Analysis of the Role of the EnvZ Linker Region in Signal Transduction Using a Chimeric Tar/EnvZ Receptor Protein, Tez1*

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Tez1 is a chimeric protein in which the periplasmic and transmembrane domains of Tar, a chemosensor, are fused to the cytoplasmic catalytic domain of EnvZ, an osmosensing histidine kinase, through the EnvZ linker. Unlike Taz1 (a similar hybrid with the Tar linker), Tez1 could not respond to Tar ligand, aspartate, whereas single Ala insertion at the transmembrane/linker junction, as seen in Taz1A1, restored the aspartate-regulatable phenotype. Analysis of the Ala insertion site requirement and the nature of the insertion residue on the phenotype of Tez1 indicated that a junction region between the transmembrane domain and the predicted helix I in the linker is critical to signal transduction. Random mutagenesis revealed that P185Q mutation in the Tez1 linker restored the aspartate-regulatable phenotype. Substitution mutations at Pro-185 further demonstrated that specific residues are required at this site for an aspartate response. None of the hybrid receptors constructed with different Tar/EnvZ fusion sites in the linker could respond to aspartate, suggesting that specific interactions between the two predicted helices in the linker are important for the linker function. In addition, a mutation (F220D) known to cause an OmpCe phenotype in EnvZ resulted in similar OmpCe phenotypes in both Taz1A1 and Tez1, indicating the importance of the predicted helix II in signal propagation. Together, we propose that the N-terminal junction region modulates the alignment between the two helices in the linker upon signal input. In turn helix II propagates the resultant conformational signal into the downstream catalytic domain of EnvZ to regulate its bifunctional enzymatic activities.

EnvZ, a histidine kinase osmosensor, locates on the inner membrane of Escherichia coli. It is composed of a periplasmic domain, transmembrane domains, and a cytoplasmic domain (1, 2). The cytoplasmic domain has been further dissected into three subdomains, the linker region, domain A, and domain B, in which the latter two form the catalytic core of EnvZ, harboring both kinase and phosphatase functions (3). NMR structures of both domain A and domain B have been solved (4, 5). Biochemical analysis has revealed that the domain A of the EnvZ is responsible for the dimerization, phosphotransfer and phosphatase functions, and domain B binds ATP and catalytically assists the enzymatic function of domain A (3, 6). The spatial arrangement between these two domains appears to be crucial for the modulation of EnVZ enzymatic activities (6). Previous studies suggest that EnvZ senses the extracellular osmolarity changes, transmits the signal through its transmembrane domain, and then modulates the kinase/phosphatase ratio of the cytoplasmic catalytic domain, which controls the cellular concentration of phosphorylated OmpR to mediate the reciprocal expression of the two major outer membrane porin proteins OmpF and OmpC (2, 7–11).

Although the exact ligand for EnvZ remains unknown, two kinds of chimeric receptors have been constructed in which the periplasmic and transmembrane domains of chemoreceptor Tar (sensor for aspartate) or Trg (sensor for ribose) are fused with the catalytic core of EnvZ (through the Tar or Trg linker, respectively). The resultant Tax (12) and Trz (13) are able to respond to aspartate and ribose in the medium, respectively, to activate ompF-lacZ in a concentration-dependent manner, suggesting that the chemoreceptors and EnvZ share a common signal transduction mechanism. Similar chimeric receptors have also been constructed using the periplasmic and transmembrane domain of Tar and the cytoplasmic domain of the human insulin receptor (14) or the periplasmic domain of the histidine kinase NarX and the cytoplasmic domain of Tar (15), further supporting a notion that there is a common mechanism widely used by signal transduction across the membrane in both prokaryotes and eukaryotes. Extensive studies on signal transduction through the transmembrane domain of chemotaxis receptors have been carried out, and various mechanisms have been proposed (16–21). However, it remains unknown how the signal is propagated through the membrane to the cytoplasm. Particularly, it is of great interest how the linker region, a structural element connecting the transmembrane domain with the cytoplasmic signaling domain, propagates signal into the cytoplasm.

The linker region (also called HAMP domain) is widely found not only in histidine kinases and chemoreceptors but also in bacterial nucleotidyl cyclases and phosphatases. It is considered to function as a regulatory element in these proteins (22–24). Even though the linker regions show low primary sequence homology, they have a similar helix-turn-helix fold based on secondary structure prediction. A cysteine-scanning study has indicated that the Tar linker consists of a helix-turn-helix structure (25). However, no three-dimensional structures have yet been determined for any linker regions.

The EnvZ linker is also predicted to contain a helix-turn-helix structure (22, 23, 26) like the Tar linker. A number of mutations that block the osmosensing function of EnvZ have been mapped within the linker, suggesting its important role in signal transduction (26–30). Only substitution of hydrophobic residue to charged residue, but not to hydrophobic residue, within the two predicted helices of the linker disturbed normal
osmoregulation, suggesting that the amphipathic nature of the helices is important for signal propagation. It is important to note that the effects of the linker mutations on the function of the catalytic domain can be detected only when EnvZ is associated with the membrane (26). Once the cytoplasmic domain of EnvZ is detached from the membrane, the linker mutations no longer affect the catalytic function of EnvZ, indicating that the linker has to be fixed on the membrane to transmit the mutational effects to the catalytic domain.

To precisely elucidate the role of the EnvZ linker in signal propagation and the structural basis for its function, here we exchanged the Tar linker in the Taz construct with the EnvZ linker and examined how the linker exchange affects the aspartate-regulated EnvZ function. As shown in Fig. 1A, the sequence alignment between the EnvZ linker and the Tar linker together with the C-terminal regions of the transmembrane domain TM2 indicates that the EnvZ linker starts with one Arg residue, whereas the Tar linker starts with two Arg residues, resulting in an extra Arg residue at the transmembrane/linker junction for the Tar linker than the EnvZ linker. In the newly constructed Tar-EnvZ hybrid protein termed Tez1, the EnvZ linker starting with Arg-180 (the residue number is based on the EnvZ sequence) is fused to the Tar transmembrane domain TM2, ending at Ile-212 (the residue number is based on the Tar sequence). Tez1 could not induce ompC-lacZ in RU1012 cells even in the presence of aspartate. However, the insertion of a single Ala residue at the transmembrane/linker junction, not other regions in the linker, restored the aspartate-regulatable signal transduction. In addition, through random mutagenesis we demonstrated that position 185 (the residue number is based on the EnvZ sequence), where a highly conserved Pro residue locates, is important for the linker function. To explore the possible structural basis for proper signal transmitting, we constructed several new Tar-EnvZ hybrids fused at different sites in the linker region. We also examined the effect of the F220D mutation, known to block EnvZ osmosensing, on Tez1 and its variant, Tez1A1. On the basis of the characterization of all these hybrid receptors and their mutants, we propose models as to how signal propagation through the linker region takes place.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Strain RU1012 (MC4100 ara +, F (ompC-lacZ) 10–25 ΔenvZ::Km) (12) was used for in vivo study.

Tez1 was constructed by a two-step PCR ligation. The first PCR was carried out with primers SC110 (5′-CGTACCAGCTGTCAATAATTG-3′) and 9485 (5′-CGTCTCTGTGATAGAAGCGCC-3′) using pBR001 (12) as template. The second PCR was carried out with primers 9484 (5′-CGTGTACGCGGCGGATCTGATCCAGAACGACG-3′) and 3435 (5′-AAGGTTGCGGCAACGTTGTAATTCGATGAAAC-3′) using pBR002 (12) as template. The third PCR fragment was generated with primers SC110 and 8435 using the first and second PCR products as templates. The third PCR product was digested with XhoI and NdeI and subcloned into pTA2, replacing the original EcoRI linker and examining how the linker exchange affects the aspartate-regulated EnvZ function. As shown in Fig. 1A, the sequence alignment between the EnvZ linker and the Tar linker together with the C-terminal regions of the transmembrane domain TM2 indicates that the EnvZ linker starts with one Arg residue, whereas the Tar linker starts with two Arg residues, resulting in an extra Arg residue at the transmembrane/linker junction for the Tar linker than the EnvZ linker.

For random mutations on the linker region, mutagenesis PCR was carried out using dITP/dNTP mixture and Mn2+ as described previously (31) with primers N66 (5′-CGCTCTTTGCTGCTGACAACGGTTGG-GTTGCTGAACCAGCCG-3′) and N64. Mutagenized linker fragments were digested with EcoRI and NdeI and subcloned into Tez1 to replace the wild-type EnvZ linker. Primers 27601 (5′-GTATCCAGAGACGACTTTAGGATGACTGCG-3′) and 27602 (5′-GAGATCCAGACACTTTAGGATGACTGCG-3′) were used in site-directed mutagenesis to separate the two suppressor mutations P185Q (Tez3) and R184Q (Tez4), respectively. Primers 26473 (5′-CGTATCTAGACGGTATCCTGAACCGTAACG-3′) and 26474 (5′-TTCGAGTACTGCAANNTTTCGATGACG-3′) were used for constructing Tez1P185X mutants by site-directed mutagenesis using Tez1 as template. Note that the residue numbers used are based on the EnvZ sequence.

Preparation of the Membrane Fraction—Membrane fractions containing Taz1, Tez1, or Tez1 variants were prepared as described previously (9). The protein amounts were determined by Bio-Rad protein assay. Western blot analysis was carried out using an equal amount of membrane proteins (5 μg).

Autophosphorylation and OmpR Kinase Assay—Membrane fractions containing receptor proteins (5 μg) were incubated at room temperature in reaction buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 5 mM MgCl2, 5 mM β-mercaptoethanol, and 0.5 μM of (γ-32P)ATP (3000 Ci/mmol 1, 10 μCi/ml; PerkinElmer Life Sciences). Ten-μl aliquots were removed from the reaction mixture at 2 and 15 min, and the reactions were stopped by adding 2.5 μl of 5× SDS loading buffer. In the remaining reaction mixture, OmpR was added to a final concentration of 2 μM. Ten-μl aliquots were removed at 5 and 10 min, and reactions were stopped as described above. All reaction mixtures were then analyzed by SDS-PAGE followed by autoradiography.

β-Galactosidase Assay—β-Galactosidase activities of RU1012 cells grown on various Taz1 or Tez1 constructs were determined as described previously (32).

Screening for Intragenic Suppressor—Tez1 constructs harboring randomly mutagenized linker sequence were transformed into RU1012 cells. For random linker mutations, the mutagenesis PCR was carried out using dITP/dNTP mixture and Mn2+ as described previously (31) with primers N66 (5′-CGCTCTTTGCTGCTGACAACGGTTGG-GTTGCTGAACCAGCCG-3′) and N64. Mutagenized linker fragments were digested with EcoRI and NdeI and subcloned into Tez1 to replace the wild-type EnvZ linker. Primers 27599 (5′-CGATCCAGAAGACGACTTTAGGATGACTGCG-3′) and 27600 (5′-GAGATCCAGACACTTTAGGATGACTGCG-3′) were used in site-directed mutagenesis to separate the two suppressor mutations P185Q (Tez3) and R184Q (Tez4), respectively. Primers 26473 (5′-CGTATCTAGACGGTATCCTGAACCGTAACG-3′) and 26474 (5′-TTCGAGTACTGCAANNTTTCGATGACG-3′) were used for constructing Tez1P185X mutants by site-directed mutagenesis using Tez1 as template. Note that the residue numbers used are based on the EnvZ sequence.

RESULTS

Tez1 Cannot Respond to Aspartate, the Ligand for Tar—To precisely examine the role of the linker region of EnvZ in signal transduction, we replaced the Tar linker (44 residues) in the Taz1 receptor with the EnvZ linker (43 residues) to construct a new hybrid protein termed Tez1, based on the assumption that the two linkers share a similar mechanism for signal propagation. In Tez1, the EnvZ linker was connected directly to the C-terminal end of the Tar transmembrane domain TM2 (Fig. 1A). As shown in Fig. 1B, Tez1 could not respond to aspartate and resulted in an OmpC-constitutive repression phenotype (OmpC) in RU1012 cells. Two possible reasons may account for this phenotype: (i) the linker replacement might compromise the stability of the receptor protein, since the lack of an aspartate receptor in the membrane is expected to result in the
OmpR-P, the phosphorylated OmpR band. The EnvZ linker is unable to transmit the input signal to the downstream catalytic domain, and thus, cells are unable to increase the OmpR-P concentration by increasing the kinase/phosphatase ratio of the catalytic domain.

To test these possibilities, Western blot analysis of the membrane fractions from RU1012 cells harboring either Tez1 or Taz1 construct was carried out. As shown in Fig. 1C, Tez1 receptor could be expressed and localized in the membrane to a similar level as Taz1 receptor. Using the same membrane fractions containing Tez1 or Taz1 to check the autokinase and OmpR kinase assay of Tez1 and Taz1. The reactions were carried out as described under “Experimental Procedures” using membrane fractions containing the same amount (5 μg) of Tez1 (lanes 1–4) or Taz1 (lanes 5–8). The autophosphorylation reaction was stopped at 2 min (lane 1, Tez1; lane 5, Tez1) and 15 min (lane 21, Tez1; lane 6, Taz1). The OmpR kinase assay was stopped at 5 min (lane 3, Tez1; lane 7, Taz1) and 10 min (lane 4, Tez1; lane 8, Taz1). Mem-P, the phosphorylated Tez1 or Taz1 band; OmpR-P, the phosphorylated OmpR band.

Adjustment of the Transmembrane/Linker Junction in Tez1—The Tar transmembrane domain TM2 is presumed to consist of an α-helix structure (for review, see Ref. 17). The addition or deletion of an amino acid residue(s) at the transmembrane/linker junction may change the relative orientation of the EnvZ cytoplasmic domain to the Tar transmembrane domain through altering the conformation of the linker region, which in turn recovers the aspartate-regulatable OmpC expression in RU1012 cells. Therefore, we constructed three new Tez1 hybrid receptors, Tez1A1, Tez1ΔI181, and Tez1AA. In Tez1A1, one Ala residue was inserted immediately downstream of the membrane interface residue Arg-180; in Tez1ΔI181, Ile-181 residue was deleted; and in Tez1AA, two Ala residues were inserted after the 180 residue (Fig. 2).

Fig. 2. Adjustments at the transmembrane/linker junction in Tez1. A, sequence of the EnvZ linker region showing the modifications at the transmembrane/linker junction. In the Tez1A1 construct, one Ala residue was inserted immediately after Arg-180. In Tez1ΔI181, Ile-181 was deleted. In Tez1AA, two Ala residues were inserted after Arg-180. Two predicted helices (I and II) in the linker region were marked in open boxes. B, the β-galactosidase activities of E. coli RU1012 cells carrying Tez1 or Taz1. β-Galactosidase activities were measured with (black bar) or without (hatched bar) 5 mM aspartate in the growth medium. The data plotted were the average values from three independent experiments.
brane fraction at a similar level as Tez1 and retained enzymatic activities (data not shown). These results indicate that the length adjustment at the transmembrane/linker junction is crucial for the proper signal transduction from the transmembrane domain to the cytoplasmic domain.

**Specificity of Amino Acid Insertion after Arg-180**—To further examine how the Ala residue inserted in Tez1A1 plays a specific role in signal transduction, random amino acid insertion was carried out after Arg-180 to construct Tez1X. Of 15 different residues introduced at this position, only Asp and Asn in addition to Ala residue were able to resume the aspartate-regulatable regulation of ompC-lacZ in RU1012 cells as shown in Fig. 3. Notably, β-galactosidase activities in the absence of aspartate were quite high in the case of Asp and Asn residue insertion in contrast to Tez1A1. Amino acid residues such as Leu, which are preferred in helix formation, were not able to recover the aspartate-regulatable phenotype, suggesting that the Ala residue insertion after Arg-180 is not simply extending a helical structure through the TM2 transmembrane domain. Most interestingly, the Tez1R construct, in which the EnvZ linker starts with Arg-Arg as the Tar linker and also contains 44 amino acid residues, exhibited an OmpCc phenotype. The fact that the residues to be inserted at the transmembrane/linker junction are very selective indicates possible conformational requirements at the junction region for proper signal propagation.

**Effects of an Ala Residue Insertion at Different Sites in the Linker**—Next we examined whether length adjustment at other regions in the linker could recover aspartate-regulatable phenotype as seen in Tez1A1. Ala residues were inserted at three different positions (Tez1A2, Tez1A3, and Tez1A4) (Fig. 4A). In Tez1A2, an Ala residue was inserted in the predicted helix I, 7 amino acid residues downstream of the Tez1A1 fusion site, whereas in Tez1A3 an Ala residue was inserted in the predicted helix II. Note that according to the secondary structure prediction, helix II continues further into N-terminal end of the EnvZ catalytic domain A (22, 23, 26). In Tez1A4, an Ala residue was inserted immediately upstream of Arg-180. β-Galactosidase assays using RU1012 cells harboring these Tez1A variants showed that Tez1A2 and Tez1A3 exhibited an OmpC phenotype even in the absence of aspartate, whereas Tez1A4 maintained an aspartate-regulatable phenotype as Tez1A1 (Fig. 4B). This suggests that the length adjustment in the linker for recovering signaling has to be at the transmembrane/linker junction. The phenotypes of Tez1A2 and Tez1A3 also suggest that the helical orientations of the predicted two helices, helix I and helix II, play important roles in the linker function.
intragenic suppressors of Tez1. PCR random mutagenesis in the entire
intragenic linker was carried out as described under "Experimental Procedures." A
suppressor mutant thus isolated (Tez2) harbored suppressor mutations
P185Q and R184Q. These two mutations were separated by site-di-
rected mutagenesis, and the two Tez mutants were named Tez3 (Tez1
harboring the P185Q mutation) and Tez4 (Tez1 harboring the R184Q
mutation). An Ala residue was inserted after Arg-180 within Tez3,
resulting in Tez3A, β-galactosidase activities of Tez2, Tez3, Tez4, and
Tez3A as well as Tez1 and Tez1A1 were measured with (black bar)
or without (hatched bar) 5 mM aspartate in the growth medium. B, the
β-galactosidase activities of E. coli RU1012 cells carrying various
Tez1P185X mutants. Random mutagenesis at Pro-185 was carried out
using Tez1 as template. β-Galactosidase activities of various
Tez1P185X receptors were measured with (black bar) or without
(hatched bar) 5 mM aspartate in the growth medium. The data plotted
were the average values from three independent experiments.

Specific Interactions between helix I and helix II in the
linker—Both Tar and EnvZ linkers could serve as a functional unit in signal transduction. Although they share little primary sequence homology, a similar helix (helix I)-turn-helix (helix II)
structure fold is adapted by the two linkers, which may under-
line a similar structural basis for signal transduction. To ex-
amine whether the conservation of helical amphipathicity or
specific residue interactions are required for the linker func-
tion, two different hybrid receptors were constructed in which
helix I from the Tar linker and helix II from the EnvZ linker
were connected in two different manners in the loop region as
shown in Fig. 6A. Both constructs (Tez5 and Tez6) exhibited
OmpC" phenotypes, which are similar to that of a previously
caracterized Tar/EnvZ hybrid receptor, Taz2-1 (9) (Fig. 6). We
also constructed three other Tar/EnvZ hybrid receptors, Tez7,
Tez8, and Tez9, in which the fusions were made within helix II
(Fig. 6). Unlike Tez5, Tez6, Taz2-1, and Taz1, these hybrid
constructs exhibited OmpC" phenotypes even in the presence
of 5 mM aspartate (Fig. 6B). Therefore, both helix I and helix II
in the linker have to be derived from the same protein for the
normal linker function. Specific interactions between the two
helices in the linker seem to be critical to signal transduction,
although further investigation is needed to pinpoint the resi-
dues that are involved in the interactions.

Effects of F220D Mutation on the Phenotypes of Tez1A1 and
Tez1 Receptors—Based on the LEARNCOIL analysis, part of
helix II of the EnvZ linker (residues 216–222) may continue
to the downstream helical region of the EnvZ domain A to
form a coiled-coil structure in a dimer configuration. A similar
prediction has been made for other histidine kinases, suggest-
ing that a coiled-coil structure may be a common feature for
most of the histidine kinases (34). In helix II of EnvZ, it has
been shown that substitution of a hydrophobic to a charged
amino acid residue at position 220 (F220D) blocked EnvZ os-
molarity sensing, resulting in an OmpCc phenotype (26). One predicted
helices (I and II) in the linker region of Tar and EnvZ were marked as
open boxes. B, the β-galactosidase activities of E. coli RU1012 cells
carrying Tar/EnvZ hybrid receptors as shown in A. β-Galactosidase
activities of various hybrid receptors as shown in A were measured with
(black bar) or without (hatched bar) 5 mM aspartate in the growth
medium. The data plotted were the average values from three inde-
pendent experiments.
In the present study, the phenotypes of Tez1 and its variants in comparison to that of Tar suggest that although the EnvZ linker and the Tar linker share similar secondary structures, they need to adapt proper phase configurations with respect to the upstream transmembrane domains and the downstream catalytic domains for signal transduction. The transmembrane/linker junction is crucial for the linker function, as present results showed that adjustment at the junction resulted in all possible signaling modes: the constitutive “off” phenotype, the ligand-responsive “off” to “on” regulatory phenotype, and the constitutive “on” phenotype. The phenotypes of Tez1 further indicated that a specific residue is needed at the junction for proper signal transduction in addition to the possible length requirement for the linker function. Because both the EnvZ linker and the Tar linker are able to propagate external signals to regulate the function of the downstream EnvZ catalytic domain, it seems evident that EnvZ and Tar share a similar mechanism for signal transduction.

Insertion of an Ala residue at different sites in the EnvZ linker led to different phenotypes for Tez1, indicating that the length requirement is not the only determining factor for the linker function. The aspartate responsiveness of Tez1A1 and Tez1A4 further confirms the importance of the transmembrane/linker junction. In addition, the phenotypes of Tez1A1 and Tez1A2, in which an Ala residue was inserted seven residues apart, suggest that unlike what has been proposed for the Tar linker (25), a junction region (Arg-180 to Leu-186) may exist at the N-terminal end of the EnvZ linker that does not simply adapt a helical structure connecting the transmembrane domain TM2 with the predicted helix I of the EnvZ linker to form a single long helical structure. Within this junction region, the residue at position 185 seems to be crucial for the linker function, because substitution mutations at Pro-185 lead to all the three possible phenotypes for Tez1. Note that it has been shown that substitution mutations at Arg-180 (R180C and R180W) in EnvZ led to an OmpC c phenotype (11, 30), and the P185L mutation in EnvZ led to an OmpC phenotype (11), again confirming the notion that the junction

![Image of a model for signal transduction through the cytoplasmic domain of EnvZ.](http://www.jbc.org/)
region is crucial for signal transduction. The aspartate-regulatable phenotype of Tez3A, which contains an Ala residue insertion at the transmembrane/linker junction together with the P185Q mutation in Tez1 (the residue number is based on the EnvZ sequence) further implies that the junction region functions as a single structural unit for modulating the conformation of the EnvZ linker. Specific amino acid requirements, as seen in Tez1X and Tez1P185X mutants, together with the fact that the linker has to be fixed on the membrane for its proper function indicates that the interaction between the membrane and the junction region is crucial for proper signal propagation. Alternatively, because both Tar and EnvZ have an unusually long transmembrane TM1 region, possible interactions between the junction region and the portion of TM1 facing the cytoplasmic space under different signaling conditions may also play an important role in modulating the linker conformation.

Consistent with the previous observation (26), the helical orientations of both helix I and helix II in the EnvZ linker may be important for the linker function, since the Ala insertion in either helix I or helix II locked signal transduction at the “on” signaling mode. The phenotypes of different Tar-EnvZ hybrid receptors in the present paper (Tez5, -6, -7, -8, and -9) as well as those of Taz1 and Taz2-1 in the previous works (9, 12) suggest that both the Tar linker and the EnvZ linker need to be intact for fulfilling their function, and specific interactions between helix I and helix II in the linker is important for signal propagation. Particularly, helix II may serve as an effector element within the linker for further signal propagation, as it may form a long coiled-coil structure together with helix I of domain A of EnvZ based on LEARNOIL prediction. The F220D mutation in helix II of either the EnvZ, Tez1, or Tez1A1 linker causes an OmpC phenotype in all cases, implying that the conformation of the junction region is, thus, altered to modulate the specific interactions between helices I and II within the linker. Helix II of the linker further propagates the conformational signals to helix I of domain A through the long coiled-coil structure. As a result, the relative positioning between domains A and B is changed to reset the kinase/phosphatase ratio of EnvZ to a higher value, which results in higher cellular concentrations of OmpR-P to activate the ompC gene transcription and to repress the ompF gene transcription (Fig. 8B).

In the model presented in Fig. 8, the two helices in the linker together with their counterparts within a receptor dimer are presumed to form a parallel four-helix bundle similar to those observed in the basic helix-loop-helix structure of transcription factors such as Max and Myc (36–39). The fact that the linker function requires its attachment to the membrane indicates that the cytoplasmic membrane may be essential for maintaining a stable four-helix bundle structure in the linker. The junction region in respect to the transmembrane domains may also be important for the linker structure and the relative helical alignment within the linker four-helix bundle. However, it is still possible that helix I of the linker lies parallel to the plane of the membrane surface, which dissociates from the membrane to transiently interact with helix II in the linker upon signal input as proposed by Williams and Stewart (24). In that case, the relative flexibility of the junction region may also allow the N-terminal regions of helix I to interact with each other in an antiparallel fashion. Further structural studies are needed to distinguish those possibilities.

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