

COMMUNICATION

Motifs of Two Small Residues can Assist but are not Sufficient to Mediate Transmembrane Helix Interactions**Dirk Schneider^{1,2} and Donald M. Engelman^{1*}**

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Both experimental and statistical searches for specific motifs that mediate transmembrane helix–helix interactions showed that two glycine residues separated by three intervening residues (GxxxG) provide a framework for specific interactions. Further work suggested that other motifs of small residues can mediate the interaction of transmembrane domains, so that the AxxxA-motif could also drive strong interactions of α -helices in soluble proteins. Thus, all these data indicate that a motif of two small residues in a distance of four might be enough to provide a framework for transmembrane helix–helix interaction. To test whether GxxxG is equivalent to (small)xxx(small), we investigated the effect of a substitution of either of the two Gly residues in the glycoporphin A GxxxG-motif by Ala or Ser using the recently developed GALLEX system. The results of this mutational study demonstrate that, while a replacement of either of the two Gly by Ala strongly disrupts GpA homo-dimerization, the mutation to Ser partly stabilizes a dimeric structure. We suggest that the Ser residue can form a hydrogen bond with a backbone carbonyl group of the adjacent helix stabilizing a preformed homo-dimer. While (small)xxx(small) serves as a useful clue, the context of adjacent side-chains is essential for stable helix interaction, so each case must be tested.

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Discovering the principles of folding and interaction of membrane proteins is one of the important challenges in contemporary membrane biology. Though the integration of proteins into membranes and the helix interactions can be cooperative,¹ studies with bacteriorhodopsin have shown that the integration of individual transmembrane (TM) helices can be separated in space and time from the formation of higher-order TM structures. Thus, the folding of a membrane protein can be described as a succession of (i) an incorporation of individual helices into a membrane and (ii) an interaction of the helices to form stable higher-order oligomeric structures.² Helices form closely packed interfaces with each other, and the association is stabilized mainly by van der Waals interactions.³ Other factors, like extra-membrane links, lipids or strong hydrogen bonding of polar residues, may enhance

stability, but are not necessarily required for interaction.⁴

The recent finding that amino acid motifs can mediate the interaction of α -helical TM helices is an important step toward identifying the location of interacting helical interfaces and for structural prediction of helix–helix interactions. Two motifs that drive TM helix–helix interactions have been well studied: a heptad motif of Leu residues⁵ and the GxxxG-motif.⁶ The GxxxG-motif was identified in a statistical analysis as well as in a genetic screen for strong TM helix–helix interactions in a random library.^{6,7}

Originally, the GxxxG-motif was found to be important for the dimerization of the glycoporphin A (GpA) TM helices,^{8–10} and it was shown that substitutions of either of the two Gly residues strongly disrupts the homo-dimerization of the TM domains.^{9,10} The motif places two helices in a very close contact to each other, allowing van der Waals interactions between the two helices.¹¹ Interestingly, the GxxxG-motif is important for TM protein interactions and can mediate α -helical interaction

Abbreviations used: TM, transmembrane; wt, wild-type; GpA, glycoporphin A.

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in soluble proteins.¹² In addition to the GxxxG-motif, Kleiger *et al.* identified the AxxxA-motif in α -helical interactions in soluble proteins.¹² These results suggest that the GxxxG-motif may be defined more generally as (small)xxx(small). In fact, other motifs of two small residues at a distance of four residues were also found to be over-represented in TM domains.⁷ Two small residues at the same helical interface would allow two α -helices to come into close contact, potentially resulting in a larger contact area at the helix interface. This hypothesis was supported recently by a study on the ErbB receptor and on integrin TM domains, which demonstrated that the motifs SxxxG and AxxxG^{13,14} can mediate an interaction of two TM helices, though the interactions were weaker than that measured for the GpA TM homodimerization.

A structural analysis of the known GpA structure suggested that the interaction of the two helices may be stabilized also by hydrogen bonding. The β -hydroxyl group of Thr87 is close to the backbone carbonyl group of Val84 on the opposing helix and it was argued that these two groups may form a hydrogen-bond.¹⁵ Additionally, the Gly79 and Gly83 C α H-atoms may form hydrogen bonds to backbone carbonyl oxygen atoms on the adjacent helix.¹⁶ The strength of this type of bond has been estimated recently to be 0.88 kcal/mol (1 cal = 4.184 J).¹⁷ Though this bond strength is relatively weak, several such bonds could contribute significantly to the stabilization of transmembrane helix bundles.

While other motifs of small residues can drive the association of TM helices, it is unlikely that these motifs stabilize interactions by backbone C α

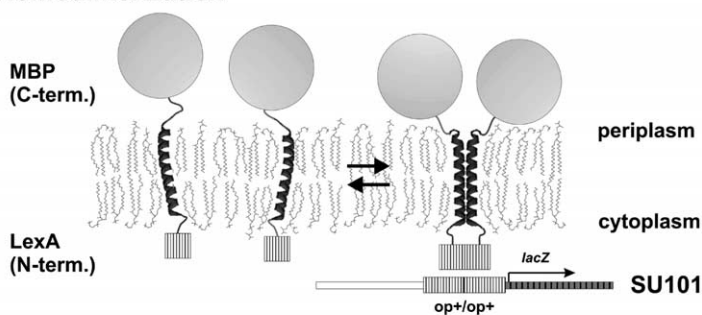
hydrogen bonding because of the increased inter-helical distance imposed on the helix-helix interaction by Ala at the interface compared to Gly. However, small residues like Ala or Ser do allow two helices to come into close contact with each other, permitting increased inter-helical contacts of other residues. Since the side-chain of the Ser residue is bulkier than the Ala side-chain, motifs with Ala should allow a closer contact of two helices than Ser-containing motifs.

But, can motifs of any two small residues mediate TM helix-helix interactions? And, is the GxxxG-motif identical with a (small)xxx(small)-motif?

To address these questions, we investigated the effect on substitutions of either of the two Gly residues in the GpA TM sequence by the small residues Ala and Ser, and by the large residue Ile. Synthetic oligonucleotide cassettes encoding the TM of interest were ligated into the plasmid pBLM100,¹⁸ and homo-dimerization of the individual TM domains was measured in the *Escherichia coli* reporter strain SU101 using the GALLEX approach. An overview of the GALLEX plasmids and strains is given in Figure 1. Membrane insertion of the chimeric proteins was tested in each case by extraction of lysozyme treated cells with NaOH as described.¹⁸ In each case, the correct orientation of the chimera in the *E. coli* inner membrane was tested by transformation of each plasmid into *E. coli* NT326 cells, which are *malE* deficient,¹⁹ and growing the cells on M9 medium with maltose as the only carbon source. Growth will occur only if the MalE-domain of the chimeric proteins is located in the periplasm and the topology is established (Figure 2(C) and (D)).

The results of the homo-dimerization

Homodimerization



Heterodimerization

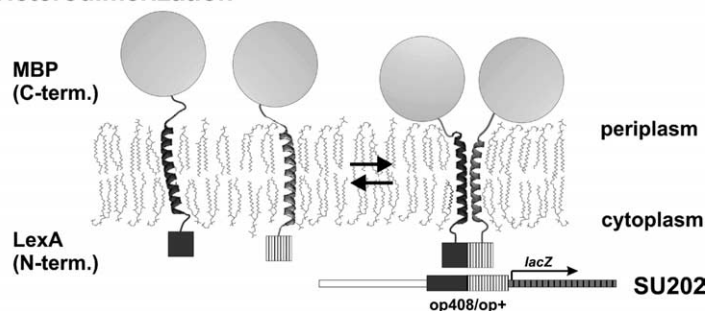


Figure 1. The GALLEX assay. A homo-dimerizing fusion protein from the plasmid pBLM100, which encodes for the wt LexA DNA-binding domain, will bind to the wtLexA promoter/operator and represses expression of *lacZ* in the genome of the reporter strain SU101. For monitoring heterodimerization, one subunit is expressed from the wt LexA plasmid and the other one from the corresponding LexA plasmid pALM148 with a mutated LexA DNA-binding domain. A heterodimerizing fusion will bind to the hybrid LexA promoter/operator and repress the expression of *lacZ* in the genome of the reporter strain SU 202. The TM domain anchors the chimera in the cytoplasmic membrane of *E. coli* with the C-terminal MBP domain located in the periplasm and the LexA DNA-binding domain in the cytoplasm.

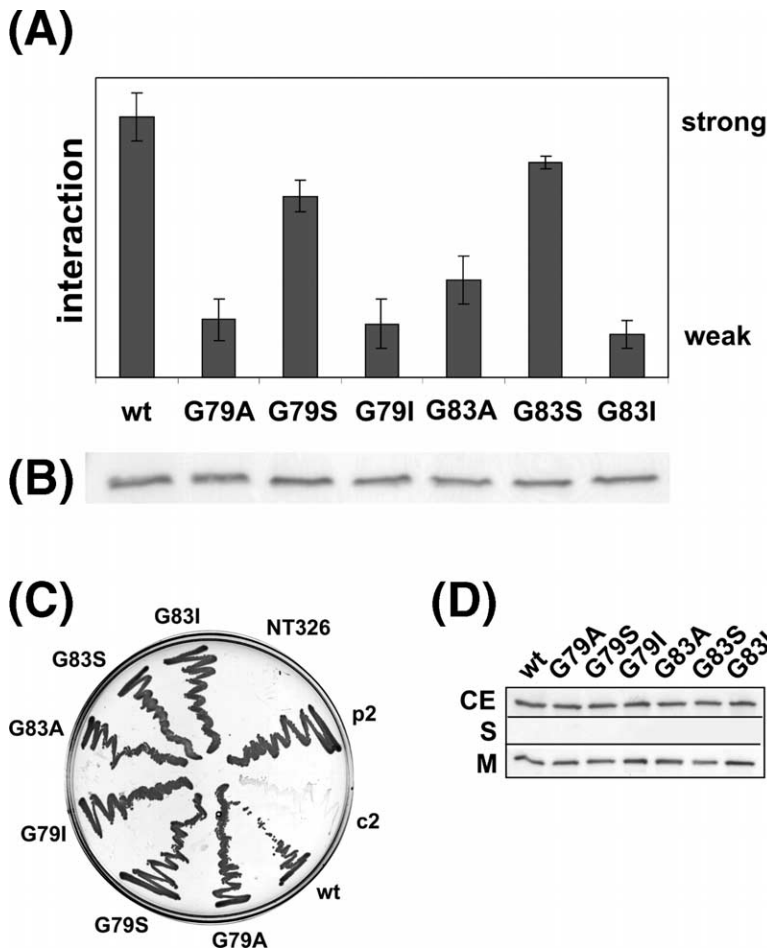


Figure 2. Homo-dimerization of GpA wt and mutated TM sequences. The β -galactosidase activity of the GpA wt TM was set "strong". Bars represent the β -galactosidase activities of three to nine independent measurements relative to GpA wt. The construction of the plasmids pBLM100 and pALM148 has been described recently.¹⁸ (A) To measure homo-association, plasmids containing GALLEX chimeras were transformed into *E. coli* SU101; for the measurement of hetero-association the plasmids were transformed into *E. coli* SU202. Cells were grown overnight in the presence of antibiotics and 0.01 mM IPTG and diluted to $A_{600}=0.1$ the next morning. Cells were harvested at $A_{600}=0.6$ and β -galactosidase activity was measured as described.²⁵ (B) Whole cell lysates were used to estimate expression levels of the chimera. Chimeric proteins were detected by Western analysis using anti-LexA-antibodies (Invitrogen) and blots were developed using NBT/BCIP (Sigma). (C) and (D) Test for insertion and orientation of the chimeric proteins in *E. coli*. (C) The *malE* complementation assay to test for LexA(GpA)MBP orientation. *E. coli* NT326 cells were transformed with

plasmids encoding for the GpA chimeric proteins and cultivated on M9 minimal medium. MBP expression from the plasmid pMal-c2 serves as a negative control, periplasmic MBP expression from pMal-p2 complements for the *malE* deficiency of NT326 cells. (D) Western analysis of *E. coli* cell extracts after extraction with NaOH. CE, whole cells; S, supernatant after extraction; M, pellet after extraction with NaOH (integral membrane proteins). The expressed proteins with a molecular mass of 54 kDa are found exclusively in the membrane protein fraction.

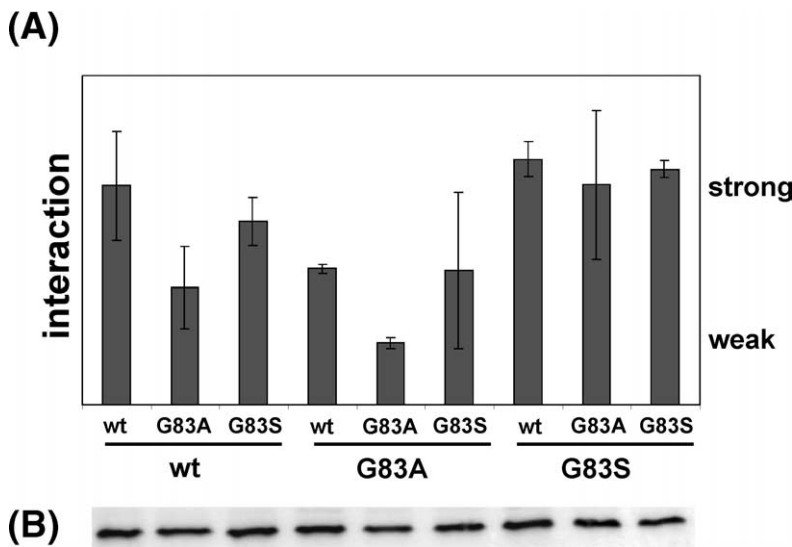


Figure 3. Hetero-dimerization of GpA wt with G83 mutated TM helices measured in *E. coli* SU202. Bars in (A) represent the β -galactosidase activities of three to six independent measurements and activities are shown relative to the strain expressing the GpA wt chimera from both plasmids. GpA TM sequences shown under the black bar are expressed from pALM148, those above the bar are expressed from pBLM100. (B) The expression level of the chimera in each strain was tested by Western analysis as described in the legend to Figure 2.

measurements (Figure 2) show that substitution of either Gly by Ile is highly disruptive, as previously observed by SDS-PAGE.⁹ Replacement of Gly79 or Gly83 by Ala led to a strong decrease in the homo-dimerization capacity of the TM domains and the disrupting effect was almost as strong as that observed for the Gly-to-Ile substitution. Although Ala has only a methyl group as side-chain, the replacement of a hydrogen atom in the Gly structure by a methyl group of Ala may interfere with the packing of GpA.^{9,20,21} The substitution of either of the two Gly residues in the GxxxG-motif by Ser also reduces the capacity of the TM helix to homo-dimerize, however, the homo-dimers appear to be more stable than after the replacement of the Gly by Ala (Figure 2). In the context of the GpA TM helix, sequence motifs of Gly and Ser mediate stronger TM helix-helix interactions than motifs of Gly and Ala.

A replacement of the Gly residues by Ser also disrupted the helix-helix interaction, though the effect was not as strong as observed after substitution to Ala. Since Ser should disrupt tight packing more than Ala, Ser must provide stability by

additional factors. The only difference between Ala and Ser is the presence of an OH group in the Ser C^β carbon atom. It was found in earlier studies that Ser residues can form hydrogen bonds in membranes stabilizing oligomeric structures.²² The Ser residues may stabilize the mutated GpA helix dimer by electrostatic interactions: the Ser side-chain OH groups on both TM helices may interact with each other or the OH groups may interact with the backbone of the adjacent helix. Side-chain-side-chain interactions involving Ser residues as well as side-chain-backbone hydrogen bonding have been identified in computational studies.²² The measurement of the homo-dimerization of a given Ser-containing TM does not allow discrimination between a possible side-chain-side-chain interaction or a side-chain-backbone interaction. To discriminate between these two possibilities, oligonucleotide cassettes encoding for the GpA TM helices with Gly83 substitutions were cloned into the plasmid pBLM100 and pALM148, which allows the measurement of the hetero-dimerization of two TM helices (Figure 1). Two plasmids encoding for the wild-type (wt) and Gly83-substituted GpA,

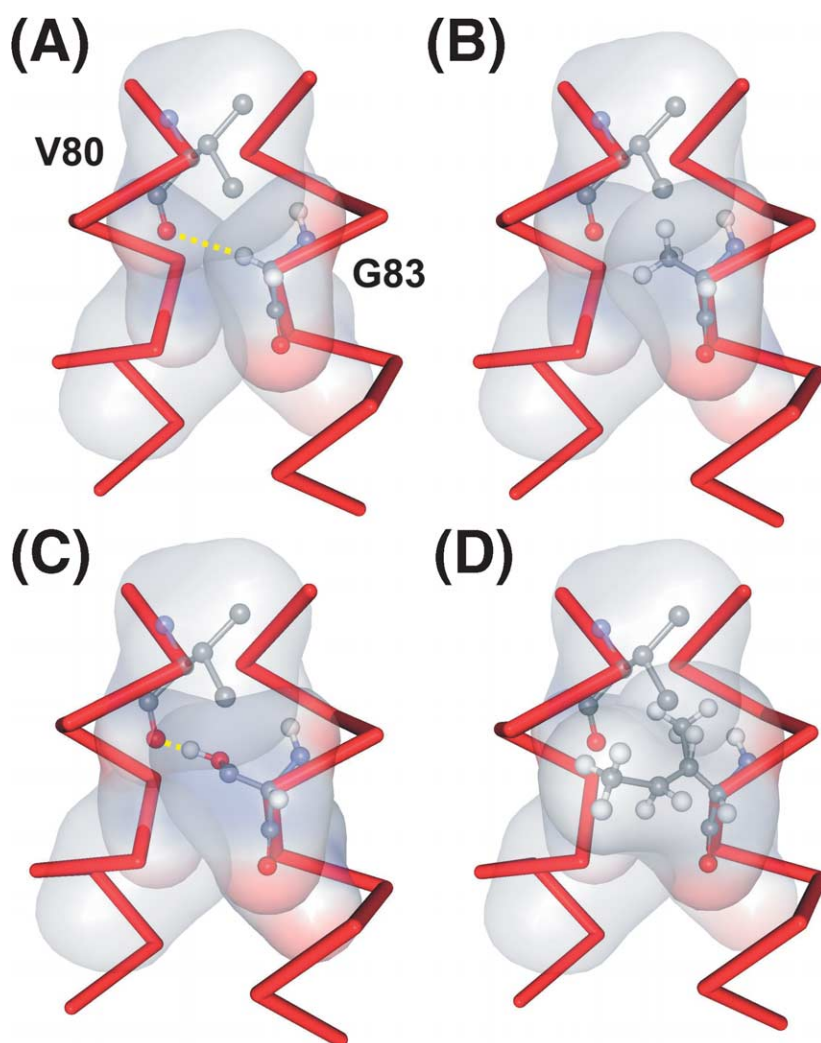


Figure 4. Packing of the GpA homo-dimer: in the wt structure (A) the C^α hydrogen atom can interact with a carbonyl group on the adjacent helix to form a hydrogen bond.¹⁶ The formation of a hydrogen bond is no longer possible in the G83A (B) or the G83I (D) mutants. After G83S substitution (C), the serine side-chain OH group can interact with an backbone carbonyl oxygen on the adjacent helix thereby stabilizing the helix-helix interaction. The van der Waals radii of the residues V80, G83 and V84 are indicated. The wt GpA structure was taken from the PDB database (1afo). Point mutations were introduced using the Swiss-PdbViewer software (<http://www.expasy.org/spdbv/>).²⁶

respectively, were transformed into *E. coli* SU202 and the capacity of the two helices to form hetero-dimers was measured.

The results shown in Figure 3 illustrate that a wt GpA TM helix forms hetero-dimers with another helix in which either Ala or Ser replaces the Gly83 residue. While the Ala residue disfavors more strongly hetero-dimer formation with either of the two G83 mutants, the G83S GpA TM domain forms a stronger hetero-dimer with the wt TM domain.

The homo-dimerization assay data (Figure 2) demonstrate that Ser residues can partly stabilize a homo-dimeric structure, while an Ala residue at either of the two Gly positions strongly disrupts the dimerization. The observation that a moderately strong dimer is formed by the wt GpA TM sequence with a TM sequence with one Gly-to-Ser substitution suggests that the Ser residue is involved mainly in electrostatic interactions with a carbonyl oxygen atom of the adjacent TM helix backbone (Figure 4). Thus, the disruptive packing effect of the Ser residue compared to Gly can be compensated partly by the formation of new hydrogen bonds.

The data presented here indicate that Ser residues can stabilize TM helix-helix associations by side-chain-backbone interactions (Figure 4). The substitution of the Gly residues by Ala disrupts the dimeric GpA structure, whereas the Ser residues compensate partly for the negative packing effect by the formation of hydrogen bonds.

The presence of a single Ser in model helices does not result in a strong homo-oligomerization,^{19,23} while multiple Ser residues can mediate the heterologous interaction of TM helices.²² The observation that single Ser residues alone cannot drive significant homo-dimer formation^{19,23} argues against the possibility that a single Ser induces specific interactions. Previous studies showed that, besides the two Gly residues, other amino acids are involved in mediating the homo-dimerization of GpA.⁸⁻¹⁰ In this context, it is most likely that in our study the remaining residues of the GpA interaction motif contribute to the formation of a dimeric structure, which is stabilized further by a Ser hydrogen bond.

In a recent study, the influence of a sequence context on the formation of TM dimers driven by polar residues was investigated,²⁴ and the authors showed that the sequence context indeed modulates association of polar residues strongly in transmembrane helices. As polar residues in TM helices do not automatically mediate interactions, our results suggest additionally that the presence of a GxxxS-motif does not automatically stand for an interaction motif. The motif can help to stabilize a dimeric structure if the dimerization is mediated further by surrounding residues. Within several subtypes of the ErbB receptor and integrins, some (small)xxx(small) motifs (GxxxA, SxxxG, SxxxA) do not induce homo-oligomerization of TM domains whereas others do.^{12,13} Even the GxxxG-motif does not mediate a strong interaction if surrounding residues disfavor such an interaction.^{9,10} As already

pointed out, motifs of small residues, and especially the GxxxG-motif, do provide a framework for TM helix-helix interactions.⁶ But, while the presence of such a motif is a useful clue, it does not automatically prove the existence of an interaction, its significance for mediating interactions must be tested in each specific case.

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