimer structures, corresponding to inactive and active receptor structures, and that either can be used.

Computations by ben Tal and colleagues further suggested that the TM domain of ErbB2 may undergo dimerization via either one of the two dimerization motifs (120). Most importantly, the calculations showed that the dimer could switch between the two structures, by having the helices slide and rotate 120° with respect to the other. This switch could occur without encountering a prohibitive energy barrier. The authors argued that this switch may explain available data about the pathogenic V664E mutation in ErbB2 (Neu). If the

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dimerization occurs via the C-terminal SmxxxSm motif, Glu664 should be exposed to lipids, which may be energetically unfavorable. In the alternate putative structure, involving the N-terminal SmxxxSm motif, Glu664 is less exposed to lipids, which is presumably less costly. Thus, in the mutant, the C-terminal motif dimer structure should be depopulated, while both structures are available to the wild-type dimer. Because the mutation increases the activity of the ErbB receptor in cellular systems (121), the authors deduced that the mutation-stabilized structure, engaging the N-terminal motif, is the active structure, and that the other is inactive.

10. The structural evidence for switches is inconclusive

As discussed above, the ErbB2 TM dimer structure confirmed the prediction that a SmxxxSm motif is important for its dimerization (92), as the amino acids participating in the dimer interface are Thr652, Ser656 and Gly660. The ErbB2 dimer utilizes the N-terminal dimerization SmxxxSm motif, and corresponds to what Ben Tal termed “the active structure”. The C-terminal SmxxxSm motif is not used for dimerization in the structure, a surprising finding since the computational work of Ben Tal predicted this to be the structure with the lowest free energy minimum. In the solved ErbB2 (HER2) structure, the position of the oncogenic Val659Glu mutation (analogous to Val664Glu in Neu) faces the interface, and the structure is consistent with the formation of stabilizing Glu-mediated hydrogen bonds that do not distort the structure. The argument of dimerization through the N-terminal motif for both the wild-type and the mutant explains the observed ErbB2 dimer stabilization in cellular systems (43;45), without a need to invoke a switch. In this regard, it should be noted that Shai and colleagues proposed that the two SmxxxSm motifs in ErbBs are responsible, alternately, for homo- and heterodimerization, rather than being involved in a homodimer switch (122).

The ErbB3 sequence has only an N-terminal SmxxxSm motif, but the dimer structure shows that it does not utilize this motif. Instead, ErbB3 forms a left-handed dimer, with contacts mediated by a very hydrophobic interface IxxLVxIFxxLxxxFLxxR (103). The ErbB3 dimer interface, which is not predicted or described by any previously recognized dimerization motif, is stabilized by van der Waals interactions between bulky hydrophobic side-chains, and perhaps also π-π and cation-π interactions. From the paradigm of dimerization motifs and the idea of a motif switch, it may be speculated that two alternative, dimeric structures exist for the ErbB dimers: one right-handed as the one captured in the ErbB2 structure, utilizing the SmxxxSm motif, and one left-handed as the one captured in the ErbB3 structure. However, an alternative and straightforward interpretation of the data is that ErbB2 and ErbB3 each have only a single unique structure.

The structure of the TM domain dimer of the erythropoietin-producing A1 (EphA1) receptor (93) also utilizes a SmxxxSm motif close to the N-terminus, with contacts mediated by Ala550, Gly554, and Gly558. However, there is also a hint of a second dimer structure that is sparsely populated. While the high resolution structure of the second conformation could not be solved, the measured chemical shifts suggest that the dimer interface is likely composed of Leu557, Ala560, Gly564, and Val567, so it again involves a SmxxxSm motif. The authors argue that there could be a switch between the two structures that is important for EphA1function; however, this remains to be demonstrated experimentally. Like EphA1, EphA2 has a GxxxG motif near the N-terminus. Yet, the dimer interface of the EphA2 TM domain dimer does not use this motif. Instead it is stabilized by the motif LAxIGxxAVxVVxxLVxxxxxFF, involving van der Waals contacts and π-π stacking interactions (104). Like the ErbB3 dimer, the EphA2 dimer structure utilizes a unique and unexpected dimerization interface that does not belong to a known motif. Here again, the sequence motif paradigm and the motif switch concept would suggest that both EphA1 and

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EphA2 can alternate between two possible structures (104). However, the simpler explanation, that the EphA1 and EphA2 dimers, each has a single unique structure, cannot be excluded without additional data.

11. Beyond the sequence motif paradigm

In our current understanding of soluble protein folding, the concept of simple sequence motifs as drivers of interactions is not widely used. The leucine zipper dimerization motif, and the related heptad repeat motif of coiled coils are notable exceptions (114). Yet, we have long hoped that interactions within membrane proteins could be defined by a set of simple sequence motifs. Indeed, the sequence motif paradigm can describe some interacting TM helices such as glycophorin A (GpA) exceptionally well. Furthermore, in many cases the principles derived from the GpA studies have been shown to be applicable to other membrane proteins (reviewed in (10)). But as more and more systems are being explored, and as more and more unique interacting helices are described, it is becoming clear that the sequence motif paradigm is incomplete. Some helices interact in membranes despite having no recognizable motifs, and perhaps more importantly, sequences with recognizable sequence motifs do not always interact using these motifs. Often SmxxxSm or “leucine-zipper” motifs are invoked to rationalize data. However, the abundance of small residues and leucines and other aliphatics is naturally very high in transmembrane sequences, giving rise to many apparent motifs (Figure 2). This would predict the occurrence of many non-specific interactions in cellular membranes if these motifs were indeed sufficient for TM helix dimerization. Thus, the simple sequence motif paradigm is an oversimplification of a system of interactions that is much more complex and less well understood than previously recognized.

The idea of the sequence motifs as drivers of interactions does not take into account all the different driving forces of TM helix interactions reviewed above. Yet, there is no doubt that all these forces contribute to the interactions. In addition, there may be interactions that are not yet well understood and described. Many questions remain, and the conceptual framework behind the membrane protein folding problem is still not fully developed.

There is a growing body of data available on TM domain dimerization, including genetic screens that have identified many interacting sequences in bacterial membranes (37;38;74;75;75), as well as a still small but growing number of membrane protein structures (22;92–98;104–107). While there are currently too few structures to support the widespread use of homology modeling for membrane structure prediction, we are hopeful that the available data hide the underlying set of principles governing TM helix interactions, and that these principles can be extracted.

Some successes have been reported for molecular dynamics simulations of TM helix dimerization (123), especially if course grained models are used. These studies have been successful in modeling the TM domain dimers of glycophorin A (124;125), the ErbB receptors (126–128), and BNIP3 (129). However, neither experimental data on dimerizing sequences nor simulations alone are likely to solve the membrane protein folding problem. Perhaps we will need novel computational approaches that utilize training and feedback, based on available data, including the large amount of genetic selection data, to delineate the rules needed to describe and predict the folding of membrane proteins. We are looking forward to novel prediction tools that are as sophisticated as ROSETTA and other state-of-the-art docking programs (3) used for soluble proteins to provide information on the unique physical chemistry of protein folding in membranes.

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12. Conclusion

The idea of sequence motifs has been very useful in revealing some of the ways in which TM helices can interact. However, recent data has made it clear that the presence of known sequence motifs alone does not guarantee interactions. Furthermore, TM sequences that do not contain any recognizable motifs can interact. Experimental evidence has accumulated to strongly suggest that the search for sequence motifs, as mediators of TM helix dimerization, cannot solve the membrane protein folding problem alone. By analogy with soluble proteins, we expect that structure determination and computational approaches will lead the effort to solve the membrane protein folding problem.

Highlights

Transmembrane helix dimerization studies shed light on membrane protein folding

Helix dimerization in membranes has been described within the sequence motif paradigm

The sequence motif paradigm in membrane protein folding is incomplete

The search for sequence motifs cannot solve the membrane protein folding problem

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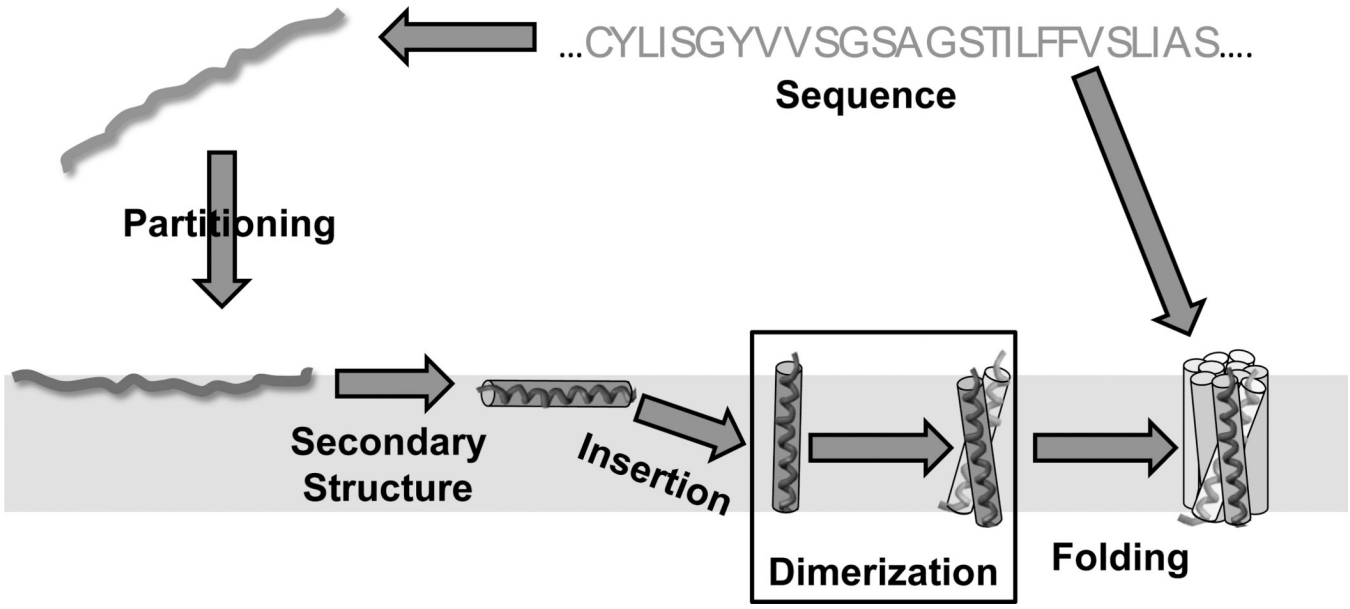

Figure 1. The membrane protein folding problem

In the pursuit of a solution to the membrane protein folding problem, it has been useful to separate the sequence-structure relationship into individual steps that can be experimentally characterized and quantitated on their own. For example, in this five stage model, sequence hydrophobicity drives partitioning and insertion, while lateral interactions between inserted segments drive dimerization and folding. In this review, we discuss what has been learned from studying the simplest folding reaction in membranes: the dimerization of membrane-spanning α-helices.

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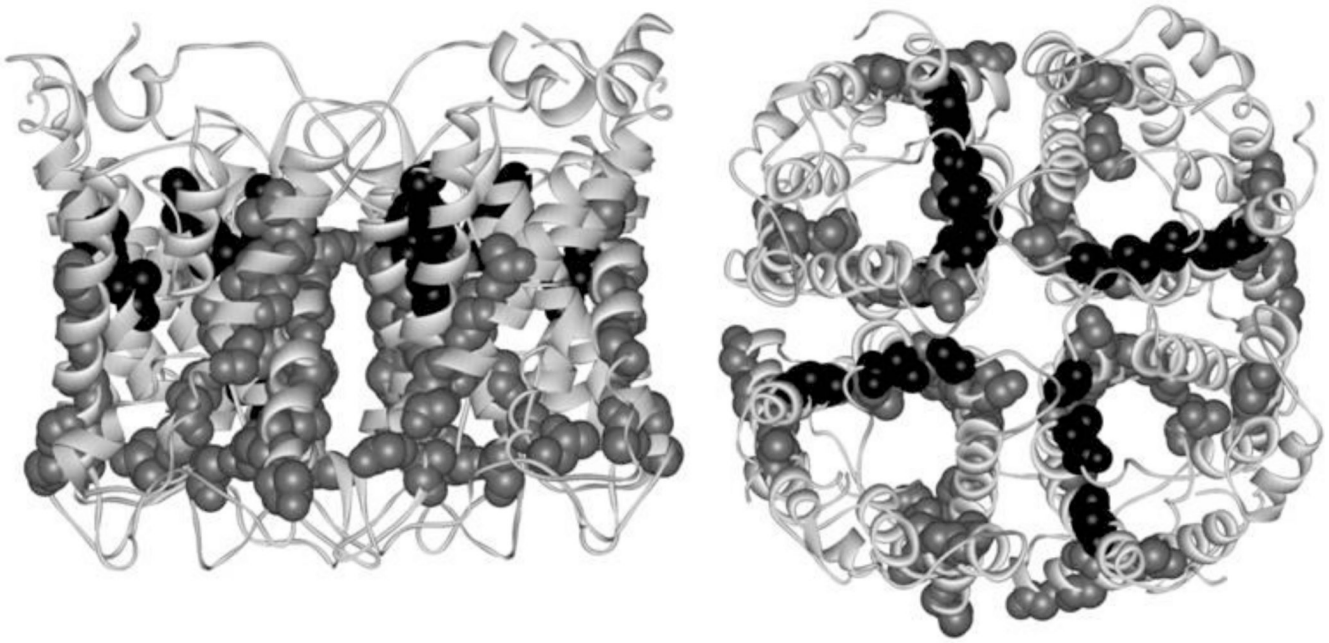

2A.

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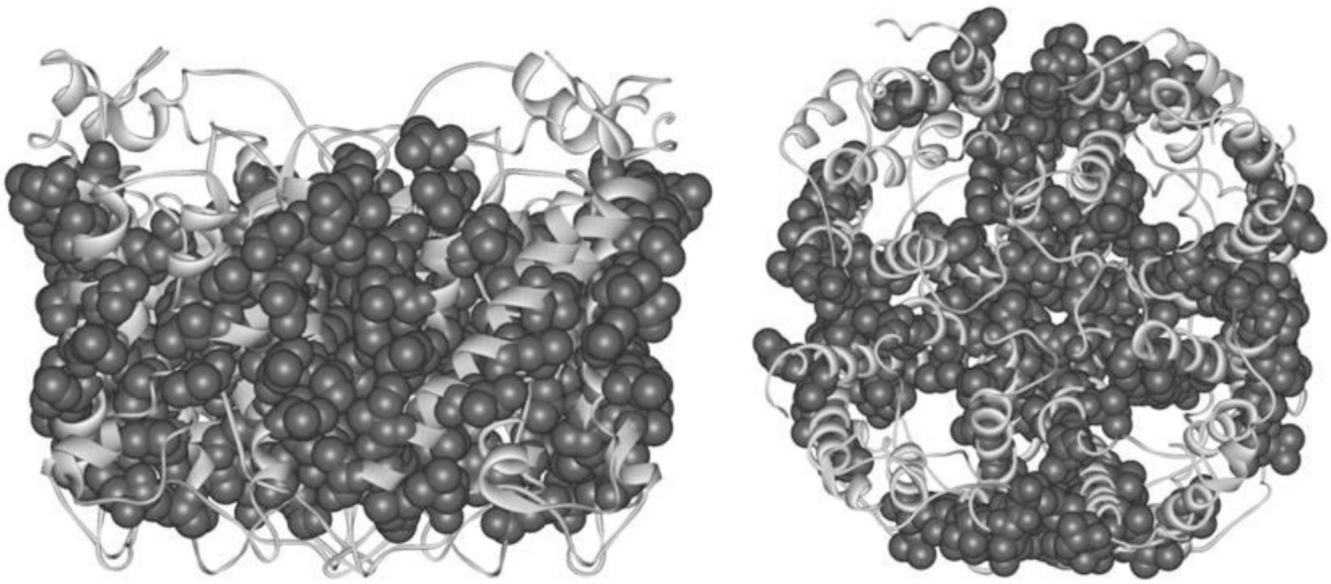

2B.

Figure 2. The abundance of several motifs in a typical membrane protein

The example protein shown here is the tetramer of aquaporin 1, but all membrane proteins have similar composition. **A:** All residues in the TM helices that are part of a pattern of SmxxxSm, where Sm = Gly, Ala, Thr or Ser, are shown in gray. SmxxxSm motifs that pack against one another (and thus might be involved in lateral interactions) are shown in black. Most motifs are not involved in interactions between helices. **B**. Leucine zipper-like motifs are very abundant in membrane proteins. Shown in the figure are all leucine, Ile or Val residues that are separated by an *i*, *i*+3 or *i*, *i*+4 pattern consistent with a coiled-coil or leucine zipper.

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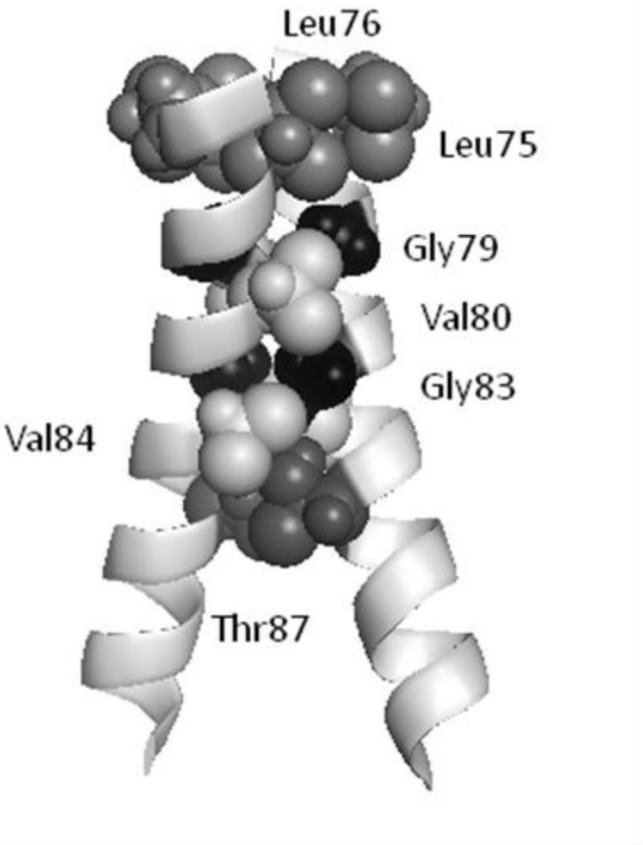

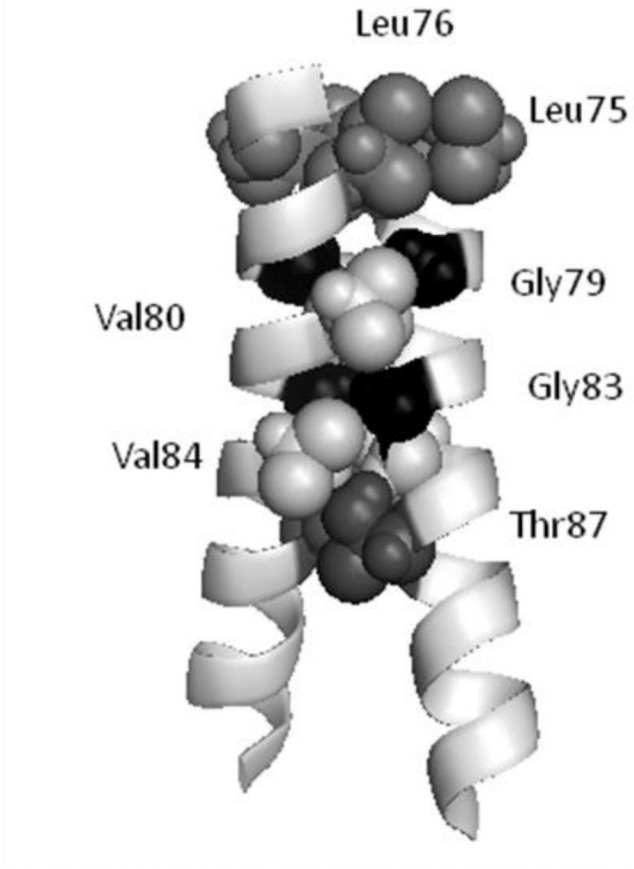

3A

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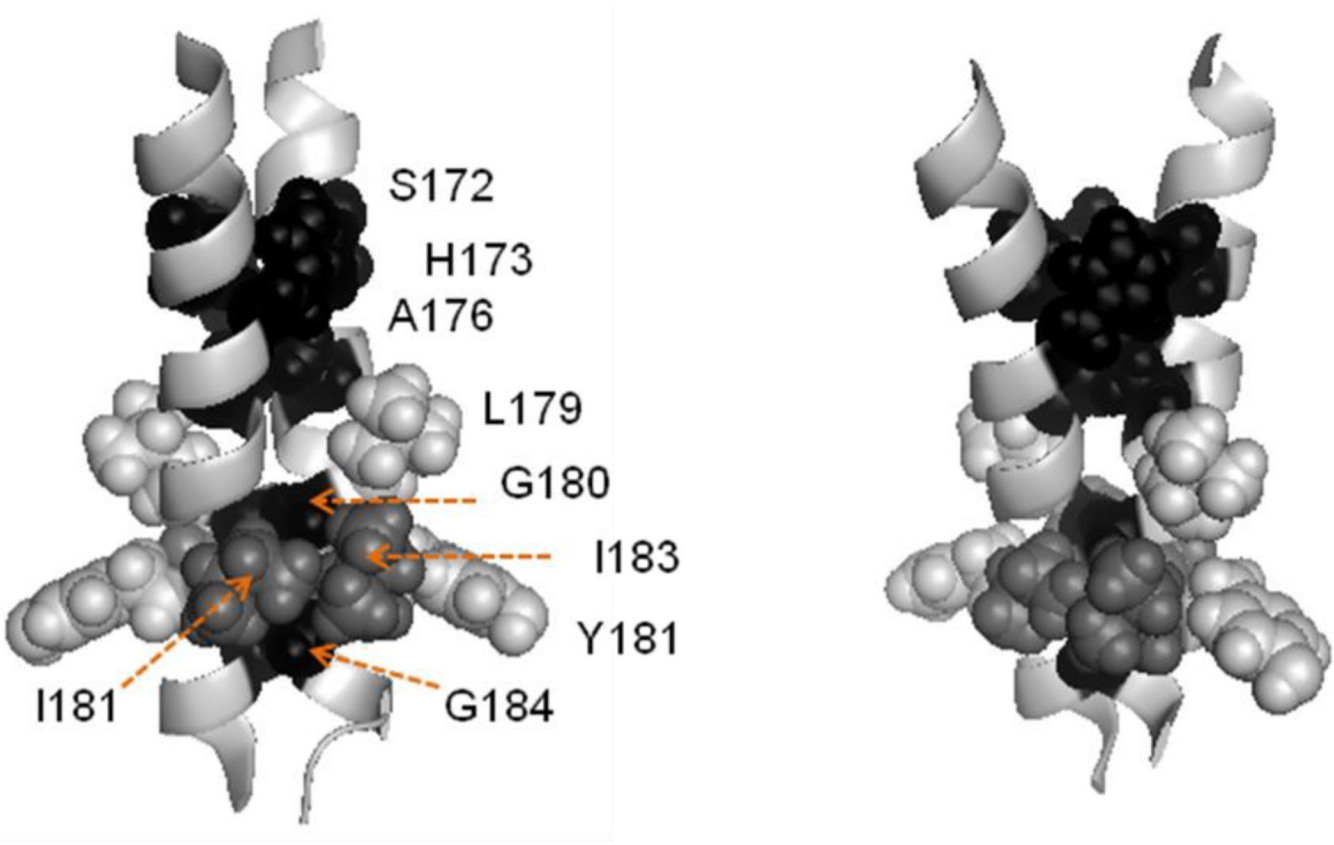

3B

Figure 3. Examples of NMR dimer structures

A. The structure of the glycophorin A transmembrane TM homodimer (22). The side-chains of the seven residues that are most sensitive to mutations are shown (55). The degree of shading is proportional to the sensitivity of the dimer to mutations: black (highly sensitive: Gly79 and Gly83), dark gray (moderately sensitive: Leu75, Leu76 and Thr87), light gray (somewhat sensitive: Val80 and Val84). **B.** Structure of the BNIP3 homodimer (95) The side-chains of residues that are sensitive to mutations are shown (95). The degree of shading is proportional to the sensitivity of the dimer to mutations: black (highly sensitive: Ser172, His173, Ala176, Gly180, and Gly184), dark gray (moderately sensitive: Ile181 and Ile183), light gray (somewhat sensitive: Leu179 and Tyr181).