Influence of hydrophobic matching on association of model transmembrane fragments containing a minimised glycophorin A dimerisation motif

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Abstract **The principles that govern the folding and packing of membrane proteins are still not completely understood. In the present work, we have revisited the glycophorin A (GpA) dimerisation motif that mediates transmembrane (TM) helix association, one of the best-suited models of membrane protein oligomerisation. By using artificial polyleucine TM segments we have demonstrated in this study that a pattern of only five amino acids (GVxxGVxxT) promotes specific dimerisation. Further, we have used this minimised GpA motif to assess the influence of hydrophobic matching on the TM helix packing process in detergent micelles and found that this factor modulates helix–helix association and/or dissociation between TM fragments.**

Keywords: Transmembrane helix; Helix packing; Hydrophobic mismatch; Membrane protein association; Glycophorin A

1. Introduction

Transmembrane (TM) segments of integral membrane proteins are commonly composed of hydrophobic α-helices 15–27 amino acids long [1]. Sequence-specific interactions between these regions support the folding and assembly of many integral membrane proteins into functional proteins [2,3]. These interactions are based on reciprocal recognition of structurally complementary surfaces of the TM helices. One of the best-suited models of a membrane protein that oligomerises (more specifically, dimerises) through interactions of its TM α-helices is glycophorin A (GpA) [4,5]. The wide use of this protein as a model membrane protein is based on its intrinsic simplicity, since its single TM fragment drives detergent resistant homodimerisation of the protein. Thus, the dimerisation process and the factors that could modulate it can be quantitatively analysed using sodium dodecyl sulfate polyacryamide-gel electrophoresis (SDS–PAGE). In addition, the overall results of such studies have been validated by fluorescence resonance energy transfer (FRET) techniques [6,7].

The GpA homodimer, defines a dimerisation interface that has been extensively studied by diverse techniques such as saturation mutagenesis [8], alanine-insertion scanning [9], computational modelling [10], genetic reporter systems [11,12], solution NMR in dodecylphosphocholine micelles [13] and solid-state NMR in lipid membranes [14]. The output of these studies describes a dimerisation motif in the TM fragment composed of seven residues, $L^{75}IxxGVxxGVxxT^{87}$ (x being a hydrophobic residue), that is responsible of the dimerisation process.

In addition to sequence, the equilibrium established between the monomeric and the oligomeric forms of TM fragments could be displaced depending on several factors that can modify the energetic balance and in turn the oligomerisation process. Among those factors, the mismatch between the hydrophobic length of the TM fragment and the membrane hydrophobic thickness has been considered to play a key role in membrane organisation and function [15]. However, the study of membrane response to hydrophobic mismatch is difficult since well-defined systems are needed in which both lipid and protein hydrophobic length can be varied in a systematic manner. In this regard, only synthetic consensus membrane spanners have been used in lipid model membranes in a range of mismatch situations. The main response of this synthetic peptide–lipid system to hydrophobic mismatch was the exclusion of a significant amount of peptide from the bilayer [16], at least when the hydrophobic length of the peptide was less than the hydrophobic thickness of the lipid bilayer [17]. Also, in a similar peptide–lipid system, theoretical simulations have estimated from fluorescence quenching studies that the free energy of peptide dimer formation can be affected by lipid structure [18].

To establish a simplified model to study the individual contribution of the amino acid residues involved in the GpA dimerisation process and the influence of hydrophobic matching in TM packing, we designed chimeric proteins harbouring artificial TM fragments based on leucine residues in which we grafted the seven key amino acids considered to form the dimerisation motif of GpA. We show that in the stringent environment of detergent micelles not all of the seven residues significantly contribute to the dimerisation, and that the level of TM association in such a minimised motif can be modulated by hydrophobic mismatch between the TM length and the size of the detergent micelles used for solubilisation.

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Abbreviations: GpA, Glycophorin A; SDeS, sodium decyl sulfate; SDS–PAGE, sodium dodecyl sulfate polyacryamide-gel electrophoresis; STS, sodium tetradecyl sulfate; TM, transmembrane

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2. Results and discussion

The initial chimera was obtained through substitution of the GpA TM domain in the His-tagged SN/GpA fusion [9] by desired polyleucine stretches (see Section 4). To investigate the individual contribution and the precise role of each of the seven residues involved in the GpA dimerisation motif in driving assembly we grafted, in stages, these residues onto an engineered hydrophobic homogeneous stretch of 18 leucines. A polypeptide segment of 18 amino acid residues in length, when folded in a α-helical conformation, is expected to span the length of the hydrophobic core of a membrane and could be defined as a model TM fragment. In addition, leucine has a high α-helical propensity in membrane environments [19] and is the most abundant residue in TM α-helical domains [20].

In agreement with previously reported data [21], purified chimeric proteins harbouring a polyleucine helix do not dimerise under SDS–PAGE analysis. Introduction of the GxxxG motif (mutant GG, Fig. 1), which has often been found to be important for mediating the interaction of TM helices (recently reviewed in [22]) does not promote dimerisation under experimental conditions used here (Fig. 1B). Since both statistical analysis of amino acid patterns in TM helices [23] and an in vivo selection system for TM oligomerisation motifs [24] highlighted the importance of the presence of β-branched residues flanking the glycines in the GxxxG motif, we tested the influence of the two valine residues present in the native GpA sequence (mutant GVGV) and observed only a low level of protein dimerisation (<15%). Interestingly, introduction of the threonine residue corresponding to position 87 in the native sequence (mutant GVGVT) renders a significant increase in protein dimerisation (70 ± 8%, Fig. 1B) up to levels of the full GpA TM sequence [25]. Further addition of the isoleucine (position 76) residue, previously included in the GpA dimerisation motif [26], did not increase the dimerisation degree (Fig. 1B). Nevertheless, the contribution of the β-branched residues flanking the glycines (the two valines in this case) to dimerisation is significant since a construct of polyleucine carrying the two glycines plus the threonine residue did not dimerise at all (mutant GGT, Fig. 1B). In addition, the second valine (Val84 in the original sequence) turned to be more relevant (compare mutants GGVT vs. GVGT, Fig. 1B). In fact, in a mutant where only the second GV pair and the threonine are present (LLGVT), the level of dimerisation is still significant (28 ± 3%). Interestingly, the dimerisation degree in this latter case is similar to the one observed in a previously reported mutant, where five leucines were inserted between residues Met81–Ala82 in the native sequence [27], positioning in both cases the same LLxxGVxxT pattern at the dimer interface.

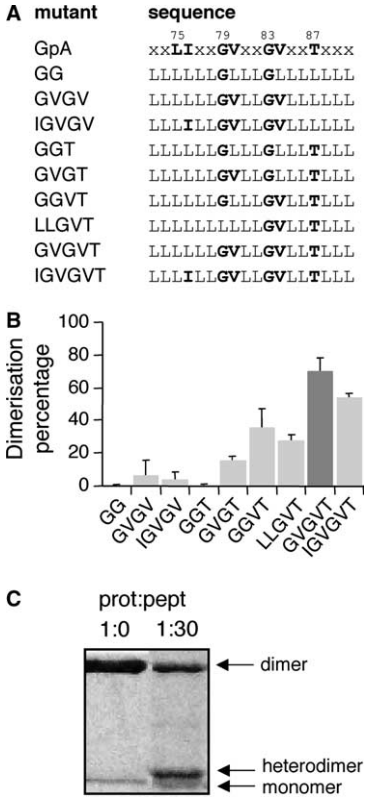

Fig. 1. Contribution of residues within the GpA dimerisation motif to the packing of model polyleucine (18L) TM fragments. (A) Amino acid sequences of the different TM fragments as expressed in the context of the protein chimera. (B) Quantification of the SDS–PAGE analysis of the chimeric proteins. The bars represent mean values and error bars denote deviations obtained from three independent protein expression/purification experiments. (C) Competition study using GpA TM peptide. Purified GVGVT chimera was mixed with GpA TM synthetic peptide [25] at protein:peptide ratio of 1:30. Samples were tested for disruption of chimeric homodimer by the peptide using SDS–PAGE.

To determine whether the high level of dimerisation found for the GVGVT motif is induced by interactions similar to those that mediate GpA TM dimerisation, we performed competition experiments between this polyleucine contaning chimeric protein and a synthetic peptide harbouring the full GpA TM sequence. This peptide has been shown to reproduce the dimerisation events to the same extent as those achieved by the original protein [25,28]. As shown in Fig. 1C, the dimerisation of the GVGVT protein is disrupted by the presence of the synthetic peptide with the concomitant formation of peptide–protein heterodimers, suggesting that the molecular events that drive the oligomerisation in the original TM sequence are present in this minimised motif.

We next sought to investigate the role of the leucine residue located at position 75 in the GpA native sequence, since our chimeric polyleucine scaffold provides intrinsically its presence. As shown in Fig. 2, mutations of Leu75 to hydrophobic residues (alanine, isoleucine, and valine) at this position do not preclude dimer formation. This suggests a secondary, if any, role for the residue located at this position, since even β-branched residues like isoleucine or valine, residues with restricted side chain flexibility, allow dimerisation in this homogeneous polyleucine scaffold, in contrast to the original GpA sequence, where the replacement of Leu75 by valine abrogated dimer formation and its substitution to alanine and isoleucine resulted in only detectable levels of dimer [8].

The three-dimensional structure of the GpA dimer modelled from solid-state NMR data [14], showed the presence of a hydrogen bond between the side chain hydroxyl group of Thr87 and the backbone carbonyl of Val84 across the dimer interface [29]. In addition, replacement of Thr87 by serine, a residue also containing a hydroxylated side chain, allowed significant dimer formation [8]. In order to address the relevance of this hydrogen bonding for protein stability in our simplified dimerisation model, Thr87 was replaced by serine (GVGVS;

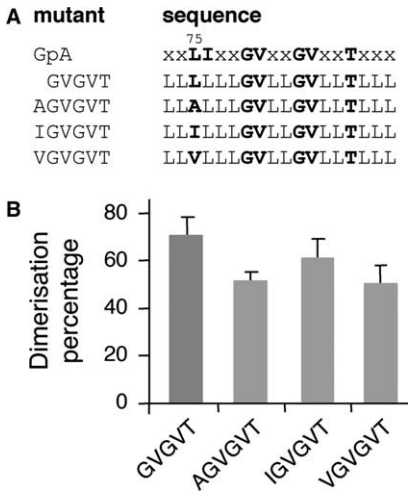

Fig. 2. Contribution of leucine 75 to the dimerisation process. (A) TM segment sequences analysed. (B) Quantification of the SDS–PAGE analysis. As in Fig. 1, the bars represent mean values and error bars denote deviations obtained from three independent experiments.

Fig. 3B). The significant level of dimer found in this construct suggests a specific role for hydroxylated side chains in dimer stabilisation. In this context, recent studies have suggested a determinant role for asparagine, a residue with a carboxamide containing side chain, as a dimerisation-promoting residue in defined environments [21,30]. To test the ability of this residue to promote TM packing in our minimised five residue motif we sought to replace the threonine residue by asparagine, both in our dimerising five-residue motif (GVGVT) and in a non-dimerising mutant (GGT), since it has been demonstrated that asparagine residues located within TM domains are sufficient to drive their interaction in apolar environments like lipid membranes and detergent micelles [21,30,31]. Interestingly, replacement of Thr87 by asparagine in both cases rather than facilitating, actually disrupts dimer formation in the stringent environment of the SDS micelles used in our experimental ap-

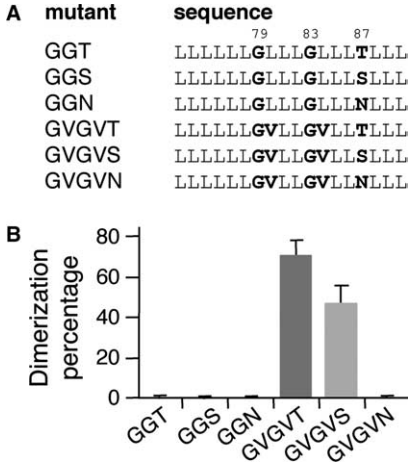

Fig. 3. Relevance of the hydroxylated residue at position 87. (A) TM segment sequences analysed. (B) Quantification of the SDS–PAGE analysis. As in Fig. 1, the bars represent mean values and error bars denote deviations obtained from three independent experiments.

proach (Fig. 3). Altogether, these results point to a robust dimerisation event between model TM fragments driven by a five residues motif (GVxxGVxxT/S) rather than the seven key residues (LIxxGVxxGVxxT) included in the previously defined GpA dimerisation motif.

Having defined the five residues motif in a polyleucine stretch as sufficient to drive dimerisation we used it as a tool to study the influence of hydrophobic matching in TM packing. The ability of only five amino acids to drive polyleucine dimerisation provide us with the possibility to study hydrophobic matching while keeping leucine residues (at least one helical turn) at both sides of the motif, even for the shortest hydrophobic stretches (see Table 1). To examine the effect of hydrophobic helix length on packing, chimeric polyleucine stretches with increasing number of residues, from 15 to 27, were prepared. The sequence and the estimated lengths of the helices in these constructs are summarised in Table 1. We focused the analysis on stretches with lengths whose presence in membrane proteins is statistically relevant [1,23], and that cover the hydrophobic thicknesses for intrinsic membrane proteins estimated from high-resolution structures [32]. In addition, recent molecular dynamics of SDS micelle formation around dimeric GpA TM helices have shown that the micelle retained its integrity over the course of the simulations [33], further validating the use of SDS micelles as membrane mimetics in TM packing studies.

The capacity of the GpA minimised motif (GVxxGVxxT) to induce dimerisation of the polyleucine segments on SDS–PAGE, (Fig. 4A), is dependent on the length of the hydrophobic TM region, indicating that, the energetic balance that drives the monomer–dimer equilibrium can be displaced as a function of the hydrophobic mismatch. Insertion of the minimised dimerisation motif in hydrophobic regions of 15 leucines long (15L) is not sufficient to induce dimer formation between these artificial TM helices. A decrease in dimerisation levels is observed for hydrophobic segments longer than 24 residues as well. Thus, it seems that critical lengths from 18 to 24 residues are needed for noticeable oligomerisation, with the highest extent of dimer formation observed in the presence of a 21-residue hydrophobic sequence in SDS micelles. Thus, if the TM fragments are both too short (15L) and too long (27L), the autoassociating equilibrium is strongly displaced towards the monomeric form of the protein.

These results can be explained by considering the energetics of the system. TM helices can respond to mismatch in a number of ways that minimise the contact between hydrophobic and non-hydrophobic surfaces [34]. Introduction of a TM fragment within the hydrophobic media when there is a hydrophobic mismatch produces distortion on the surrounding acyl

Table 1
Sequences and dimensions of the TM segments used

Chimeric protein	Sequence of the TM region	Length of the hydrophobic helix (Å)[a]
15L	$L_3GVL_2GVL_2TL_3$	22.5
18L	$L_6GVL_2GVL_2TL_3$	27
21L	$L_6GVL_2GVL_2TL_6$	31.5
24L	$L_9GVL_2GVL_2TL_6$	36
27L	$L_9GVL_2GVL_2TL_9$	40.5

[a]Assuming that each residue in a α-helix conformation covers a length of 1.5 Å.

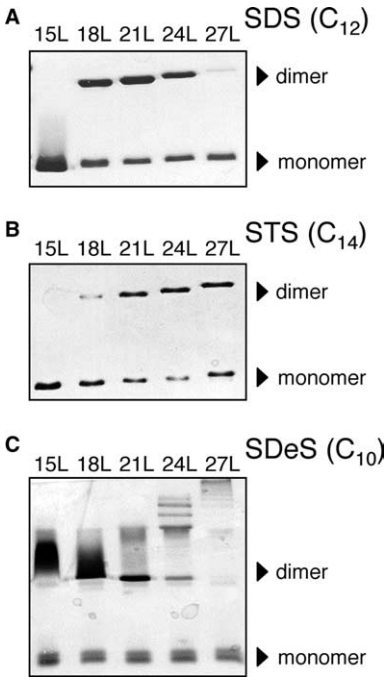

Fig. 4. Influence of hydrophobic matching on the dimerisation process of the minimised GVGVT motif grafted onto polyleucine fragments of different lengths. Chimeric proteins harbouring polyleucine stretches of 15, 18, 21, 24, and 27 leucines were purified in the presence of detergents and boiled prior to PAGE analysis. (A) Chimeric proteins were purified in the presence of SDS and analysed by PAGE. (B) Chimeric proteins were purified in the presence of STS and analysed by PAGE at 37 °C. (C) Chimeric proteins were purified in the presence of SDeS and analysed by PAGE.

chains in order to avoid unfavourable exposure of hydrophobic surfaces to a hydrophilic environment. As a consequence, an energetic cost in the system may be produced when a positive (i.e., TM hydrophobic thickness exceeds hydrophobic environment) or a negative (i.e., hydrophobic TM segment is too short to traverse the hydrophobic environment) mismatch occurs. In this context, oligomerisation between TM fragments diminishes the surface of protein–acyl chains interactions that in turn could induce a reorganisation of the media that minimise this energetic cost. Whereas, when there is a wide mismatch, gains in protein–protein interactions as well as improvement in protein–acyl chains interactions by the oligomerisation processes are not sufficient to compensate for the expanded contact between hydrophobic and non-hydrophobic surfaces, resulting in the observed bell-shape profile (Fig. 4A), in which a minor extent of dimerisation occurs in fragments of extreme lengths.

Interestingly, the same profile was found by Arkin and Brunger [1] in statistical analysis of length distribution of predicted TM α-helices, where the average length was roughly 21 hydrophobic residues for multispannig proteins, and one to two residues longer in single spanning membrane proteins. This length coincides with the minimum length of an α-helix required to traverse the ≈30 Å thick hydrocarbon core of a lipid bilayer [2], and gives the maximum dimerisation efficiency (75 ± 6% of dimer) observed for 21L constructs in the present study in SDS micelles. Furthermore, both experimental [35] and theoretical [36] studies of SDS micelles showed that the micelle hydrophobic interior is a sphere volume with a diameter of around 32 Å, which nicely matches with the 31.5 Å length of the 21L construct (see Table 1).

To test the influence of hydrophobic matching in TM helix packing we extended our mismatch studies using sodium tetradecyl sulfate (STS), an analogue of SDS with an enlarged hydrophobic region (14 carbon atoms). As shown in Fig. 4B, in coincidence with the SDS results, the construct harbouring the shortest polyleucine stretch (15L) did not dimerise in STS micelles. In contrast to the SDS results, the 18L construct displays only a modest amount of dimer (<20%), while the 27L mutant showed a significant amount of dimer (>65%). The hydrophobic thickness of a STS micelle is not known, but an enlargement of the hydrophobic region of around 3.5 Å (two carbons) relative to SDS can be assumed, based on data obtained with lipid bilayers [37], resulting in a hydrophobic thickness of 35.5 Å, that correlates with the maximum dimerisation level found for mutant 24L (with a hydrophobic helix of 36 Å in length, Table 1) in the STS experiments (Fig. 4B).

As expected, when the hydrophobic thickness of the micelle decreases, e.g., using sodium decyl sulfate (SDeS) with a hydrophobic sphere volume around 28 Å, maximum dimerisation is observed for the mutant with hydrophobic length of 27 Å, i.e., the construct 18L (Fig. 4C). The resolution and protein mobility in the polyacrylamide gels when prepared in the presence of this detergent are clearly diminished, although consistently reproducible (see Section 4), allowing a qualitative analysis. As shown in Fig. 4C, a significant amount of dimer is also detected for the shortest construct (15L), as well as some aggregates with higher molecular weights, particularly for mutants 24L and 27L. In these later cases, the response of the system to large mismatch could be that the extent of incorporation is reduced and a larger fraction is present in micellar aggregates, similarly to what has been reported for synthetic hydrophobic peptides and model lipid bilayers [16].

3. Concluding remarks

The GpA homodimeric complex results from the association between TM fragments through helix–helix contacts involving classically a motif of seven-residues ($L^{75}I^{76}xxG^{79}V^{80}xxG^{83}V^{84}xxT^{87}$). In the present study, we have demonstrated that in the case of homogeneous artificial TM fragments, helix–helix association is chiefly mediated by a five-residue motif, including the two GV pairs plus a β-hydroxylate residue (Thr or Ser). The role of the $L^{75}I^{76}$ pair was previously questioned both when the GpA motif was grafted onto the sequence of the single TM domain of the *neu* oncogene product Neu [26], and in an Ala-insertion approach [9], where it was speculated that a motif including only five residues could drive dimerisation to a significant extent. This minimised GpA dimerisation motif has been used to study the influence of hydrophobic matching in TM packing and we have found that hydrophobic matching effectively modulate association of model TM fragments.

Fluorescence quenching methods have been developed to study the behaviour of Lys-flanked polyleucine peptides when inserted into lipid bilayers [18,38,39]. These studies indicate that the mismatch between peptide helix length and bilayer width can control not only location and orientation but also helix–helix interactions. Combined with these observations, the present results further reinforce the concept that hydrophobic matching

could drive helix–helix association and/or dissociation in membrane proteins. This is an interesting issue in view of the increasing body of evidence for coexisting membrane domains/compartments with different lipid compositions, and (probably) different widths, which would suggest that both protein assembly and protein–protein interactions in membranes could be fine-tuned by the cellular microenvironment.

4. Materials and methods

4.1. Plasmid constructs

Construction of plasmids encoding the His-tagged chimeric proteins (SN/GpA) is described by [9,40]. Substitution of the GpA TM domain by the designed polyleucine stretches was carried out by adding a *Xho*I restriction site after Glu70 and a *Kpn*I site ahead of Arg96 (GpA sequence) in the pSN/GpA plasmid, in order to maintain the charge balance in the flanking regions of the designed stretches. The prototypic sequence generated was $\ldots E^{70}LEL_nGTR^{96}R\ldots$, where superscripts refer to the native GpA sequence and the subscript *n* indicates the number of leucine residues in the model TM region (i.e., 15, 18, 21, 24, or 27). Introduction of the polyleucine stretches was achieved by annealing the corresponding oligos and their insertion in the properly digested plasmid. Mutations for the introduction of the residues involved in dimerisation within the artificial TM regions were obtained by site directed mutagenesis using the QuikChange™ site directed mutagenesis kit (Stratagene, La Jolla, CA). All mutants were confirmed by DNA sequencing.

4.2. Protein expression and purification

Overexpression and purification of His-tagged SN/GpA derived proteins were performed as described [41] when SDS was used as a detergent solution. In the experiments using STS (purchased from Lancaster, England), STS (0.5%) was used all along the purification protocol instead of SDS, and purification was performed at 37 °C. In the experiments using SDeS (purchased from Fluka, Germany), the purification of the protein was carried out at room temperature in the presence of 2% SDeS. This higher percentage of detergent was used in order to be well-above the critical micelle concentration of this detergent [42].

4.3. Peptide synthesis

Peptide containing GpA TM sequence was chemically synthesised and purified as described previously [25].

4.4. SDS–PAGE analysis

Purified proteins were loaded onto 12% polyacrylamide mini-gels. For the SDS gels, standard conditions were used. In the cases where we used STS and SDeS, the gels were prepared without SDS according to previously described conditions [43]. The running and the loading buffers contained 0.1% and 2%, respectively, of the corresponding detergent. In the STS experiments, gels were run at 37 °C. All gels were stained with Coomassie blue, and the percentage of monomer, homodimer and heterodimer were estimated with an LKB Ultroscan 2202 laser densitometer with a 3390A Hewlett–Packard integrator.

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