SUPPLEMENTAL DATA

Crystal Structure of the Glycophorin A Transmembrane Dimer in Lipidic Cubic Phase

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Figure S1: Fluorescence Recovery After Photobleaching. Fractional fluorescence recovery of a bleached area is shown for different methods of reconstitution of GpA-TM into monoolein LCP. Free dye (Cy3 only) partitions to the lipid phase and shows a fluorescence recovery of 98% in 300 s, whereas detergent-free reconstitution of GpA-TM-Cy3 shows a diffusible fraction of 93% in the same timeframe. Detergent-solubilized GpA-TM shows a recovery of 84% for DDM, 82% for TDPC and 74% for LMPG.

Figure S2: Omit map of the GpA TM dimer with structure model colored according to B-factors. A simple 2mFo-DFc-type omit map for one GpA TM dimer is shown (grey, calculated in PHENIX, fractions of atoms to omit is 0.05). The model in the density is colored according to b-factors, which are low (blue) in the center of the membrane and around the dimerization motif (G79-T87) and are higher (yellow-red) in peripheral regions.

Table S1, Related to Figure 1. Data collection and refinement statistics (molecular replacement)

Glycophorin A TM dimer (5EH4)	
Wavelength (Å)	0.9537
Resolution range (Å)	35.94 - 2.81 (2.91 - 2.81)*
Space group	P 31 1 2
Unit cell	43.195 43.195 129.419 90 90 120
Total reflections	43467 (2497)
Unique reflections	3515 (345)
Multiplicity	12.3 (7.2)
Completeness (%)	98.68 (92.37)
Mean I/sigma(I)	22.93 (3.27)
Wilson B-factor	37.45
R-merge	0.3602 (1.862)
R-meas	0.3754
CC1/2	0.994 (0.524)
CC*	0.998 (0.829)
Reflections used for R-free	367
R-work	0.2278
R-free	0.2597
Number of non-hydrogen atoms	945
macromolecules	920
ligands	25
water	0
Protein residues	120
RMS(bonds)	0.002
RMS(angles)	0.64
Ramachandran favored (%)	96.4
Ramachandran allowed (%)	2.1
Ramachandran outliers (%)	0.9
Clashscore	3.02
Average B-factor	29.3
macromolecules	29.3
ligands	29.3

*Values in parentheses are for highest resolution shell. The structure was solved from two isomorphous crystals.

Table S2, Related to Figure 4. Key interatomic distance measurements for the interface in different structures of the Glycophorin A dimer. Average distances of 20 structures in the 1AFO micelle and 2KPE bicelle NMR structure bundles, the ssNMR phospholipid bilayer structure and the monoolein 5EH4 structure are given. The ranges from shortest to largest distances over 40 measurements (1AFO and 2KPE), 2 measurements (ssNMR) and 4 measurements (5EH4, dimer AB and dimer CD) are shown in parentheses.

	Average distance in Å					
	I76-O--G79-2H α	G79-O--V80-H α	V80-O--G83-2H α	V84-H α --T87-O γ	T87-O γ --V84-O	T87-O γ --G83-O
1AFO	3.5 (2.5-4.0)	2.5 (2.1-3.5)	2.8 (2.4-3.8)	2.7 (2.3-3.4)	4.2 (3.8-2.5)	3.2 (2.9-3.6)
2KPE	3.9 (3.8-4.1)	2.9 (2.8-2.9)	3.4 (3.3-3.5)	2.9 (2.8-3.2)	4.5 (4.4-5.5)	2.7 (2.6-2.7)
ssNMR	3.0 (3.0)	2.4 (2.4)	2.6 (2.6)	2.5 (2.4-2.5)	3.2 (3.2-3.3)	3.5 (3.5-3.6)
5EH4	2.8 (2.5-2.9)	2.3 (2.2-2.5)	3.1 (2.9-3.4)	2.4 (2.2-2.6)	4.1 (3.9-4.3)	2.8 (2.5-3.2)

Experimental Procedures

Production and Purification of Glycophorin A TM Peptides

The human erythrocyte glycophorin A transmembrane (GpA-TM) peptides with added C-terminal cysteine EPEITLIIFGVIAGVIGTILLISYGIRRLC and EPEITLIIFGVIAGVIGTILLISYGIRRLGSGC were produced as 9His-trpLE fusion proteins in *E. coli*. Dissolved fusion protein from inclusion bodies was purified on Nickel affinity resin, cyanogen bromide digested and reverse-phase HPLC purified following the published procedure (Sharma et al., 2013) with the following modifications.¹ The C-terminal Cys sulfhydryl group was protected using 10 mM S-methyl methanethiosulfonate (MMTS, Sigma-Aldrich) during lysis and inclusion body solubilization and peptides were at no time disulfide linked. Pure peptide was stored as lyophilized product at room temperature until used. The underlined M81I mutation was introduced to avoid internal peptide cleavage during cyanogen bromide digest.

Lipidic Cubic Phase Preparation

For detergent-assisted reconstitution into LCP, lyophilized peptide was weighed and dissolved in 100 mM of the respective detergent solution in Tris pH 8.0, 40 mM NaCl and mixed 2/3 with monoolein (Nu-Chek Prep) as described in Caffrey and Cherezov, 2009². For detergent-free reconstitution³, peptide was weighed and co-dissolved with appropriate amounts of monoolein in hexafluoroisopropanol (HFIP; sigma-Aldrich). The solvent was removed under streaming nitrogen and then under vacuum overnight. The peptide-monoolein mix was heated (52 °C) until liquid and mixed 3/2 with appropriate buffer (see respective application) for LCP formation using coupled 100 ul gastight Hamilton syringes (Formulatrix) at room temperature.

Fluorescent Labeling of GpA-TM Peptides and Fluorescence Recovery After Photo-bleaching (FRAP) Measurements

Lyophilized MMTS-protected peptide (EPEITLIIFGVIAGVIGTILLISYGIRRLGSGC) was dissolved in HFIP and deprotected using 0.125 M TCEP (0.5 M stock solution, Sigma-Aldrich). Excess TCEP was removed by lyophilizing the sample and washing the peptide in ultrapure water and labeled using 2x molar excess of thiol-reactive cyanine3 maleimide (Lumipropbe) in 3/1 HFIP/phosphate-buffered saline (PBS). Unbound label was removed by RP-HPLC and labeled peptide stored as lyophilized product.

The cubic phase was prepared as described above using a final labeled peptide concentration of 10-40 μ M and 10 mM Tris pH 8.0, 40 mM NaCl buffer in the aqueous phase with or without the indicated detergent. The samples were prepared on microscopy slides with 140 μ m thick double sticky spacers, commonly used for LCP crystallization sandwich plates,² and sealed with glass coverslips. The buffer mentioned above was used as precipitant solution for each measurement. FRAP experiments were performed on a Zeiss LSM 780 confocal microscope at 514 nm excitation bleaching a small region of interest (ROI). Five pre-bleach images were recorded in 0.78 s intervals, the ROI was bleached for 9.5 s and recovery was recorded for a total of 300 s. The fractional fluorescence recovery was

obtained by normalizing the mean fluorescence signal in the ROI to pre-bleach intensities. Potential bleaching effects during the measurement were compensated by subtracting background fluorescence changes in non-bleached areas. FRAP curves were plotted in Prism 6.

Crystallization of GpA-TM

The GpA-TM peptide without GSG linker (EPEITLIIFGVIAGVIGTILLISYGIRRLC) was used for crystallisation trials to reduce flexibility of terminal regions. The peptide was reconstituted into monoolein LCP using the detergent-free method to a final concentration of 40 mg/ml. The LCP mixture was dispensed in 200 nl drops onto 96-well glass plates (Molecular Domensions) with 1000 ul of precipitant solution using a mosquito LCP (TTP Labtech) robot at room temperature. Plates were sealed and kept at 20 °C in a Gallery 700 incubator coupled to a Minstrel HTUV imaging system (Rigaku) for monitoring crystal formation.

Data Collection and Structure Determination

Crystals were harvested from the crystallization plates and immediately frozen in liquid nitrogen using 30% glycerol in precipitant solution as cryoprotectant. Data were collected on the MX2 beamline of the Australian Synchrotron at a wavelength of 0.9537 Å at 100 K. Data were indexed and scaled using HKL2000.⁴ Structure factor amplitudes were obtained using Truncate (CCP4 Program Suite).^{5,6} The GpA-TM monomer structure was solved with Phaser⁷ by molecular replacement using the DAP12 transmembrane trimer structure⁸ (pdb 4WOL, a chain) as the search model. Iterative rounds of refinement were performed in PHENIX⁹ (xyz coordinates, real space, individual B-factors, optimize X-ray/stereochemistry weight, TLS parameters) and model building in Coot.¹⁰ The Glycophorin A TM dimer structure was solved in Phaser using the monomer structure as a search model, refined in PHENIX and built in Coot. The structure was solved and refined using the twin law $-h, -k, l$. In early stages of refinement, noncrystallographic (NCS) symmetry was defined assuming all four chains in the asymmetric unit were equivalent and secondary structure restraints were used. After several rounds, NCS restraints were eliminated and only xyz coordinates and individual B-factors were used for further refinement leading to a drop in the R-free value. N- and C-terminal residues in the monomer structure (5EH6) and side chains R96, R97 and L98 could not be built without significant increase in the R-free value. All residues of the dimer structure (5EH4) were built in all four chains including the engineered C-terminal Cys with thiomethane protection group (SCH). Monoolein ((2S)-2,3-dihydroxypropyl (9Z)-octadec-9-enoate) was modeled in density in the center of the four chains.

Supplemental References

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