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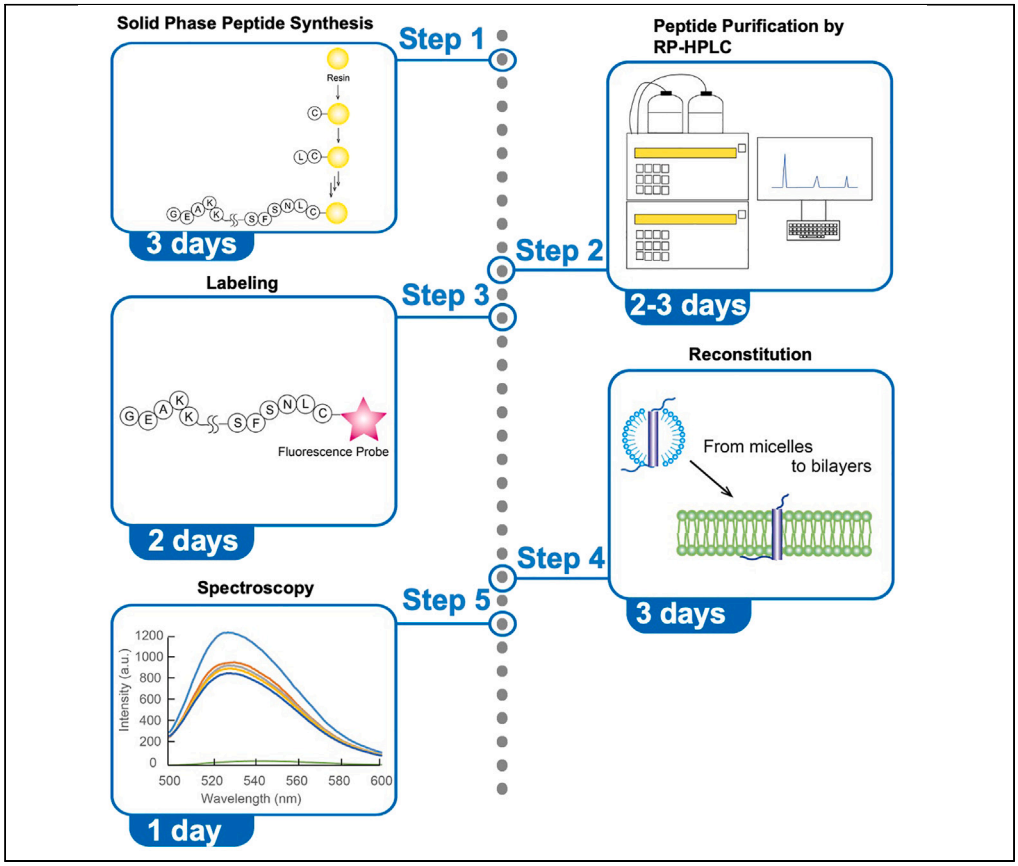
Characterizing interaction between the juxtamembrane region of the single transmembrane protein and membrane using chemically synthesized peptides



In membrane proteins, a transmembrane region and a juxtamembrane region play important roles in its function. Here, we present a protocol for characterizing membrane protein dynamics between the juxtamembrane region of the single transmembrane protein and acidic membrane. We describe steps for solid-phase peptide synthesis, peptide purification, and labeling. We then detail reconstitution of the transmembrane peptide into lipid bilayers and its evaluation and structural analysis.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Chemical synthesis of transmembrane-juxtamembrane peptide

Reconstitution of transmembrane peptide into lipid bilayers

Spectroscopies on the transmembrane-juxtamembrane region of membrane protein

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Characterizing interaction between the juxtamembrane region of the single transmembrane protein and membrane using chemically synthesized peptides

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SUMMARY

In membrane proteins, a transmembrane region and a juxtamembrane region play important roles in its function. Here, we present a protocol for characterizing membrane protein dynamics between the juxtamembrane region of the single transmembrane protein and acidic membrane. We describe steps for solid-phase peptide synthesis, peptide purification, and labeling. We then detail reconstitution of the transmembrane peptide into lipid bilayers and its evaluation and structural analysis.

For complete details on the use and execution of this protocol, please refer to Prasada Rao et al.[1]

BEFORE YOU BEGIN

Knowing the structure of a target protein is a huge advantage to proceed with the analysis of its function and dynamics. For membrane protein, however, its hydrophobic and aggregation-prone nature has been an obstacle to obtaining structural information. Especially, a membrane protein with a single transmembrane region, a single transmembrane protein, has been recognized as one of the difficult targets and has not been crystalized for its structural analysis. Since there has been no structure in full length reported, structural biology on the single transmembrane protein has been performed in the "putting together" strategy[2] where analyses are separately performed for an extracellular, an intracellular or a transmembrane region and results are to "put together" to understand a molecular mechanism or dynamics for the function.

This protocol introduced here is built up on a basis of an experimental strategy for structural analysis on a transmembrane peptide with the sequence of transmembrane region and juxtamembrane region, a region adjoining to the transmembrane region, using spectroscopies such as FT-IR (Fourier Transform Infrared spectroscopy) and fluorescence spectroscopies. This protocol has also been utilized for structural analyses in transmembrane peptides using solid state NMR.[3,4] A critical point in the protocol is "reconstitution", which is a conventional name for peptide insertion into membrane bilayers although it is not really "reconstituting". Among multiple ways of the reconstitution procedure, here we introduce the detergent dialysis strategy. This strategy provides reproducible results on polarized FT-IR measurements for measuring the tilt of the transmembrane helix relative to the membrane, which can be the first step for further analysis in the system.

The membrane-reconstituted system provides a selection of lipids in variety. Any type of lipids can be incorporated into the membrane. In this protocol, a procedure to change the lipid component *in*

situ is also introduced. This procedure allows analyzing a behavior, namely an association with the membrane, of the juxtamembrane region, depending on a change in membrane component.[3,4]

Here, we briefly mention the background of our research where the reconstituted system was applied. During meiosis, proteins on the nuclear envelope (NE), namely, SUN- and KASH-domain proteins, promote telomere movement, thus, chromosome motion. Budding yeast SUN-domain protein, Mps3, forms multiple meiosis-specific ensembles on NE, which show dynamic localization for chromosome motion. In the original research where this protocol herein is followed,[1] we showed that the phosphorylation in the luminal juxtamembrane region, which is comprised of 30 amino acid residues adjoining the transmembrane region, of Mps3 juxtaposed to the inner nuclear membrane is required for meiosis-specific localization of Mps3 on NE. In this protocol, we demonstrate a way to show a theoretical and chemical background regarding the phosphorylation-induced structural change in Mps3. As a result, we found that negative charges introduced by meiosis-specific phosphorylation in the luminal region of Mps3 alter its interaction with negatively charged lipids by electric repulsion in reconstituted liposomes.

The membrane reconstituting system demonstrated here must provide an experimental strategy for a deeper understanding of biological chemistry on the membrane. This technique can also be applied to other single transmembrane proteins such as receptor tyrosine kinases,[3–5] cytokine receptors[6] and proteins involved in regulated intramembrane proteolysis (amyloid precursor protein[7]).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals		
NovaPEG Rink Amide resin LL	Novabiochem	Cat#15402-35
OxymaPure	Watanabe Chemical Industries	Cat#A00699
Diisopropylcarbodiimide (DIC)	Watanabe Chemical Industries	Cat#A00011
Piperazine	Kanto Chemicals	Cat#13129-1A
1-Hydroxybenzotriazole (HOBt)	Peptide Institute	Cat#1022
Diethyl ether	Nacalai Tesque	Cat#15401-45
Acetonitrile	FUJIFILM Wako Chemical Corporation	Cat#014-00381
Dimethylformamide (DMF)	Kanto Chemicals	Cat#10344-2B
N-methylpyrrolidone (NMP)	Nacalai Tesque	Cat#23033-83
Diisopropylethylamine (DIEA)	Nacalai Tesque	Cat#14014-84
Trifluoroacetic acid	Nacalai Tesque	Cat#34831-25
Triisopropylsilane(TIS)	TCI	Cat#T1533
3,6-Dioxa-1,8-octanedithiol	TCI	Cat#D2649
octyl-β-D- glucoside	Anatrace	Cat#O311
1,2-Dioctanoyl-sn-glycero-3-phospho-(1′-myo-inositol-4′,5′-bisphosphate) (ammonium salt)	Avanti Polar Lipids	Cat#850-185P
1-Palmitoyl-2-oleoyl-glycero-3-phosphocholine	Avanti Polar Lipids	Cat#850-457P
1-Palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (sodium salt)	Avanti Polar Lipids	Cat#840034P
Fmoc-Ala-OH	Peptide Institute	Cat#2301
Fmoc-Cys(Trt)-OH	Peptide Institute	Cat#2323
Fmoc-Glu(OtBu)-OH	Peptide Institute	Cat#2308
Fmoc-Asp(OtBu)-OH	Peptide Institute	Cat#2305
Fmoc-Phe-OH	Peptide Institute	Cat#2315
Fmoc-Gly-OH	Peptide Institute	Cat#2309
Fmoc-His(Trt)-OH	Peptide Institute	Cat#2310
Fmoc-Ile-OH	Peptide Institute	Cat#2311
Fmoc-Lys(Boc)-OH	Peptide Institute	Cat#2313
Fmoc-Leu-OH	Peptide Institute	Cat#2312

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fmoc-Met -OH	Peptide Institute	Cat#2314
Fmoc-Asn(Trt)-OH	Peptide Institute	Cat#2325
Fmoc-Pro-OH	Peptide Institute	Cat#2316
Fmoc-Gln(Trt)-OH	Peptide Institute	Cat#2326
Fmoc-Arg(Pbf)-OH	Peptide Institute	Cat#2324
Fmoc-Ser(tBu)-OH	Peptide Institute	Cat#2415
Fmoc-Thr(tBu)-OH	Peptide Institute	Cat#2416
Fmoc-Val-OH	Peptide Institute	Cat#2419
Fmoc-Trp(Boc)-OH	Peptide Institute	Cat#2424
Fmoc-Tyr(tBu)-OH	Peptide Institute	Cat#2418
AlexaFluor 568 C5 maleimide	Invitrogen	Cat#A20341
MOPS	Nacalai Tesque	Cat# 23438-35
ODS column (Cosmosil 5C18-ARII 10 × 250 mm)	Nacalai Tesque	Cat#38023-11
C4 column (Cosmosil 5C4-AR300 10 × 250 mm)	Nacalai Tesque	Cat# 38047-11
Software and algorithms		
NAMD	Theoretical and Computational Biophysics group at University of Illinois at Urbana-Champaign	https://www.ks.uiuc.edu/Research/namd/
Fityk	Fityk	https://fityk.nieto.pl
Other		
Liberty Blue Microwave assisted peptide synthesizer	CEM	
FT-IR spectrometer	Jasco	FT-IR4600st
Diaphragm type vacuum pump	Eyela	EVP-1200
ISOLUTE double fritted filtration column	Biotage	Cat#120-1026-E
Mass spectrometer	Bruker	autoflex
Lyophilizer	Eyela	FDU-1110
High-speed shaker	Eyela	Cat#CM-1000
Falcon conical tube 50 mL	Corning	Cat#352070
Microtube 1.5 mL	Eppendorf	Cat#0030125150
Microtube 0.5 mL	Treff	Cat#96.04625.9.01
Extruder	Avanti Polar Lipids	Cat#610023-1EA
Polycarbonate membrane 0.1 μm	Avanti Polar Lipids	Cat#610005-1EA

MATERIALS AND EQUIPMENT

Reagents for the peptide synthesizer: deprotecting cocktail

Reagent	Final concentration	Amount
Piperazine	10%	50 g
1-hydroxybenzotriazole (HOBt)	0.1 M	6.8 g
N-methyl-2-pyrrolidone (NMP)	N/A	Adjust to 500 mL
Total		**500 mL**

Keep at 4°C. However, prepare the solution in the quantity that is required, and immediate use is recommended.

Reagents for the peptide synthesizer: activating cocktail1

Reagent	Final concentration	Amount
Diisopropylcarbodiimide (DIC)	0.5 M	12.6 g
Dimethylformamide (DMF)	N/A	Adjust to 200 mL
Total		**200 mL**

Keep at 4°C. However, prepare the solution in the quantity that is required for the synthesis and immediate use is recommended.

Reagents for the peptide synthesizer: activating cocktail2		
Reagent	Final concentration	Amount
1-hydroxybenzotriazole (HOBt)	0.5 M	13.5 g
Dimethylformamide (DMF)	N/A	Adjust to 200 mL
Total		**200 mL**

Keep at 4°C. However, prepare the solution in the quantity that is required for the synthesis and immediate use is recommended.

Reagents for the peptide synthesizer: activating cocktail3		
Reagent	Final concentration	Amount
OxymaPure	0.5 M	2.1 g
Dimethylformamide (DMF)	N/A	Adjust to 30 mL
Total		**30 mL**

Keep at 4°C. However, prepare the solution in the quantity that is required for the synthesis and immediate use is recommended.

A cocktail for peptide deprotection and cleavage		
Reagent	Final concentration	Amount
Trifluoroacetic acid (TFA)	90% (v/v)	1850 μL
ddH_2O	5% (v/v)	50 μL
Triisopropylsilane (TIS)	2.5% (v/v)	50 μL
3,6-Dioxa-1,8-octanedithiol	2.5% (v/v)	50 μL
Total		**2 mL**

⚠ CRITICAL: Be sure to do everything in the hood when reagents are mixed. Ware glasses and gloves.

Dialysis buffer pH7.0		
Reagent	Final concentration	Amount
MOPS	1 mM	4.2 g
KCl	100 mM	149 g
ddH_2O	N/A	20 L
Total		**20 L**

Keep at 4°C.

STEP-BY-STEP METHOD DETAILS

Peptide synthesis on the peptide synthesizer

Timing: 3 days

This section describes peptide synthesis on a microwave-assisted peptide synthesizer. In this research, microwave-assisted solid phase peptide synthesis (SPPS) was carried out using a Liberty Blue peptide synthesizer (CEM, NC). The biggest benefit of using the peptide synthesizer is that once it is started, there is no need to look after until it completes the synthesis. Since it was planned to perform fluorescence measurements, cysteine (Cys) residue, where a fluorescence probe was attached, was added to the C-terminus of the peptide. The sequence for the transmembrane-juxtamembrane region of Mps3 (Mps3TMJM) was as follows:

GEAKKLKWRTYIFYGGLFFVFYFFGSFLMTTVKNNDLESHSSGATSSPGKSFSNL-C.

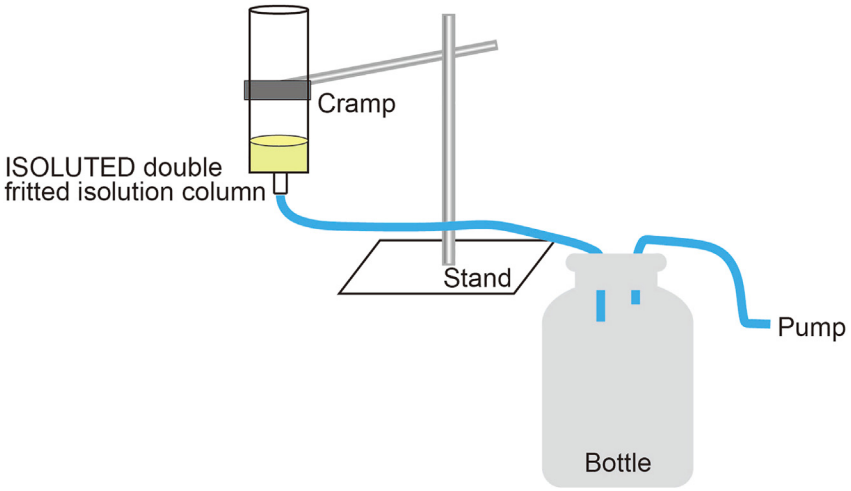

Figure 1. Flow for extraction of the peptide component and the experimental setups

1. Scale for the synthesis is set to 0.1 mmol. Weigh 400 mg of the resin (NovaPEG Rink Amide resin; 0.25 mmol/g).
2. Setting the resin on the synthesizer.
   a. Put the resin into a double-fritted filtration column and add dimethylformamide (DMF, 2 mL). Make sure that both ends of the column are capped.
   b. Vortex it for swelling the resin for 10 min at 25°C and then let the resin precipitate by gravity.
   c. Then the solvent is removed by flowing through the bottom end of the column. An experimental setup is shown in Figure 1. The resin is ready to be placed, which can be accomplished by using a spatula, in the reaction vessel on the synthesizer.
3. Type in the peptide sequence and make a protocol for the peptide elongation on the synthesizer.
   a. The scale for the synthesis is set to 0.1 mmol.
   b. For the deprotection scheme, the temperature is set to be 75°C for 15 s and to 90°C for 50 s 10% piperazine with 0.1 M 1-hydroxybenzotriazole (HOBt) in NMP is employed as a Fmoc group deprotecting reagent.
   c. For the coupling scheme, the temperature is set to be 75°C for 15 s and 90°C for 110 s. All of the residues except His, Cys, Arg residues, Diisopropylcarbodiimide (DIC) and HOBt (0.5 mmol each) are employed as activating reagents and a single coupling is performed.
   d. For His and Cys residues, DIC and HOBt (0.5 mmol each) are employed as activating reagents and the single coupling reaction is performed at 25°C for 120 s and at 50°C for 480 s.
   e. For Arg residue, DIC and OxymaPure (0.5 mmol each) are employed as activating reagents and the double coupling is performed at 75°C for 15 s and at 90°C for 180 s.
4. Run peptide synthesis on the synthesizer. Average time for a single cycle except His, Cys, Arg residues, is about 5 min. For His and Cys, the cycle takes about 10 min. For Arg, the cycle takes about 12 min.
5. Post-treatment after the synthesis.
   a. The peptide resin in the reaction vessel is collected in a glass vial.
   b. Pour methanol into the vial to wash the peptide resin and then remove methanol by decantation. "Pipetting" can be applied for an effective wash.
   c. Do this washing cycle three times. Dry the resin under vacuum overnight.

Note: In the original research,[1] the peptide elongation was successfully performed using the microwave-assisted peptide synthesizer. Applying microwave in deprotection and coupling steps accelerates peptide synthesis and increases the yield. The procedure mentioned here requires a microwave irradiating device. Although it is time-consuming, a similar outcome in peptide synthesis can be achieved manually without the microwave. Increasing the temperature to 37°C in the coupling step might be a tip for better yield.

Deprotection and cleavage of the peptide from the resin

Timing: 4 h

In the previous section, the peptide chain elongation was accomplished on the resin. Here, the peptide component is cleaved from the resin and is deprotected. Be sure to do everything in the hood, and wear glasses and gloves since the reagent TFA is hazardous.

6. Preparation of a cocktail for deprotection and cleavage.
   a. In a glass tube, mix water (50 μL), triisopropylsilane (TIS,50 μL), 3,6-Dioxa-1,8-octanedithiol (50 μL), and Trifluoroacetic acid (TFA, 1850 μL). The total volume is 2 mL.
7. Cleavage and deprotection of the peptide from the resin.
   a. Weigh 100 mg of the peptide resin in the Falcon tube (15 mL in size).
   b. Add the cleavage cocktail (2 mL) to the resin in the Falcon tube and shake on the high-speed mixer for 2.5 h at 25°C. The speed of the shake is not critical as long as resins are moving around in the solvent.

   Optional: The mixture can be stirred using a stirring bar magnet with a magnetic stirrer.

8. Extraction of the peptide component.
   a. Prepare a ISOLUTE double fritted filtration column on a cramp and place a Falcon tube (15 mL in size) under it (Figure 2: Experimental setup for resin removal).
   b. Pour the reaction mixture to the reaction vessel, leaving the resin component on the filter. The reaction mixture solution is passed through the filter into the Falcon tube.
   c. Preparation of Ar gas. Connect one side of a gas hose to an Ar gas cylinder and place a 200 μL pipet tip to the other end that goes into the Falcon tube (Figure 2: Experimental setup for solvent removal).
   d. Direct a slow stream of Ar gas to remove the solvent component of the reaction mixture. Extreme caution must be paid not to expose yourself to splash from the tube. Exposing TFA on your skin causes burn injury. The amount of the Ar gas to blow up the solvent must carefully be adjusted for your safety. A strong flow of the gas must remove the pipet tube placed at the end of the hose, which may also cause the splash.
   e. To precipitate the peptide component, add cold diethyl ether (up to the top of the Falcon tube, which is about 15 mL) to the resultant solid in the tube, then fluffy white solid, peptide components, appears.
   f. Spin down the white solid and decant out the supernatant, diethyl ether. This procedure is to wash out small fragments of protecting groups that have been cleaved from the peptide and captured by the scavengers. Add diethyl ether again, vortex, spin down the solid, and decant out the supernatant. Do this cycle for three times.
   g. To the resultant solid, peptide components, add a mixture of acetonitrile and water (1/1, in a total of 8 mL) and lyophilize overnight at 25°C. A fluffy powder of the crude is obtained (44.2 mg).

Purification of the peptide

Timing: 1 week

In this step, the desired peptide is purified using reversed-phase (RP) HPLC. Purification of transmembrane peptides is one of the most difficult steps in the related research area. A typical strategy for the purification of a transmembrane peptide is chosen to start finding RP-HPLC purification conditions, which is to employ a C4 column together with a mixture of 2-propanol, acetonitrile, and ddH_2O (containing 0.1%TFA) as an eluent.[8] Also, in the original research, standard conditions for peptide purification with RP-HPLC, which is to use an ODS column together with a mixture of acetonitrile and water (0.1% TFA) as an eluent, were tested. Both conditions were employed for the

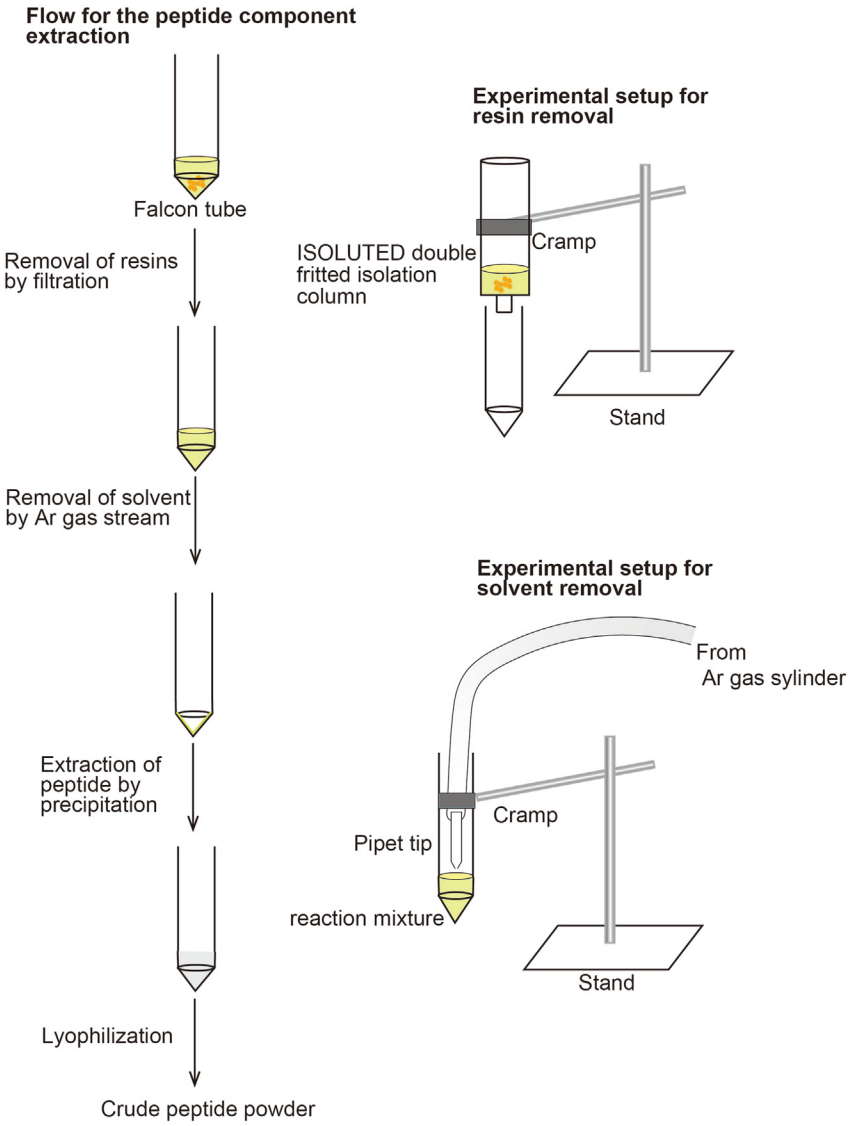

Figure 2. Experimental setup for extracting peptide components

purification in this research. Since the latter, the standard condition for peptide purification provides fewer steps to obtain the purified peptide powder, it is explained in this protocol.

9. Setting up conditions for RP-HPLC.
   a. Checking the solubility of the crude powder in a mixture of acetonitrile and water. Take a small amount (less than 0.1 mg is enough) of the crude powder in a microtube (1.5 mL in size). Add 100 μL of ddH_2O and then 10 μL of acetonitrile.

Note: In the case that the crude powder does not dissolve into the mixture solution, add more acetonitrile. This procedure provides a rough idea for the initial concentration of acetonitrile in the eluent system for RP-HPLC. Since solubility of crude powder depends on how well the synthesis and the deprotection reaction proceeded, it is difficult to estimate the solubility. As it is written above, the best strategy is to mix the crude powder and water firstly and then add acetonitrile little by little until the powder disappears in the solution. In this research, the initial concentration of acetonitrile was set to 30%.

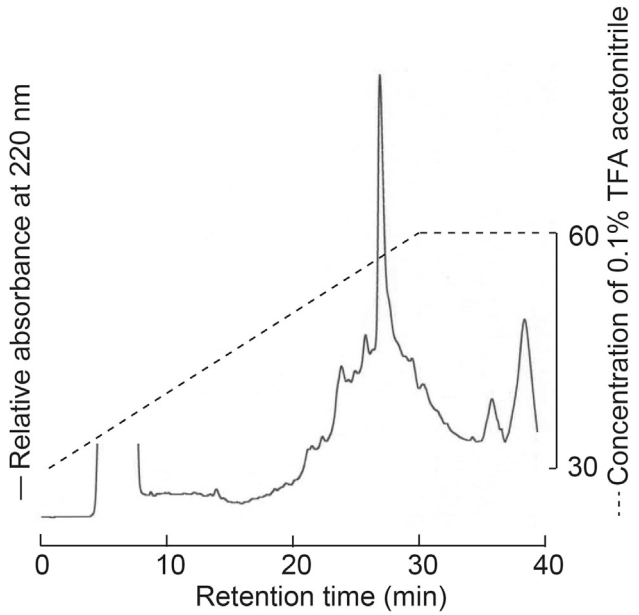

Figure 3. RP-HPLC chromatogram for the crude powder of Mps3TMJM peptide

Conditions can be found in the text. The desired product is obtained at a peak of 27 min (Mass found: 5315.0; Mass calculated for $[M + H^+]$: 6314.16).

b. Prepare ddH_2O containing TFA (0.1% v/v) and acetonitrile (HPLC grade) containing TFA (0.1% v/v). The volume for each solvent should be more than 1 L.
c. Attach the ODS column (10 mm × 250 mm) in the system.
d. Setting a gradient program on the HPLC machine. Set the initial concentration of the acetonitrile solution to 30%, where the concentration is increased to 60% in 30 min. Set the flow rate to 2.5 mL/min. The detecting wavelength should be set to 220 nm.
e. Run the program several times (twice should be enough to set the column ready for purification) without injecting the sample.

Optional: If the alternative conditions, using an eluent system with 2-propanol, are decided to be employed, the acetonitrile solution is changed to a mixture of 2-propanol and acetonitrile (1/1) containing 0.1% TFA (v/v). The C4 column can also be used instead.

10. Injection of the crude powder and checking the elution profile.
    a. Take 0.1 mg of the crude powder in a microtube (1.5 mL in size) and dissolve it into the eluent mixture with the initial acetonitrile concentration. The volume can vary from 100 μL to 500 μL. The volume does not matter as long as the entire crude powder dissolves.
    b. Prepare microtubes (1.5 mL in size) in hand and be ready to manually collect peaks that appear on the HPLC elution profile (Figure 3).

    Note: Here, fractions are collected by hands. However, this can be automated if there is an equipment for the purpose.

    c. There is no need to collect the whole eluent at the peak of interest. Collect the eluent that comes out at the peak top. Typically, ~10 drops (~50 μL) of the eluent that comes out including the peak top are enough for mass analysis.

Note: The collection of the eluent is for analysis by mass spectrometry, MALDI-TOF-MS. It is desirable to collect a fraction with peptide components being condensed. This is the reason that the elution at the peak top is collected.

Note: In the case where peaks on the elution profile overlap or no apparent peak are collectible, the gradient program should be changed. In this experiment, a gradient of acetonitrile containing 0.1% TFA over water from 30% to 60% in 30 min was employed. By increasing the length of time for the gradient from 25 min to 40 min or so, peaks on the profile must come apart. However, there is a chance that increasing the time may broaden peaks on the profile, making it difficult to fractionate.

d. The microtube with the collected sample is dumped into liquid nitrogen to be frozen. And the sample is subjected to lyophilization.
e. Matrix preparation for mass analysis. Have a matrix powder (0.5 mg is enough) in a microtube (0.5 mL in size) and add a mixture of acetonitrile and water (1:1, 200 μL).
f. After the lyophilization from step d, a tiny amount (~50 μg) of powder remains in each microtube. Add 10 μL of the supernatant of the matrix prepared in the previous procedure (10e). And mix it with the powder.
g. Place 2 μL of the sample solution on a sample plate for mass measurement. And perform mass analysis. Repeat f and g for all of the fractionated samples.
h. Purification.
    i. Take 0.1 mg of the crude powder in a microtube (1.5 mL in size) and dissolve it into the eluent mixture with the initial acetonitrile concentration. The volume can vary from 100 μL to 500 μL.
    ii. Inject the solution.
    iii. Collect the peak at the retention time for the desired product.
    iv. Repeat collecting the peak and lyophilize to get the fluffy powder of the desired peptide in a quantity of ~2 mg. It may take a few tens of injections.

Note: The amount of the crude powder for the injection can vary depending on how (well) the synthesis has proceeded. Typically, ~ 1 mg of the crude powder is dissolved for each injection.

Note: In a case where proper separation is not possible on the chromatogram, please refer to references for more options.[8,9]

Peptide derivatization with a fluorescence probe

Timing: 3 days

In this research, a behavior (association with membrane) of the C-terminal juxtamembrane sequence is analyzed by fluorescence spectroscopy.[4] For this purpose, a fluorescence probe (Alexa Fluor 568) is introduced to the side chain of Cys residue at the C-terminus.

11. Derivatization.
    a. Weigh 1 mg of the peptide resin in a microtube (1.5 mL in size).
    b. Weigh Alexa Fluor 568 C5 Maleimide (0.28 mg) in a microtube (0.5 mL in size) and dissolve it with DMF (100 μL).
    c. Pour (pipet in) Alexa568- DMF solution into the microtube with peptide.
    d. Add diisopropylethylamine (DIEA, 2 μL) to the solution above to start the reaction at 25°C.

Note: The reaction solution should be covered with aluminum foil (or be kept in the dark) to prevent the fluorescence from photobleaching.

e. Preparation of RP-HPLC. Conditions must be the same as the ones with the purification mentioned above but the detecting wavelength should be changed to 575 nm for detecting absorbance from the fluorescent dye.
f. After 2 h from the derivatization is started, take 2 μL, which is enough for detection in mass analysis, of the reaction solution and inject it into the RP-HPLC.
g. Run RP-HPLC and collect the peak, which must be located at a similar elution time to that of the unlabeled peptide. Again, collect the eluent at the peak top.
h. Measure the mass of the component in the fraction.
i. When it is decided to finish the derivatization, or 24 h after the reaction is started, start purification with RP-HPLC. Conditions for the purification are the same as above.
j. A few injections must be enough for the purification of the derivatized product. The eluted solution can directly be frozen for lyophilization. Be sure to cover the sample with aluminum foil to prevent it from photobleaching.

Peptide insertion to lipid bilayers (reconstitution)

Timing: 4 days

In this step, the purified peptide is inserted into lipid bilayers, which can also be called reconstitution. Technically, it is just mixing peptides and lipids. There are multiple procedures that have been used for this purpose. Here a technique of reconstitution with detergent dialysis is introduced since this strategy has provided a high reproducibility in our research for more than a decade. The reproducibility is obtained through the measurement of transmembrane helix orientation relative to the membrane normal by the polarized FT-IR experiment described in the next section. Obviously, this measurement provides one of the results for structural analysis of the transmembrane peptide.

12. Peptide reconstitution into 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) / 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) bilayers for polarized FT-IR.
    a. Weigh peptide (1.5×10^{-7} mol, 1 mg), POPC (1.2×10^{-5} mol, 8.8 mg), POPS (0.3×10^{-5} mol, 3.0 mg), and octyl-β-D-glucoside (50 mg) in a glass vial and mix them in a mixture chloroform and methanol (10:3).
    b. Remove the organic solvents under a stream of Ag gas. And dry under vacuum for 18 h.
    c. To the solid, add MOPS buffer (pH: 7.0; 1 mL) and stir using a magnetic stirrer at 37°C for 4 h.

Note: The reconstitution procedure must proceed above the phase transition temperature of lipids. Since the phase transition temperature of POPC and POPS are −2°C and 14°C, respectively, precise control of the temperature is not necessary as long as all of the procedure is performed at 25°C. However, here in this research, everything was done at 37°C.

    d. Put the solution into a dialysis bag (cutoff: 3000 Da) and start dialysis. For each dialysis, the volume of the buffer is 3 L. Keep the temperature at 37°C. Here a water bath was used to keep the temperature.
    e. Dialysis is performed for 2 days. On day 1, change the dialysis buffer after 1 h and then after 3 h. On day 2, the dialysis buffer should be changed two times.
    f. On day 3, the sample solution is ready for analysis of polarized FT-IR. Take out the solution from the dialysis bag and put it into a vial or a microtube. The sample can be kept at 4°C. But it is preferable to proceed with the measurement.
13. Peptide reconstitution into POPC/POPS bilayers for fluorescence spectroscopy.

a. Weigh peptide (1.5×10^{-9} mol, 10 μg), POPC (1.2×10^{-7} mol, 88 μg), POPS (3×10^{-7} mol, 30 μg), and octyl-β-D- glucoside (37.5 mg) in a microtube (1.5 mL in size) and mix them in a mixture chloroform and methanol (10:3) in the volume of 3 mL.

Note: In this stage, the peptide and lipids used are in the order of micrograms. Since fluorescence measurements are performed several times, it is recommended to weigh the peptide and lipids for multiple experiments. In this research, samples enough for 20 experiments are measured, split, and kept in a −40°C freezer (after removing the organic solvents by a stream of Ar gas).

b. Remove the organic solvents under a stream of Ar gas. And dry under reduced pressure for 18 h.

Note: Be sure to keep the sample in the dark by covering it with aluminum foil from now on.

c. To the solid, add MOPS buffer (pH 7.0; 750 μL) and stir using a magnetic stirrer at 37°C for 4 h.
d. Put the solution into a dialysis bag and start dialysis. Keep the temperature at 37°C.
g. Dialysis is performed for 2 days. On day 1, change the dialysis buffer after 1 h and then after 3 h. On day 2, the dialysis buffer should be changed two times.
e. On day 3, take out the solution from the dialysis bag and put it into a microtube (1.5 mL in size). This sample for the fluorescence measurement can be kept at 4°C for no more than 48 h.

14. Making large unilamellar (LUV) vesicles for fluorescence spectroscopy.
    a. Prepare an extruder system with a polycarbonate filter of 100 nm pore size.

Note: The pore size of the polycarbonate filter can also be 200 nm, which does not affect the results in this experiment. The only procedure that was performed at room temperature, 20°C–25°C, was this extrusion.

b. Extrude the sample solution 21 times and the sample is ready for fluorescence measurement.

Note: The size and quality of the LUVs can be confirmed by a light scattering experiment although it was not performed in this research.

Evaluation of the reconstitution and the first structural analysis by polarized FT-IR

15. Polarized attenuated total reflection (ATR) FT-IR experiment.

Note: IR spectroscopy detects the absorption of electromagnetic radiation of matter due to different vibrational modes of the chemical bonds. For a protein sample, absorption by main chain amide bonds provides information on its secondary structure. Further, polarized ATR, which is implemented in FT-IR, can be utilized for determining the orientation of a transmembrane peptide or protein in an aligned membrane sample. In the polarized ATR experiment, orientational order parameters are obtained from the dichroic ratio of the amide I or the amide II band. The dichroic ratio is defined as the ratio of absorption for parallel and perpendicular polarized incident light. From the orientational order parameter, a tilt angle of a transmembrane helix relative to a membrane normal can be calculated. Its theory is briefly described in the analysis section.

a. Obtaining a back gtound spectrum for the parallel polarized light
    i. Setting up the instrument. Place a germanium internal reflection element (a germanium plate) without any sample.

ii. Set the polarizer to 90 degrees to produce parallel polarized light. Close the sample room and fill it with nitrogen gas (to remove air and water from the room).
iii. Scan it 1,000 times, which gives the background spectrum for the parallel polarized light.

b. Set the polarizer to 0 degrees to produce perpendicular polarized light. Do the same procedure above (a. i, ii, and iii.) to obtain a background spectrum for the perpendicular polarized light.

c. Setting sample on the germanium plate
i. Sample preparation. Take the sample solution (50 μL) and spread it on the germanium plate.
ii. Direct a slow flow of nitrogen gas at an oblique angle to the IR plate to blow up water components to form an oriented multilamellar peptide-lipid film.

d. Take another 50 μL of the sample solution and spread it on the IR plate over the film that has been created and repeats the same procedure written above (a. i, ii, and iii.). And, again, do the same procedure (the third time) to form the layered film.

e. Obtaining FT-IR spectrum for the parallel polarized light
i. Measurement. Place the germanium plate with the peptide-lipid film having been layered. Set the polarizer to 90 degrees to produce parallel polarized light. Close the sample room and fill it with nitrogen gas.
ii. Scan it 1000 times, which gives the resulting spectrum for the parallel polarized light.

f. Obtaining FT-IR spectrum for the perpendicular polarized light
i. Set the polarizer to 0 degrees to produce perpendicular polarized light. Close the sample room and fill it with nitrogen gas.
ii. Scan it 1000 times, which gives the resulting spectrum for the perpendicular polarized light.

g. Subtract the background from the resulting spectrum for each obtained in e. and f. to get final results. These spectra should be exported as a comma-separated value file (.csv) for the next analysis step.

16. Data analysis on results from polarized FT-IR.

Note: In this step, spectra obtained are analyzed for getting insight into the secondary structure of the protein and a tilt of the transmembrane helix relative to the membrane normal. For this purpose, the software, Fityk,[10] is utilized. Fityk is a free software for data processing and curve fitting. Here we perform, curve fitting to derive a peak component representing the secondary structure from the native data that has been experimentally obtained. Details on the use of Fityk is passed on to its manual but, in this manuscript, procedures and important points for the analysis are introduced.

a. In the spectrum, focus on the region from 1600 to 1700 cm^{-1}, which represents amide I frequency. Peaks in the region can be assigned for the secondary structure: α-helix: 1648–1660 cm^{-1}; aggregated strand: 1610–1628 cm^{-1}, 1675–1695 cm^{-1}; β-sheet: 1625–1640 cm^{-1}, 1675–1695 cm^{-1}. Since peak overlap is expected, peak fitting is required.
b. Start Fityk and load the spectrum in csv format.

Note: For curve fitting, a number of peaks are introduced as the sum of the introduced peaks fit the experimentally obtained spectrum. It is important to be aware of the number of peaks that should be introduced. Each introduced peak must be assignable to the secondary structure since the focusing region on the spectrum is limited to peaks from amide I.

c. Firstly, choose the shape of the peak that is to be introduced. In this research, "Gaussian" was chosen.
d. Press the "auto-peak" button a number of times. A peak should appear at a wavenumber on the spectrum where there is a maximum or a "shoulder" exists. In Figure 4, these peaks are shown in blue.

e. Press the "Start-fitting" button, then the curve fit is accomplished. In Figure 4, this fitted curve is curve is shown in red.
f. Export the result for an output.
g. Obtaining the area for the introduced peak. The area of the peak from the α-helix is required for calculating the dichroic ratio. Click on the peak representing α-helix and read the value in the window. All procedures for the analysis must be repeated for spectra from parallel and perpendicular polarized light.
h. Calculate the dichroic ratio.

Note: For the next step, calculating the angle between the helix director and the membrane normal, a method described by Liu et al.[11] is followed. Here, requirements for obtaining a desired value are briefly described. The observed dichroic ratio (R^{ATR}) is used to calculate the order parameter S_{meas} using the equation below,

$$S_{meas} = \frac{E_x^2 - R^{ATR}E_y^2 + E_z^2}{E_x^2 - R^{ATR}E_y^2 - 2E_z^2}$$

where the electric field amplitudes are given such as $E_x = 1.399$, $E_y = 1.514$, $E_z = 1.621$. These values are based on the assumption that the thickness of the deposited films on the germanium plate is much larger than the penetration depth (~1 μm) of the evanescent wave.

The measured order parameter S_{meas} is related to three nested order parameters describing the average distribution of the helix relative to the membrane normal (S_{hel}), the orientation of the transition dipole moment for the amide I bands relative to the helix axis (S_{dip}), and disorder in the orientation of the membrane (S_{mem}),

$$S_{meas} = S_{hel}S_{dip}S_{mem} = \left[\frac{3\langle cos^2\theta\rangle}{2} - \frac{1}{2}\right]\left[\frac{3cos^2\alpha}{2} - \frac{1}{2}\right]S_{mem}$$

where θ is the angle between the helix director and the normal of the internal-reflection element, and α is the angle between the helix director and the transition-dipole moment of the amide I vibration mode. Following Smith et al., a value of $\alpha = 41.8°$ is used. This value is based on the polarized ATR FT-IR experiment on bacteriorhodopsin and has been used for multiple analyses. The use of this value of α implies a value of S_{mem} between 0.8 and 0.9. And in this calculation, the value 0.85 is used.

i. Calculate the angle between the helix director and the membrane normal.

Fluorescence measurements for looking at the interaction between the juxtamembrane region and membrane

Timing: 5 h

In this research, to determine the role of putative phosphorylation in the luminal juxtamembrane region of Mps3, a biophysical analysis was performed. Chemically, the phosphate group provides a negative charge at the site, which must have some effect on the interaction between the juxtamembrane region and the membrane. The biophysical experiment here was aimed to see whether there was such an interaction and if there was an electrostatic interaction, how it affected the structure of the protein.

Here, a simple strategy, focusing on electrostatic repulsion, is briefly explained. The luminal juxtamembrane region of Mps3 has a net charge of +1, which may have effect on an interaction between this region and the acidic membrane. However, the existence of multiple Ser and Thr residues may induce an interaction between the juxtamembrane region and the membrane through hydrogen bonding with the lipid head group. A hypothesis was that the juxtamembrane region in the wild-type

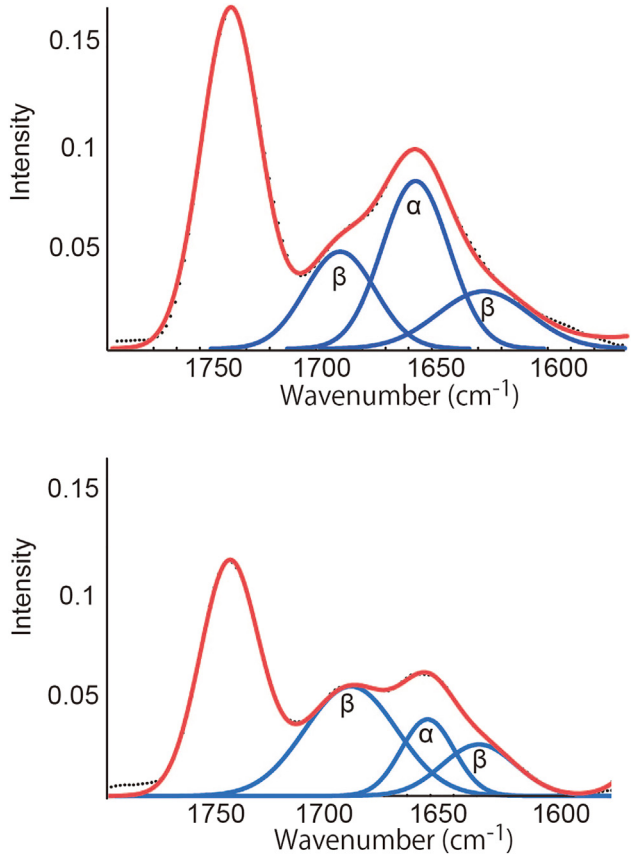

Figure 4. Typical FTIR spectra obtained with light polarized parallel (top) or perpendicular (bottom) to the membrane normal

Only the amide I region of the IR spectrum is shown for clarity. The frequency of the amide I band at ~1657 cm^{-1} is characteristic of the α-helical secondary structure. The original spectrum (shown in red) is deconvoluted into spectra (shown in blue) that can be assigned to the secondary structure. The data were adopted from Prasada Rao et al.[1]

sequence weakly binds to the membrane surface. The introduction of phosphate groups to Thr-Ser-Ser sequence increases negative charge, which must cause an electrostatic repulsion between this region and the membrane surface, leading to this region being released from the membrane.

As it has been mentioned above, a fluorescence probe, Alexa568 was introduced at the C-terminus of the juxtamembrane region. Changing a membrane component *in situ* must have an effect on the fluorescence spectrum if the juxtamembrane region is bound to the membrane. The change in the membrane component, namely a charge on the liposome surface, is accomplished by the addition of 1,2-dioctanoyl-sn-glycero-3-phospho-(1′-myo-inositol-4′,5′-bisphosphate) (P(4,5)P2).[3]

17. Preparation of PI(4,5)P2.

> ***Note:*** Here PI(4,5)P2 is added to liposome suspension to change the membrane component. PI(4,5)P2 molecules diffuse into the liposomes. Since PI(4,5)P2 dealt with here is a tiny amount, a measurable quantity is divided and separated into multiple microtubes. The sample solution of peptide liposome is poured into each microtube and vortexed to take PI(4,5)P2 into liposomes. This procedure is repeated to increase the concentration of PI(4,5)P2 for each measurement, which does not change the volume of the solution and allows us to measure the fluorescence intensity from the peptide in PI(4,5)P2 concentration-dependent manner.

a. Weigh 58 μg of PI(4,5)P2 on a microbalance. The amount of PI(4,5)P2 is determined to obtain 4.8 μM concentration in 750 μL solution as mentioned in the next procedure 18b.
b. Add a mixture of chloroform and methanol (1:1, 2 mL) and split the PI(4,5)P2 solution into 20 microtubes (1.5 mL in size).
c. Remove the organic solvent for all of the microtubes and keep them in a freezer (−20°C).

18. Fluorescence measurement.
a. Turn on the fluorescence spectrometer and the temperature regulation unit. The temperature should be fixed (in this research, it was fixed at 37°C). The excitation wavelength is set to 568 nm and the scanning wavelength should be set from 580 to 660 nm.
b. Pipet the sample solution (500 μL) into a quartz cuvette with a path length of 1 cm. In the sample preparation section, it was mentioned that the sample volume was set to 750 μL. Here only 500 μL of the sample is used.
c. Place the cuvette into the fluorescence spectrometer and wait 20 min.
d. Scan.
e. After scanning, the sample solution is recovered from the cuvette and pipet back to the original microtube where the other 250 μL of the solution is left.
f. Take all of the sample solutions (750 μL) and pour it into the microtube with PI(4,5,)P2 that has been prepared in the previous section.
g. Vortex the microtube to let PI(4,5)P2 diffuse into liposome for 2 min. This procedure produces the sample solution with 4.8 μM PI(4,5)P2. Do not sonicate the solution. The sonication destroys LUVs.
h. Pipet out 500 μL of the sample solution and put it into the cuvette. Place the cuvette and wait for 20 min. Scan the sample.
i. Repeat steps from f. to h. to increase the concentration of PI(4,5)P2.
j. Export results for output.

Note: A brief note for interpretation of data. Measured fluorescence intensity from the juxtamembrane region of the peptide must change with an increase of PI(4,5)P2 if the juxtamembrane region is bound to the membrane (Figure 5). Conversely, the intensity does not change in the case where the juxtamembrane region is being released from the membrane. In the original research[1] where this protocol was used, fluorescence intensity rarely changed when negative charges were introduced in the juxtamembrane region suggesting that the introduction of phosphate groups in this region must release itself from the membrane.

Molecular dynamics simulation

Timing: 5 days or more, depending on the calculation ability of your workstation or computer

To get more insight into the juxtamembrane region, a molecular dynamics simulation was performed. Although a lot of structural and dynamic features of protein are available from the calculation, interaction between the juxtamembrane region and the membrane is focused on in this study.

19. Preparing files for MD.
a. Preparation of a starting structure. Since there is no structure reported for the transmembrane and juxtamembrane region for Mps3, it was decided to artificially build a starting structure (pdb file) on the Pymol program.
b. In Pymol, using "Build" module, each amino acid is selected one by one from the N-terminus. Since this module allows us to assign the secondary structure to a certain sequence, a putative transmembrane region is set to α-helical and a putative juxtamembrane region is set to an extended structure. The sequence for the transmembrane region can be decided by a suggestion from the UniProt website.
c. Since the monomer is dealt with here in this study, just simply, save the structure as a pdb file.

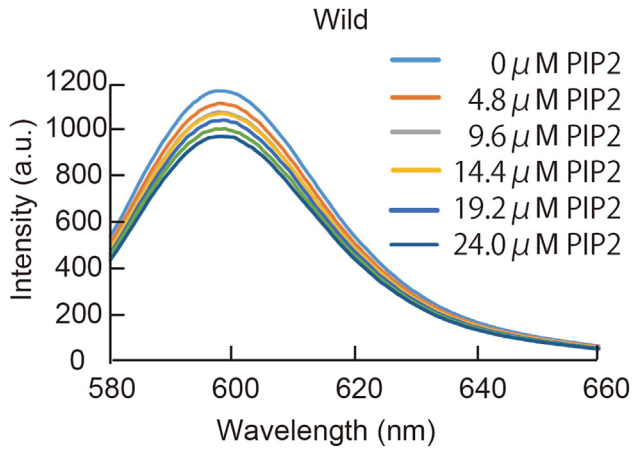

Figure 5. Fluorescence spectra obtained in this research

Spectra obtained for the wild-type sequence are shown. As the concentration of PI(4,5)P2 increases, the fluorescence intensity decreases. Interpretation is mentioned in Note below. The data were adopted from Prasada Rao et al.[1]

Note: Preparation of files on Charmm-gui website.[12] For preparation of files for MD calculation, Charmm-gui website can be utilized. Since this website provides a simple procedure where following the instruction leads you to be ready for running MD simulation, only important points are introduced here.

d. For running all atom simulation, choose "Input Generator", "Membrane Builder" and then "Bilayer builder" to load your starting structure.
e. Load the starting structure. For the next few steps on the website, nothing has to be changed.
f. For determination of orientation for the transmembrane helix relative to the membrane in the web page with "Orientation options", check "Align the First Principal Axis Along Z". This option aligns the principal axis of the molecule to the Z axis.
g. Click on "Next Step", which leads you to see how the peptide is oriented in the membrane. In the case that the orientation shown is not desirable, go back to the previous page and play with "Positioning Options". In the end, the desired orientation is to have the center of the transmembrane region closely match the center of the membrane and have the transmembrane region closely be perpendicular to the membrane.

Note: In the case that you are introducing a symmetric membrane, the direction of the transmembrane helix inserted in the membrane has nothing to do with the calculation. However, if it is planned to introduce an asymmetric membrane, the direction matters. The "Flip Molecule along the Z axis" option in "Positioning Options" must work for choosing the direction.

Note: Since the principal axis of the molecule is aligned to the Z axis and the direction of the transmembrane region is determined, the transmembrane region must be in the membrane. The next thing to be considered is to place the center of the transmembrane region at the center of the membrane. This can be accomplished by checking "Translate Molecule along Z axis" and typing a value in Angstrom for vertical movement.

h. After obtaining the desired orientation, click "Next step" to move on to determine lipid components. "Length of X and Y" can be ~70 Å since a single molecule is dealt with here.
i. Selection of lipids. In this calculation, "standard" lipids such as POPC and POPS are selected.
j. In the rest of the pages, there is nothing to be considered but to follow and click "Next Step". In the end, conditions such as salt are set to 0.15 M NaCl and temperature is set to 303 K.
k. Download files for NAMD, being ready for starting calculation on NAMD.

20. Running the calculation on NAMD.[13]
    a. After downloading the file created by the Charmm-gui website, extract the file in your working directory on your workstation or computer.
    b. Open the extracted folder and go to "namd" directory or folder.
    c. Here in this "namd" directory, there are six input files named "step6.1(~6) equilibration.inp". These files allow to perform gradual equilibration of the initially assembled system. Type in commands below one by one:

```
./namd2 +idlepoll +p32 +devices 0,1 step6.1_equilibration.inp > WT6.1.log

./namd2 +idlepoll +p32 +devices 0,1 step6.2_equilibration.inp > WT6.2.log

./namd2 +idlepoll +p32 +devices 0,1 step6.3_equilibration.inp > WT6.3.log

./namd2 +idlepoll +p32 +devices 0,1 step6.4_equilibration.inp > WT6.4.log

./namd2 +idlepoll +p32 +devices 0,1 step6.5_equilibration.inp > WT6.5.log

./namd2 +idlepoll +p32 +devices 0,1 step6.6_equilibration.inp > WT6.6.log
```

Note: Two parts, "+p32" and "+devices 0,1", depend on your workstation setup. This example shows that 32 threads and 2 GPUs assigned as 0 and 1 are utilized for this calculation.

    d. Before moving to the next step for the production run, open "step7.1_production.inp"(input file) by a text editor and set the value for calculation time (duration). In NAMD, the duration is set by the number of steps, "numsteps" in the input file. The default value is 500000 for 1 ns calculation. In our case, the time was set to 50 ns.

Note: Regarding the duration, obviously, longer is better. However, it depends completely on the ability of your machine setup.

    e. Type in the commands below to start the calculation:

```
./namd2 +idlepoll +p32 +devices 0,1 step7.1_production.inp > WT7.1.log
```

    f. When it is decided to finish the calculation, the trajectory can be visualized by software such as VMD.[14] VMD is also equipped with some analysis tools. For more information on VMD, please refer to its manual.

EXPECTED OUTCOMES

Reconstitution and Polarized FT-IR: For the sequence of the transmembrane-juxtamembrane region of Mps3, the dichroic ratio obtained from the measurements was found to be 2.90, suggesting that the transmembrane region is inserted in the membrane with its tilt angle of ~30 degrees relative to the membrane normal. In a case where a transmembrane peptide is not inserted properly, a dichroic ratio can be as low as 1 suggesting that there is no orientation, or reconstitution is not accomplished. Also, a peak that is assigned to α-helix is not found on the spectrum, suggesting that the peptide is aggregating. In this case, as such, the reconstitution procedure, especially the choice of detergent and the solubilization step, must be reconsidered.

Fluorescence measurements: As it has been mentioned above, in the case where there is an electrostatic interaction between the membrane surface and the juxtamembrane region, incorporation of PI(4,5)P2 in the system provides a change on the spectrum as shown in Figure 5. The incorporation of PI(4,5)P2 must shift its equilibrium toward the side of the juxtamembrane region being bound with the membrane, resulting in an increase in the fluorescence intensity and a blue shift of the spectrum. Conversely, no change is observed on the spectrum when there is no interaction between the membrane and the juxtamembrane region or when the juxtamembrane region is released from the

membrane. There may also be a chance that a decrease in the fluorescence intensity is observed.[5] In this case, an interpretation can be made such that the fluorescence probe is self-quenching due to an association of the juxtamembrane region, or if it accompanies a redshift of the spectrum, the region is dissociating from the membrane. Additional experiments must be required to get insight into the mechanism of this behavior.

LIMITATIONS

A major limitation of this reconstituting experiment involves the sequence of a target peptide. A transmembrane sequence has hydrophobic and aggregating nature. These characteristics may cause precipitation of the peptide itself during detergent removal by dialysis. Unfortunately, there is no solution to this problem but changing the sequence or adding hydrophilic residues to both the N- and the C-terminal.

TROUBLESHOOTING

Problem 1

Difficulty in dissolving crude powder into solution for injection to the HPLC machine (steps 9).

Potential solutions

In the case that the crude powder does not dissolve into a mixture of acetonitrile and water, increase the volume of acetonitrile. Since solubility of crude powder depends on how well the synthesis and the deprotection reaction proceeded, it is difficult to estimate the solubility. As it is written above, the best strategy is to mix the crude powder and water firstly and then add acetonitrile little by little until the powder disappears in the solution. However, there is a possibility that increasing the acetonitrile does not result in dissolving the crude powder. In that case, change the solvent system. As mentioned in step 9, the system with 2-propanol may provide better solubility of the crude powder and separation on the HPLC profile. If this system does not provide better results, use conditions described in Sato et al.[8]

Problem 2

Peak overlapping on RP-HPLC elution profile causes poor isolation (step 9–10).

Potential solutions

This is a typical problem encountered in the purification of a transmembrane peptide by RP-HPLC. As it's been mentioned in the Purification of the peptide section, using the C4 column, instead of the ODS column, and employing a mixture of 2-propanol, acetonitrile, and water as the elution solvent system may provide a better-isolating pattern. In the case where these conditions do not improve the isolation, the solvent system can be changed to a mixture of formic acid,2-propanol, and water. The use of this formic acid system for purifying transmembrane peptide is described in detail by Sato et al.[8]

Problem 3

When the peptide is purified with the solvent system with 2-propanol and acetonitrile, the sample melts during the lyophilization (step 9).

Potential solutions

This is the problem that you encounter when the system above is employed for purification. Before moving into lyophilization process, it is better to remove solvents, namely, 2-propanol, acetonitrile and water in the fractionated solution by using evaporator. After the removal, add acetonitrile and water (1/1) and freeze the solution for lyophilization.

Problem 4

When fluorescence labels are introduced to the peptide, the HPLC profile does not change. How is the reaction monitored (step 11).

Potential solutions

When transmembrane peptide is dealt with, retention time on the elution profile for the derivatized peptide is similar to that of the starting material, the purified peptide. In this case, the progress of the derivatization cannot be monitored through RP-HPLC alone. This is the reason that the mass measurement of the eluted component is performed. The mass value of the starting material is expected NOT to be detected when the reaction ends. The derivatization should be continued for up to 24 h.

Problem 5

ATR FT-IR measurement does not provide a spectrum showing TM helix orientation (steps 15 and 16).

Potential solutions

This is caused by the fact that the TM region is not "well inserted" into lipid bilayers, which involves a failure in the reconstitution procedure. Removal of detergent by dialysis must be performed carefully. The temperature should be set above the phase transition temperature of the lipids used. Enough time, such as two days at least, should be spent on dialysis. Also, a choice of detergent is critical. In this protocol, octyl-β-D-glucoside was introduced. A criterion for the choice, other than its ability to dissolve a hydrophobic peptide or protein, is that the critical micelle concentration (CMC) of the detergent should be high enough to be removed by dialysis. The CMC value of octyl-β-D-glucoside is 25 mM, being easily dialyzed during the procedure.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the technical contact, Takeshi Sato (takeshi@mb.kyoto-phu.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze datasets or code.

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AUTHOR CONTRIBUTIONS

T.S. and A.S. wrote this manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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