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The membrane environment modulates self-association of the human GpA TM domain—Implications for membrane protein folding and transmembrane signaling

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ABSTRACT

The influence of lipid bilayer properties on a defined and sequence-specific transmembrane helix–helix interaction is not well characterized yet. To study the potential impact of changing bilayer properties on a sequence-specific transmembrane helix–helix interaction, we have traced the association of fluorescent-labeled glycophorin A transmembrane peptides by fluorescence spectroscopy in model membranes with varying lipid compositions. The observed changes of the glycophorin A dimerization propensities in different lipid bilayers suggest that the lipid bilayer thickness severely influences the monomer–dimer equilibrium of this transmembrane domain, and dimerization was most efficient under hydrophobic matching conditions. Moreover, cholesterol considerably promotes self-association of transmembrane helices in model membranes by affecting the lipid acyl chain ordering. In general, the order of the lipid acyl chains appears to be an important factor involved in determining the strength and stability of transmembrane helix–helix interactions. As discussed, the described influences of membrane properties on transmembrane helix–helix interactions are highly important for understanding the mechanism of transmembrane protein folding and functioning as well as for gaining a deeper insight into the regulation of signal transduction via membrane integral proteins by bilayer properties.

1. Introduction

Within biological membranes, α-helical membrane proteins are in contact with other TM proteins as well as with lipids [1]. While in some cases individual lipids are tightly bound to a membrane protein at specific sites, the physiological functions of protein–lipid interactions often remain rather enigmatic [2,3]. Global bilayer properties can dramatically influence the function of individual membrane proteins, and, e.g., several proteins function optimally at a bilayer thickness which matches the hydrophobic length of the membrane integrated protein domain, whereas the function is strongly reduced in membranes possessing either a much shorter or a much longer hydrophobic core [1,2,4]. Indeed, when reconstituted into pure phospholipid bilayers, the activities of the cytochrome c oxidase, the F_1F_0-ATPase [5], the Ca-ATPase [6], the Na,K-ATPase [7], the MscL ion channel [8], the melibiose permease [9] or the diacylglycerol kinase [10] are modulated by the thickness of the hydrophobic lipid bilayer core.

Within a single eukaryotic cell, many internal membrane systems exist and upon their synthesis at the endoplasmic reticulum (ER), membrane proteins have to be sorted to their final subcellular destination in the exocytotic pathway. Individual cellular membrane systems have different thicknesses [11], and even within a single membrane, different regions with different lipid compositions can exist, such as cholesterol-rich lipid microdomains [12–14]. Differences in the bilayer thicknesses might be critical for subcellular sorting, folding and functioning of α-helical membrane proteins [15,16], and thus might have to be considered in *in vitro* studies on membrane protein folding, assembly, stability and function. In recent studies interactions of artificial membrane spanning helices, which do not contain any special helix–helix interaction motifs, have been analyzed in different bilayers to define nonspecific forces involved in folding and stability of TM proteins [17]. Hydrophobic mismatch between the hydrophobic length of a TM α-helix and the hydrophobic thickness of a lipid bilayer can cause unspecific oligomerization/aggregation although the individual helices have no specific interaction propensity [17,18]. The influence of the bilayer thickness on a structurally stable TM oligomer, which is stabilized by a defined interaction motif, has not been analyzed yet. Oligomerization of the M2 proton channel TM helix from the influenza A virus has been studied to some extent and appears to be most efficient under

Abbreviations: GpA, human glycophorin A residues 69–101; TM, transmembrane; FRET, Förster resonance energy transfer; Fl, carboxyfluorescein; TAMRA, 5- (and 6)-carboxytetramethylrhodamine; PC, phosphatidylcholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DDM, *n*-dodecyl-β-D-maltoside; DPC, dodecyl phosphocholine; HEPES, *N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid; GP, generalized polarization

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hydrophobic matching conditions [19]. However, a clearly defined helix–helix interaction motif has not been identified and mutations at the M2 TM helix–helix interface do not destabilize the oligomeric TM structure [20]. Furthermore, the individual helix–helix interactions appear to be weak and the M2 proton channel TM structure is rather flexible.

Studies in the presence of cholesterol containing model membranes have indicated that cholesterol can promote unspecific interactions of TM helices by increasing the bilayer thickness [17,18]. Furthermore, cholesterol also influences the order of lipid acyl chains, which could also have an influence on TM helix–helix interactions [17,18]. Besides global bilayer properties, more specific factors, such as distinct packing interactions, interactions between polar residues, as well as defined interaction motifs, such as the well-characterized GxxxG-motif, are involved in determining interactions between individual TM helices and the final structure of a TM protein. In recent years, the TM helix of human glycophorin A (GpA) has become a paradigm for studying the sequence-specific interaction between TM helices. The seven residues motif L^{75}IxxGVxxGVxxT^{87} of the GpA TM helix has been identified to be important for dimerization of the GpA TM helix [21,22]. While interactions of the GpA TM domain within the inner membrane of *E. coli* have been studied extensively by genetic systems [23–27], these approaches did not allow studying the influence of changing bilayer property on the interaction of the GpA TM helices. Most *in vitro* studies with the GpA TM domain have been performed in detergent, and homodimerization of the GpA TM domain is significantly influenced by the detergent environment [28–31]. Since the GpA TM helix forms stable dimers in liposomes [32] and the dimeric structure does not significantly alter in different lipid bilayers [33–35], model membranes can be used to study the influence of global bilayer properties on the defined, sequence-specific GpA TM helix–helix interaction. Based on several measurements, it was originally assumed that GpA is always dimeric in membranes. However, a recent study has shown that dimerization strongly depends on the detergent properties [28,36]. Furthermore, the actual fraction of dimeric GpA can be small in bacterial as well as in eukaryotic membranes and the fraction dimeric GpA strongly depends on the protein concentration within cellular membranes [27,37,38].

In the present work, interactions of fluorescent-labeled GpA TM peptides were studied by fluorescence spectroscopy in model membranes to test the influence of the bilayer thickness on a TM helix dimer stabilized by a defined helix–helix interaction motif (GxxxG-motif). The observations of this study strongly suggest that the lipid bilayer thickness can influence the monomer–dimer equilibrium of the GpA TM domain significantly. Furthermore, the presence of cholesterol in model membranes promotes self-association of TM helices by increasing the bilayer thickness and – more importantly – by changing the lipid acyl chain ordering. As discussed, these results not only severely affect membrane protein folding studies but may have a significant impact on *in vivo* studies regarding sorting and functioning of membrane proteins.

2. Materials and methods

2.1. Sample preparation

Peptides corresponding to the human GpA TM domain (SEPEITLIIFGVMAGVIGTILLISYGIRRLIKK) were custom-synthesized (Peptide Specialty Laboratories GmbH, Heidelberg, Germany). The N-terminus of the peptides was labeled on the resin with 5-carboxyfluorescein (Fl) and 5-carboxytetramethylrhodamin (TAMRA) (Merck Biosciences), respectively, after cleavage of the N-terminal Fmoc protecting group. Labeled peptides were cleaved from the resin in 95% trifluoroacetic acid (TFA), 4% H_20, 1% Triisopropylsilan for 2 h at room temperature. Subsequently, peptides were precipitated with isopropylether in 30% acetonitrile, 0.1% TFA and lyophilized. The peptides were further purified by RP-HPLC on a C18-column (Varian) using a linear gradient from 10% acetonitrile, 0.1% TFA to 80% acetonitrile and 0.1% TFA. The purity of the peptides was confirmed by HPLC and mass spectrometry.

Fluorescence measurements were performed on vesicles at a constant peptide–lipid ratio. The lipids were dissolved in chloroform or chloroform/methanol (2/1), and the peptides were dissolved in 2,2,2 trifluoroethanol. The following lipids (Avanti Polar Lipids, Alabaster, AL, USA) were used in this study: 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine (diC(14:1) PC), 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine (diC(16:1) PC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (diC(18:1) PC), 1,2-dieicosenoyl-*sn*-glycero-3-phosphocholine (diC(20:1) PC) and 1,2-dierucoyl-*sn*-glycero-3-phosphocholine (diC(22:1) PC).

Peptides were incorporated into the lipid vesicles by mixing the lipids and peptides according to the indicated peptide–lipid ratio. Upon removal of the organic solvents, the dried lipid/peptide films were hydrated by vortexing for 5 min with 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl. The samples were then subjected to 8 cycles of freeze–thawing. The resulting vesicles were used without further manipulation for fluorescence measurements.

2.2. Steady-state fluorescence measurements

Steady-state fluorescence measurements were performed at room temperature in an Aminco Bowman series 2 luminescence spectrometer having both the excitation and emission band-pass filter set at 4 nm. Fluorescence was measured from 480 to 650 nm in liposomes containing defined concentrations of donor- and acceptor-labeled peptides after donor excitation at 439 nm. Liposomes containing only donor-labeled peptides served as the no-FRET control. All experiments were performed at least three times and average results are given.

Energy transfer E was calculated by means of donor fluorescence intensity at 525 nm in the absence and presence of the acceptor according to

$$E = (F_D - F_{DA}) / F_D, \quad (1)$$

where F_D and F_{DA} are the donor intensities of samples containing only donor-labeled peptides and samples with both donor- and acceptor-labeled peptides, respectively.

To determine lipid packing in the various diC(X:1) PCs lipids, 0.5 mole % of the laurdan fluorescence probe was added to the lipid before drying. The samples were prepared by hydrating the lipid films in 10 mM HEPES pH 7.4, 150 mM NaCl and subjected to at least five freeze–thaw cycles prior to fluorescent measurement. Cholesterol containing membranes were prepared similarly. Fluorescence emission spectra of laurdan in phospholipid bilayers were measured between 400 and 550 nm after excitation of the laurdan probe at 350 nm. Based on the contribution of its blue emission band ($I_B = 435$ nm) and the red emission band ($I_R = 490$ nm) to the total fluorescence emission, generalized polarization (GP) values were calculated, which describe the relative order of a membrane [39,40]:

$$GP = (I_B - I_R) / (I_B + I_R) \quad (2)$$

2.3. Calculation of association free energy derived from FRET measurements

In lipid bilayers the measured energy transfer E is given by

$$E = E_d + E_s \quad (3)$$

where E_d is the FRET caused by sequence-specific dimerization and E_s is the spontaneous FRET contribution arising due to spontaneous

proximity of donor and acceptors randomly distributed in the lipid bilayer. In each experiment, the observed FRET efficiency E was corrected with regard to FRET arising due to random distribution of donor and acceptor in the lipid bilayers.

Assuming a random distribution of the acceptors in two dimensions, the values of E_s can theoretically be estimated using the equations of Wolber and Hudson [41],

$$E_s = 1 - [0.65 \times \exp(-4.75 \times C) + 0.35 \times \exp(-2.06 \times C)] \quad (4)$$

where C is the two dimensional concentration of the acceptor (per unit area). The area per lipid molecule was 35 Å^2, accounting for the fact that the lipids are organized as a bilayer [42]. Further in this analysis, the other necessary parameter, distance of closest approach between donor and acceptor were assumed as being zero.

The apparent association constant K_a and the corresponding apparent Gibbs free energy of dimerization ΔG^o, are given by

$$K_a = [D] / [M]^2 \quad (5)$$

$$\Delta G^o = -RT \ln K_a \quad (6)$$

where $[D]$ and $[M]$ are the mole fractions of the dimer and the monomer in the bilayers, respectively.

The dimer fraction (f_D) can be calculated with Eq. 6, assuming a monomer–dimer equilibrium and a binomial distribution of donors and acceptors in the dimer, because an oligomer without any acceptors does not contribute to FRET [31,43].

$$f_D = 2[D] / [T] \quad (7)$$

$$[D] = E_d \cdot [T] / 2X_A \quad (8)$$

$$[T] = [M] + 2[D] \quad (9)$$

where $[T]$ is the total peptide mole fraction, and X_A is the mole fraction of acceptor-labeled molecules, $X_A = [a]/\{[d]+[a]\}$, where as $[a]$ and $[d]$ are the respective donor and acceptor mole fraction.

3. Results

In recent years, sequence-dependent oligomerization of individual TM α-helices has been studied to some extent, whereas the influence of lipid bilayer properties on a defined helix–helix interaction is far less characterized. But how do bilayer properties, such as the hydrophobic thickness, affect formation and stability of specific TM helix dimers? To answer this question, we chemically synthesized the TM domain of GpA and labeled the peptide N-terminally with either fluorescein (Fl) or 5-(and 6-)carboxytetramethylrhodamine (TAMRA). Fig. 1 shows fluorescence excitation and emission spectra of Fl- and TAMRA-labeled GpA peptides in *n*-dodecyl-β-D-maltoside (DDM) micelles. The emission spectrum of the Fl-labeled donor peptide (Fig. 1A) shows a significant overlap with the excitation spectrum of the TAMRA-labeled GpA acceptor peptide (Fig. 1B). Based on the NMR structure of the GpA peptide, the distance between the amino termini of the individual TM helices in the dimer is less than 10 Å [21], which is far below the reported Förster radius (R_0) of the Fl/TAMRA FRET pair (49–54 Å) [44]. Thus, FRET measured as donor emission quenching and/or sensitized acceptor emission (Fig. 1C) is a direct measure of the stable dimer population and is not limited to the distance between the fluorophores. While determination of the GpA TM dimer structure in different bilayers by NMR has indicated that the GpA structure does not change in response to variations in bilayer thickness [33–35], in a few computational studies slight changes in the helix–helix crossing angle, caused by changes in the bilayer thickness, have been postulated [45,46]. However, the predicted small changes of the helix crossing angle would result in separation of the GpA TM helix N-terminus of less than 2 Å, and this difference in the distance would translate into changes of the FRET efficiency of below 1%, which is within the tolerance of the performed FRET measurements. Thus, any observed changes in the FRET efficiency stem exclusively from changes in the monomer–dimer equilibrium, whereas small changes of the helix–helix crossing angle cannot be traced by the applied method.

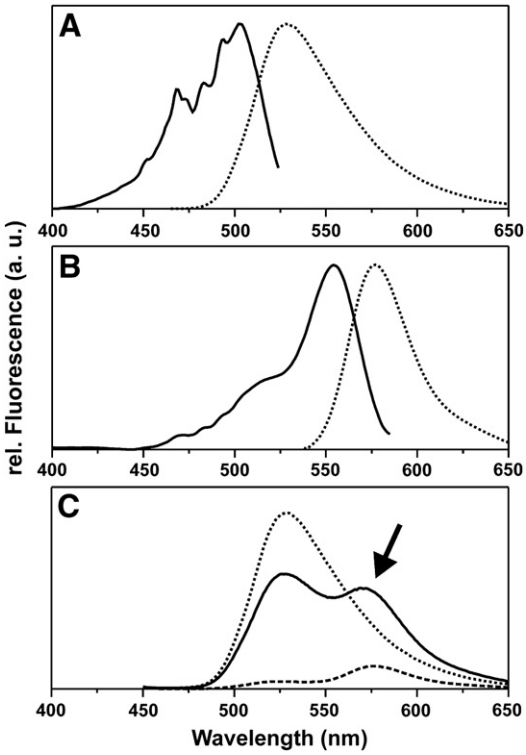

Fig. 1. Excitation (solid lines) and emission spectra (dashed lines) of Fl (donor)- and TAMRA (acceptor)-labeled GpA peptides and FRET pairs. (A) Fl-GpA, excitation λ = 439 nm, emission λ = 530 nm. (B) TAMRA-GpA, excitation λ = 530 nm, emission λ = 590 nm. (C) Fluorescence emission spectra of donor- and acceptor-labeled peptides (solid lines) as well as control samples containing only donor-labeled peptides (dotted lines) and only acceptor-labeled peptides (dashed lines) after excitation at 439 nm. The arrow indicates sensitized fluorescence emission of the acceptor fluorophore after excitation of the donor. Spectra were measured in 10 mM HEPES buffer containing 150 mM NaCl and 5 mM DDM at pH 7.4.

3.1. GpA dimerization in lipid bilayers

To ensure that the fractional association of the GpA TM helix can be analyzed in lipid bilayers, self-association of the GpA TM domain was first followed in diC(20:1) PC at varied peptide to lipid ratio at constant (1:1) Fl-GpA/TAMRA-GpA molar ratio using the EmEx FRET method as described in detail recently [43,47]. Fig. 2 shows the energy transfer efficiencies at different peptide to lipid ratios. Based on the observations of this experiment we selected a lipid–protein ratio of 625:1 for the subsequent studies, since at this ratio we have a clear fluorescence signal with little disturbance by the liposomes and changes in the FRET signal can still be observed. However, in our experiments we observed the FRET efficiencies approaching up to ~0.7 (Fig. 2) whereas a dimerization-related FRET efficiency cannot exceed 0.5, when it is probed with an equimolar donor–acceptor mixture. Therefore, we always calibrated our raw data for the contribution from proximity effect (Eq. 4) and quantified the dimer fraction in each lipid environment. As a result, a FRET intensity of 0.5 corresponds to a dimer fraction of 1.

To study the influence of the membrane hydrophobic thickness on the GpA TM helix oligomerization in detail, the hydrophobic thickness of the lipid bilayers was gradually changed from 20 to 34 Å using

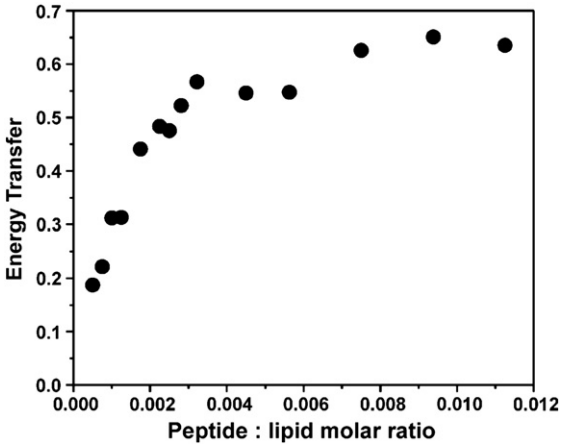

Fig. 2. FRET analysis of GpA TM domain interaction in diC(20:1) PC lipid bilayer. Energy transfer as a function peptide to lipid molar ratio as measured with equimolar mixture of Fl-GpA and TAMRA-GpA TM domain.

mono unsaturated phosphatidylcholines (PC) having 14 to 22 acyl chain carbons: diC(*X*:1) PCs, with *X* = 14, 16, 18, 20 and 22, respectively (see Materials and methods). Fig. 3A shows the fluorescence emission spectra of the analyzed FRET pair in the different diC(*X*:1) PC membranes. The sensitized emission at 570 nm increases with increasing acyl chain length and remains just about constant at diC(20:1) PC and diC(22:1) PC, which corresponds to thicknesses of the hydrophobic bilayer region of about 30.5 and 34 Å, respectively (Table 1). Fig. 3B shows the individual fractions of GpA dimers formed in the various PC membranes having increasing acyl chain lengths.

In diC(14:1) PC membranes, about 35% of the GpA TM helices are present as a dimer, and this fraction increases up to 56% in thicker membranes composed of diC(*X*:1) PCs (*X* = 20, 22). These results indicate that the GpA monomer–dimer equilibrium is significantly shifted towards the dimeric structure in diC(*X*:1) PC (*X* = 20, 22) compared to thinner membranes diC(*X*:1) PC (*X* = 14, 16, 18). However, the thus far presented results do not rule out the possibility of the GpA peptide forming higher ordered oligomers different from the dimers in response to variations of the bilayer thickness, and thus, the observed FRET differences could merely reflect changes in the oligomeric structure. For analyzing the oligomeric state of single TM helices in detergent or lipid vesicles, the dependence of energy transfer between the donor and acceptor dye on the mole fraction of the acceptor can be used to determine the number of peptides

Table 1
Hydrophobic thickness of PC bilayers in the fluid phase and apparent free energies of association of the GpA dimer determined in the respective membrane environments.

Lipid	Hydrophobic thickness (Å)[a]	ΔG^{app} (kJ/mol)	
		−Cholesterol	+Cholesterol
diC(14:1) PC	20	−20.79 ± 0.04	−25.31 ± 0.11
diC(16:1) PC	23.5	−22.55 ± 0.07	−26.19 ± 0.08
diC(18:1) PC	27	−23.35 ± 0.05	−28.79 ± 0.03
diC(20:1) PC	30.5	−23.87 ± 0.05	−28.19 ± 0.13
diC(22:1) PC	34	−23.80 ± 0.04	−21.88 ± 0.04

[a] Hydrophobic thickness defined by average distance between C = O groups [52].

associated within an oligomer [29,43,47,48]. In Fig. 3C, the determined FRET efficiencies are shown as a function of the acceptor mole fraction measured in membranes having the thinnest diC(14:1) PC and thickest diC(22:1) PC hydrophobic core region. As can be seen, the FRET efficiency linearly depends on the acceptor mole fraction, which clearly demonstrates that the formed oligomer is a dimer, as derived, e.g., in [29,43,47,48]. As a further control, the helicity and orientation of the GpA TM peptide reconstituted in different lipid bilayers was tested by circular dichorism (CD) and orientated circular dichorism (OCD) (Fig. S1).

3.2. Effect of cholesterol on GpA oligomerization

The above-presented results strongly suggested that the GpA monomer–dimer equilibrium is dramatically influenced by the bilayer thickness. To further analyze the influence of the bilayer thickness on the GpA TM helix dimerization, we characterized membranes with 40 mole % cholesterol since the presence of cholesterol is known to increase the hydrophobic thickness of diC(*X*:1) PC membranes [49]. In Fig. 4A, emission spectra of the GpA FRET pair in diC(*X*:1) PCs membranes containing 40 mole % cholesterol is shown, and Fig. 4B shows the dimer fractions derived from the FRET measurement. The observed sensitized emission at 570 nm increases depending on the PC chain length as previously observed in pure diC(*X*:1) PC membranes. Surprisingly, the FRET efficiency dropped dramatically in diC(22:1) PC membranes in the presence of cholesterol. The calculated dimer fractions (Fig. 4B) indicate that increasing the membrane thickness of diC(22:1) PC membranes by adding cholesterol dramatically affects GpA dimerization. The control experiments shown in Fig. 4C further show that addition of cholesterol does not cause unspecific TM helix aggregation and does not influence the formation of solely the dimer.

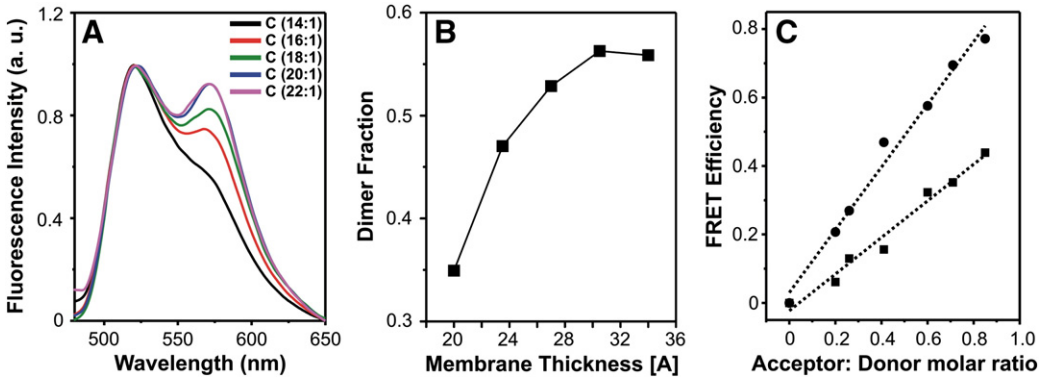

Fig. 3. Self-association of GpA peptides in lipid bilayers. Fluorescence emission was measured in lipid bilayers with Fl-labeled peptide alone and with the Fl- and TAMRA-labeled peptide pair (1:1 ratio). Energy transfer was calculated from the Fl-fluorescence decrease at 525 nm. The lipid/peptide ratio was kept constant at 625:1 mol/mol. (A) FRET spectra recorded for Fl- and TAMRA-labeled peptides in monounsaturated PCs (diC (*X*:1) PCs). (B) Fraction dimer plotted against the acyl chain length of the various PC lipids. (C) FRET efficiencies as a function of acceptor mole fraction are shown for diC(14:1) PC (■) and diC(22:1) PC (●) membranes, respectively. The total peptide and lipid concentrations were kept constant, whereas the ratio of acceptor and donor peptide varied between 0.2 and 0.85. The linear dependence of the FRET efficiency on the acceptor mole ratio demonstrates exclusive dimer formation.

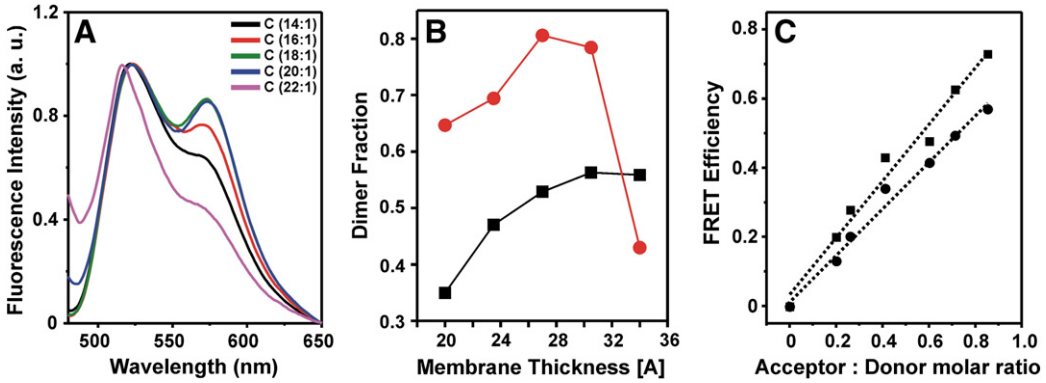

Fig. 4. The effect of cholesterol on GpA TM helix–helix association. (A) Fluorescence emission spectra recorded with Fl- and TAMRA-labeled peptides in diC(*X*:1) PC membranes containing 40 mole % cholesterol. The lipid/peptide ratio was kept constant at 625 mole/mole. (B) Fraction dimer plotted against the acyl chain length of the various monounsaturated PCs. (C) FRET efficiencies as a function of acceptor mole fraction are shown for diC(14:1) PC (■) and diC(22:1) PC (●) membranes, respectively, in the presence of 40 mole % cholesterol. The total peptide and lipid concentrations were kept constant, whereas the ratio of acceptor and donor peptide varied between 0.2 and 0.85. The linear dependence of the FRET efficiency on the acceptor mole ratio shows exclusive dimer formation.

In general, the presented results demonstrate that the presence of cholesterol influences GpA dimerization in membranes. Interestingly, the calculated dimer fractions in the various diC(*X*:1) PC membranes were always increased in membranes containing cholesterol as opposed to pure PC membranes (Fig. 3), with the exception of diC (22:1) PC. The observed disproportional high increase in the dimer fraction cannot exclusively be explained by the slight increase in the hydrophobic thickness of the cholesterol containing PC bilayers, and thus, other parameters must also have caused the increased interaction propensity of the GpA TM helix, which could involve alterations in the lipid acyl chain order.

3.3. Influence of lipid acyl chain order on GpA dimerization

To further characterize the impact of the bilayer thickness and the lipid acyl chain order on the GpA TM helix–helix interaction in more detail, we measured FRET efficiencies in DMPC membranes at increasing temperatures. To rule out uneven diffusion, the peptides were reconstituted at the lipid fluid phase.

Fig. 5A and B shows fluorescence emission spectra of the labeled GpA peptides in DMPC liposomes at 10 °C and at 35 °C, at which the DMPC membrane is in a gel and fluid phase, respectively. The solid lines show the samples containing only Fl-GpA peptides and dashed lines show the samples containing both Fl- and TAMRA-labeled GpA. FRET was monitored at temperatures 10 °C below up to 10 °C above the DMPC chain-melting transitions (t_m ~24 °C). The fractions of dimeric GpA remains approximately constant up to 15 °C and decreased steeply at higher temperatures in parallel with changes in the lipid phase behavior, where DMPC shows ripple (~16 °C) and main-phase transition (~24 °C). At low temperatures, when the membrane is in a gel phase, the dimer fraction was 0.69 which decreased to ~0.1 at higher temperatures when the membrane is in the fluid phase (Fig. 6A). Changes in the dimer fraction could be caused by difference in the hydrophobic thickness of the DMPC membrane in the gel and fluid phase. However, while the hydrophobic thickness of the DMPC bilayer in its fluid phase is about 23 Å and increases by 4–5 Å in the gel phase, this small change alone cannot explain the disproportional high increase in the FRET efficiency at low temperatures (compare Fig. 4 and Table 1). Besides differences in the membrane thickness, the lipid acyl chains are also more ordered in the lipid gel phase compared to the fluid phase. Thus, the results shown in Figs. 6A and 5C indicate, that besides changes in the membrane thickness, changes in the acyl chain ordering during the lipid phase transition dramatically affect the GpA monomer–dimer equilibrium.

To exclude that the observed effects are mainly caused by a temperature-dependent destabilization of the GpA TM helix dimer structure, the dimer fractions were not only followed in a DMPC membrane but also determined at different temperatures in a diC (14:1) PC bilayer (Fig. 6A). In contrast to DMPC, in diC(14:1) PC lipid bilayers, which have a lipid phase transition below 0 °C, the calculated fractions of dimeric GpA were not drastically affected by the

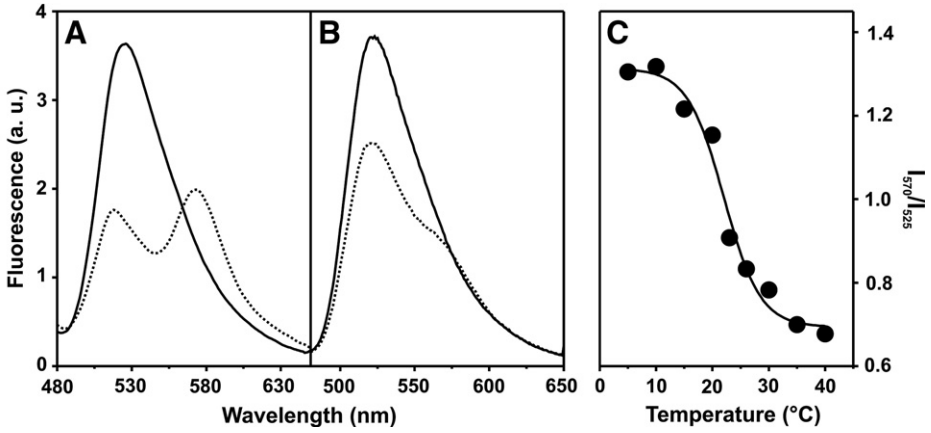

Fig. 5. Effect of temperature on the GpA TM helix–helix association in DMPC bilayers. Fluorescence spectra of donor- (solid line) and donar-acceptor-labeled (dashed line) GpA peptides in the DMPC at 10 °C (A) and 35 °C (B), corresponding to the gel and fluid membrane phase, respectively. (C) Temperature-dependent ratio of sensitized and donor emission (570/525 nm). The lipid/peptide ratio was kept constant at 372 mole/mole.

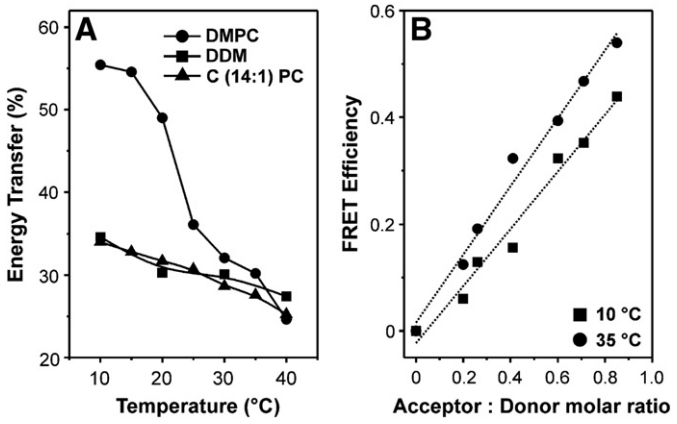

Fig. 6. Influence of the temperature on GpA dimerization in membranes. (A) Fraction dimer plotted against temperature, in DMPC bilayer (●), diC(14:1)PC bilayers (Δ) and DDM micelles (■) (■). (B) FRET efficiency as a function of acceptor mole fraction measured in DMPC bilayers at 10 and 35 °C. The total peptide and lipid concentrations were kept constant, while the ratio of acceptor and donor peptide varied between 0.2 and 0.85. The linear dependence of the FRET efficiency on the acceptor mole ratio indicates formation of solely the dimer.

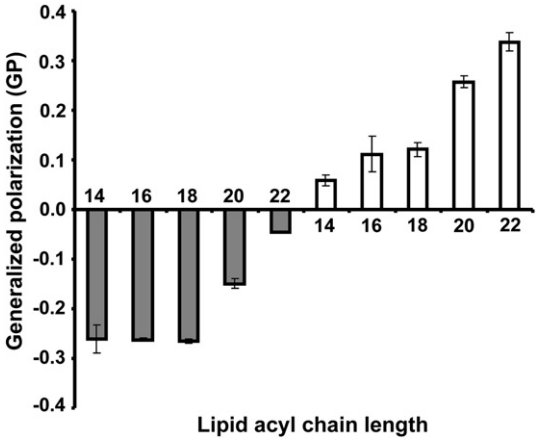

Fig. 7. Generalized polarization (GP) of laurdan in diC(*X*:1) PCs lipids and diC(*X*:1) PCs containing 40 mole % cholesterol, where '*X*' represents the lipid acyl chain length (14, 16, 18, 20 or 22 carbons, respectively). GP values were calculated according to Eq. 2 (see Materials and methods) by following the laurdan emission between 400 and 550 nm after excitation at 350 nm. The laurdan/membrane lipid ratios were 1:500. Values are the mean ± standard deviation.

temperature change from 10 to 40 °C. These results demonstrate that the observed temperature effect on the GpA dimerization is caused by the changing bilayer properties rather than by a temperature-dependent disruption of the GpA dimer. Furthermore, the control experiments shown in Fig. 6B demonstrate that changes in the lipid phase do not result in formation of different higher ordered GpA TM oligomers since formation of only the dimeric, parallel GpA species was observed. Since most studies on dimerization of the GpA TM helix (and of most TM helices) are performed in detergents, we also monitored the temperature-dependent dimerization of the GpA TM domain in the widely used detergent DDM (Fig. 6A). Following dimerization of the GpA TM domain in DDM at the various temperatures shows that dimerization is also not significantly affected by the temperature, as observed in diC(14:1) PC lipid bilayers. In order to further define a potential influence of the lipid acyl chain order on dimerization of the GpA TM domain, we subsequently analyzed lipid packing in the before analyzed diC(*X*:1) PC (*X* = 14, 16, 18, 20 or 22) phospholipid bilayers (compare Fig. 3) using the fluorescence dye laurdan [39,51]. The laurdan molecule is dynamically anchored in the hydrophobic core of the lipid bilayer by hydrophobic interactions between its lauric acid tail and the lipid alkyl tails, whereas its fluorescing moiety is located in the glycerol region of the phospholipid head groups [50]. The fluorescence emission spectrum of laurdan is sensitive to the polarity and to the phase of phospholipid bilayers, which is described by the generalized emission polarization value (GP) (see Materials and methods). GP values obtained by spectroscopy can theoretically have values ranging from ≥+0.5, when lipids are most ordered, down to ≤–0.5, when lipids are least ordered [39,40]. Fig. 7 shows the GP values, which reflect lipid packing and acyl chain ordering, determined for the different PC membranes used in this study. The GP values of the diC(*X*:1) PC lipids indicate significant differences in lipid packing depending on the lipid acyl chain length. The GP values of the pure diC(*X*:1) PC lipids shift to more positive values in parallel with increasing lipid acyl chain length of the unsaturated lipids. Between the various pure diC(*X*:1) PC lipids and cholesterol containing diC(*X*:1) PC lipids, we observed large GP differences, which strongly suggests increased ordering of the lipid acyl chains in the presence of cholesterol (Fig. 7). These observations further demonstrate that the acyl chains are more ordered in cholesterol containing PC membranes, and the acyl chain order increases in parallel with increasing acyl chain length. Thus, the observed dramatic increase of the fraction dimeric GpA in the cholesterol containing PC membranes (Fig. 4) is most likely mainly caused by cholesterol-induced ordering of the lipid acyl chains.

Altogether, the results strongly suggest that the lipid environment is important for mediating and stabilizing the GpA TM helix–helix interactions, and especially the acyl chain order may influence the interaction propensity of individual TM helices dramatically.

4. Discussion

4.1. The membrane thickness severely influences GpA TM helix dimerization

The TM domain of human GpA forms a parallel homodimer in micelles and DMPC bilayers [28,29,32,34,51]. To study the influence of the bilayer thickness on a sequence-specific stable homodimerization of a TM helix, we analyzed the dimerization of Fl- and TAMRA-labeled GpA TM peptides. GpA dimerizes in all tested lipid bilayers, and dimerization is most efficient in diC(20:1) PC and diC(22:1) PC, which corresponds to hydrophobic thicknesses of 30.5 and 34 Å, respectively [52]. Based on the NMR structure of the GpA TM dimer [21] and the OPM database [53,54], the hydrophobic region of the GpA TM α-helix is about 31.3 ± 2.2 Å, which corresponds well to the observed maximum dimer fraction in membranes having a hydrophobic core of 30.5–34 Å (Fig. 3, Table 1).

To better understand GpA TM helix dimerization in different lipid environments, the relative contributions of peptide–peptide versus peptide–lipid interactions have to be considered. TM helices can adapt to hydrophobic mismatch conditions by different mechanisms, including changes in the tilt angle, membrane deformation or change of a TM helix aggregation state [2]. In the present study, we show that GpA forms stable dimers in all tested lipid bilayers, although differences in the dimer fraction were observed between thinner and thicker membranes (Fig. 3). The presented data indicate that hydrophobic mismatch destabilizes the GpA TM dimer. For interaction of the individual TM helices, these have to align in a TM conformation which allows for optimal packing of the helices. In other words, the helices should have the correct tilt angle within a membrane, which is about 20° for the GpA TM helix [21]. If the hydrophobic thickness of the lipid bilayer core does not promote this tilting angle, TM helix–helix interactions will involve adjusting the tilt angle of the monomer within the bilayer, which most likely also involves membrane deformation [2]. Thus, hydrophobic mismatch condition will destabilize the TM helix dimer, as observed in this study. Hydrophobic matching presumably promotes the most favorable protein-protein

interaction by lowering the energy needed to form and stabilize the correct TM helix tilt angle.

Apparent free energies of GpA TM helix dimerization in the different lipid bilayers were calculated using Eq. 6 and are summarized in Table 1. Stronger associations with more negative ΔG^{app} values were observed under hydrophobic matching condition. While in diC(14:1) PC the ΔG^{app} was about –21 kJ mol^{-1}, this value increases to about –24 kJ mol^{-1} in diC(20:1) PC.

4.2. Cholesterol promotes TM helix–helix interactions by changing the acyl chain order

When GpA dimerization was monitored in cholesterol containing lipid bilayers (Fig. 4B), a maximum dimer fraction was observed in diC(18:1) PC, whereas in the absence of cholesterol, dimer formation was most efficient in diC(20:1) PC (Fig. 3). While these observations further support the notion that the hydrophobic thickness of a membrane can severely affect dimerization of the GpA TM domain, the fractions of dimeric GpA measured in the presence of cholesterol were almost always significantly higher in the tested diC(*X*:1) PC membranes (Fig. 4) compared to the respective measurements without cholesterol (Fig. 3). While addition of cholesterol slightly increases the bilayer thickness [49], this slight increase cannot solely explain the observed dramatically increased dimerization propensity of the GpA peptides, and other factors must be involved in promoting TM helix–helix interactions in cholesterol containing membranes. Besides increasing the bilayer thickness, cholesterol also affects the order of the lipid acyl chains. Cholesterol particularly stabilizes the liquid order phase, in which the atoms in the hydrophobic core are more tightly packed than in the liquid disordered phase [49]. Thus, acyl chain ordering might also promote TM helix–helix interactions, as previously observed with artificial TM segments, which did not contain defined helix–helix interaction motifs [17]. In cholesterol containing membranes, the free energies of dimerization were higher and increased from about –25 kJ mol^{-1} in diC(14:1) PC to –28 kJ mol^{-1} in diC(20:1) PC. Addition of cholesterol to all tested lipid bilayers, except diC(22:1) PC, resulted in significantly stronger dimerization of the GpA TM domain compared to pure PC bilayers. The obtained free energy values reported in Table 1 clearly suggest that formation of a liquid-ordered phase in cholesterol containing membranes [55,56] promotes self-association of the GpA TM domain.

The dimerization propensities of the GpA TM observed in DMPC membranes at different temperatures (Figs. 5 and 6) strongly suggest that the acyl chain order can indeed dramatically influence the interaction propensity of individual TMs. Furthermore, the acyl chain order of individual PCs increases with increasing acyl chain length, and cholesterol clearly increased the lipid acyl chain order in the analyzed PC membranes (Fig. 7). Thus, the observed increase of the GpA TM domain interaction propensity in cholesterol containing membranes was most likely caused by both, a slight increase in the bilayer thickness, as well as by a change in the acyl chain order. Furthermore, the observed differences in the GpA TM interaction propensities observed in pure PC membranes with different PC acyl chain length were most likely also caused by both, an increasing bilayer thickness as well as increasing acyl chain order with increased acyl chain length (Fig. 7).

TM helix oligomerization involves removal of lipids from the monomer at the interface contacting the adjacent helix [57–59]. When lipid acyl chains are less flexible, this will directly affect the interaction of individual lipids with a TM helix. The lipid packing density in the liquid-ordered phases increases the possibility of forming voids at the lipid–protein interface, which might contribute to favorable TM helix dimerization. As a result, at low temperatures and in cholesterol-rich membranes the interaction of individual lipids with a TM helix could be disturbed, favoring the formation of TM helix oligomers. Furthermore, the presence of gel phase and liquid-ordered lipids eventually also increases the local concentration of peptides in a bilayer and thus lead to an increase in oligomer formation.

The above described findings clearly suggest that differences in the acyl chain order can have significant effects on a TM α-helix oligomerization.

4.3. Implications for membrane protein folding and TM signaling

The observations discussed above demonstrate that thickness and acyl chain order parameters of a lipid bilayer can dramatically influence the propensity of a TM helix pair to properly interact and to form higher ordered oligomeric structures. In some studies, it has already been shown that bilayer properties can severely influence integration of membrane proteins into membranes as well as the structure and function of membrane proteins [60,61]. But why do larger α-helical membrane proteins not fold and function properly in the "wrong" bilayer environment? Based on the results of this study, folding of polytopic α-helical membrane proteins, which can involve multiple TM helix–helix interactions, can already be disturbed on the level of an individual TM helix–helix interaction.

The thickness of biological membranes increases along the secretory pathway from the ER membrane having the smallest thickness, via the Golgi apparatus to the plasma membrane with the thickest thickness [62–65]. This increase in the bilayer thickness can determine proper sorting of membrane proteins, and the hydrophobic core of individual membrane proteins often matches the thickness of the hydrophobic core of the membrane in which the protein naturally functions [15,16,62]. But since all α-helical membrane proteins are first synthesized into the ER membrane, this observation raises the question which structure, e.g., a plasma membrane protein adapts upon synthesis into the ER membrane. The observations of this study indicate that such a protein has eventually not reached its final structure since hydrophobic mismatch conditions do not promote proper interactions of individual TM helices.

Transport of a membrane protein from the ER via the Golgi apparatus to the plasma membrane could thus involve further maturation of the membrane protein structure. This could, e.g., also explain why some membrane proteins misfold to a large extent following synthesis into the ER membrane [66]. Hydrophobic mismatch in the ER membrane does not allow proper interactions of individual TM helices, which increases the danger of unspecific aggregations. Furthermore, assuming that some proteins are locked in an intermediate folding step until delivery to their final membrane also implies that these proteins are eventually not functional. In several cases, it could be an advantage for a cell that, e.g., certain plasma membrane channels or transporters are not functional in the ER or Golgi membrane.

However, it has to be noted that this does most likely only account for some membrane proteins. Some membrane proteins can influence a bilayer thickness themselves [11], and in some cases the membranes adapt to hydrophobic mismatch conditions by compression or stretching of the lipid bilayer [2].

As has been intensely discussed in recent years, certain areas of membranes are enriched with specific lipids as well as with cholesterol, and especially proteins involved in TM signaling are enriched in such cholesterol-rich lipid domains [67,68]. The reason, why these proteins function especially in these domains is however largely unknown. The observations of this study indicate that some membrane proteins could adopt their final functional structure in these lipid domains due to an increased bilayer thickness as well as due to changes in the lipid acyl chain order. Thus, these proteins reach their final and functional structure within the lipid domains, and regulating the partitioning into such domains could trigger a protein function. This is especially obvious in the case of single span membrane proteins, such as receptor tyrosin kinases or integrins [67,69,70], where specific interactions of the TM domains are most

likely involved in TM signaling [71–75]. Here, TM helix–helix interactions could be promoted by the defined lipid environment, and the local bilayer properties might directly influence the signaling potential of individual TM proteins.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2010.06.027.

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Further reading

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