Published in final edited form as:

Angew Chem Int Ed Engl. 2017 February 06; 56(7): 1756–1759. doi:10.1002/anie.201609708.

GXXXG-mediated parallel and antiparallel dimerization of transmembrane helices and its inhibition by cholesterol: Single-pair FRET and 2D IR studies**

Dr. Yoshiaki Yano,

Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501 (Japan)

Kotaro Kondo,

Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501 (Japan)

Yuta Watanabe,

Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501 (Japan)

Tianqi O. Zhang,

Department of Chemistry, University of Wisconsin Madison, WI 53706 (USA)

Jia-Jung Ho,

Department of Chemistry, University of Wisconsin Madison, WI 53706 (USA)

Dr. Shinya Oishi,

Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501 (Japan)

Prof. Nobutaka Fujii,

Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501 (Japan)

Prof. Martin T. Zanni, and

Department of Chemistry, University of Wisconsin Madison, WI 53706 (USA)

Prof. Katsumi Matsuzaki

Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501 (Japan)

Abstract

Small-residue-mediated interhelical packings are ubiquitously found in helical membrane proteins, although their interaction dynamics and lipid dependence remain mostly uncharacterized. We used a single-pair FRET technique to examine the effect of a GXXXG motif on the association of de-novo designed (AALALAA)₃ helices in liposomes. Dimerization occurred with sub-second lifetimes, which was abolished by cholesterol. Utilizing the nearly instantaneous time-resolution of 2D IR spectroscopy, parallel and antiparallel helix associations were identified by vibrational couplings across helices at their interface. Taken together, the data illustrate that the GXXXG motif controls helix packing but still allows for a dynamic and lipid-regulated oligomeric state.

**This work was supported by a JSPS KAKENHI Grant-in-Aid for Scientific Research (C) (25460034) (Y.Y.) and a SUNBOR GRANT (Y.Y.).

Correspondence to: Katsumi Matsuzaki.

Abstract

Unique interaction inside membrane: Model transmembrane helices containing a GXXXG motif were synthesized to explore interplay of the motif and lipid composition on the intramembrane helix associations. Single-pair FRET with controlled association topology and isotope-edited 2D-IR experiments revealed the dynamic nature, distinct interfaces, and high cholesterol sensitivity of the GXXXG-mediated interactions, which can actively regulate the functions of membrane proteins.

Keywords

FRET; GXXXG motif; IR spectroscopy; Membrane proteins; Single-molecule studies

Small amino acid residues such as Gly, Ala, and Ser are ubiquitously found at contact interfaces of transmembrane helices in clusters of small residues with 2 or 3 interval residues (such as GXXXG and GXXXGXXG) with various topologies and orientations.^[1–3] Because these small residues do not have sufficient side chain hydrophobicities to compensate for the partitioning of the polar main chain of the helix into membranes,^[4] the polar surfaces can preferentially pack with each other to avoid lipid exposure. The impact of small residues on transmembrane helix–helix interactions is likely not only of a passive nature, but also involves active forces, such as van der Waals interactions and $C_{\alpha}H \cdots O=C$ hydrogen bonds.^[5, 6] The energetic stability of small-residue packing markedly depends on the protein sequence and membrane lipids,^[6] although the regulation rules are poorly understood.

While it is clear from the sequence homology that small-residue packing is important for stabilizing interhelical interactions in membranes, the role that these sequences play in dynamics is less well understood. Rearrangements of transmembrane helices due to transient helix–helix interaction are known to occur in membrane proteins.^[5] For example, G-protein-coupled receptors and growth factor receptors fluctuate among distinct conformations and oligomeric states in millisecond and subsecond timescales, respectively.^[7–10] Single-molecule and ensemble methods, such as fluorescence, atomic force microscopy, and NMR, have enabled the observation of membrane protein dynamics in model membranes and living cells.^[11–13] However, while the existence of dynamics has been established, obtaining specific structural information is difficult in membrane-bound systems, obscuring the link between structure and dynamics.

To investigate the effects of sequence motifs and lipid compositions on helix associations, we utilize a de-novo designed hydrophobic peptide (AALALAA)₃. The peptide assumes a stable transmembrane helix with a tendency to form antiparallel dimers due to electrostatic attraction between the helix macrodipoles.^[14] Recently, single-pair fluorescence resonance

energy transfer (sp-FRET) experiments in liposomes revealed that cholesterol stabilizes an hourglass-shaped antiparallel dimer by ‘lipophobic’ forces.^[15] Using this transmembrane helix as a host, we investigated the effect of the guest GXXXG motif at the center of the sequence (AALALAA-AGLALGA-AALALAA).

To monitor the relative helix topology in the liposomal sp-FRET, two helices labeled with the FRET donor Cy3B and the acceptor Cy5 were disulfide-linked between the N and N, or N and C termini (Supporting Information, Figure S1). After dilution of the dimer helices into vesicles (1 dimer per 10 vesicles on average), reduction of the disulfide resulted in two transmembrane helices with controlled topology (Figure 1a). As a proof-of-concept, antiparallel and parallel host helix dimers were prepared in palmitoyl oleoyl phosphocholine (POPC)/cholesterol (7/3) vesicles, and observed with total internal reflection fluorescence microscopy (Figure 1b). As expected, most of the bright spots including a donor and acceptor pair exhibited FRET fluctuation due to transient associations in antiparallel topology (86%, $n = 28$),^[15] whereas no FRET was observed for parallel topology (0%, $n = 28$) in the time resolution (17 msec).

In POPC, no association was detected for the host helices, whereas the introduction of the GXXXG motif evoked transient dimerizations in both topologies (Figures 2a,b). The addition of cholesterol completely inhibited these Gly-mediated associations (Figure 2a). The association of glycoporphin A with a GXXXG motif was also weakened by cholesterol and other membrane components.^[16, 17] On the other hand, cholesterol was shown to promote the dimerization of the glycoporphin A transmembrane domain.^[18] Therefore, the inhibitory effect of cholesterol does not appear to universally apply to all types of transmembrane helix associations with GXXXG. A possible mechanism is preferable interaction between GXXXG and cholesterol to suppress helix–helix contact, as reported for the C99 domain of the amyloid precursor protein.^[19, 20] However, the degree of FRET from a fluorescent sterol, dehydroergosterol, to NBD-labeled helices suggested no association between them (Supporting Information, Figure S2).

To obtain more detailed structural insights, we performed 2D-IR and polarized-attenuated total internal reflection (PATR) FTIR in POPC for GXXXG helices (NBD-AALALAA-AGLALGA-AALALAA) that were $^{13}\text{C}=^{18}\text{O}$ labeled at each residue in the central heptad (A8, G9, L10, A11, L12, G13, and A14). Oriented bilayers and vesicle suspensions were prepared for the PATR FTIR and 2D-IR measurements, respectively (Supporting Information, Supporting Methods). The isotope amide I' peaks were 60–70 cm^{-1} red shifted ($\sim 1,590 \text{ cm}^{-1}$) compared with unlabeled peaks (Supporting Information, Figure S3), as reported.^[21] The position-dependent tilt of amide bonds relative to the bilayer normal, calculated from amide I' PATR dichroic ratios, was best described assuming a helix orientation angle (between the helix axis and the bilayer normal) of $\sim 5^\circ$, when the residues at interhelical contacts (G9 and L10, see below) were excluded from the fitting (Figure 3a). The position dependence of the tilt angle suggests the presence of restricted rotation around the helix axis. On the other hand, two limiting cases can explain the estimated orientation angle: the angle was indeed 5° with small angle distribution, or the angle distribution was larger than 5° although the mean of the angle was ca. 5° . Using 2D IR spectroscopy, we also measured interhelical vibrational coupling between residues upon self-association, as

determined by the shift in frequency as compared with isotope-diluted samples (Figure 3b, black bars). 2D IR spectroscopy is more advantageous than FTIR for vibrational coupling measurements because the isotope bands appear more prominently relative to weaker background bands.^[22] A positive shift at G9 is consistent with a parallel contact,^[22] whereas a negative shift at L10 suggests an antiparallel approach with head-to-tail dipole geometry.^[22] Indeed, spectral simulations with crossing angles of $+10^\circ$ and -170° gave the G9 and L10 shifts, respectively (Figure 3b). Note that the parallel and antiparallel contacts did not have identical interfaces and affect L10 and G9 shifts, respectively, because 1) the two residues were 100° apart in the rotation angle and 2) the simulations implied different contact interfaces. Although the 2D-IR data do not exclude the possibility of other undetectable contact interfaces (Supporting Information, Supporting Discussion), we conclude that interhelical contacts with both topologies are populated, supporting the findings from the smFRET experiments. A similar parallel interface was independently predicted by a surface-based packing algorithm (Supporting Information, Supporting Discussion and Figure S4).

The lifetimes of monomers and dimers were estimated by HaMMY fitting^[23] to the apparent FRET efficiency (Figure 4). Helix macrodipole attraction in antiparallel orientation resulted in a faster association and slower dissociation of the helices. Approximate energetic contributions of the GXXXG motif are summarized in Table 1, obtained from sp-FRET, ensemble FRET, or self-quenching (Supporting Information, Figure S5) experiments. We have observed that the single-molecule and ensemble measurements give the same association energy values,^[15] although the introduction of Cys and linker residues for the topology control can slightly alter the G_a ($\sim 3 \text{ kJ mol}^{-1}$) (Table 1). In POPC, the motif contributed to G_a of -17 and -15 kJ mol^{-1} (in doubled mole fraction units) for parallel and antiparallel associations, respectively, comparable with the effect of a Gly mutation on glycophorin A dimerization.^[24] However, in the presence of cholesterol, the contribution became weaker in both topologies.

Here we propose a possible model in which cholesterol inhibits the association of the GXXXG helices. The host helices form cholesterol-induced hourglass-shaped dimers with a crossing angle of $50\text{--}60^\circ$ to release the higher lateral pressure by cholesterol in the hydrocarbon core by the penetration of water between helices.^[15] On the other hand, the GXXXG dimers have small crossing angles ($\sim 10^\circ$) (Figure 3a) therefore should be mechanically unstable in the presence of cholesterol. Furthermore, introduction of Gly residues enhance flexibility of the main chain, thus cannot release the cholesterol-originated lateral pressure even by forming hourglass-shaped dimers. In addition, the flexibility also diminishes the value of macrodipole as a driving force of dimer formation. Consistent with the model, a small broadening of amide I' band was observed for GXXXG helices (Supporting Information, Figure S6).

It is interesting to note that the dissociation rates of the GXXXG dimers studied here in POPC ($k_{\text{off}} = 3\text{--}7 \text{ s}^{-1}$) are similar or even higher than those of aqueous coiled-coils,^[25, 26] despite a comparable bulk K_d of $200\text{--}10 \text{ }\mu\text{M}$ (Supporting Information, Supporting Discussion).

In conclusion, two emerging techniques, the liposomal sp-FRET with controlled association topology and isotope-edited 2D-IR, shed light on the dynamic nature of small-residue-mediated helix associations with a defined interface, which is useful for the rapid rearrangement and transient association of membrane proteins. The motif drove both parallel and antiparallel associations, whereas cholesterol impaired the motif-mediated attraction. In animal cells, cholesterol levels are tightly controlled among organelles,^[27] suggesting a mechanism for membrane proteins in which the strength of small-residue interaction is regulated by cholesterol in organelles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Zhang SQ, Kulp DW, Schramm CA, Mravic M, Samish I, DeGrado WF. *Structure*. 2015; 23:527–541. [PubMed: 25703378]
2. Walters RF, DeGrado WF. *Proc. Natl. Acad. Sci. U.S.A.* 2006; 103:13658–13663. [PubMed: 16954199]
3. Russ WP, Engelman DM. *J. Mol. Biol.* 2000; 296:911–919. [PubMed: 10677291]
4. White SH, Wimley WC. *Annu. Rev. Biophys. Biomol. Struct.* 1999; 28:319–365. [PubMed: 10410805]
5. Langosch D, Arkin IT. *Protein Sci.* 2009; 18:1343–1358. [PubMed: 19530249]
6. Teese MG, Langosch D. *Biochemistry*. 2015; 54:5125–5135. [PubMed: 26244771]
7. Kofuku Y, Ueda T, Okude J, Shiraishi Y, Kondo K, Mizumura T, Suzuki S, Shimada I. *Angew. Chem. Int. Ed.* 2014; 53:13376–13379. *Angew. Chem.* 2014; 126:13594–13597.
8. Low-Nam ST, Lidke KA, Cutler PJ, Roovers RC, van Bergen en Henegouwen PM, Wilson BS, Lidke DS. *Nat. Struct. Mol. Biol.* 2011; 18:1244–1249. [PubMed: 22020299]
9. Hern JA, Baig AH, Mashanov GI, Birdsall B, Corrie JE, Lazareno S, Molloy JE, Birdsall NJ. *Proc. Natl. Acad. Sci. U.S.A.* 2010; 107:2693–2698. [PubMed: 20133736]
10. Kasai RS, Suzuki KG, Prossnitz ER, Koyama-Honda I, Nakada C, Fujiwara TK, Kusumi A. *J. Cell. Biol.* 2011; 192:463–480. [PubMed: 21300851]
11. Kasai RS, Kusumi A. *Curr. Opin. Cell Biol.* 2014; 27:78–86. [PubMed: 24480089]
12. Jefferson RE, Blois TM, Bowie JU. *J. Am. Chem. Soc.* 2013; 135:15183–15190. [PubMed: 24032628]
13. Zocher M, Zhang C, Rasmussen SG, Kobilka BK, Muller DJ. *Proc. Natl. Acad. Sci. U.S.A.* 2012; 109:E3463–3472. [PubMed: 23151510]
14. Yano Y, Matsuzaki K. *Biochemistry*. 2006; 45:3370–3378. [PubMed: 16519531]
15. Yano Y, Kondo K, Kitani R, Yamamoto A, Matsuzaki K. *Biochemistry*. 2015; 54:1371–1379. [PubMed: 25629582]
16. Hong H, Bowie JU. *J. Am. Chem. Soc.* 2011; 133:11389–11398. [PubMed: 21682279]
17. Yano Y, Furukawa N, Ono S, Takeda Y, Matsuzaki K. *Biopolymers*. 2016; 106:484–490. [PubMed: 26285787]
18. Anbazhagan V, Schneider D. *Biochim. Biophys. Acta.* 2010; 1798:1899–1907. [PubMed: 20603102]
19. Barrett PJ, Song Y, Van Horn WD, Hustedt EJ, Schafer JM, Hadziselimovic A, Beel AJ, Sanders CR. *Science*. 2012; 336:1168–1171. [PubMed: 22654059]
20. Song Y, Hustedt EJ, Brandon S, Sanders CR. *Biochemistry*. 2013; 52:5051–5064. [PubMed: 23865807]
21. Manor J, Mukherjee P, Lin YS, Leonov H, Skinner JL, Zanni MT, Arkin IT. *Structure*. 2009; 17:247–254. [PubMed: 19217395]

22. Hamm, P., Zanni, M. Concepts and methods of 2D infrared spectroscopy. 1. Cambridge University Press; 2011.
23. McKinney SA, Joo C, Ha T. Biophys. J. 2006; 91:1941–1951. [PubMed: 16766620]
24. Hong H, Blois TM, Cao Z, Bowie JU. Proc. Natl. Acad. Sci. U.S.A. 2010; 107:19802–19807. [PubMed: 21041662]
25. Wendt H, Berger C, Baici A, Thomas RM, Bosshard HR. Biochemistry. 1995; 34:4097–4107. [PubMed: 7696274]
26. De Crescenzo G, Litowski JR, Hodges RS, O'Connor-McCourt MD. Biochemistry. 2003; 42:1754–1763. [PubMed: 12578390]
27. Ikonen E. Nat. Rev. Mol. Cell. Biol. 2008; 9:125–138. [PubMed: 18216769]
28. Yano Y, Takemoto T, Kobayashi S, Yasui H, Sakurai H, Ohashi W, Niwa M, Futaki S, Sugiura Y, Matsuzaki K. Biochemistry. 2002; 41:3073–3080. [PubMed: 11863446]

Figure 1.

The concept (a) and proof-of-concept (b) of controlling the helix topology in liposomal sp-FRET experiments. (a) Liposomes containing disulfide-bridged dimers are attached to a cover glass via biotin–avidin interaction. Reduction of the disulfide with 2-mercaptoethanol results in two helix monomers that can noncovalently dimerize with a defined topology. Note that the flip-flop of the helix does not occur.^[28] (b) Fluorescence time traces for host helix dimers in POPC/cholesterol (7/3) vesicles (25°C). Arrowheads indicate transient FRET signals due to helix association. Right panels show histograms of the apparent FRET efficiency ($F_{\text{acceptor}}/(F_{\text{donor}}+F_{\text{acceptor}})$).

Figure 2. GXXXG-mediated association of transmembrane helices (25°C). (a) Representative sp-FRET trajectories under different association topologies and lipid compositions. (b)(c) Histograms of the apparent FRET efficiency for parallel (b) and antiparallel (c) associations of GXXXG helices in POPC.

Figure 3.

Helix orientation and association interface measured with $^{13}\text{C}=^{18}\text{O}$ isotope-labeled helices.

(a) Position-dependent orientation angle of amide I bands (circles), determined from dichroic ratios of amide I' bands for PATR FTIR spectra. The curve fitting did not consider the data for G9 and L10. The best fit (cyan) gave a helix orientation angle of 4.5° . (b) Frequency shift of 100% isotope samples from isotope-diluted samples (25% isotopes) determined from the diagonal cut of 2D-IR spectra (black bars). Cyan and purple bars indicate simulated shifts for the parallel and antiparallel dimers, respectively.

Figure 4. Kinetics of the monomer–dimer transition of GXXXG helices in POPC. Distributions of rate constants for association and dissociation processes were estimated by the HaMMY program, and the histograms were fitted with Gaussians to estimate the average lifetimes.

Table 1

Energetic contribution of GXXXG motif at 25°C

		POPC		POPC/cholesterol (7/3)	
		Parallel	Antiparallel	Parallel	Antiparallel
Host	G_a	ca. -4 [a]	-13.2 ± 0.2 [b]	ca. -8 [a]	-19.1 ± 0.2 [c] (-22.6 ± 0.1 [b])[d]
GXXXG	G_a	-21.4 ± 1.2 [c]	-28.3 ± 1.4 [c]	-15 or weaker [e]	-15 or weaker [e]
approx.	G_a (kJ mol ⁻¹)	-17 (-7 kT)	-15 (-6 kT)	-7 or weaker	+4 or more positive

[a] Estimated from self-quenching of NBD-labeled helices (Figure S5).

[b] Determined by ensemble FRET[15].

[c] Determined by topology controlled sp-FRET.

[d] The slight difference in the G_a values (~ 3 kJ mol⁻¹) is due to the introduction of Cys and linker residues for topology control in the sp-FRET experiments.

[e] Estimated from the detection limit of $k_{off} = 1/(17 \times 10^{-3}) = 59$ s⁻¹ and assuming k_{on} of GXXXG parallel dimer (2.3 s⁻¹).