Biochimica et Biophysica Acta 1818 (2012) 974–983

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Review

Transmembrane domains interactions within the membrane milieu: Principles, advances and challenges ☆

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ARTICLE INFO

Article history:
Received 15 August 2011
Received in revised form 17 November 2011
Accepted 22 November 2011
Available online 3 December 2011

Keywords:
Helix–helix interaction
Transmembrane domain
ToxR
TOXCAT
GALEX
Recognition within the membrane

ABSTRACT

Protein–protein interactions within the membrane are involved in many vital cellular processes. Consequently, deficient oligomerization is associated with known diseases. The interactions can be partially or fully mediated by transmembrane domains (TMD). However, in contrast to soluble regions, our knowledge of the factors that control oligomerization and recognition between the membrane–embedded domains is very limited. Due to the unique chemical and physical properties of the membrane environment, rules that apply to interactions between soluble segments are not necessarily valid within the membrane. This review summarizes our knowledge on the sequences mediating TMD–TMD interactions which include conserved motifs such as the GxxxG, QxxS, glycine and leucine zippers, and others. The review discusses the specific role of polar, charged and aromatic amino acids in the interface of the interacting TMD helices. Strategies to determine the strength, dynamics and specificities of these interactions by experimental (ToxR, TOXCAT, GALLEX and FRET) or various computational approaches (molecular dynamic simulation and bioinformatics) are summarized. Importantly, the contribution of the membrane environment to the TMD–TMD interaction is also presented. Studies utilizing exogenously added TMD peptides have been shown to influence *in vivo* the dimerization of intact membrane proteins involved in various diseases. The chirality independent TMD–TMD interactions allows for the design of novel short D- and L-amino acids containing TMD peptides with advanced properties. Overall these studies shed light on the role of specific amino acids in mediating the assembly of the TMDs within the membrane environment and their contribution to protein function. This article is part of a Special Issue entitled: Protein Folding in Membranes.

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doi:10.1016/j.bbamem.2011.11.029

1. Introduction

Cells sense and respond to environmental stimuli through cell surface transmembrane proteins. Membrane proteins represent about 20–30% of the genome in a variety of different organisms [1–3]. These proteins are capable of transmitting different signals from the extracellular environment to the intracellular compartments of the cells. These signals are crucial for many vital cellular processes such as homeostasis and signal transduction [4–8]. The signal transduction cascades are usually initiated by ligand binding, which is believed to induce conformational changes in the receptor that stimulates receptor clustering or oligomerization. Oligomerization was found to be extensively involved in the function of many proteins and therefore it was not surprising to find that deficient oligomerization accounts for several diseases ranging from cancer to amyloidal diseases [9–12]. The complexity and sophistication of the biological membrane are overwhelming. The dynamics, versatile composition and multiple processes that occur simultaneously within this unique environment are beginning to be understood. Although our understanding of the activity and organization of the membrane has evolved tremendously since the relativity simple models of Sanger and Nicholson, our understanding of key features and aspects of these areas is still rather limited. These areas encompass many topics such as protein assembly within the membrane, trafficking and maintenance of homeostasis with each of these topics deserving a full review on its own. Here we will focus on protein–protein interactions within the membrane milieu which are mediated mainly through transmembrane domains (TMD–TMD interactions). We hope to shed more light on this important and intriguing area by focusing on TMD biological significance, sequence motifs, technical advances in the field, and the complex interaction with the different hydrophobic moieties.

2. Sequences mediating TMD–TMD interactions

2.1. Conserved motifs that drive TMD–TMD interactions

Non-covalent association of native TMDs was reported to be mediated by several motifs including: (i) a GxxxG motif, which is the most common motif for interaction of two TMD helices and was first found in the glycophorin A (GPA) TMD [13–15]; (ii) a heptad motif of leucines through their side-chain residue packing interactions [16]; (iii) polar residues through the formation of hydrogen bonds [17–21]. A specific case is the QxxS motif which has been found via bioinformatical analysis of the transmembrane no. 1 of the bacterial aspartate receptor (TAR-1), showing that it is highly expressed in bacterial TMDs, significantly more than its anticipated abundance. This motif has been extended to Polar-xx-Polar motif, in which polar amino acids include Ser, Thr, Glu, Gln, Asp, and Asn [21,22]; (iv) An Aromatic-xx-Aromatic, in which Trp gives the best dimerization; (v) a Gly zipper (GxxxGxxxG) motif, found as the primary packing interface for the TMD of the myelin protein zero, which is the major integral membrane protein of peripheral nerve myelin in higher vertebrates [23]; (vi) A Leu zipper motif that controls the dimerization of the transmembrane domain of the platelet-derived growth factor β-receptor (PDGFβR) receptor [24], or the dimeric receptor tyrosine kinase DDR1 [25]; and (vii) A Ser/Thr rich sequences which was found by a randomized library of TMD helices that was generated, in which glycine was omitted [20]. This motif was found later in the transmembrane domain of Hepatitis C virus (HCV) nonstructural protein 4B (NS4B), which is an integral membrane protein, playing an important role in the organization and function of the HCV replication complex. However, the involvement of additional motifs or key factors that mediate protein–protein interactions within the membrane merit needs further investigation.

2.2. The role of polar residues in TMD–TMD interaction

Generally speaking, once proteins become inserted into the membrane and their secondary structure is created, tertiary contacts are formed between TMD helical segments. This process is believed to be largely driven by the maximization of Van der Waals contacts through complementary knobs-into-holes type interactions [13,14,26,27]. However, it became evident that interhelical association can also be stabilized by formation of hydrogen bonds. In certain geometries, a single membrane-buried hydrogen bond may contribute as much stabilization energy as that provided by the sum of the interhelical Van der Waals contacts along the full lengths of two interacting TMDs [28]. Such hydrogen bonding is formed between a pair of TMDs through one or more polar residues.

The significance of polar residues for the association of TMDs has been studied both *in vitro*, by examining TMDs synthetic peptides with polar residues in their sequence, and *in vivo*, by analysis of *de novo* designed TMD helices [17–20]. These studies revealed that amino acids containing two polar side-chain atoms (such as Asn and Gln) have a greater tendency to drive TMD association than residues containing only one polar side-chain atom (Thr or Ser) [17–20]. The amino acids with two polar atoms can act simultaneously as a good hydrogen bond donor and acceptor and therefore form a more stable oligomer than the polar residues Ser and Thr, which contain only one polar side-chain atom. Nevertheless, the presence of polar residues within the TMD is not always sufficient for TMD assembly and depends on the exact position of the polar residue along the TMD [29]. When discussing the effect of Glu and Asp within the membrane environment, it is important to bear in mind that the core of the biological membrane has a low dielectric constant. This imposes protonation of the carboxylic group. Thus, the Glu and Asp residues are not charged within the membrane environment but rather contain two polar side-chain atoms such as Gln and Asn [17–20].

Von-Heijne and collaborators [30], utilized in vitro translation of model integral membrane protein constructs in the presence of rough microsomes, and showed that Asn-Asn or Asp–Asp-mediated interactions with a neighboring transmembrane helix can enhance the membrane insertion efficiency of a marginally hydrophobic transmembrane segment. Their data suggest that inter-helix hydrogen bonds can form during Sec61 translocon-assisted insertion and thus could be important for membrane protein assembly.

It has been also found that non-polar-to-polar mutations in the TMDs of membrane proteins are associated with several diseases [11,31]. For example, a specific Val→Glu mutation within the TMD of the ErbB2 oncogene product (Neu) [27,32] is known to induce ErbB2 dimerization and activation [33,34]. Such activation of ErbB2 has been detected in a large fraction of breast and ovarian cancers

[32]. Another example is the replacement of the two polar residues, of the QxxS motif, by non-polar residues significantly reduced the dimerization ability of Tar-1 [21,35,36]. The role of the polarity of amino acids within the QxxS motif has been also extensively studied and is discussed in detail in the proceeding paragraphs.

2.3. The role of positively charged residues in TMD–TMD interaction

Positively charged amino acids, which are localized within the TMDs of membrane proteins, are known to have both functional and structural roles in the activity of these proteins. Examples include their involvement in substrate recognition [37], their ability to determine the electrogenicity of the H,K-ATPases [38], and their involvement in mitochondrial citrate transport [39]. Moreover, mutations that introduce positively charged residues into TMDs have been previously shown to be involved in human genetic diseases [31,40], including cystic fibrosis, Charcot–Marie–Tooth disease, Wilson's disease, dominant rental hypomagnesaemia [31,40,41], and many others. TMDs are known to be involved in self- and hetero-assembly of membrane proteins. The charged amino acids may also affect the structure of the protein. Therefore, such mutations might interfere with the interactions and proper assembly of the TMDs. Nevertheless, it seems that despite significant progress in understanding the mechanism of TMD–TMD interactions, the direct effect of positively charged residues on these interactions have not been extensively studied *in vivo*.

As discussed above, the core of the biological membrane has a low dielectric constant. This imposes the alteration of physical and chemical properties of all polar amino acids [18,19]. As a result, Arg and Lys can either stabilize dimerization by forming hydrogen bonds or shift the equilibrium toward monomers by inducing repulsion. This can be affected by their protonation state. It is most probable that positively charged residues have a disruptive effect on dimerization only when they are localized to the interacting surfaces of the helices, as was previously shown by synthetic peptides derived from the TMD region of the sodium pump γ subunit [11]. Interestingly, most of these disease-causing mutations introduce Arg and not Lys into the TMDs. Moreover, evidence has been presented that positively charged residues interact with negatively charged ones in the membrane [42].

In another study, a single amino acid substitution, from Ala to Lys, within the TMD No 1 of the full-length Tar receptor, resulted in the loss of the chemotaxis function induced by the protein. However, neither the membrane integration nor the aspartate-binding ability of the mutated receptor has been affected [43].

Recently, Hristova and Wimley [44] reviewed the current knowledge about the energetics of Arg insertion into the bilayer hydrocarbon core, and discussed discrepancies between experimental and computational studies of the insertion process. While simulations suggest that it should be very costly to place Arg into the hydrocarbon core, experiments, such as those described above, show that Arg is found there. Nevertheless, both types of studies suggested that Arg insertion into the bilayer involves substantial bilayer deformation, with multiple hydrogen bonds between the Arg guanidinium group and lipid polar groups. An explanation for such a difference might be due to the fact that simulations overestimate the cost associated with bilayer deformation and underestimate the ability of the bilayer to adapt to charged and polar groups.

2.4. The role of aromatic residues in TMD–TMD interaction

Aromatic residues serve as key structural elements that mediate the molecular recognition and the self-assembly of many membrane proteins including amyloid polypeptides, bacterial toxins and others [45–49]. Theoretical and empirical studies have shown that aromatic rings tend to form high-order clusters of four different types: parallel displaced, T-shaped, parallel staggered or Herringbone. All these four geometries are referred to as π–π interactions or π-stacking [50–52]. Studies have shown that a mutation of a single aromatic amino acid can abolish the ability of the corresponding amyloid peptide to form amyloid fibrils [53]. Sequence analysis of membrane immunoglobulin and T-cell receptor revealed highly conserved aromatic amino acids within the TMDs of these proteins. These may point out to the involvement of these residues in TMD–TMD interactions [54]. Further support for this assumption comes from the studies by Langosch and coworkers who demonstrated, using a randomized TMD library, that Trp is enriched in self-interacting TMDs containing a heptad repeat backbone sequence [55]. In another study TMD–TMD interactions were shown to be mediated by π–π interactions of parallel aromatic residues as in the case of the WxxW motif. In this case stacking of the Trp aromatic residues serves to stabilize the TMD–TMD interaction [21,35]. A modification of this interaction was later on shown to involve cationic–π interactions. In this case, a motif composed of Ala and Ile was replaced in specific positions with charged or hydrophobic residues. This work shows that the interaction between the charged residues and the hydrophobic ones greatly enhance dimer formation [56].

3. Strategies for studying TMD–TMD interactions within membrane proteins

Despite the abundance and importance of TMD–TMD interactions, biochemical research trying to elucidate their structure function relationship has been facing many technical obstacles [57]. One major obstacle is purification and crystallization. Due to their hydrophobicity, membrane proteins are relatively insoluble in aqueous solution, making detergent choice a key factor in this process. Most of the 231 unique membrane protein structures (as of 3/2010) are of bacterial membrane proteins (MPs) expressed in bacteria, or eukaryotic MPs from natural sources. However eukaryotic membrane proteins, especially those with more than three membrane crossings rarely succumb to any suitable expression in bacterial cells [58–60]. As helix–helix interactions within the membrane are many times flexible by nature, it is hard to follow such interactions in the intact protein or within a rigid crystal (reviewed by [61]). In order to address issues of flexibility in interaction within the membrane milieu, a variety of techniques were developed in recent years as summarized below.

3.1. The ToxR, TOXCAT and GALLEX systems

The ToxR system [62] can detect weak protein–protein interactions within the membrane environment of *E. coli*. The method is based on the functional organization of a plasmid that encodes the ToxR-TM-MalE chimeric protein, in which: the cytoplasmic domain ToxR is linked via a TMD of choice to the periplasmic MalE moiety. The ToxR transcription activator is a membrane protein with a short predicted TMD. External stimuli are thought to induce oligomerization of the ToxR. The oligomeric ToxR molecule binds to a tandemly repeated DNA element found within the *ctx* promoter, thus initiating transcription of the *ctx* genes (*lacZ* in the indicator cells) (Fig. 1A). In the ToxR system the TMD of the ToxR is replaced by the studied TMD. The amount of homodimerization is quantified by measuring the activity of the β-galactosidase (β-Gal) reporter gene and dividing the activity by the cell content (OD_{590}) (Miller units). The results are normalized between the positive and negative controls, ToxR-GPA and ToxR-A_{16}, respectively [42,62].

The ToxR system was designed to detect homo-oligomerization, but was modified further to detect hetero-oligomerization as well [63]. The idea is to exogenously introduce TMD peptides into the ToxR system and to monitor their effect on the level of oligomerization, detected by the activity of β-galactosidase (Fig. 1B). Interaction between a TMD peptide and the TMD that is inserted in the chimera protein (ToxR-TM-MalE) will result in reduction of the initial oligomerization signal [63]. The reduction results due to the creation of a hetero-oligomer that does not produce a β-Gal signal. Since the

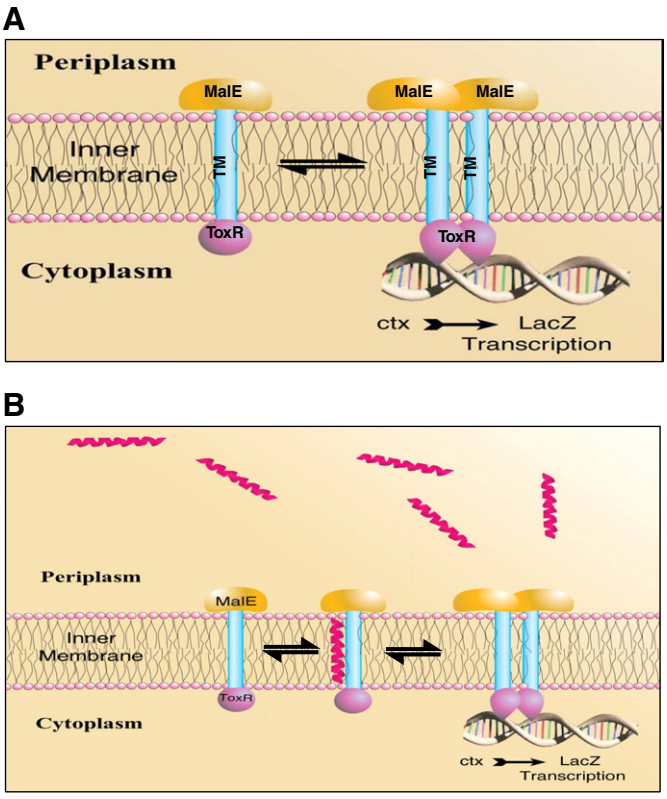

Fig. 1. (A) Schematic illustration of the ToxR homo-oligomerization system. The association of the TMDs activates ToxR, which only then can bind the *ctx* promoter and can initiate the lacZ transcription process [62]. (B) Schematic illustration of the ToxR hetero-oligomerization system. Hetero-association of the exogenous peptides with the TMD of the ToxR system prevents the activation of ToxR by shifting the equilibrium toward monomeric ToxR, thus reducing lacZ transcription and hence its signal. The inserted TMD of choice is illustrated as a blue cylinder and the exogenously added TMD peptide (e.g. Tar-1) is a pink helix [63].

TMD peptides are very hydrophobic and tend to aggregate, Lys residues are added to their N and C-termini. These Lys tags confer water solubility to many of the hydrophobic TMDs without affecting correct oligomerization and insertion into the membrane [64–66]. Nevertheless, stock solutions of highly insoluble TMDs are prepared in DMSO and added to the cells such that the final concentration of DMSO does not exceed 2%.

A similar and more sensitive system had been developed by Russ and Engelman and is based on the expression of the chloramphenicol acetyltransferase therefore termed TOXCAT [67]. Both systems have been widely used, with the TOXCAT developed to assess changes in free energy as a result of TMD–TMD dimerization, as described above.

To assess hetero-assembly in a more direct way, another system has been developed —the GALLEX system first reported by Schneider and Engelman [68]. This approach is similar to the previous methods but is based on a mutated LexA repressor that consists of two subunits transcribed from two different plasmids. In this system, β-Gal is constitutively expressed by *E. coli* cells. The self-interacting GpA is used as a positive control yielding high repression of β-Gal expression and the mutated GpA G83I is used as a non-interacting pair yielding maximal β-Gal activity. This system was recently used to assess changes in the membrane fluidity on TMD–TMD interaction (see details in the proceeding paragraphs).

3.2. Analytical fluorescence resonance energy transfer (QI-FRET)

QI-FRET has been recently developed to follow dynamic TMD–TMD interactions within the membrane milieu [69]. In this technique, plasma membrane vesicles and two fluorescently tagged TMDs are scanned and visualized by confocal microscopy. Statistical analysis of the FRET between the two fluorophores can indicate the strength of the interaction. Moreover, this method can be used to calculate free energy differences between specific mutants as has been shown for GpA TMDs.

3.3. Stop flow fluorescence analysis

A stop flow fluorescence analysis has been demonstrated by Tang et al., [70]. This assay is based on the fluorescence of two distinct probes Trp and Phe-cn that are present in a model TMD peptide. The fluorescence of both probes is sensitive to the environment. Phe-cn is also sensitive to the protein or peptide conformation. Thus, following the rapid mixing of peptides with POPC/POPG vesicles, the kinetics were observed (through the above mentioned probes) and revealed three distinct phases: membrane binding, insertion and dimerization, with the latter being in the time scale of seconds.

3.4. High throughput combinatorial peptide library

He et al. [71] utilized high throughput selection approach to study the single TMD of a receptor tyrosine kinase (RTK), in order to identify specific dimerization sequence motifs that bind to this TMD. For that purpose they designed a 3888-member combinatorial peptide library based on the TMD domain of Neu (ErbB2) as a model RTK. The library contains many closely related, Neu-like sequences, including thousands of sequences with known dimerization motifs. They then used an SDS-PAGE-based screen to select peptides that dimerize better than the native Neu sequence, and assayed the activation of chimeric Neu receptors in mammalian cells with TMD sequences selected in the screen. Interestingly, despite the very high abundance of known dimerization motifs in the library, they found only a very few dimerizing sequences. One of the advantages of this approach is that it yields direct information about the specificity of such TMD–TMD interactions.

3.5. Molecular dynamics simulations and computational design

The lack of sufficient structural data has led many researchers to use molecular dynamics (MD) simulations in order to obtain insights about different aspects of the membrane protein interface (see review [72]). In a recent study Zhang and Lazaridis [73] tested the influence of a non-interfacial residue (Lys 40) on the association of the TMDs of the major coat protein (MCP) from the bacteriophage M13. By using MD simulation and comparing MCP, GPA and different sequence chimeras of their TMD, the researchers were able to show that this specific polar residue has a tremendous impact on the association ability of the MDP TMDs. The usage of MD simulations allows for an explanation of this phenomenon: simulated protonation and deprotonation of Lys 40 caused major configuration changes within the tilt and position of the TMD relative to the membrane bilayer. Thus, the researchers conclude that different flanking non-interfacial ionizable residues in the TMD can influence dimer association by inducing large conformational changes of TMDs [73].

Very recent studies compared the self-assembly dynamics of Leukocyte integrin αβ2 and αβ3 TMD helices in a model membrane using coarse-grained MD simulations [74]. They found that although αβ3 TMD helices associate more easily, packing is suboptimal. In contrast, αβ2 TMD helices achieve close-to-optimal packing. This suggests that αβ2 TMD packing is more specific, possibly due to the inter-helix hydrogen bond. Disruption of this inter-helix hydrogen bond in αβ2 via the β2T686G mutation results in poorer association and a similar profile as αIIβ3.

The *de novo* design of protein-binding peptides by computational design has been also described. For example, Sammond et al. [75] used as a model system the GoLoco motif from the G-protein regulator

and H-Ras effector, RGS14. This motif binds to the heterotrimeric G-protein R subunit Gα(i1) in its inactive, GDP-complexed state. They described a strategy that iterates between structure and sequence optimization to redesign the C-terminal portion of the RGS14 GoLoco motif peptide so that it adopts a new conformation when bound to Gα(i1). An X-ray crystal structure of the redesigned complex closely matches the computational model, with a backbone root-mean-square deviation of 1.1 E.

In another case, MD simulations combined with conformational considerations were used in order to probe TMD–TMD interactions without prior experimental data. The researchers tested their model against an experimental data of GpA dimers and further used it to probe the association of ErbB2 and ErbB3. Using this method they were able to suggest conformational considerations for the association between these two receptors under different conditions [76,77]. This system can prove valuable when there is lack of experimental data and as a general guideline for probing unknown interactions.

Another example for the usage of MD simulations is to compare structural aspects of different dimerizing motifs. In an article by Sal-Man et al., a novel dimerizing motif WxxW was identified and compared by MD simulations to the already known dimerization motif QxxS. Using this approach it was revealed that the backbone of the TMD constrains the WxxW motif while it has little effect on dimerization of the QxxS motif [35].

3.6. Bioinformatic tools

Large scale bioinformatical scanning of TMD libraries can be a useful tool to identify novel interacting elements or motifs. Engelman and coworkers used a novel formalism called TMSTAT to calculate the expectancies of all pairs and triplets of residues in individual sequences, taking into account differential sequence composition and the substantial effect of finite length in short segments. The major finding was patterns of small residues (Gly, Ala and Ser) at i and i+4 found in association with large aliphatic residues (Ile, Val and Leu) at neighboring positions (i.e. i+/−1 and i+/−2). The most over-represented pair is formed by two glycine residues at i and i+4 (GxxxG, 31.6%), and it is strongly associated with the neighboring β-branched residues Ile and Val [78]. Another study utilized this approach to identify cationic interaction together with GxxxG motifs as possible interacting elements [42]. In this study, GxxxG with either K/R or D/E at positions −10 to −1 away were examined for their natural occurrence within TMD sequences. The data revealed that some of the sequences were significantly over expressed in the natural libraries. Testing their ability to interact showed that at least one pair was able to promote efficient heterogenic interactions. In another study statistical analyses were undertaken for putative transmembrane α-helices obtained from a database representing the subset of membrane proteins available in Swiss-Prot. The average length of a transmembrane α -helix was found to be 22–21 amino acids with a large variation around the mean. The same study also described the preferred positions for hydrophobic, polar, tyrosin and trypotophan [79]. Other examples include the identification of the QxxS, and the WxxW dimerization motifs [21,35].

4. Examples signifying the importance of TMD–TMD interactions for protein function

4.1. The EGF receptor

One of the most investigated examples is the family of ErbB growth factor receptors. This family of receptor tyrosine kinases consists of four members that through their combinatorial association are able to recognize a wide array of ligands [80,81]. Although the main driving force for their specificity in binding their ligand is the recognition by the extracellular regions, it was also postulated that the TMD of these proteins are able to self-associate and influence biological activity. For example, a specific Val→Glu mutation within the TMD of the ErbB2 protein is known to induce uncontrolled dimerization and activation of the receptor, resulting in production of an oncogene (Neu) [32–34]. Furthermore, Mendrola et al., [27] has shown that the TMDs of these receptors can self-associate where the association was the strongest for ErbB4 and the weakest was ErbB3. Further analysis of these interactions revealed that this interaction is mediated by several GxxxG motifs and that their role is in both hetero- and homodimerization. While it was established that the GxxxG in the C terminus TMD of ErbB1 and ErbB2 was involved in homodimerization, Gerber et al., [82] showed that another GxxxG motif located at the N terminus of the TMD is involved in heteroassembly between ErbB1 and ErbB2. Thus, it is proposed that these regions account for the fine tuning of these receptors in response to different ligands.

In a different aspect, the dimerization of these regions was also suggested to increase the stability of these proteins. The oncogenic mutation V644E in the rat homolog of ErbB2 was shown to have a reduced ability to self-associate in membranes in comparison to the wt TMD [83]. However the mutant isolated TM domain has been shown recently to cross-linked to a higher degree than the wild-type TM domain in cells, which contradicts the ToxCAT data [84]. In another recent study by Chen et al., the researchers calculated the decrease in the free energy due to TMD dimerization and found it to be in the order of 2.5–3 kcal/mol [85]. Similar results were observed in studies of GpA, correlating free energy changes due to dimerization with increased protein stability [86]. Taken together, these findings suggest that such energetic changes have a significant influence on biological activity.

4.2. The integrins

Integrins are a family of membrane receptors that mediate cell adhesion. Several studies have shown that their TMDs are capable of forming oligomers and that these interactions are based on the GxxxG motifs present within these regions [87]. Mutational analysis revealed that the interaction between the TMDs of the integrins αIIBβ3 is sensitive to other residues as well, probably allowing for subtle conformational changes within this region to affect stability ([88] see below). In a different study, Yin et al., showed that an exogenous peptide that corresponds to the TMD of the integrin αIIb is able to activate these integrins *in vitro* [89]. The model proposed for integrin activity suggests that in their resting state, the heterodimer TMDs are packed closely together, while in their activated state the heterodimer TMDs dissociated and there is a greater tendency towards homodimer formation. By adding an exogenous TMD peptide that is capable of interacting with the native protein TMD, interfering with heterodimer formation, the equilibrium is shifted towards the activated state. As integrins are plasma membrane proteins important for cell adhesion, such a peptide was shown to activate platelet aggregation in a ligand independent fashion.

In depth examination of the interaction between the αIIb and β3 was performed to confirm this model [87,90]. In these studies mutational analysis was used to assess the heterodimer formation ability, as well as the effect of such mutations on the activation level of these proteins. The findings that β3 mutations along the TMD heterodimer interface reduce heterodimer association but activate the local adhesion kinase, a down-stream effector of the integrin receptor, support this theory. Thus it seems that the interface of these proteins is optimized for heterodimer assembly. Further mutational analysis done by Li et al., [88] showed that the interaction between the TMDs of the integrins αIIBβ3 is sensitive to other residues as well. In this specific case the important residues are at positions −1 and +4 from the GxxxG motif, allowing for fine tuning of this interaction.

Further comprehensive mutational analysis along the TMD interface of the integrins reveals that all mutations along the β3 interface are associated with reduced heterodimer stability [90].

4.3. The bacterial aspartate receptor (Tar)

The *Escherichia coli* aspartate receptor (Tar) is one of the known examples of proteins whose TMD is involved in oligomer stabilization. This receptor is one of the main chemotaxis receptors found in bacteria and it mediates chemotactic response, mainly to aspartate, glutamate, and maltose [91–93]. Tar forms a homodimer complex in which each subunit is composed of two TMD helices (Tar-1 and Tar-2) separated by a substantial periplasmic domain [94,95]. Disulfide cross-linking studies suggested that the Tar-1 TMD interacts with the corresponding Tar-1' TMD of another Tar protein to form the dimer. There is no direct contact between the two Tar-2 helices in the Tar dimer [94]. In support of this, a synthetic peptide corresponding to the Tar-1 TMD could form an SDS-resistant homodimer, whereas the Tar-2 TMD remains monomeric [64]. The Tar-1 does not contain the well-characterized GxxxG motif. Studies utilizing ToxR system and various biophysical approaches revealed that the ^{22}QxxS^{25} sequence is the minimal motif sufficient for Tar-1 TMD–TMD self-assembly. These two polar residues are located on the same helix interface and most probably stabilize association by forming hydrogen bonds. Interestingly, creating a GxxxG motif, instead of the polar motif, almost completely abolished dimerization. Swapping positions between two wild-type polar residues did not affect dimerization, implying a similar contribution from both positions [22]. Moreover, Sal-Man et al. [21] demonstrated that this short polar motif is sufficient to induce dimerization of a non-dimerizing backbone (containing Leu and Ala residues). Statistical analysis of a bacterial TMD database revealed that this motif appears to be significantly over-represented compared with its theoretical expectancy [22]. Taken together, these results suggest that the QxxS motif plays a general role in TMD assembly. Mutational analysis was then performed in order to examine the involvement of specific residues within this motif in TM self-assembly. One or both polar residues (Gln and Ser), were mutated to various amino acids (polar, positively charged, or aromatic). The data revealed that substitution of polar residues at positions 22 and 25 for non-polar residues dramatically impaired the self-association ability of Tar-1. However, replacement of the original polar residues by other polar amino acids preserved or increased assembly depending of the number of hydrogen bonds created: Gln and Asn which can act simultaneously as a good hydrogen bond donor and acceptor, induced a stronger association than the polar residues Ser and Thr, which contain only one polar side-chain atom [22]. Furthermore, introducing an arginine into the Tar-1 interacting interface significantly reduces its ability to self-associate, thus implying that arginine, which contains three polar atoms within the low dielectric environment of the membrane, shifts the oligomer equilibrium towards monomers by inducing repulsion [96].

5. The role of chirality in membrane protein assembly: peptide inhibitors as a case study

The current dogma asserts that chirality is vital for proper assembly of proteins. However, this is mainly due to observations of soluble proteins. Challenging this dogma by short interfering TMD peptides containing D- and L-amino acids suggests that the membrane environment allows for leniency in the assembly of TMDs with mirror images and even diastereomers with both D- and L-amino acids in the same peptide. This has been demonstrated for the first time by studies showing that the all-D amino acid GPA transmembrane domain, as well as its two all-D amino acids mutants, specifically associated with an all-L wildtype GPA TMD within the membrane of *E. coli*. MD techniques revealed a possible structural explanation to the observed interaction between all-D and all-L transmembrane domains. A very strong correlation was found between amino acid residues at the interface of both the all-L homodimer structure and the mixed L/D heterodimer structure, suggesting that the original interactions are conserved. The results suggest that GPA helix–helix recognition within the membrane is chirality independent [97].

In a second study by Gerber et al. [98] the replacement of two Val within the TMD of GpA with their D enantiomers was used to assess the role of chirality in this dimerization of GPA TMDs. Although this replacement caused major alteration in the structure of the peptide, it was still active and was able to interact with its respective TMD. The heterodimer seems to maintain all the interactions within the native GPA homodimer. Thus, the dimerization of GPA through its TMD proved sterically tolerant to assembly with its mirror image TMD [98].

In a third study, Gerber et al. investigated the role of chirality in the assembly of the TMDs of the T-cell receptor (TCR) complex. This work was based on a peptide that was previously reported to uncouple TCR-α chain from CD3 and by that inhibits the T-cell activation signal [99]. When testing different enantiomers of this peptide, both all-L and all-D peptides were found to co-localize with TCRα in the immune synapse and inhibit antigen-specific T-cell activation. The inhibition is carried out in a sequence specific manner, as demonstrated by an inactive 2G CP mutant. *In vivo*, both all-L and all-D CP enantiomers inhibited adjuvant arthritis (AA) in rats [100]. Furthermore, similarly to the case of GPA TMD, the secondary structure of CP was disrupted by replacing two positive residues, needed for the interaction of CP with the TCR complex, by their D-amino acids enantiomers. Various biophysical and biological studies revealed that the diastereomer, despite its significantly altered structure, preserved its ability to bind to the native CP and to be effective also *in vivo*: it inhibited AA in rats and delayed type hypersensitivity in BALB/c mice. Importantly, the new diastereomer manifested greater immunosuppressive activity than wild-type CP, both *in vivo* and *in vitro*, which can be attributed to the greater solubility and resistance to degradation of peptide [101].

Using a similar approach Sal-Man et al. showed that a D enantiomer TMD peptide of the *E. coli* aspartate receptor was able to inhibit chemotaxis mediated by this receptor [36]. Finally, a D analogue of TMD number 5 of the multidrug resistant transporter P-glycoprotein was shown to inhibit the efflux activity of this transporter. This inhibitory activity was sufficient to sensitize doxorubicin resistant cancer cells to this specific drug [102].

What is the mechanism that allows for chirality independent TMD–TMD interactions? Remarkably, a simple rotation of the tilt angle may compensate for the replacement of an all-L chain with an

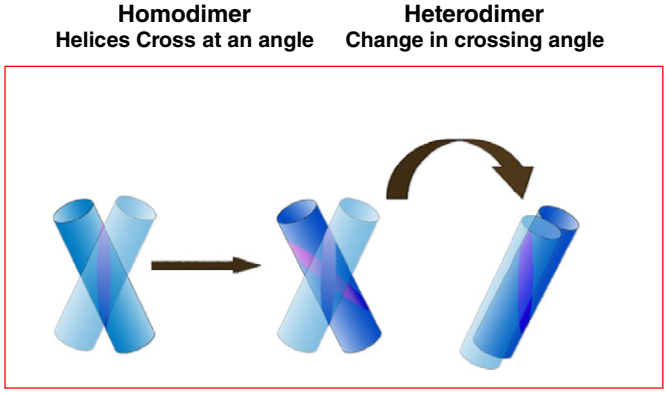

Fig. 2. A model describing the assembly of an all-L helix with its all-D enantiomer. The surface of interaction between the two all-L helices is seen in pink. When one of the chains is replaced with and all-D enantiomer TMD the interaction surfaces cross each other and assembly is impossible. However, by changing the tilt angle between the helices it is possible to re-orient of the interaction surfaces to face each other [97,100].

all-D chain (Fig. 2). For example, the basic TMD homodimer, based on the model of GPA, is built of two all-L chains crossing at an angle to one another. A replacement of one chain to its all-D enantiomer will result with an interaction surface, which has the same sequence as the wild type, although in mirror image orientation. By changing the tilt angle between the two helices, it is possible to bring the interaction surfaces to a similar orientation as in the wild-type homodimer (Fig. 2). A similar approach can explain TCR inactivation by these D-analogue peptides (Fig. 3).

The surprising efficiency of the all-D peptides might have important therapeutic implications, since D-peptides are not hydrolyzed by endogenous proteases leading to an increased half life time in the biological system of interest. Moreover, the fact that these are highly hydrophobic peptides that are rapidly sequestered to the hydrophobic membrane interface might also affect and potentially increase their retention time within the body.

6. Membrane influence on TMD–TMD interactions

One of the main difficulties in studying TMD–TMD interactions is the usage of detergents to assess different aspects of these interactions. It is becoming apparent that in some cases there are large differences in the state of the protein under investigation in micelles and membranes as can be reflected by their simulated structure (see [61]). In an attempt to systematically explore such differences in dimerization propensity in micelles vs. biological membranes, Doung et al. compared GpA dimerization by TOXCAT analysis to sedimentation analysis in SDS [86]. An important observation in this study was that a decrease in the hydrophobicity of the TMD of GpA lowers dimer stability under solvation by detergent, but does not affect dimer stability in the biological membrane as revealed by TOXCAT. Thus, the researchers concluded that the TMD within the detergent assumes a different conformation that would be less favorable under the 2-step model of insertion into the biological lipid bilayer [103]. Earlier, when developing the TOXCAT assay, Russ and Engelman reported on another discrepancy between biophysical analysis and this *E. coli* based reporter system [67]. In this study the researchers observed that while Tyr substitutions at non-interfacial positions of the GpA TMDs were none-disruptive in the TOXCAT assay, such mutations had a disruptive effect on dimerization as revealed by SDS-PAGE.

Membrane physical aspects such as fluidity can also affect TMD–TMD interactions. In a recent study by Anbazhagan et al., it was shown that the local anesthetic phenylethanol decreased the order of the acyl chains within a membrane bilayer, thus increasing its fluidity. As a consequence, TMD–TMD dimerization of the GpA, ErbB2, ErbB3 and FGFR TMDs was significantly lower as determined by the TOXCAT assay [104]. This study suggest that affecting the membrane property, and indirectly affecting protein dimerization, might be an explanation for the mode of action for anesthetic, which is still an open question.

Another intriguing aspect of TMD–TMD interactions involves lipid solvation. In a study by Cunningham et al., an inverse correlation was observed between lipid accessibility (i.e. lipid protein interface) and dimerization ability, as defined experimentally by TOXCAT assays. In this study, the Val at positions 80 and 84 of the GPA were mutated to other hydrophobic residues such as Ile, Leu and Ala in different combinations. Although these substitutions were adjacent to the GxxxG motif, some had a large impact on TMD–TMD interactions, with some pairs giving even better dimerization than the Val-Val wt pair. Based on the two step model of TMD assembly mentioned above and the structural arrangement of the different mutants, the researchers conclude that the increased ability or inability of the lipid environment to solvate the α-helical structure may greatly decrease or increase the helix–helix interactions, respectively. In other words, the more the side chains are exposed to the lipid interface they have less ability to establish TMD–TMD interactions.

7. Short TMD peptides as novel inhibitors of membrane protein function

Due to the biological importance of TMD regions, much research has focused on the ability of exogenously added peptides to influence

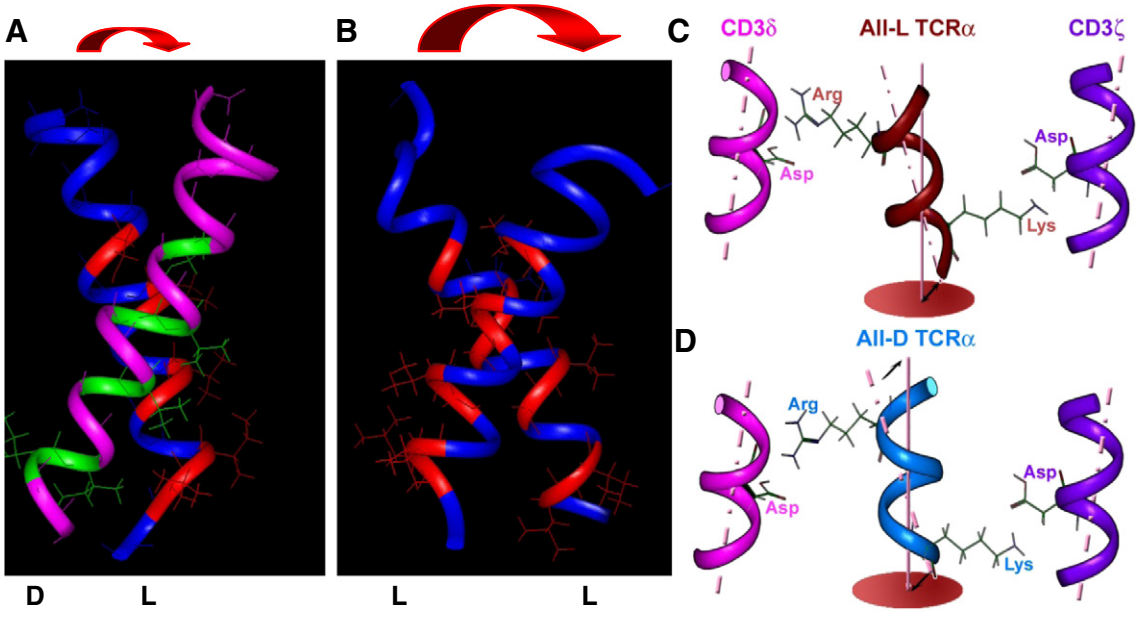

Fig. 3. The change in tilt angle accommodates for all-L replacement with all-D TMD. (A) GPA heterodimer structure after molecular dynamics procedure [97]. The all-L helix is in blue with the "interaction motif" labeled in red. The all-D helix is in pink with the "interaction motif" is labeled in green. (B) GPA homodimer structure after the molecular dynamics procedure [63]. The all-L helix is in blue with the "interaction motif" labeled in red. (C) The TCR complex consists of several TMDs, nevertheless it follows the same rules. The TCRα is known to interact with CD3δ and CD3ζ through salt bridges. A is presented for the 3 α-helices and oriented them in such a manner to allow the salt bridges to form. The all-L TCRα helix is tilted with respect to the CD3 helices. (D) The all-L TCRα with all-D TCRα are reoriented by changing the tilt angle, until the Arg and Lys side chains were placed at similar positions in space as the original all-L TCRα side chains [100].

the dimerization of membrane proteins *in vivo*. This method is based on the ability of these short TMDs to bind their respective TMD within the membrane and affect their assembly or dimerization (for a more detailed review on this approach see Ref. [57]). In addition to the studies on integrins (above), other reports showed that exogenously added TMD peptides influence the dimerization of several other TMDs such as the TMD of GPA and the TMDs of members from the growth factor receptor family ErbB [63,82]. As the TMD–TMD interactions within the ErbB family were shown to be important for protein stability, peptide inhibition of the ErbB2 signaling was also shown and is dependent on the GxxxG of the peptide [85,105].

Other examples of TMD peptides that inhibit a membrane protein function are those of the phospholamban peptide that perturbed the SERCA Ca^{++} channel [106], the core TCRα TMD (Core peptide, Cp) that inhibited the T-cell activation through the TCR complex [21] and a TMD peptide that inhibited the multidrug transporter P-glycoprotein in a chiral independent manner ([102], see below). The design of β-peptide foldamers targeting the TMDs of complex natural membrane proteins has been a formidable challenge. Degrado and coworkers [107] designed a series of β-peptides to stably insert in TMD orientations in phospholipid bilayers. The secondary structures and orientation of the peptides in the phospholipid bilayer was characterized using biophysical methods. Computational methods were then devised to design a β-peptide that targeted a TMD helix of the integrin α(IIb)β(3). The designed peptide (β-CHAMP) interacts with the isolated target TMD of the protein and activates the intact integrin *in vitro*.

In summary, this review summarizes our knowledge on the sequences mediating TMD–TMD interactions, as well as the specific role of polar, charged and aromatic amino acids in the interface of the interacting TMD helices. Strategies to determine the strength, dynamics and specificities of these interactions include both experimental and computational approaches. Importantly, the contribution of the membrane environment to the TMD–TMD interaction is also discussed. Besides shedding light on the role of specific amino acids in mediating TMDs specific assembly, short exogenously added synthetic TMD peptides can influence *in vivo* the dimerization of intact membrane proteins involved in various diseases. Finally, the finding of chirality independent TMD–TMD interactions allows for the design of novel short D- and L-amino acids containing TMD peptides with advanced properties.

Acknowledgements

Yechiel Shai has The Harold S. and Harriet B. Brady Professorial Chair in Cancer Research. This study was supported in part by the Israel Science Foundation, Josef Cohn Minerva Center for Biomembrane Research and the Minerva Foundation.

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