Supplementary Materials for

Rhomboid-catalyzed intramembrane proteolysis requires hydrophobic matching with the surrounding lipid bilayer

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**Detailed Materials and Methods**

**Material**
Peptides were custom synthesized by the Core Unit Peptide Technologies at the University of Leipzig, Leipzig, Germany. Sequences were: LacYTM2: KRHDINHISK SDTGIIFAAI SLFSLLFQPL FGLLSKK; LacYTM2(EDANS/DABCYL): KRHDINE(EDANS)ISK SDTGK(DABCYL)IFAAI SLFSLLFQPL FGLLSKK. Cholesterol, 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (POPE), and 1-oleoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine (L-PE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The lipids DLPC-d_{46}, DMPC-d_{54}, DPPC-d_{62} were perdeuterated in both acyl chain while the mixed chain lipids POPC-d_{31}, POPE-d_{31} and POPG-d_{31} were perdeuterated in the palmityl sn-1 chain. All the deuterated lipids were purchased from Avanti Polar Lipids (Alabaster, AL) through Otto Nordwald GmbH (Hamburg, Germany). n-Dodecyl-β-D-Maltoside (DDM) was bought from Glycon (Luckenwalde, Germany). All other chemicals were purchased of the highest purity if not stated otherwise. The organic solvents were of MS grade for MS experiments and HPLC grade for all other experiments.

**Recombinant expression of GlpG-His6**
The sequence of the *E. coli* GlpG wildtype (UniProt ID P09391) was cloned into *E. coli* expression vector pET-25b(+) (Novagen) with a C-terminal hexahistidine-tag (GlpG-His6). Single mutations (S201A, R137A, and G261A) in the GlpG sequence were introduced by site-directed mutagenesis according to Stratagene’s QuikChange™ protocol (Agilent Technologies, Santa Clara, CA, USA). The expression vector was transformed into chemically competent BL21(DE3)pLysS cells (Novagen), grown in LB medium (Miller) containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol at 37°C. Expression of the protein was induced with 0.3 mM isopropyl-thiogalactopyranoside (IPTG) at OD_{600nm} of 0.3 and expressed overnight (~20 h) at 16°C. Cells were harvested by centrifugation at 2,500 g for 15 min at 4°C and resuspended in 20 mM HEPES pH 7.4, 150 mM NaCl, 5 mM MgCl_{2}, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM β-mercaptoethanol. Prior to lysis, 200 μg/mL lysozyme, 1 mM PMSF and benzonase (2.5 ku, Merck KGaA, Darmstadt, Germany) were added and cells were lysed using Emulsiflex (Avestin, Ottawa, Canada) with a maximum pressure of 15 kPSI (100 MPa). Crude membranes were obtained by ultracentrifugation at 100,000 g for 45 min at 4°C. The membrane pellet was resuspended in 50 mM HEPES pH 7.4, 150 mM NaCl, 5 mM MgCl_{2}, 10% glycerol, 1 mM PMSF, 5 mM β-mercaptoethanol. GlpG-His6 was solubilized from the crude membranes with 1.5% DDM for 1 h on a rotating wheel at 4°C. Extraction of GlpG-His6 from membrane debris was done by ultracentrifugation at 100,000 g for 1 h at 4°C. Cleared extract was batch incubated with Ni-NTA beads (Macherey-Nagel, Düren, Germany) for 1 h on a rotating wheel at 4°C for His-tag affinity purification. Bound GlpG-His6 was washed with 50 mM HEPES pH 7.4, 300 mM NaCl, 10% glycerol, 50 mM imidazole, 0.05% DDM with at least 10 column volumes and eluted with 50 mM HEPES pH 7.4, 300 mM NaCl, 10% glycerol, 400 mM imidazole, 0.05% DDM. Determination of fractions containing purified GlpG-His6 was established by SDS-PAGE running 12% acrylamide gels, which were visualized through Coomassie staining. GlpG protein quality was further tested using MALDI-TOF-MS. If required, GlpG-His6 protein was concentrated using an Amicon® Ultra Filter (Merck KGaA, Darmstadt, Germany) with a 10 kDa MWCO at 4°C.

**Cell-free expression of Ser-GlpG-His6**

*Preparation of plasmid DNA*
The *E. coli* GlpG WT sequence was cloned from the pET-25b(+) (Novagen) plasmid into the pIVEX2.3d plasmid (Biotechrabbit GmbH, Hennigsdorf, Germany) using PIPE cloning (38). To improve expression yields, a Ser-Tag (5’AAATCATCATCATCATCA-3’) was N-terminally fused to the GlpG WT sequence. C-terminally, the His-tag including a spacer sequence was fused to the GlpG wt sequence (5’-CCCGGGGGGGTTCTCATCATCATCATCATATTAA-3’).

For PIPE cloning, S7 fusion polymerase (Mobidiag, Espoo, Finnland) and the following primers were used according to the manufacturer’s protocol:

| Name        | Sequence                                      |
|-------------|------------------------------------------------|
| IPIPE_forward | 5’-CGGCACATATGAAATCATCATCATCATGATGATTACCTCTTTT GCTAACCC-3’ |
| IPIPE_reverse | 5’-GAACCCCCCCCGGGTTTTCGTTTTCGCCGATT-3’ |
| VPIPE_forward | 5’AATGCACCAAACGGAAACGGGGGGGGTTTC-3’ |
| VPIPE_reverse | 5’-GGGTTAGCAAAGAGGTAAATCATCAATGATGATGATGATGATTTC ATATGTGCG-3’ |

**Cell-free expression**

Cell-free Expression of Ser-GlpG-His6 was performed as previously described with the following specifications (39). The cell-free reaction was performed with an 18 mM Mg²⁺ concentration and for 24 h at 34°C in a total volume of 2 ml.

**GlpG purification and refolding procedures**

After cell-free expression, two different approaches were executed to obtain a renatured and active GlpG protease.

In the first approach, the precipitated GlpG protein of the cell-free reaction was pelleted by centrifugation (10,000 g, 10 min, 20°C) and washed three times with a 50 mM Tris, 150 mM NaCl, pH 7 buffer. Subsequently, GlpG was solubilized in 1.5% DDM in a 50 mM Tris, 150 mM NaCl, pH 7 buffer for 3 h at room temperature on a rotating wheel. Solubilized protein was then separated from insoluble protein using centrifugation (10,000 g, 15 min, 20°C) and finally applied to IMAC purification using an Äkta purifier system (GE Healthcare, Chicago, USA). After loading the solubilized protein onto the HisTrap column (GE Healthcare, Chicago, USA), the column was washed with at least 5 column volumes (CV) of a 50 mM Tris, 150 mM NaCl, 50 mM imidazol, 0.05% DDM, pH 7 buffer. Elution was performed using a 50 mM Tris, 150 mM NaCl, 500 mM imidazol, 0.05% DDM, pH 7 buffer. Finally, GlpG in DDM micelles were obtained.

In the second approach, solubilization in SDS, purification and reconstitution was performed as described (24, 39) with slide adaptations. Briefly, 18 ml of 50 mM sodium phosphate, 15 mM SDS and 50 mM DTT, pH 6.5 was added to the cell-free reaction mix and dialyzed for 24 h against 50 mM sodium phosphate, 15 mM SDS, pH 6.5 for the solubilization of the GlpG protein. Subsequently, the solution was adjusted to pH 8 and applied to IMAC purification using an Äkta purifier system and a HisTrap column (both from GE Healthcare, Chicago, USA). Elution was performed by a pH shift to 4.5. Finally, the purified GlpG was dialyzed for 60 h against 50 mM sodium phosphate, 2 mM SDS, 1 mM EDTA, pH 8.9 to reduce the SDS concentration before reconstitution of GlpG into DMPC membranes as described below.

Both, Ser-GlpG-Hi6 in DDM micelles and Ser-GlpG-Hi6 reconstituted in DMPC membranes were then tested on purity by SDS-PAGE and protease activity by substrate cleavage rates as described below.

**Reconstitution of GlpG into lipid membranes**
Two component lipid systems were mixed in 1:1 (v/v) of chloroform/MeOH (POPE/POPG, DPPC/Chol) and evaporated until dry using a rotary evaporator at 40°C. The dried two-components samples and one-component systems (POPC, DLPC, DMPC) were dispersed in either pH 7 buffer (50 mM Tris, 150 mM NaCl) or pH 4 buffer (50 mM NaAcetate, 150 mM NaCl) or for cell-free GlpG samples with pH 7 buffer (50 mM NaP, 1 mM EDTA, pH 7.8) to 10 mg/ml lipid. The lipid dispersions were extruded through 2 stacked 100 nm polycarbonate filters to produce large unilamellar vesicles using the lipid extruder (Lipex Biomembranes, Vancouver, BC, Canada) as described in the literature (40). DHPC was added to produce bicelles, at molar ratio detergent:lipid excess of 4-fold DMPC and DLPC and 6-fold for the rest of the lipids and lipid mixtures. DMPC and DLPC turned immediately transparent after DHPC addition. For the other lipid samples an incubation at 50°C for 30 min was required to acquire a clear solution. For substrate containing samples, LacYTM2 was added as powder in a substrate/GlpG molar ratio of 1.2 at the same time as DHPC addition. For activity measurements, the peptide substrate LacYTM2 (EDANS/DABCYL) were dissolved in a stock solution (1 mg/ml) in pH 4 buffer (50 mM NaAcetate, 150 mM NaCl, 10 mg/ml DHPC, 0.05 wt% DDM) and added before GlpG. GlpG was added in DDM, either adjusted to pH 4 or in elution buffer (50 mM Hepes, 300 mM NaCl. 10% (v/v) glycerol, 400 mM imidazole, 0.05% DDM, pH 7.4) to the bicelles. Cell-free GlpG was added in NaP buffer (50 mM NaP, 2 mM SDS, 1 mM EDTA, pH 7.8). Samples were diluted to 0.5-1 mg/ml GlpG if needed by detergent buffer at pH 4 or pH 7 that contained extra 0.05 wt% DDM for protein stability. GlpG-free samples were diluted to the same volume with the detergent buffers. The samples where then incubated for 20 min in a 42°C water bath and 20 min on ice for three times to integrate GlpG and the substrate into the bicelles. Afterward, 100 mg/ml BioBeadsSM2 (Bio-Rad, CA, USA) were added and samples were shaken overnight at 4°C to form multilamellar vesicles. The procedure was repeated for 4 h until the solution was turbid, normally two biobead cycles were required. The biobeads were removed by centrifugation (20 min at 21,500 × g) through 100 µm EASYstrainer filter (Greiner, Frickenhausen, Germany) at 4°C. 2H NMR samples were inserted into 4 mm MAS rotors and kinetic samples were stored in Eppendorf containers at -20°C before measurement.

Stationary 2H NMR spectroscopy and determination of order parameters and lipid geometry
2H NMR spectra were acquired on a Bruker 750 Avance I NMR spectrometer with a resonance frequency of 115.1 MHz for 2H. The NMR had a double channel solids probe equipped with a 5 mm solenoid coil. The phase-cycled quadrupolar echo sequence was used for signal acquisition (41). Pulse program included two π/2 pulses of 2.5-4 μs length separated by a 30 μs delay. The spectral width was ±250 kHz and the recycle delay was 1 s. All measurements were performed at 37°C. All analysis and parameters calculated from the NMR spectra was performed in programs written in the Mathcad software as described before (42, 43). The 2H spectrum was depaked and afterwards the order parameters were calculated (42,43). Projected chain lengths were calculated according to the mean-torque model (21).

Stationary 31P NMR measurements
After 2H NMR measurements samples were measured with stationary 31P NMR using a Bruker Avance III 600 MHz spectrometer using a PASEX probe head (XY) with a resonance frequency of 242.9 MHz. Hahn echo pulse sequence was used for the acquisition of the 31P NMR spectra with 10 μs length for the 90° pulse. An echo delay of 50 μs, a spectral width of 50 kHz, and relaxation delay of 3 s was used. Low power broadband 1H decoupling was applied during acquisition (ω1/2π = 2.5 kHz). All measurements were performed at 37°C. A program written in Mathcad 14.0 (Parametric Technology Corporation, Needham, MA, USA) were used to simulate the 31P NMR spectrum to extract the chemical shift anisotropy (Δσ), contributions for each numerical lineshape and orientation of the vesicles. Due to the anisotropic susceptibility of lipids, in some preparations
a slight orientation of lipids with the long axis perpendicular to the external magnetic field leading to a slight deformation of the vesicles is observed (44). POPE/POPG samples were simulated with two axially symmetric components and one isotropic component. The other samples were simulated with one axially symmetric component and one isotropic component.

$^{31}$P magic-angle spinning (MAS) NMR measurements
$^{31}$P MAS NMR spectra were acquired on a Bruker Avance Neo 700 MHz NMR spectrometer using a double resonance MAS probe with a 3.2 mm spinning module and a MAS frequency of 10 kHz. Typical 90° pulse lengths were 4 µs, the recycle delay was 3.5 s, and $^1$H low power decoupling ($\omega_H/2\pi = 2.1$ kHz) was applied during acquisition. $T_1$ and $T_2$ relaxation times were measured using standard inversion recovery and CPMG pulse sequences, respectively.

Protein and peptide quantification

Dissolution of the MLV sample
The MLV sample was resolved in pH 7 buffer (50 mM Tris, 150 mM NaCl) or pH 4 buffer (50 mM NaAcetate, 150 mM NaCl), respectively. A sample aliquot was mixed in a 1:1 (v/v) ratio with MLV resolving buffer (50 mM Tris, 150 mM NaCl, 50 mM SDS, pH 7) for complete dissolution of the MLV sample. Afterwards, either the GlpG protein or the LacYTM2 peptide amount was quantified as described below.

GlpG protein quantification
The concentration of the GlpG protein was determined by absorption measurements at 280 nm at a Nanodrop 1000 spectrometer (Nano Drop Technologies, Wilmington, DE, USA). Subsequently, the GlpG protein amount in the MLV sample was calculated using the Lambert-Beer law.

LacYTM2 peptide quantification
The concentration of the LacYTM2 peptide was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA USA) according to the manufacturer’s protocol and measured at an Infinite M200 plate reader (Tecan, Männedorf, Switzerland). The concentration of the LacYTM2 (EDANS/DABCYL) kinetic peptide variant was determined by absorption measurements of the DABCYL group at 453 nm. The final peptide concentration was then calculated from a calibration curve (linear model) recorded with the LacYTM2 (EDANS/DABCYL) peptide in DMSO.

Lipid quantification using HPTLC
The concentration of the lipids was measured using High-performance thin-layer chromatography (HPTLC) and densitometric analysis of the visualized lipid spots.

Quantitative lipid extraction
The MLV sample was resolved in pH 7 buffer (50 mM Tris, 150 mM NaCl) or pH 4 buffer (50 mM NaAcetate, 150 mM NaCl), respectively. For quantitative lipid extraction from the resolved MLV samples, Bligh and Dyer extraction (45) was chosen. Briefly, 100 µl of a buffer-resolved MLV sample was mixed with 200 µl CHCl₃/MeOH (1:1 (v/v)) for 30 sec. Subsequently, phase separation was obtained by centrifugation at 20°C and 10,000 g for 5 min. The CHCl₃ phase containing the lipids was carefully transferred into a new glass vial. To increase the quantity of lipid extraction, the extraction was repeated twice with the remaining sample with 100 µl CHCl₃ per repeated extraction step. The CHCl₃ phases were pooled and placed into the vacuum centrifuge RC 10.22 (Jouan, Saint-Herblain, France) to evaporate the organic solvent. The concentrated lipid film was then stored at -20°C until further analysis using HPTLC.
For lipid quantification, the concentrated lipid film was resolved in CHCl₃ and spotted on a HPTLC silica gel 60 plate (Merck KGaA, Darmstadt, Germany) using a CAMAG® Linomat 5 sample application system (CAMAG, Berlin, Germany) in addition to a lipid calibration standard on the same HPTLC plate. TLC was developed in a glass chamber (CAMAG, Berlin, Germany) with a mobile phase for lipid separation: chloroform / ethanol / water / triethylamine (30:35:7:35, v/v/v/v) for either 2-3 h (PC lipids) or 45 min (PE/PG lipids). Lipid spot visualization was achieved by primuline staining (100 mg primuline (Sigma-Aldrich, Taufkirchen, Germany) in 200 ml acetone / H₂O (4:1 (v/v)) and subsequent excitation under UV light. The lipid concentration in the MLV sample was finally calculated using the calibration curve (non-linear model) recorded with an appropriate lipid standard.

Dynamic light scattering (DLS)
The DLS measurements were performed at the Malvern Panalytica Zetasizer Ultra instrument (Malvern, UK) in backscatter mode. MLV samples were resuspended in 1 ml of millipore water. After filtering through a 100 µm cell sieve samples were transferred into a 4 ml polystyrene cuvette and measured.

Enzymatic cleavage assays in membranes
For functional studies, both GlpG and the peptide substrate LacYTM2 (EDANS/DABCYL) (molar ratio 1:1.2) were reconstituted in different lipid membranes as MLVs at pH 4, where GlpG protease activity is prevented. The fluorescence assay was performed with a GlpG concentration of 6 µM in a final volume of 50 µl at pH 7 and pH 4 at 37°C. Progress of substrate cleavage over time was monitored by measuring the EDANS fluorescence using Infinite M200 plate reader (Tecan, Männedorf, Switzerland). Progress curves were then analyzed and fitted using R (Version 4.1.0, R Foundation for Statistical Computing, Vienna, Austria) with the following exponential equation:

\[ I(t) = I_{\text{max}} - I_0 \cdot \exp\left(-t/\tau\right) \]

with \( I(t) \) being the EDANS fluorescence signal as a function of time, \( I_0 \) is the fluorescence at time point zero (= offset), \( I_{\text{max}} \) is the maximal fluorescence, and \( \tau \) a characteristic time constant.

Activity measurements in DDM micelles
GlpG WT or mutants (S201A, R137A, or G261A) were incubated with the kinetic peptide substrate LacYTM2 (EDANS/DABCYL) in DDM micelles and substrate cleavage was monitored over time. In each assay, 0.4 µM purified GlpG in DDM micelles were incubated with 10 µM kinetic substrate LacYTM2 (EDANS/DABCYL) in assay buffer (20 mM HEPES, 150 mM NaCl, 0.5% (v/v) DMSO, 0.05% DDM, pH 7.4) in a total volume of 50 µl at 37°C. Substrate cleavage was monitored by measuring the EDANS fluorescence using an Infinite M200 plate reader (Tecan, Männedorf, Switzerland). In contrast to the cleavage reaction in membranes, here, the progress curves did not reach the equilibrium. Therefore, the initial slope of the reaction was calculated from a linear regression of the measured data and was used as an indicator of GlpG protease activity. Subsequently, GlpG activities were plotted as jitter bar graphs and tested for significant differences using Welch two sample two-tailed t-test (both in R version 4.1.0, R Foundation for Statistical Computing, Vienna, Austria).

Activity measurements of cell-free (CF) expressed Ser-GlpG-His6
Protease activity of CF GlpG in DDM or CF GlpG in DMPC membranes were investigated by incubating GlpG with the kinetic peptide substrate LacYTM2 (EDANS/DABCYL) and compared to Rec. GlpG in DDM or DMPC, respectively.
In each assay, 0.4 µM purified GlpG was incubated with 10 µM kinetic substrate LacYTM2 (EDANS/DABCYL) in assay buffer (20 mM HEPES, 150 mM NaCl, 0.5% (v/v) DMSO, 0.05%
DDM, pH 7.4) in a total volume of 50 µl at 37°C. Substrate cleavage was monitored over time by measuring the EDANS fluorescence using an Infinite M200 plate reader (Tecan, Männedorf, Switzerland). Progress curves followed a nearly linear relationship. The slope of each reaction was calculated from a linear regression of the measured data and was used as an indicator of GlpG protease activity. Subsequently, GlpG activities were plotted as jitter bar graphs and tested for significant differences using Welch two sample two-tailed t-test (both in R version 4.1.0, R Foundation for Statistical Computing, Vienna, Austria).

**1H and 31P NMR spectroscopy in solution**

Using 31P solution NMR spectroscopy, detailed analysis on the lipid composition of the MLV samples in the presence and absence of GlpG was performed subsequently to 2H NMR measurements.

**solNMR sample preparation**

MLV samples were resolved in a cholate detergent solution (200 mM sodium cholate, 50 mM HEPES, 5 mM EDTA in D2O:H2O 1:8, pH 7.6-7.7) for solution NMR spectroscopy. In some cases, additional ultrasonication was required to obtain a clear solution. Trimethylsilylpropanoic-2,2,3,3-d4 acid (TSP-d4) and D2O were added to the sample for indirect referencing of the 31P chemical shift and locking of the sample, respectively.

**NMR spectroscopy**

31P and 1H solution NMR was performed at 37°C using a Bruker 600 MHz Avance III NMR spectrometer using a 5 mm PASEX probe. 1H NMR spectra were recorded as single pulse experiment using a 90° pulse of 13.5 µs. 31P NMR spectra were acquired using an Ernst angle optimized recycle delay/T1 ratio of 1 for an excitation pulse of 68° for 31P (7 µs). Additionally, Waltz16 decoupling with a decoupling field strength of \( \omega_{\text{H}} = 4 \) kHz was applied during the entire experiment to boost sensitivity using the nuclear Overhauser effect until a sufficient signal to noise (S/N) ratio was achieved. Spectra were processed and analyzed using TopSpin 4.0.8 (Bruker Biospin GmbH, Ettlingen, Germany).

To determine the quantity of the residual detergent DHPC in the MLV samples, NMR spectra were recorded for all different membrane systems used in this study with an inverse gated decoupling pulse sequence using an 90° excitation pulse of 10.1 µs, a recycle delay of 9 s and Waltz16 decoupling during acquisition to reach a very high S/N ratio (> 640). Spectra were processed and analyzed using TopSpin 4.0.8.

**Mass spectrometry**

**HPTLC-ESI-MS**

For qualitative lipid analysis, ESI-MS was performed after HPTLC. The lipid spots on the TLC were extracted for 45 s using a Plate Express™ TLC plate reader (Advion, Ithaca, NY, USA) with methanol and directly applied to an amazon SL ESI-MS spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Lipid ESI-MS was performed with the following parameters: spray voltage 4.5 kV, end plate offset 500 V, nebulizer gas 7.3 psi, drying gas (N2) 4 l/min, dry temperature 180°C, flow rate 3 µl/min, sheath gas (He) flow rate 25 a.u. The spectra were recorded using the alternating positive/negative mode including enhanced resolution. Measurements were analyzed using Bruker Daltonics software.

**MALDI-TOF-MS**

For MALDI-MS analysis of purified GlpG wt or mutants in DDM micelles, aliquots were desalted using C18 ZipTip™ Pipette Tips C18 (Merck KGaA, Darmstadt, Germany) according to the manufacturer’s protocol. MS was performed using alpha-Cyano-4-hydroxycinnamic acid (HCCA)
matrix (Merck KGaA, Darmstadt, Germany) on an autoflex speed mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) in linear mode. Measurements were analyzed by Bruker Daltonics software.

For MALDI-MS analysis of MLV samples, sample aliquots containing 100 µg GlpG protein were resolved in reconstitution buffer and subjected to a Bligh and Dyer extraction (45). Briefly, 100 µl of a buffer-resolved MLV sample was mixed with 200 µl CHCl3/MeOH (1:1 (v/v)) for 30 s. Subsequently, phase separation was obtained by centrifugation at 20°C and 10,000 g for 5 min. The CHCl3 phase containing the lipids was carefully removed. The remaining interphase (MeOH/water) was air-dried overnight. On the next day, the air-dried protein pellet was resolved in 40 µl 25% acetonitrile/75% methanol/1% trifluoroacetic acid. MS was performed using HCCA matrix (Merck KGaA, Darmstadt, Germany) on an autoflex speed mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) in linear mode. Measurements were analyzed by Bruker Daltonics software.

SDS-PAGE analysis
GlpG/membrane samples were mixed in a 1:1 (v/v) ratio with MLV resolving buffer (50 mM Tris, 150 mM NaCl, 50 mM SDS, pH 7) for complete dissolution of the MLV sample and allowing SDS-PAGE analysis. GlpG/DDM micelle samples did not need further preparation and could be directly loaded onto SDS gels. Samples containing 1.5 µg GlpG protein were separated by SDS-PAGE using either 16% Novex Tris-Glycine precast gels (Thermo Scientific, Waltham, MA USA) or using 4-20% Mini-PROTEAN® TGX™ precast protein gels (BioRad, Hercules, CA, USA) according to the manufacturer’s protocols. Subsequently, gels were applied either to conventional Coomassie staining, TAMRA fluorescence measurements or Western blot analysis depending on the assay purpose was performed as described below.

Western blot analysis
To investigate the presence of an intact C-terminus (His6-tag) of full length (FL) and cleaved GlpG, western blot analysis using the HisProbe™-HRP (Thermo Scientific, Waltham, MA USA) was performed on two selected GlpG/membrane samples. After SDS-PAGE analysis, the gel was tank blotted onto a nitrocellulose membrane (0.45 µm pore size, BioRad, Hercules, CA, USA) for 2 h with 25 V, 400 mA and 15 W. After western blotting, the HisProbe™-HRP was applied to the membrane and developing was subsequently performed on the using the Pierce™ DAB substrate kit according to the manufacturer’s protocols (both from Thermo Scientific, Waltham, MA USA).

GlpG protease activity assay using TAMRA in-gel fluorescence
GlpG protease activity was assayed in GlpG/membrane samples for all different membrane systems tested in this study (PC membranes and POPE/POPG membrane) after 2H NMR spectroscopy measurements using the ActivX TAMRA-FP Serine Hydrolase Probe (Thermo Scientific, Waltham, MA USA). As controls, appropriate GlpG/membrane samples were used which did not undergo 2H NMR spectroscopy.

MLV sample aliquots containing 1.5 µg GlpG protein were resolved in pH 7 buffer (50 mM Tris, 150 mM NaCl) and incubated with 0.5 µM ActivX TAMRA-FP Serine Hydrolase Probe (Thermo Scientific, Waltham, MA USA) in a total volume of 10 µl at 30°C for 1 h 45 min. The reaction was stopped by the addition of SDS sample buffer and immediate heating at 95°C for 4 min. Afterwards, SDS-PAGE was performed as described above. TAMRA in-gel fluorescence was measured using a G:BOX Imaging System (Syngene, Cambridge, UK). As a protein loading control, SDS-gels were stained with Coomassie Brilliant Blue R 250 after fluorescence measurements.
**Fig. S1.**

$^2$H NMR spectra of membranes in the presence and absence of GlpG. A different sample conditions for WT GlpG. Columns indicate the lipid mixture and the deuterated lipid. Rows indicate the pH of the sample, and the presence or absence of GlpG and/or LacYTM2 substrate. **B** $^2$H NMR spectra of POPE-$d_{31}$/POPG 3:1 in the presence of different variants of GlpG at pH 4, **C** $^2$H NMR spectra of POPE/POPG-$d_{31}$ 3:1 in the presence of different variants of GlpG at pH 4. All measurements were performed at 37°C.
Fig. S2.
**Lipid to protein/peptide (L/P) ratios of reconstituted membrane samples.** Lipid to protein and lipid to peptide ratios were measured from different GlpG protein and/or LacYTM2 peptide reconstituted membranes after $^2$H NMR measurements. Sample preparations intended a lipid to protein/peptide ratio of **A** L/P: 100 (GlpG protein samples in black, blue and green), L/P: 83 (LacYTM2 peptide samples in red) or **B** L/P: 100, 200 and 350 (GlpG protein samples in blue) and ended up in the respective values as shown in the plots. Data are presented as mean ± SEM.
Fig. S3.

DHPC detergent analysis of membrane samples using $^{31}$P NMR. Membrane samples were solubilized in cholate detergent and investigated for remaining DHPC after reconstitution. For a sufficient resolution of the $^{31}$P NMR spectra and to separate the lipids from the DHPC signals, the spectra from the membrane samples (colored) are overlaid with respective spectra from lipid-DHPC controls (gray). Lipid signals of the membrane samples were measured up to very high S/N ratios ($\geq 640$) and the amount of lipid and DHPC was quantified by peak integration. No DHPC was detectable in the reconstituted membrane samples except for a very little amount (~1.5% DHPC) in the case of POPC.
Fig. S4.
DDM detergent analysis of membrane samples by $^1$H NMR. Different membrane samples were solubilized in cholate detergent and investigated for remaining DDM after reconstitution using $^1$H NMR. A Reference sample of DDM/DMPC (4:1). B Sample of GlpG reconstituted into DMPC (90%) with 10% PE/PG mix. C Sample of S201A GlpG reconstituted into POPE/POPG membranes. Asterisks and red shaded areas indicate prominent signals from DDM.
Fig. S5.

Lipid analysis of GlpG reconstituted POPE/POPG membranes. A $^{31}$P NMR spectra of GlpG/membrane preparations solubilized in cholate in comparison with a Lyso-PE (L-PE 18:1) control sample. GlpG samples were reconstituted with different lipid to protein ratios (L/P ratios) and with either POPE or POPG deuterated as indicated. B TLC-ESI-MS from a representative GlpG reconstituted POPE-d/POPG membrane sample. TLC Spots are indicated as follows: FFAs (free fatty acids, POPG, POPE, Lyso-PE (L-PE). POPE and Lyso-PE spots were investigated using ESI-MS upon extraction from the TLC plate. The ESI-MS spectrum of POPE confirms deuteration of POPE. For the Lyso-PE, the m/z value and the absence of the deuteration pattern in the MS spectrum strongly indicates a L-PE 18:1 species resulting from POPE cleavage at the sn-1 position.
Fig. S6.

$^2$H NMR spectra and lipid acyl chain length, $^{31}$P NMR spectra and functional analysis of POPC/POPG (3:1, mol/mol) membranes in the absence and in the presence of GlpG. A $^2$H NMR spectra of POPC/POPG multilamellar vesicles at a temperature of 37°C in the absence and in the presence of GlpG, either POPC or POPG is $^2$H labeled, indicated by “–d$_{31}$”. B Acyl chain length of either POPC or POPG in the mixture determined from the $^2$H NMR spectra according to the mean torque model (21). C Stationary $^{31}$P NMR spectra of the vesicles in the absence and in the presence of GlpG also at 37°C with numerical lineshape simulations shown in red. D Activity assay of GlpG in POPC/POPG membranes and in pure POPC membranes for comparison showing identical kinetics in the EDANS fluorescence increase for both membrane systems.
**Fig. S7.**

**Stationary $^{31}$P solid-state NMR spectra of different GlpG-containing membranes.** Black lines indicate original NMR spectrum and colored line the simulated spectrum. A Membranes containing WT GlpG. Sample conditions are indicated in the title. B Membranes containing mutated GlpG S201A, R137A and G261A. C Phase contribution from the different components determined from numerical lineshape simulations. Chemical shift anisotropy ($\Delta \sigma$) and magnetic orientation ($\alpha$) is indicated next to spectrum. Due to the anisotropic susceptibility of lipids, in some preparations a slight deformation of the vesicles due to the preferential orientation of lipids with the long axis perpendicular to the external magnetic field is observed. C Phase contributions to the $^{31}$P NMR spectra for all preparation. All measurements were performed at 37°C.
Fig. S8.
Dynamic light scattering results of POPE/POPG (3:1, mol/mol) multilamellar vesicles in the absence (gray) and in the presence of GlpG (violet). Shown are the intensity-based size distributions of these multilamellar vesicles. Data were recorded using the backward scatter only mode. Three individual runs from the same preparation for each sample are shown.
Fig. S9.

$^{31}$P NMR spectra of POPE/POPG membranes (3:1 mol/mol) in the presence of increasing concentrations of lyso-PE at 37°C. NMR spectra were recorded in the absence (A) and in the presence of 7 mol% (B), 13 mol% (C), and 38 mol% lyso-PE (D). Experimental spectra are shown in black and best fit lineshape simulations in red (44).
Fig. S10.

$^{31}$P magic-angle spinning (MAS) NMR spectra and relaxation time measurements of membrane models in the absence and in the presence of GlpG. 

A $^{31}$P MAS NMR spectra at of POPC (left) POPC/POPG (3:1, mol/mol) (middle), and POPE/POPG (3:1, mol/mol) (right) membranes in the absence (top) and in the presence (bottom) of GlpG. Spectra were recorded at a MAS frequency of 10 kHz and a temperature of 37°C. Red lines indicate the lineshape deconvolution for the POPC/POPG sample in the presence of GlpG.

B $T_1$ relaxation times for each lipid component in the absence (gray) and in the presence of GlpG (color). In the POPE/POPG membrane in the presence of GlpG, both bars are identical as the POPE and the POPG signal could not be separated. Error bars represent the uncertainty from fitting experimental integrals.
Control assays of the GlpG mutants S201A, R137A and G261A. A GlpG protease activity of purified GlpG WT and variants (S201A, R137A or G261A) were determined in DDM micelles using the substrate LacYTM2 (EDANS/DABCYL). Protease activities were normalized to GlpG WT activity and compared with all GlpG variants. Each single mutation (S201, R137A or G261A) in the GlpG resulted in almost complete loss of protease activity. Data are presented as mean ± SD. B Lipid to protein ratios were measured from GlpG (wt or variants) reconstituted POPE/POPG membranes after ²H NMR experiments. Sample preparations intended a lipid to protein ratio of 100 and ended up as shown. Data are presented as mean ± SEM. C ³¹P NMR spectra of GlpG WT or variant (S201A, R137A or G261A) reconstituted POPE/POPG membrane samples solubilized in cholate.
Fig. S12.

Structural integrity of the GlpG protein core after ²H NMR experiments. A TAMRA-probe activity assay was performed on several GlpG reconstituted samples using different membranes after ²H NMR experiments. Each SDS-PAGE gel was performed with a marker (lane M), control GlpG/membrane sample (lane con), GlpG/membrane sample reconstituted at pH 7 (lane 1), GlpG/membrane sample reconstituted at pH 4 (lane 2), GlpG/LacYTM2/membrane sample reconstituted at pH 4 (lane 3). The samples were brought to pH 7 and the assay performed as described before. Gels were first investigated for TAMRA fluorescence (TAMRA) and stained afterwards with Coomassie Brilliant Blue (CBB) for comparison. The TAMRA probe fluorescence for all tested GlpG/membrane samples was similar to the respective controls indicating a correctly folded tertiary structure of the protease. Since GlpG appeared in two distinct bands on the gel in several cases, further investigations on the GlpG truncation were executed. B Western blot analysis using an anti-His-tag-probe was performed on two selected GlpG/membrane samples and demonstrated that full length (FL) GlpG and cleaved GlpG contain an intact C-terminus. C MALDI-TOF mass spectrum of a GlpG/DMPC membrane sample performed after Bligh and Dyer extraction using a HCCA matrix. The spectrum indicates a mass difference between GlpG FL and cleaved GlpG of about 8.5 kDa. Taken together, these data provide evidence that the truncated GlpG protein is cleaved in the cytosolic N-terminal domain in the region of residues 77-78 and thus the transmembrane protein core of GlpG remains intact during ²H NMR experiments.
Table S1.
Average order parameters, projected chain lengths ($L_c$), number of gauche defects per chain, and area per molecule for differently deuterated lipids in the presence and absence of different GlpG variants and LacYTM2 substrate at different pH values at 37°C.

| Lipid           | pH | GlpG | LacYTM2 | $S_{CD}$ | $L_c$ (Å) | # gauche defects | A/Å² | ΔA/Å²* |
|-----------------|----|------|---------|---------|----------|-----------------|------|--------|
| DLPC-$d_{46}$   | 7  | -    | -       | 0.149   | 9.6      | 4.0             | 66.1 |        |
| DLPC-$d_{46}$   | 4  | -    | -       | 0.150   | 9.7      | 3.9             | 65.8 |        |
| DLPC-$d_{46}$   | 7  | WT   | -       | 0.158   | 9.9      | 3.7             | 64.3 | -1.8   |
| DLPC-$d_{46}$   | 4  | WT   | +       | 0.156   | 9.8      | 3.8             | 64.7 | -1.1   |
| DLPC-$d_{46}$   | 4  | -    | +       | 0.149   | 9.6      | 4.0             | 66.1 |        |
| DMPC-$d_{54}$   | 7  | -    | -       | 0.166   | 11.8     | 4.3             | 63.1 |        |
| DMPC-$d_{54}$   | 4  | -    | -       | 0.165   | 11.7     | 4.4             | 63.4 |        |
| DMPC-$d_{54}$   | 7  | WT   | -       | 0.167   | 11.8     | 4.3             | 63.0 | -0.1   |
| DMPC-$d_{54}$   | 4  | WT   | -       | 0.164   | 11.7     | 4.4             | 63.5 | 0.1    |
| DMPC-$d_{54}$   | 4  | WT   | +       | 0.160   | 11.7     | 4.4             | 63.6 | 0.2    |
| DMPC-$d_{54}$   | 4  | -    | +       | 0.159   | 11.6     | 4.5             | 63.9 |        |
| DPPC-$d_{62}$/Chol 2:1 | 7  | -    | -       | 0.353   | 17.9     | 1.0             | 47.4 |        |
| DPPC-$d_{62}$/Chol 2:1 | 4  | -    | -       | 0.358   | 18.0     | 1.0             | 47.1 |        |
| DPPC-$d_{62}$/Chol 2:1 | 7  | WT   | -       | 0.347   | 17.8     | 1.2             | 47.7 | 0.3    |
| DPPC-$d_{62}$/Chol 2:1 | 4  | WT   | -       | 0.355   | 18.0     | 1.0             | 47.2 | 0.1    |
| DPPC-$d_{62}$/Chol 2:1 | 4  | WT   | +       | 0.344   | 17.7     | 1.3             | 48.0 | 0.9    |
| DPPC-$d_{62}$/Chol 2:1 | 4  | -    | +       | 0.357   | 18.0     | 1.0             | 47.2 |        |
| POPC-$d_{31}$   | 7  | -    | -       | 0.137   | 12.2     | 6.2             | 69.2 |        |
| POPC-$d_{31}$   | 4  | -    | -       | 0.138   | 12.3     | 6.1             | 68.9 |        |
| POPC-$d_{31}$   | 7  | WT   | -       | 0.138   | 12.4     | 6.1             | 68.6 | -0.6   |
| POPC-$d_{31}$   | 4  | WT   | -       | 0.135   | 12.2     | 6.2             | 69.6 | 0.7    |
| POPC-$d_{31}$   | 4  | WT   | +       | 0.137   | 12.3     | 6.2             | 69.1 | 0.2    |
| POPC-$d_{31}$   | 4  | -    | +       | 0.145   | 12.6     | 5.9             | 67.2 |        |
| POPE-$d_{31}$/POPG | 7  | -    | -       | 0.187   | 14.0     | 4.6             | 60.6 |        |
| POPE-$d_{31}$/POPG | 4  | -    | -       | 0.190   | 14.1     | 4.5             | 60.2 |        |
| POPE-$d_{31}$/POPG | 7  | WT   | -       | 0.154   | 12.9     | 5.6             | 65.6 | 5.0    |
| POPE-$d_{31}$/POPG | 4  | WT   | -       | 0.156   | 13.0     | 5.5             | 65.3 | 5.1    |
| POPE-$d_{31}$/POPG | 4  | WT   | +       | 0.149   | 12.8     | 5.7             | 66.5 | 6.3    |
| POPE-$d_{31}$/POPG | 4  | -    | +       | 0.178   | 13.7     | 4.9             | 61.9 |        |
| POPE/POPG-$d_{31}$ | 4  | -    | -       | 0.187   | 14.0     | 4.6             | 60.6 |        |
| POPE/POPG-$d_{31}$ | 4  | WT   | -       | 0.157   | 13.1     | 5.5             | 65.0 | 4.4    |
| POPE-$d_{31}$/POPG 3:1 | 4  | S201A | -       | 0.161   | 13.2     | 5.4             | 64.5 | 4.3    |
| System          | Mutation | Charge | Width  | Height | IP |
|-----------------|----------|--------|--------|--------|----|
| POPE-d31/POPG 3:1 | R137A    | 0.159  | 13.0   | 5.5    | 65.0 | 4.8 |
| POPE-d31/POPG 3:1 | G261A    | 0.146  | 12.6   | 5.9    | 67.3 | 7.1 |
| POPE/POPG-d31 3:1 | S201A    | 0.154  | 12.9   | 5.6    | 65.9 | 5.3 |
| POPE/POPG-d31 3:1 | R137A    | 0.160  | 13.1   | 5.4    | 64.8 | 4.2 |
| POPE/POPG-d31 3:1 | G261A    | 0.149  | 12.8   | 5.7    | 66.4 | 5.9 |
| POPC-d31/POPG 3:1 | -        | 0.131  | 12.0   | 6.4    | 70.8 |
| POPC-d31/POPG 3:1 | WT       | 0.139  | 12.3   | 6.1    | 68.9 | -1.9 |
| POPC/POPG-d31 3:1 | -        | 0.136  | 12.2   | 6.2    | 69.6 |
| POPC/POPG-d31 3:1 | WT       | 0.135  | 12.2   | 6.3    | 69.7 | 0.2 |

* compared to the absence of GlpG
| Value                      | DLPC       | DMPC       | DPPC/Chol  | POPC       | POPE/POPG  |
|----------------------------|------------|------------|------------|------------|------------|
| \( \tau / s \)            | 1139 ± 129 | 117 ± 17   | > 10835 ± 4298 | 309 ± 92   | 393 ± 126  |
| \( \Delta \text{RFU / RFU} \) | 145 ± 17   | 127 ± 19   | 17 ± 7     | 141 ± 1    | 150 ± 8    |
| Substrate/Protein Ratio    | 1.27 ± 0.06| 1.05 ± 0.05| 0.96 ± 0.03| 0.98 ± 0.03| 1.08 ± 0.13|
| Lipid/Protein Ratio        | 178 ± 5    | 235 ± 12   | 72 ± 5     | 93 ± 3     | 154 ± 13   |

*Data are presented as median ± SEM*