Biochimica et Biophysica Acta 1838 (2014) 2313–2318

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Proline localized to the interaction interface can mediate self-association of transmembrane domains ☆

Neta Sal-Man [a,b,*], Doron Gerber [a,c], Yechiel Shai [a]

[a] Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel
[b] Department of Microbiology and Immunology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel
[c] The Mina & Everard Goodman Faculty of Life Sciences, The Nanotechnology Institute, Bar-Ilan University, Ramat Gan 52900, Israel

ARTICLE INFO

Article history:
Received 19 February 2014
Received in revised form 1 May 2014
Accepted 9 May 2014
Available online 17 May 2014

Keywords:
Helix–helix interaction
Transmembrane domain
TM–TM
ToxR
Recognition within the membrane
Peptide membrane interaction

ABSTRACT

Assembly of transmembrane domains (TMDs) is a critical step in the function of membrane proteins. In recent years, the role of specific amino acids in TMD–TMD interactions has been better characterized, with more emphasis on polar and aromatic residues. Despite the high abundance of proline residues in TMDs, contribution of proline to TMD–TMD association has not been intensively studied. Here, we evaluated statistically the frequency of appearance, and experimentally the contribution of proline, compared to other hydrophobic amino acids (Gly, Ala, Val, Leu, Ile, and Met), with regard to TMD–TMD self-assembly. Our model system is the assembly motif (^{22}QxxS^{25}) found previously in TMDs of the *Escherichia coli* aspartate receptor (Tar-1). Statistically, our data revealed that all different motifs, except PxxS (P/S), have frequencies similar to their theoretical random expectancy within a database of 41916 sequences of TMDs, while PxxS motif is underrepresented. Experimentally, using the ToxR assembly system, the SDS-gel running pattern of biotin-conjugated TMD peptides, and FRET experiments between fluorescence-labeled peptides, we found that only the P/S motif preserves the dimerization ability of wild-type Tar-1 TMD. Although proline is known as a helix breaker in solution, Circular Dichroism spectroscopy revealed that the secondary structure of the P/S and the wild-type peptides are similar. All together, these data suggest that proline can stabilize TM self-assembly when localized to the interaction interface of a transmembrane oligomer. This article is part of a Special Issue entitled: Interfacially Active Peptides and Proteins. Guest Editors: William C. Wimley and Kalina Hristova.

1. Introduction

Membrane receptor proteins play a significant role in mediating signal transduction across the membrane bilayer. This vital information is usually transferred as a result of conformational changes of the protein. Oligomerization is one of the most documented examples of such a change [1,2]. Receptor oligomerization is mainly mediated by the extracellular or intracellular domains. However, considerable data have been accumulated concerning the causal involvement of the transmembrane domains (TMD) in this process as well [3–7]. In contrast to the soluble regions of membrane proteins, our knowledge of the factors that control protein–protein interaction and recognition of the membrane-embedded domains is still limited.

In recent years, the role of specific residues in TMD–TMD interactions has been better characterized and various patterns of polar and hydrophobic amino acid motifs have been proposed [8–13]. However, despite the presence and the involvement of proline in the function of membrane proteins [14–18], its direct contribution to helix–helix interaction within the membrane, compared with other hydrophobic amino acids, has not been intensively studied. Structurally, proline is unique among the 20 amino acids because its side chain cycles back to the backbone amide, thus lacking the proton necessary for hydrogen bond formation, therefore inducing a kink in the protein backbone structure. In water-soluble proteins and peptides, proline is generally considered a helix breaker. A few antimicrobial peptides are known to contain conserved proline residues that confirm structural flexibility to the peptides and allow them to get only partially inserted into the membrane environment. In membrane embedded proteins, however, the structure induced by proline can vary from breaks to merely kinks of the helix and is determined by the structural context of the TMD and the specific position of the proline residue [11,19–23].

Proline is widely distributed in the putative TMDs of many integral membrane proteins such as the low-density lipoprotein receptor, the insulin receptor, and many transport proteins [14,15,24]. It has been suggested that proline in TMDs of transport proteins serves as a "switch" between the different conformations adopted by the protein in different steps of its transport cycle by cis-trans isomerization of proline [16,19,25,26]. Interestingly, analysis of TMD sequences from Human Gene Mutation Database reveals that mutations of proline have one of the highest phenotypic propensities [27]. This suggests

☆ This article is part of a Special Issue entitled: Interfacially Active Peptides and Proteins. Guest Editors: William C. Wimley and Kalina Hristova.

* Corresponding author at: Department of Microbiology and Immunology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel. Tel.: +972 8 6477295; fax: +972 8 6477162.
E-mail address: salmanne@bgu.ac.il (N. Sal-Man).

http://dx.doi.org/10.1016/j.bbamem.2014.05.006
0005-2736/

that proline located within the TMD plays a functional or structural role in the activity of membrane proteins.

The first indication of the involvement of proline in TMD self-assembly was reported in a study that examined patterns that stabilize helical oligomers in a Glycophorin A randomized library [28]. Excluding glycine from the design of this library resulted in enrichment of proline together with serine and threonine in motifs that exhibit strong helix association. Additional evidence that supports the assumption that proline may be involved in TMD association is the finding that proline has the second highest packing value in membrane proteins following glycine [21], which indicates that it is often tightly packed in the structures of natural proteins.

To explore the contribution of proline to TMD associations, compared to other hydrophobic amino acids, we first statistically evaluated the appearance of ZxxS motifs (where Z = Gly, Ala, Leu, Ile, Val, Mat, or Pro) in a database that contains 41,916 bacterial TMD with lengths ranging between 15 and 30 amino acids. A similar motif (QxxS) has been shown to form a homodimer through by forming hydrogen bonds within the *Escherichia coli* aspartate receptor TMD (Tar-1) [29]. In this study we also used several complementary methods including (i) the ToxR assembly system, which can detect protein–protein interactions within the *E. coli* membrane environment; (ii) the SDS-gel running pattern of biotin-conjugated TMD peptides; (iii) the FRET experiment between fluorescence-labeled peptides; and (iv) analysis of the frequencies of occurrence of the ZxxS motifs in a bacterial TMD database where Z is proline or one of the other hydrophobic amino acids (Gly, Ala, Val, Leu, Ile, and Met). In addition, the secondary structure of a synthetic WT TMD was compared to that of the proline mutant. The results are discussed with regard to the contribution of proline to TMD self-assembly.

2. Materials and methods

2.1. Construction of the ToxR chimeras

A NheI-BamHI TM-DNA cassette encoding 16 residues of the Tar-1 WT TMD (^{13}MVLGVFALL**Q**LI**S**GSL^{28}) was inserted between the ToxR transcription activator and the *E. coli* maltose binding protein (MalE) within the ToxR-MalE plasmid. Point-mutations of Tar-1 were done in Gln at positions 22 (Table 1). The sequences of all the constructs were confirmed by DNA sequencing. The nomenclature of the TMDs represents the two amino acids located in the positions of the original polar residues of the QxxS motif.

2.2. In vivo detection of homo-dimerization of TMD domains within the membrane

The ToxR transcription activator can be successfully used to assess weak protein–protein interactions within the *E. coli* membrane. A Tar-1 TMD encoding the DNA cassette was grafted between the ToxR transcription activator and the maltose binding protein in the ToxR-MalE plasmid. The plasmid was then transformed into *E. coli* FHK12 cells that contain β-galactosidase, under the control of a *ctx* promoter. Dimerization of the TMDs, in this system, results in association of the ToxR transcription activator, which then becomes active and is able to bind the *ctx* promoter [30]. Quantification of the level of homo-dimerization was done by measuring the activity of the β-galactosidase reporter gene and by normalizing it to the cell protein content (OD_{590}) (miller units). The baseline activity of a negative control ToxR'A_{16}, which remains a monomer, was subtracted from all the results. The transformed cells were grown in the presence of chloramphenicol for 18 h at 37 °C. β-galactosidase activities were quantified in crude cell lysates after adding *o*-nitrophenylgalactosidase and by monitoring the reaction at 405 nm for 20 min, at intervals of 30 s at 28 °C by a Molecular Devices kinetic reader [30,31]. Specific β-galactosidase activities were calculated from the V_{max} of the reaction.

Table 1
Sequences of the TM domain that were inserted between the ToxR transcription activator and the maltose-binding protein in the ToxR-MalE plasmid.

TM Domain	Sequence[a,b,c,d]
Tar-1 WT	^{13}MVLGVFALL**Q**LI**S**GSL^{28}
Tar-1 A/S	^{13}MVLGVFALL**A**LI**S**GSL^{28}
Tar-1 G/S	^{13}MVLGVFALL**G**LI**S**GSL^{28}
Tar-1 L/S	^{13}MVLGVFALL**L**LI**S**GSL^{28}
Tar-1 V/S	^{13}MVLGVFALL**V**LI**S**GSL^{28}
Tar-1 I/S	^{13}MVLGVFALL**I**LI**S**GSL^{28}
Tar-1 M/S	^{13}MVLGVFALL**M**LI**S**GSL^{28}
Tar-1 P/S	^{13}MVLGVFALL**P**LI**S**GSL^{28}

[a] Amino acids are numbered according to their position in the WT protein (swissprot p07017).
[b] The amino acids in the positions of the dimerization motif are in bold.
[c] Mutations in the Tar-1 TMD are bold and underlined.
[d] The nomenclature of the TMDs represents the two amino acids replacing the original polar residues glutamine and serine of the WT sequence at positions 22 and 25, respectively.

2.3. ToxR-TM-MalE chimera protein expression levels

Western blot analyses were performed for any mutant tested. Aliquots of 10 μl FHK12 cells, each with a different plasmid or in the presence of a different peptide, were mixed with a sample buffer, boiled for 5 min, separated on 12% SDS-PAGE, and then transferred to the nitrocellulose membrane. The primary antibody used was anti-Maltose binding protein. The detection was done with a "Phototope-HRP Western Blot Detection System" from Cell Signaling Technology.

2.4. Maltose complementation assay

Membrane insertion and correct orientation were examined as previously described [32]. Briefly, PD28 cells, transformed with the different plasmids, were cultured overnight. The cells were then washed twice with PBS and used to inoculate M9 minimal medium including 0.4% maltose at a 200-fold dilution. The growth of the cells was measured at different time points by a spectrophotometer at 650 nm.

2.5. Peptide synthesis and purification

Peptides were synthesized by the Fmoc solid-phase method on a Rink amide MBHA resin. The peptides were cleaved from the resin by trifluoroacetic acid (TFA) and were purified by RP-HPLC on a C_4 reverse phase Bio-Rad semi-preparative column (250 × 10 mm, 300 Å pore size, 5 μm particle size). The purified peptides were shown to be homogeneous (>95%) by analytical HPLC. The peptides' compositions were confirmed by electrospray mass-spectrometry. Lysine residues were added to the N- and C-termini of the peptides to confer water solubility to the hydrophobic TMDs [33,34]. It was previously shown that hydrophobic peptides conjugated to lysines tags were correctly oligomerized and inserted into the membrane [33–35].

2.6. Circular Dichroism (CD) Spectroscopy

The CD spectra of the peptides were measured in an Aviv 202 spectropolarimeter. The spectra were scanned with a thermostated quartz optical cell with a path length of 1 mm. Each spectrum was recorded at 1-nm intervals with an average time of 10 s, at a wavelength range of 260 to 190 nm. The peptides were scanned at a 100 μM concentration in 1% LPC micelles. Fractional helicities [36,37] were calculated as follows:

$$\frac{[\theta]_{222}-[\theta]^{0}_{222}}{[\theta]^{100}_{222}-[\theta]^{0}_{222}}, \quad (1)$$

where $[\theta]_{222}$ is the experimentally observed mean residue ellipticity at 222 nm, and values for $[\theta]^{0}_{222}$ and $[\theta]^{100}_{222}$, corresponding to 0% and 100% helix content at 222 nm, are estimated to be −2000 and −32,000 deg cm^2/dmol, respectively [36].

2.7. Statistical analysis

The analysis of the frequencies of occurrence of the GxxS, AxxS, LxxS, IxxS, VxxS, MxxS and PxxS motifs was performed on a bacterial TMD database. The source of the TMD sequences was the annotated non-redundant database Swiss-Prot. The database contains 41,916 bacterial TMD with lengths ranging between 15 and 30 amino acids. The occurrences of the different motifs in the database were counted. The average expected frequency of occurrences was calculated by counting the number of motif occurrences in 100 randomized databases. Randomization of the database was achieved by combining TMDs' sequences of a particular length into one long string of characters, which was then shuffled randomly and re-cut into the original length.

2.8. Peptide labeling with biotin and fluorescent dyes

The Fmoc protecting group was removed from the N-terminus of the resin-bound peptides by incubation with piperidine (20% in DMF) for 12 min, whereas all the other reactive amine groups of the attached peptides were kept protected. The resin-bound peptides were washed twice with dimethylformamide (DMF), and then treated with N-hydroxysuccinimide esters of Biotin, fluorescein, or Rhodamine-TAMRA (1 mg each), in anhydrous DMF containing 2% N,N-diisopropylethylamine (DIEA), leading to the formation of a resin-bound N-terminally labeled peptide. After 24 h, the resin was washed thoroughly with DMF and then with methylene chloride (DCM). The labeled peptides were cleaved from the resin and purified as described above (peptide synthesis and purification section).

2.9. SDS-PAGE of biotin-labeled peptides

Biotin-labeled peptide samples (1 μg) were dissolved in SDS-containing sample buffer, boiled for 5 min, separated on 16% SDS-PAGE gels, and then transferred to PVDF membrane. The detection was done using anti-biotin antibody.

2.10. Förster Resonance Energy Transfer (FRET) measurements

FRET experiments were performed by using fluorescein-labeled peptides serving as energy donors and Rhodamine-TAMRA labeled peptides serving as energy acceptors [38]. Fluorescence spectra were obtained at room temperature, with the excitation set at 439 nm, and emission was measured at 520 nm. Liposomes were prepared by mixing dry lipids in $CHCl_3$/MeOH (2:1 v/v) to make PE/PG (7:3 w/w) composition. The solvents were then evaporated under a nitrogen stream, and the lipids were subjected to a vacuum for 1 h. The resulting lipid dispersions were resuspended in the 10 mM sodium phosphate buffer (pH 7) with 500 mM NaCl and 0.1 μM of donor- and/or acceptor-labeled peptides by vortexing, and sonicated in a bath-type sonicator (G1125SP1 Sonicator, Laboratory Supplies Co., Inc., New York) until the turbidity had cleared. Peptide-to-lipid ratio was 1:500. Liposomes containing only donor-labeled peptides served as the no FRET control. The efficiency of energy transfer (E) was determined by measuring the decrease in the quantum yield of the donor as a result of the presence of the acceptor. E was determined experimentally from the ratio of the fluorescence intensities of the donor in the presence (I_{DA}) and the absence (I_D) of the acceptor, at 520 nm. The percentage of transfer efficiency, (E), is given by:

$$E(\%) = [(I_D - I_{DA})/I_D]x100. \quad (2)$$

Subtracting the signal produced by the acceptor-labeled analog alone corrected the contribution of the acceptor emission as a result of direct excitation. The contribution of buffer and vesicles was subtracted from all measurements.

3. Results and discussion

3.1. Homo-oligomerization of different Tar-1 TMDs mutants

In this study we mutated the Gln of the assembly motif to different hydrophobic amino acids. The sequences of the mutated TMDs are listed in Table 1. Using the ToxR assay, we found that all the listed TMD mutants, except for the mutant with Gln to Pro substitution (Tar-1 P/S), exhibited similar low dimerization activity (around 30%), which was probably retained from the hydrogen bond between the serine residues (Fig. 1). Interestingly, replacement of Gln by Pro showed dimerization ability similar to that of Tar-1 wild type.

To exclude the possibility that the difference between the dimerization activities of the constructs resulted from different expression levels of the chimera proteins, or alternatively, from a failure of the constructs to properly insert into the membrane, we performed Western blotting and maltose complementation assays (Fig. 1B and C, respectively). The expression levels of the chimera proteins were similar to the Tar-1 WT. Correct integration of the ToxR-TM-MalE chimera proteins into the inner membrane of *E. coli* was assessed by examining the ability of the mutants to functionally complement a MalE-deficient *E. coli* strain (PD28) [32]. Since PD28 cells are unable to grow on minimal medium containing maltose as the only carbon source, only cells that express the chimera protein in the right orientation (MalE pointing toward the periplasm) will be able to utilize maltose and thus allow cell growth. All constructs exhibited similar rates of cell growth, indicating proper membrane integration (Fig. 1C). A construct with a deleted TMD (ΔTMD) served as a negative control, since it was expected to reside in the cytoplasm and therefore was unable to complement the MalE deficiency.

3.2. Statistical analysis

A correlation between over- or under-representation of specific motifs in TMDs and helix–helix interactions was previously found [39]. Therefore, we analyzed the frequency of occurrence of the ZxxSer sequences (where Z = Gly, Ala, Leu, Ile, Val, Mat, or Pro). The analysis was done in a broad set of bacterial TMDs. Our results indicate that all different motifs, except PxxS, are within the range of their random distribution ratio within a database of 41,916 sequences of TMDs (Table 2). The PxxS motif, however, was found to be significantly less than its average random expected occurrence (Table 2). A similar difference was found for the GxxxG and the QxxS motifs [29]. Taken together, these results suggest that the PxxS motif has a specific function, possibly as a dimerization motif, although other yet unknown functions are also possible.

3.3. Secondary structure determination using Circular Dichroism (CD) spectroscopy

Proline is known as a helix breaker, although mainly when present in soluble proteins. Therefore, we determined whether the secondary structures of Tar-1 WT, P/S and A/S peptides are different in micelles (1% SDS) by using circular dichroism spectroscopy. The CD spectral profiles of both Tar-1 WT and P/S peptides revealed similar structures with double minima at ~208 nm and 222 nm, characteristic of an α-helical secondary structure (Fig. 2). Thus, the mutation of Gln to Pro did not alter the TMD structure. However, Tar-1 A/S peptide, showed a different and more complex spectra which has a partial α-helical structure.

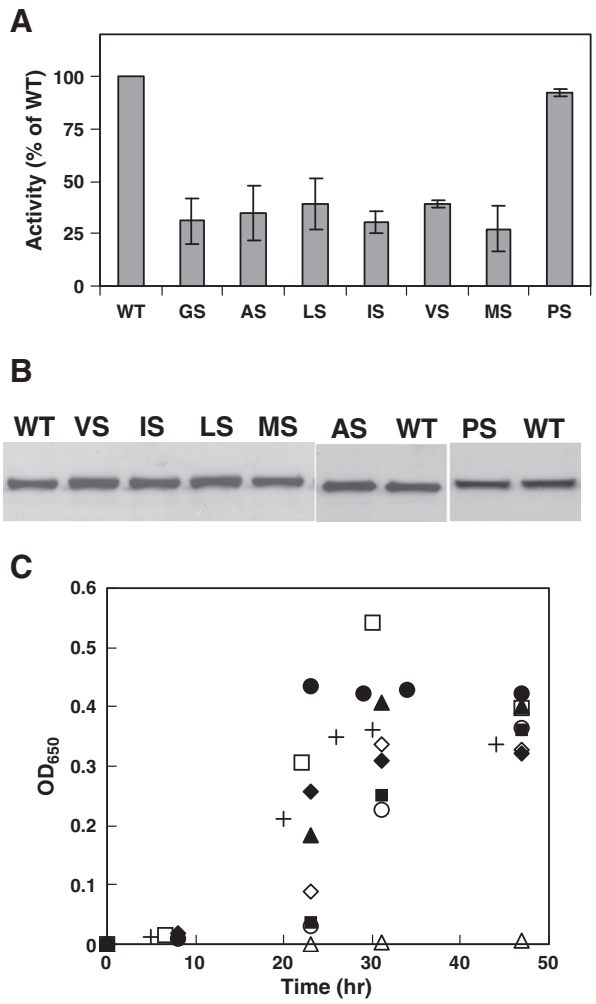


Fig. 1. *Hydrophobic mutations in the Tar-1 wild-type TMD domain.* (A) Cells expressing ToxR-TM-MalE chimeras were examined for dimerization activity (normalized relative to the WT Tar-1 TMD activity). All values are the average of at least three independent repeats. Error bars represent the estimated standard deviation. The exact TMD sequences are indicated in Table 1. (B) Comparison of the expression levels of the ToxR-TM-MalE chimera proteins (65 kDa). Samples of FHK12 cells containing different sequences of Tar-1 within the ToxR-MalE chimera protein were lysed in sodium dodecyl sulfate-sample buffer, separated on 12% SDS-PAGE, and then immunoblotted using anti-MBP antibody. The chimera protein mutants showed expression levels similar to the WT TMD. (C) Correct integration of the ToxR-TM-MalE chimera proteins was tested by their ability to functionally complement the MalE deficiency of PD28 cells. PD28 cells were transformed with Tar-1 WT (◊), Tar G/S (●), Tar A/S (□), Tar L/S (▲), Tar I/S (○), Tar V/S (+), Tar M/S (■), Tar P/S (♦), and ΔTM (△) plasmids, and were grown in minimal medium containing maltose. All constructs showed growth curves similar to Tar-1 WT, indicating proper membrane integration. The negative control with deleted TMD (ΔTM) showed no growth.

Table 2
Actual and the average expected number of occurrences of the specific motifs in a bacterial TM database.

Motif	Occurrences	Expected	STD[a]	P value[b]	Ratio
GxxS	3231	3546	52	0	0.91
AxxS	4141	4363	50	3E−10	0.95
LxxS	6639	6524	68	0.016	1.01
IxxS	4804	4487	54	2.2E−16	1.07
VxxS	4192	4081	62	0.011	1.03
MxxS	1377	1339	38	0.16	1.03
PxxS	923	1148	32	0	0.8

[a] STD is the standard deviation of the expectation distribution curves.
[b] Significance is indicated by the p values calculated as ERFC(x).

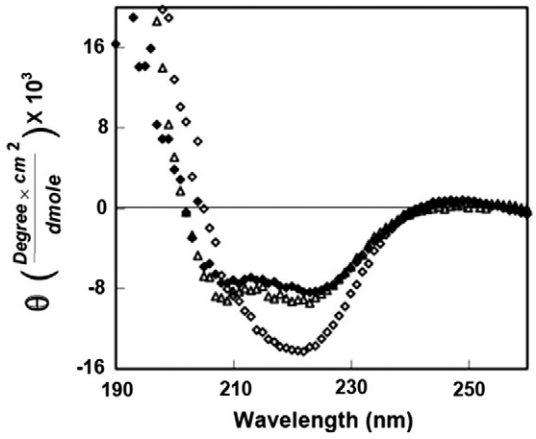
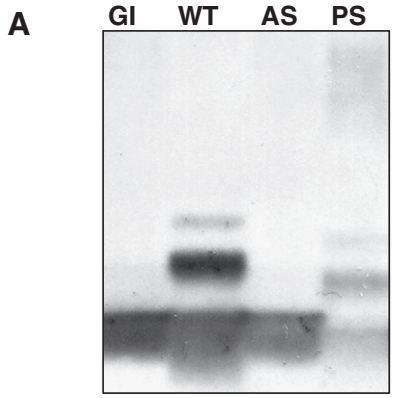


Fig. 2. *CD spectra of the peptides in SDS micelles.* Far-UV Circular Dichroism spectra of Tar-1 TM WT peptide (△), P/S peptide (♦) and A/S peptide (□) in 1% SDS in 1% SDS. Spectra were measured on an Aviv spectropolarimeter at 0.2-nm intervals with a 10-second average time, using a 0.1-cm light path.

3.4. The self-assembly of Tar-1 WT and its mutants revealed by SDS gels

SDS gels were used to analyze the oligomeric state of Tar-1 WT peptide compared to Tar-1 P/S peptide. We used Tar-1 A/S peptide as a control, since this TMD sequence demonstrated significantly reduced dimerization ability compare to the WT and P/S TM sequences, when examined using the ToxR assembly system. Tar-1 G/I peptide was also used as a control for monomers since both amino acids of the motif were mutated [40]. The TMD peptides were first labeled with biotin, which allowed the detection of the oligomeric state of the peptides using anti-biotin antibody (peptide sequences are presented in Fig. 3B). The peptides were separated on 16% SDS-PAGE, and then immunoblotted against biotin. Analysis of the running patterns revealed



B

Tar-1 WT	Biotin-MVLGVFALL**Q**LI**S**GSL-NH_2
Tar-1 GI	Biotin-MVLGVFALL**G**LI**I**GSL-NH_2
Tar-1 AS	Biotin-MVLGVFALL**A**LI**S**GSL-NH_2
Tar-1 PS	Biotin-MVLGVFALL**P**LI**S**GSL-NH_2

Fig. 3. *Peptide self-assembly detected by SDS gel.* (A) Biotin-labeled TMD peptides were subjected to immunoblotting with anti-biotin antibody. 1 μg of each peptide was loaded. (B) Sequences of the different TMD peptides. The amino acids in the positions of the dimerization motif are in bold. Mutations in the Tar-1 TMD are underlined.

differences between the peptides. The Tar-1 WT peptide, demonstrated three oligomeric assemblies, corresponding in size to monomer, dimer, and tetramer (Fig. 3A). The ratio between the different bands correlates well with the dimerization activity that was previously detected for Tar-1 WT TMD relative to Glycophorin A (50–60% [40]). In comparison, a singular band was detected for the Tar-1 G/I and the Tar-1 A/S peptides. The size of the band corresponds to a monomer (Fig. 3A). This correlates with the low dimerization activities of the corresponding mutants within the ToxR system. Interestingly, P/S peptide showed a running pattern similar to the Tar-1 WT peptide containing three major bands: monomer, dimer, and tetramer. In addition, we observed a smeared band at the upper part of the gel that most likely resulted from non-specific aggregation of the peptide. We believe that due to this aggregation tendency of the P/S peptide, its biotin signal is much weaker compared to the other peptides. Note, that the three P/S bands run slightly faster than the corresponding bands of the Tar-1 WT peptide. This is probably due to a slight charge difference between the P/S and the Tar-1 WT peptides [41]. All together, the running pattern observed for the TMD peptides correlates with the dimerization levels observed by the ToxR assembly system. Unfortunately, the ToxR assembly system can only distinguish between monomers and higher assemblies but cannot indicate the exact number of the helices participating in the complex.

3.5. Self-assembly measured by energy transfer

To further investigate the contribution of proline to the TMD–TMD interactions we used fluorescence energy transfer (FRET) assay. The peptides were fluorescently labeled with fluorescein as the donor fluorophore and rhodamine-TAMRA as the acceptor fluorophore and the energy transfer between the peptides were measured. The assay was performed in a lipid environment of negatively charged PE/PG lipids (7:3 w/w). This phospholipid composition is typical of the *E. coli* inner membrane [42]. The Tar-1 WT fluorescein-peptide showed about 45% energy transfer in the presence of the Tar-1 WT rhodamine-peptide at an acceptor-to-lipid ratio of 1:500 (Fig. 4A), indicating an interaction between the two peptides. In contrast, the energy transfer measured between the fluorescein-labeled Tar-1 A/S peptide and the rhodamine-labeled Tar-1 A/S peptide was only 18%. This result is in agreement with the low ability of the Tar-1 A/S TMD mutant to self-assemble in the ToxR system (Fig. 3B). The highest energy transfer was observed for the Tar-1 P/S mutant peptide (~55%, Fig. 4C). This result verified that TMD that contains proline in its interaction interface can self-associate and stabilize oligomer complexes.

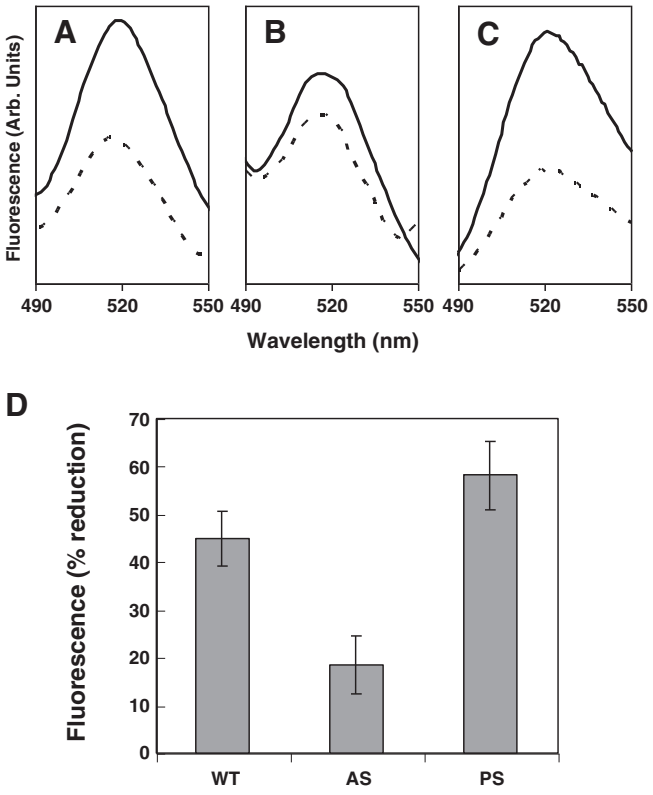


Fig. 4. *Fluorescence energy transfer (FRET) analysis of the Tar-1 peptides.* (A) Representative fluorescence emission spectra ($\lambda_{exc} = 439$ nm, $\lambda_{em} = 520$ nm) of the 0.1 μM Tar-1 WT fluorescein-peptide in the absence (continuous thick line) and in the presence of the 0.1 μM Tar-1 WT-rhodamine peptide (dashed line). A 1:500 acceptor-to-lipid molar ratio was used. (B) Fluorescence emission spectra of the 0.1 μM Tar-1 A/S-fluorescein peptide in the absence (continuous thick line) and in the presence of the 0.1 μM Tar-1 A/S-rhodamine peptide (dashed line). (C) Fluorescence emission spectra of the 0.1 μM Tar-1 P/S-fluorescein peptide in the absence (continuous thick line) and in the presence of the 0.1 μM Tar-1 P/S-rhodamine peptide (dashed line). (D) Percentage of decrease in fluorescent intensity of Tar-1 WT, A/S, and P/S peptides.

4. Concluding remarks

In this study we used the well-defined structural arrangement of Tar-1 TMD to disclose the contribution of a proline residue to TMD self-assembly. Based on our data we concluded that proline residue can stabilize TMD dimerization when localized to the interaction interface of the dimer. Our conclusion is based on the following results: (i) Mutation of the Gln residue of the QxxS dimerization motif to Pro had only a minor effect on the oligomerization, as has been monitored by the ToxR assembly system. Mutations in this position to any other hydrophobic amino acid, however, resulted in a significant reduction in the propensity of the TMD to dissociate; (ii) A Tar-1 peptide containing Gln to Pro replacement (Tar-1 P/S) exhibited a running pattern similar to the Tar-1 WT TMD peptide in SDS-PAGE, indicating the formation of similar oligomers by the two peptides. A TMD peptide that contained a mutation of the Gln to Ala (Tar-1 A/S), however, run as a monomer; and (iii) FRET experiments demonstrated that Tar-1 WT and Tar-1 P/S peptides self-associate in vitro within model phospholipid membranes, whereas the Tar-1 A/S TMD peptide exhibits significantly reduced self-association. These results, together with the statistical analysis, suggest that proline can be involved in the TMD–TMD interactions of many membrane proteins. A possible explanation for this finding is as follows: The results of the CD spectroscopy demonstrated that insertion of Pro residue into the TM domain had no effect on the secondary structure of the helix. Furthermore, the insertion of a glycine residue, which also permits helix bending, could not stabilize helix association. These data suggest that the role of proline is not to distort the helix backbone but rather to free its carbonyl oxygen atom. This may allow the formation of interhelical hydrogen bond with the polar side chain of serine, located on the corresponding TM helix.

Acknowledgments

We would like to thank Dr. D. Langosch at Heidelberg University, who provided the ToxR-GPA and A_{16} plasmids as well as the FHK12 and PD28 *E. coli* strains. This study was supported by the Israel Science Foundation (to YS). Yechiel Shai is the incumbent of the Harold S. and Harriet B. Brady Professorial Chair in Cancer Research.

References

[1] D.M. Engelman, Membranes are more mosaic than fluid, Nature 438 (2005) 578–580.
[2] A. Fink, N. Sal-Man, D. Gerber, Y. Shai, Transmembrane domains interactions within the membrane milieu: principles, advances and challenges, Biochim. Biophys. Acta 1818 (2012) 974–983.
[3] P.R. Gouldson, C. Higgs, R.E. Smith, M.K. Dean, G.V. Gkoutos, C.A. Reynolds, Dimerization and domain swapping in G-protein-coupled receptors: a computational study, Neuropsychopharmacology 23 (2000) S60–S77.

[4] R. Li, J.S. Bennett, W.F. Degrado, Structural basis for integrin alphaIIbbeta3 clustering, Biochem. Soc. Trans. 32 (2004) 412–415.
[5] E.E. Matthews, D. Thevenin, J.M. Rogers, L. Gotow, P.D. Lira, L.A. Reiter, W.H. Brissette, D.M. Engelman, Thrombopoietin receptor activation: transmembrane helix dimerization, rotation, and allosteric modulation, FASEB J. 25 (2011) 2234–2244.
[6] T. Sato, T.C. Tang, G. Reubins, J.Z. Fei, T. Fujimoto, P. Kienlen-Campard, S.N. Constantinescu, J.N. Octave, S. Aimoto, S.O. Smith, A helix-to-coil transition at the epsilon-cut site in the transmembrane dimer of the amyloid precursor protein is required for proteolysis, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 1421–1426.
[7] G. von Heijne, Recent advances in the understanding of membrane protein assembly and structure, Q. Rev. Biophys. 32 (1999) 285–307.
[8] J.R. Herrmann, J.C. Panitz, S. Unterreitmeier, A. Fuchs, D. Frishman, D. Langosch, Complex patterns of histidine, hydroxylated amino acids and the GxxxG motif mediate high-affinity transmembrane domain interactions, J. Mol. Biol. 385 (2009) 912–923.
[9] A.W. Partridge, R.A. Melnyk, C.M. Deber, Polar residues in membrane domains of proteins: molecular basis for helix–helix association in a mutant CFTR transmembrane segment, Biochemistry 41 (2002) 3647–3653.
[10] T. Reddy, S. Manrique, A. Buyan, B.A. Hall, A. Chetwynd, M.S. Sansom, Primary and secondary dimer interfaces of the fibroblast growth factor receptor 3 transmembrane domain: characterization via multiscale molecular dynamics simulations, Biochemistry 53 (2014) 323–332.
[11] A. Senes, D.E. Engel, W.F. DeGrado, Folding of helical membrane proteins: the role of polar, GxxxG-like and proline motifs, Curr. Opin. Struct. Biol. 14 (2004) 465–479.
[12] G. Zhao, E. London, Strong correlation between statistical transmembrane tendency and experimental hydrophobicity scales for identification of transmembrane helices, J. Membr. Biol. 229 (2009) 165–168.
[13] F.X. Zhou, H.J. Merianos, A.T. Brunger, D.M. Engelman, Polar residues drive association of polyleucine transmembrane helices, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 2250–2255.
[14] F.S. Cordes, J.N. Bright, M.S. Sansom, Proline-induced distortions of transmembrane helices, J. Mol. Biol. 323 (2002) 951–960.
[15] C.M. Deber, A.G. Therien, Putting the beta-breaks on membrane protein misfolding, Nat. Struct. Biol. 9 (2002) 318–319.
[16] D.M. Rosenbaum, S.G. Rasmussen, B.K. Kobilka, The structure and function of G-protein-coupled receptors, Nature 459 (2009) 356–363.
[17] Y. Ugolev, T. Segal, D. Yaffe, Y. Gros, S. Schuldiner, Identification of conformationally sensitive residues essential for inhibition of vesicular monoamine transport by the noncompetitive inhibitor tetrabenazine, J. Biol. Chem. 288 (2013) 32160–32171.
[18] K.A. Williams, C.M. Deber, Proline residues in transmembrane helices: structural or dynamic role? Biochemistry 30 (1991) 8919–8923.
[19] S.C. Li, N.K. Goto, K.A. Williams, C.M. Deber, Alpha-helical, but not beta-sheet, propensity of proline is determined by peptide environment, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 6676–6681.
[20] M.S. Sansom, H. Weinstein, Hinges, swivels and switches: the role of prolines in signalling via transmembrane alpha-helices, Trends Pharmacol. Sci. 21 (2000) 445–451.
[21] M. Eilers, A.B. Patel, W. Liu, S.O. Smith, Comparison of helix interactions in membrane and soluble alpha-bundle proteins, Biophys. J. 82 (2002) 2720–2736.
[22] M. Eilers, S.C. Shekar, T. Shieh, S.O. Smith, P.J. Fleming, Internal packing of helical membrane proteins, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 5796–5801.
[23] S. Yohannan, D. Yang, S. Faham, G. Boulting, J. Whitelegge, J.U. Bowie, Proline substitutions are not easily accommodated in a membrane protein, J. Mol. Biol. 341 (2004) 1–6.
[24] M.B. Ulmschneider, M.S. Sansom, Amino acid distributions in integral membrane protein structures, Biochim. Biophys. Acta 1512 (2001) 1–14.
[25] L.K. Iyer, S. Vishveshwara, A model for transmembrane helix with a cis-proline in the middle, FEBS Lett. 374 (1995) 21–24.
[26] A. Tolia, K. Horre, B. De Strooper, Transmembrane domain 9 of presenilin determines the dynamic conformation of the catalytic site of gamma-secretase, J. Biol. Chem. 283 (2008) 19793–19803.
[27] A.W. Partridge, A.G. Therien, C.M. Deber, Missense mutations in transmembrane domains of proteins: phenotypic propensity of polar residues for human disease, Proteins 54 (2004) 648–656.
[28] J.P. Dawson, J.S. Weinger, D.M. Engelman, Motifs of serine and threonine can drive association of transmembrane helices, J. Mol. Biol. 316 (2002) 799–805.
[29] N. Sal-Man, D. Gerber, Y. Shai, The identification of a minimal dimerization motif QXXS that enables homo- and hetero-association of transmembrane helices in vivo, J. Biol. Chem. 280 (2005) 27449–27457.
[30] D. Langosch, B. Brosig, H. Kolmar, H.J. Fritz, Dimerisation of the glycophorin A transmembrane segment in membranes probed with the ToxR transcription activator, J. Mol. Biol. 263 (1996) 525–530.
[31] H. Kolmar, C. Frisch, G. Kleemann, K. Gotze, F.J. Stevens, H.J. Fritz, Dimerization of Bence Jones proteins: linking the rate of transcription from an *Escherichia coli* promoter to the association constant of REIV, Biol. Chem. Hoppe Seyler 375 (1994) 61–70.
[32] B. Brosig, D. Langosch, The dimerization motif of the glycophorin A transmembrane segment in membranes: importance of glycine residues, Protein Sci. 7 (1998) 1052–1056.
[33] R.A. Melnyk, A.W. Partridge, C.M. Deber, Retention of native-like oligomerization states in transmembrane segment peptides: application to the *Escherichia coli* aspartate receptor, Biochemistry 40 (2001) 11106–11113.
[34] X. Han, L.K. Tamm, A host–guest system to study structure–function relationships of membrane fusion peptides, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 13097–13102.
[35] F. Liu, R.N. Lewis, R.S. Hodges, R.N. McElhaney, Effect of variations in the structure of a polyleucine-based alpha-helical transmembrane peptide on its interaction with phosphatidylethanolamine bilayers, Biophys. J. 87 (2004) 2470–2482.
[36] C.S. Wu, K. Ikeda, J.T. Yang, Ordered conformation of polypeptides and proteins in acidic dodecyl sulfate solution, Biochemistry 20 (1981) 566–570.
[37] N. Greenfield, G.D. Fasman, Computed circular dichroism spectra for the evaluation of protein conformation, Biochemistry 8 (1969) 4108–4116.
[38] E. Li, M. You, K. Hristova, FGFR3 dimer stabilization due to a single amino acid pathogenic mutation, J. Mol. Biol. 356 (2006) 600–612.
[39] A. Senes, M. Gerstein, D.M. Engelman, Statistical analysis of amino acid patterns in transmembrane helices: the GxxxG motif occurs frequently and in association with beta-branched residues at neighboring positions, J. Mol. Biol. 296 (2000) 921–936.
[40] N. Sal-Man, D. Gerber, Y. Shai, The composition rather than position of polar residues (QxxS) drives aspartate receptor transmembrane domain dimerization in vivo, Biochemistry 43 (2004) 2309–2313.
[41] A. Rath, F. Cunningham, C.M. Deber, Acrylamide concentration determines the direction and magnitude of helical membrane protein gel shifts, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 15668–15673.
[42] N. Shaw, Lipid composition as a guide to the classification of bacteria, Adv. Appl. Microbiol. 17 (1974) 63–108.