The Composition Rather than Position of Polar Residues (QxxS) Drives Aspartate Receptor Transmembrane Domain Dimerization *in Vivo*†

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ABSTRACT: Transmembrane (TM) helix association is an important process affecting the function of many integral membrane proteins. Consequently, aberrations in this process are associated with diseases. Unfortunately, our knowledge of the factors that control this oligomerization process in the membrane milieu is limited at best. Previous studies have shown a role for polar residues in the assembly of synthetic peptides *in vitro* and the association of *de novo*-designed TM helices *in vivo*. Here we examined, for the first time, the involvement of polar residues in the dimerization of a biological TM domain in its natural environment. We analyzed both the involvement of polar residues in the dimerization process and whether their influence is position-dependent. For this purpose, we used the TM domain of the *Escherichia coli* aspartate receptor (Tar) and 10 single and double mutants. Polar to nonpolar mutations in the sequence demonstrated the role of the QxxS motif in the dimerization of the Tar TM domain. Moreover, 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. Interestingly, mutants that contain two identical strong polar residues, EE and QQ, demonstrated a substantially higher level of dimerization than a QE mutant, although all three TM domains contain two strong polar residues. This result suggests that, in addition to the polarity of the residues, the formation of symmetric bonds also plays a role in dimer stability. The results of this study may facilitate a rational modulation of membrane protein function for therapeutic purposes.

One of the possible pathways by which information is believed to be communicated into cells is via changes in the oligomerization of membrane receptor proteins. This information is crucial for important cellular processes such as homeostasis and signal transduction (*1*−*4*). Furthermore, oligomerization appears to play a role in receptor trafficking (*5*, *6*). Consequently, deficient oligomerization is associated with several diseases ranging from cancer to amyloidal diseases (*7*−*11*). Membrane receptor oligomerization could involve the association of the extracellular, intracellular, or transmembrane (TM) domains (*12*−*14*). Although extensive studies have addressed the effect of oligomerization of water-soluble regions, our knowledge of the factors that govern oligomerization and recognition of the membrane-embedded domains is limited at best.

Recent studies have suggested that the noncovalent association of TM segments can be mediated by (i) a heptad motif of leucines through a side chain packing interaction (*15*), (ii) a GxxxG motif, which was first found in the glycophorin A (GPA) TM domain (*16*−*18*), or (iii) polar residues through intramembranous hydrogen bonds (*19*−*22*).

The significance of polar residues for the association of TM domains has been previously studied both *in vitro*, by examining TM domain synthetic peptides with polar residues in their sequence, and *in vivo*, by analysis of *de novo*-designed TM helices (*19*−*22*). These studies revealed that amino acids containing two polar side chain atoms (as asparagine and glutamine) have a greater tendency to drive TM association than residues containing only one polar side chain atom (threonine or serine) (*19*, *22*). Glu and Asp should be protonated within the low-dielectric membrane environment, and therefore are considered strong polar residues (*19*, *22*).

In this study, we utilized the *Escherichia coli* aspartate receptor (Tar), which is one of the main chemotaxis receptors found in bacteria. This receptor forms a homodimer in which each subunit is composed of two TM helices (Tar-1 and Tar-2) separated by a substantial periplasmic domain (*23*, *24*). Disulfide cross-linking studies suggested that Tar-1 interacts with Tar-1′ from the corresponding monomer and that there is no direct contact between the Tar-2 helices of the two monomers (*23*). A recent study showed that a synthetic peptide corresponding to the Tar-1 TM domain forms an SDS-resistant homodimer, whereas the Tar-2 TM domain remains monomeric (*25*). These observations suggest that Tar-1 is the domain that mediates the dimerization of the Tar receptor. Moreover, the Tar-1 TM domain does not contain a GxxxG motif or a classical heptad motif of leucines, but rather contains polar residues in its TM sequence, and therefore, we found this TM domain to be an appropriate system for investigating the contribution of polar residues in the self-dimerization of TM domains, *in vivo*.

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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[a]	sequence[b]
Tar-1 WT	^{13}MVLGVFALL*Q*LI*S*GSL^{28}
Tar-1 G/I	^{13}MVLGVFALL*G*LI*I*GSL^{28}
Tar-1 Q/I	^{13}MVLGVFALL<u>*Q*</u>LI<u>*I*</u>GSL^{28}
Tar-1 G/S	^{13}MVLGVFALL*G*LI<u>*S*</u>GSL^{28}
Tar-1 S/Q	^{13}MVLGVFALL<u>*S*</u>LI*Q*GSL^{28}
Tar-1 Q/E	^{13}MVLGVFALL<u>*Q*</u>LI<u>*E*</u>GSL^{28}
Tar-1 E/S	^{13}MVLGVFALL*E*LI<u>*S*</u>GSL^{28}
Tar-1 E/E	^{13}MVLGVFALL<u>*E*</u>LI*E*GSL^{28}
Tar-1 Q/Q	^{13}MVLGVFALL<u>*Q*</u>LI<u>*Q*</u>GSL^{28}
Tar-1 S/S	^{13}MVLGVFALL<u>*S*</u>LI<u>*S*</u>GSL^{28}
Tar-1 T/T	^{13}MVLGVFALL<u>*T*</u>LI<u>*T*</u>GSL^{28}

[a] The nomenclature of the TM domains provides the two amino acids replacing the original polar residues glutamine and serine of the WT sequence at positions 22 and 25, respectively. [b] Amino acids are numbered according to the position in the WT protein (Swissprot entry p07017). Mutations in the Tar-1 TM domain are underlined. Mutatable positions are italicized.

Here we introduced the Tar-1 TM domain and 10 single and double mutants into the ToxR−MalE system, which can assess TM−TM interaction within the *E. coli* natural membrane. Using this system, we explored the role of polar residues ($Q^{22}xxS^{25}$) in the dimerization process of the Tar-1 TM domain *in vivo* and investigated whether the contribution of the polar residues is position-dependent.

EXPERIMENTAL PROCEDURES

Construction of the ToxR Chimeras. An *Nhe*I−*Bam*HI TM−DNA cassette encoding 16 residues of the Tar-1 wild-type (WT) TM domain (^{13}MVLGVFALLQLISGSL^{28}) was inserted between the *E. coli* maltose binding protein (MalE) and the ToxR transcription activator in the ToxR−MalE plasmid. The mutants contained the same sequence as the Tar-1 WT TM domain except for the replacement of Gln and Ser at positions 22 and 25, respectively (highlighted in the sequence; see Table 1). All the constructs were confirmed by DNA sequencing.

ToxR−TM−MalE Protein Expression Levels. We performed Western blot analysis to determine whether the change in two polar amino acids in the sequence of the TM domain affected the expression level of the chimera protein. Specifically, aliquots of 10 μL of FHK12 cells, each transformed with a different plasmid, were mixed with a sample buffer, boiled for 5 min, subjected to 12% SDS−PAGE, and then transferred to nitrocellulose. The primary antibody that was used was anti-maltose binding protein. The detection was done with the Phototope-HRP Western Blot Detection System from Cell Signaling Technology.

Maltose Complementation Assay. Membrane insertion and correct orientation were examined as previously described (*26*). Briefly, PD28 cells transformed with the different plasmids were cultured overnight. The cells were washed twice with PBS and used to inoculate M9 minimal medium that included 0.4% maltose at a 200-fold dilution. The growth of the cells was assessed at different time points by cell density at 650 nm.

Specific Homodimerization of TMs in Vivo. Plasmids were transformed into *E. coli* FHK12 cells, which contain β-galactosidase under the control of a *ctx* promoter. In this system, dimerization of the TM domains results in association and activation of the ToxR transcription factor, which then becomes active and is able to bind the *ctx* promoter (*27*). Quantification of the amount of homodimerization was done by measuring the activity of the β-galactosidase reporter gene and dividing the activity by cell content (OD_{590}) (Miller units). The results were normalized between positive and negative controls such as ToxR′GPA_{13} and ToxR′A_{16}, respectively (*27*). 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 the addition of *o*-nitrophenylgalactosidase and by monitoring the reaction at 405 nm for 20 min at intervals of 30 s at 28 °C with a Molecular Devices kinetic reader (*27*, *28*). Specific β-galactosidase activities were computed from the V_{max} of the reaction. All the activities were further normalized according to the expression levels of the proteins, as obtained from Western blotting.

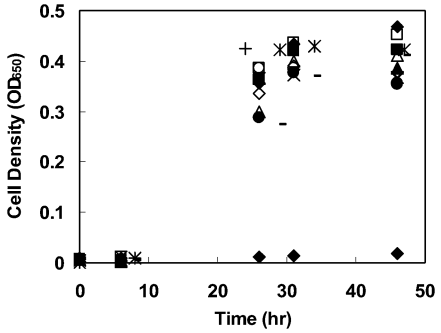

FIGURE 1: Membrane insertion and orientation. Correct integration of the ToxR−TM−MalE chimera proteins was examined by their ability to functionally complement the MalE deficiency of PD28 cells. PD28 cells were transformed with the different plasmids and grown in minimal medium containing maltose. Only cells that expressed periplasmatic MalE were able to grow with maltose as the only carbon source. All constructs exhibited similar growth curves, except the negative control with the deleted TM domain (ΔTM), indicating proper membrane integration: WT (■), E/E (▲), Q/E (●), E/S (◇), S/Q (□), Q/Q (△), T/T (○), S/S (×), G/I (+), Q/I (−), G/S (*), and ΔTM (◆).

RESULTS

To investigate the self-association of the aspartate receptor TM domains, we utilized the ToxR−MalE system (*27*), which can detect TM−TM interaction within the plasma membrane of *E. coli*. The dimeric state of the chimera protein is indicated by the activity (V_{max}) of the β-Gal reporter gene.

Membrane Insertion and Orientation. 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) (*26*). Since PD28 cells are unable to grow on minimal medium with maltose as the only carbon source, only cells that express the chimera protein in the right orientation (MalE pointed 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 (Figure 1). A construct with a deleted TM domain (ΔTM) served as a negative control, since it was expected to reside in the cytoplasm and therefore was unable to complement the MalE deficiency.

Dimerization of the Tar-1 TM Domain. Replacement of the ToxR TM domain with the Tar-1 WT TM domain

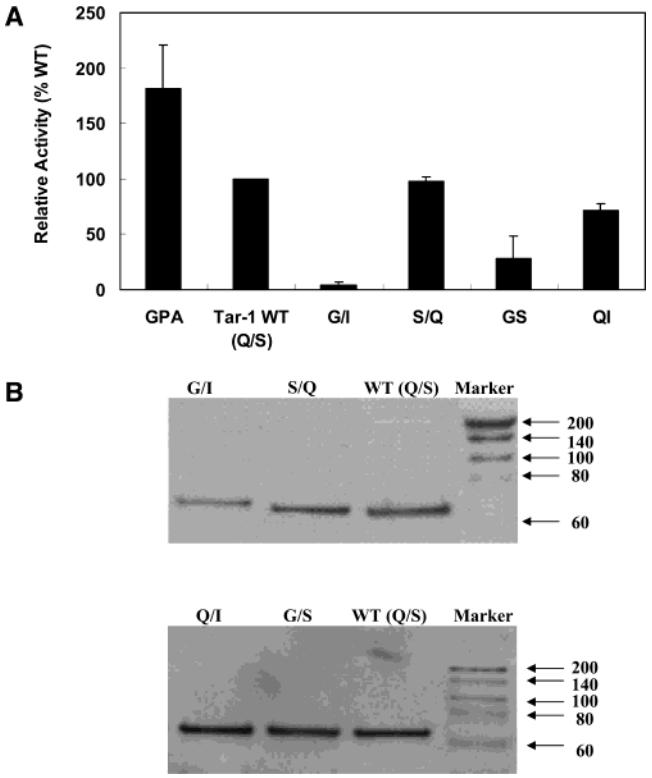

FIGURE 2: Mutations of the Tar-1 TM domain modulate its extent of dimerization in *E. coli* membranes. (A) Cells expressing a ToxR−MalE chimera were examined for lacZ activity (normalized relative to the WT Tar-1 TM domain activity). All values are the averages of at least three independent assays. Error bars represent the estimated standard deviation. The exact sequences are indicated in Table 1. The results were further normalized according to protein expression levels (panel B). (B) Comparison of the expression levels of 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 immunoblotted using anti-MBP antibody (New England Biolabs). Sizes are indicated in kilodaltons at the right.

resulted in an activity of 52 ± 9%, compared with the activity of GPA which served as a positive control for high-level dimerization ability (*16*−*18*) (Figure 2). This significant level of interaction indicated that 16 amino acids from the Tar-1 TM domain were enough to drive the self-association of the Tar-1 TM domains within the membrane environment.

Polar to nonpolar mutation (Tar G/I) almost completely abolished dimerization ability, rendering the TM domains largely monomeric in the membrane (Figure 2). This replacement of the two original polar residues with Gly and Ile created a GxxxG dimerization motif (*18*) within the Tar-1 TM domain. The weak dimerization ability of this mutant implies that the presence of a GxxxG motif is not sufficient to promote dimerization of the Tar-1 TM domains in this specific location. This result demonstrates the crucial role of polar residues in the dimerization of Tar-1 TM domains, since simultaneous mutations of these two polar amino acid residues abrogated almost entirely self-association. To address the contribution of each polar residue individually, we examined the dimerization ability of Q/I and G/S single mutants. The Q/I mutant exhibited a reduced dimerization activity compared with that of Tar-1 WT (∼70%), suggesting that the presence of glutamine alone is not sufficient for reaching WT dimerization levels. The role of serine in the dimerization of the TM domain was examined with the G/S mutant. This mutant exhibited significantly weaker dimerization ability than the WT, although it was still higher than that of the G/I double mutant. These results indicate that Gln makes a greater contribution to the dimerization process than Ser. Nevertheless, the presence of both polar residues is essential in achieving a dimerization level similar to that of wild-type Tar-1.

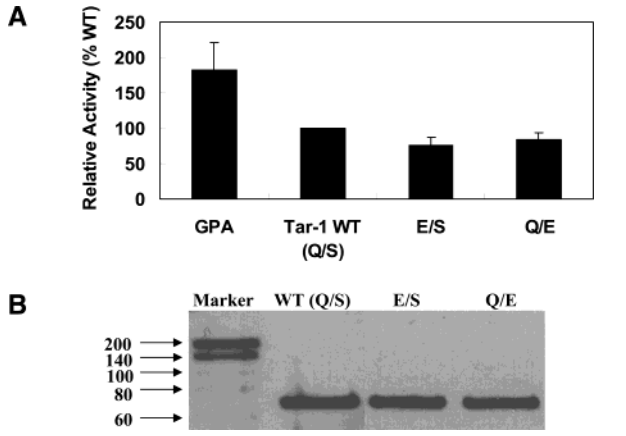

FIGURE 3: Glutamic acid mutants. (A) Replacing one of the original polar residues with glutamic acid had no significant effect on the dimerization ability of the TM domain. The details are as described in the legend of Figure 2. (B) Comparison of the expression levels of ToxR−TM−MBP chimera proteins. The chimera protein of the Q/E TM domain and the E/S TM domain exhibited expression levels similar to that of the WT TM domain.

To examine whether the specific $Q^{22}xxS^{25}$ polar residue motif was required, we compared the Tar-1 WT TM domain activity to that of Tar S/Q which contains polar residues in swapped positions ($S^{22}xxQ^{25}$). Tar S/Q exhibited a dimerization ability similar to that of wild-type Tar-1 (Figure 2), implying a similar contribution from both positions. Thus, the effect of interchanging specific polar amino acids between the two locations (positions 22 and 25) was not significant. However, the presence of polar residues at positions 22 and 25 had a major effect on the dimerization propensity of the TM domain.

Dimerization Propensity in the Presence of Glutamic Acid. To examine the effect of glutamic acid on the dimerization capacity of the TM domain, we used ToxR−MalE constructs that contain Glu instead of one of the polar residues (Q or S). The rationale of introducing glutamic acid into the TM domain was based on several studies that pointed out that glutamic acid mutations in the TM domains are associated with several diseases (*9*, *29*). For example, a specific Val → Glu mutation within the TM domain of the ErbB2 oncogene product (Neu) (*30*, *31*) is known to induce ErbB2 dimerization and activation (*32*, *33*). In this study, we observed that the replacement of one original polar residue with a Glu residue (E/S or Q/E) had no significant effect on the dimerization activity (Figure 3). In the low-dielectric membrane environment, glutamic acid most likely has a protonated carboxylic group (*19*, *22*). Thus, the Glu residue is not charged and contains two polar side chain atoms such as Gln, Asn, and His. This promotes helix association and stabilization, since Glu can act simultaneously as a good hydrogen bond donor and acceptor, in contrast to polar residues Ser and Thr that contain only one polar side chain atom. In view of this, the similar activity of the WT and

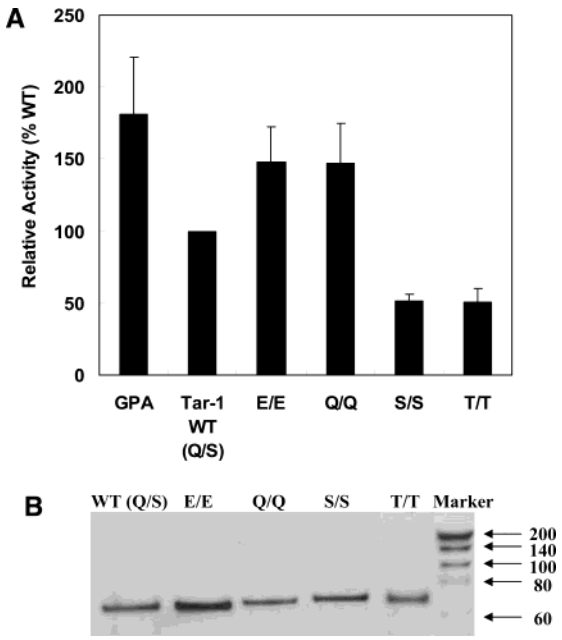

FIGURE 4: Identical polar residues at positions 22 and 25. (A) Replacement of the polar residues in positions 22 and 25 with two identical polar residues demonstrates an increased dimerization propensity for strong polar residues and a reduced propensity for residues with one polar side chain atom. The details are as described in the legend of Figure 2. (B) Comparison of the expression levels of ToxR−TM−MalE chimera proteins (65 kDa).

E/S constructs can be easily understood since substitution of Gln for Glu has a minor influence on polarity. However, the Q/E construct also exhibited dimerization ability similar to that of WT, although there is a considerable polarity difference between Ser and Glu. It is possible that the polarity of the residues at positions 22 and 25 is not the only factor that controls dimerization.

Identical Polar Residue Motif. Substitution of the two original polar residues at positions 22 and 25 with two Glu residues (E/E) resulted in an activity substantially higher than that of the wild type (Figure 4). A similar phenomenon was observed for the TM domain containing two glutamines at these positions (Q/Q). However, two serines (S/S) or two threonines (T/T) at positions 22 and 25 yielded only ~50% of the original activity. The increased activities of the TM domain with two glutamines or two glutamic acids (~150%) revealed that less than 100% of the WT chimera proteins are in a dimer form since a change in the polar residue composition shifted the monomer−dimer equilibrium toward further dimer formation. Reduced activities of the S/S and T/T mutants compared with the WT activity (Q/S) provide additional support that residues with two polar side chain atoms have a greater potential to form a stabilized helix association than residues with only one polar side chain atom.

The number of possible interhelical hydrogen bonds is not the only factor that controls the state of oligomerization, since the degree of dimerization of the two identical polar residue mutants (E/E and Q/Q) was substantially higher than that of the Q/E mutant, although all three contain two strong polar residues. It is possible that two identical polar residues create a special symmetric structure that promotes and/or stabilizes helix association.

ToxR−MalE Expression Levels. The expression levels of the chimera proteins of the different constructs were compared to the expression level of the WT TM domain. In some mutants, a significant difference in the expression levels of the construct was observed. Therefore, all the activities were normalized according to the Western blotting results.

DISCUSSION

In this study, we investigated the involvement of two polar residues, located in the *E. coli* aspartate receptor TM domain, in the dimerization process. Our data demonstrate that different residue combinations in these specific positions have a marked effect on the dimerization ability of the TM domain. More specifically, our results (i) demonstrate that the two polar residues at positions 22 and 25 are crucial for the dimerization of the Tar-1 TM domain *in vivo*, (ii) suggest that the existence of a GxxxG dimerization motif within the Tar-1 TM domain does not necessarily promote dimeriztion, (iii) reveal a similar contribution from both positions, and (iv) imply that two identical strong polar residues create symmetric hydrogen bonds that promote and/or stabilize the dimeric state of the *E. coli* aspartate receptor.

Note that previous studies have examined eukaryotic membrane protein TM domains within the bacterial membrane of *E. coli* that contains a lipid composition different from that of the mammalian membrane where these proteins are originally expressed (*16*, *27*, *31*, *34*). In this study, we investigated the *E. coli* Tar-1 TM domain within its native membrane.

Importantly, our results demonstrate that polar residues have a marked effect on the dimerization ability of the Tar-1 TM domain *in vivo*, since substitution of polar residues at positions 22 and 25 for nonpolar residues dramatically impaired the self-association ability of the TM domain. In addition, creation of a GxxxG dimerization motif instead of the $Q^{22}xxS^{25}$ motif failed to restore the dimerization activity of the Tar-1 TM domain. Examination of the involvement of Gln and Ser individually by the single mutants Q/I and G/S demonstrated that both residues participate in the dimerization process. However, Gln makes a greater contribution than Ser.

The replacement of Q with E (E/S) resulted in an activity similar to that of the WT TM domain, demonstrating that the reduced activities of G/I resulted from the absence of polar residues, which can form hydrogen bonds between the helices at these positions, rather than structure-specific requirements.

The similar activities of the WT TM domain ($Q^{22}xxS^{25}$) and the swapped TM domain ($S^{22}xxQ^{25}$) demonstrate an equal contribution at both positions for TM domain self-association. We assume that the replacement of any polar amino acid with another results in a structural change that can affect the distance between the helices and the overall geometry of the TM region. Therefore, the similar activities of the WT TM and S/Q TM domains imply that either there is freedom in the structure of this TM domain within the membrane environment or there is symmetry in the TM domain's interface. In either case, the nature of the amino acids that occupy positions 22 and 25 has a greater influence on the dimerization ability of the TM domain than the specific location at one position or the other.

The substantially higher activities of the E/E TM domain and the Q/Q TM domain compared with the activity of Tar-1

WT TM domain could not be merely due to the addition of a second strong polar residue, since the Q/E TM domain mutant exhibited dimerization levels comparable to that of WT Tar-1. These results suggest that symmetry in the TM domain's interface is another factor which can affect the dimerization level of the TM domain. As a result, we concluded that the number of possible hydrogen bonds that can be created by the polar residues is not the only factor that controls dimerization. It is reasonable to assume that the two identical polar residues facilitate self-interaction of the TM domain by forming symmetric hydrogen bonds, which allow better stabilization of the TM interactions.

In general, studies regarding the interactions that govern the oligomerization of TM domains of integral membrane proteins are of great interest and importance. This is especially true since such mutations in these domains are known to be involved in diseases (*29*). Therefore, advancing our understanding of the oligomerization process within the membrane milieu can assist us in better understanding the basic principles of membrane protein biology. In turn, this knowledge will help us, in the future, to rationally modulate the function of membrane proteins for therapeutic purposes.

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