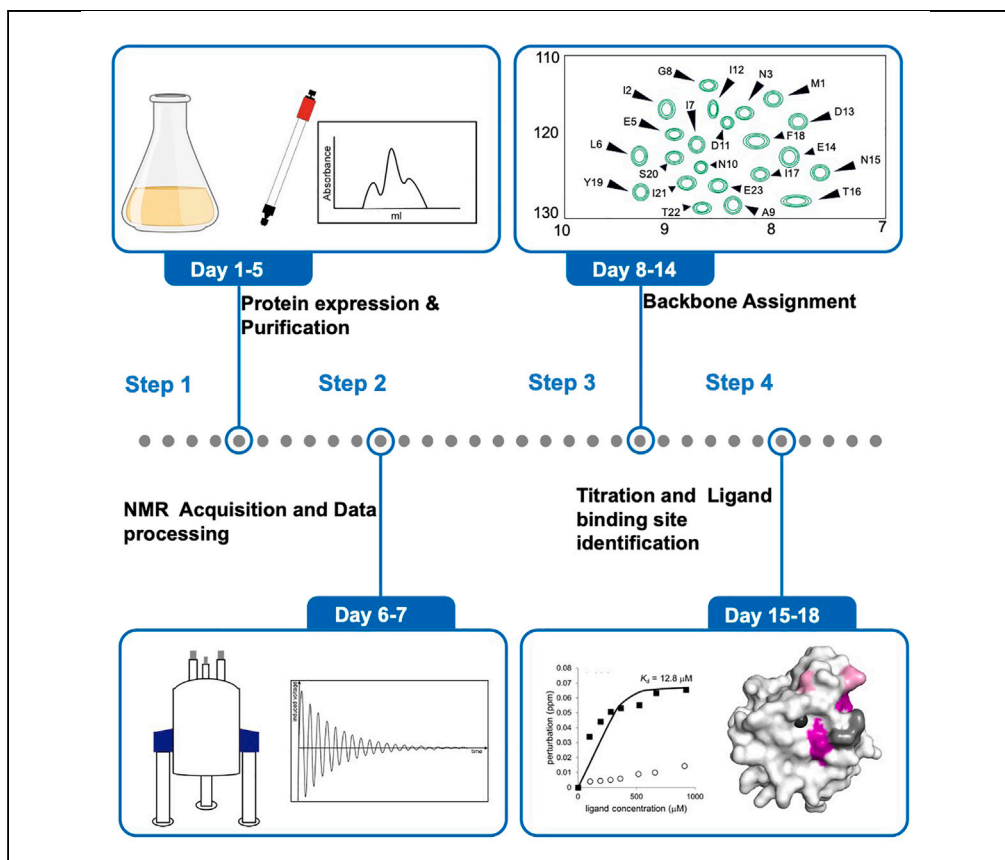


## Protocol

# Protocol to identify the ligand binding site of Mincle using NMR spectroscopy



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### Highlights

Steps for preparing protein sample for NMR analysis

The protocol to prevent protein aggregation using NDSB-195

Steps for determining the dissociation constant between protein and ligands

Mincle (macrophage-inducible C-type lectin, CLEC4E) is a C-type lectin immune-stimulatory receptor that can be targeted for inducing potent adjuvant effects. Mincle can recognize trehalose dimycolate and related glycolipids. Here, we present a protocol to identify the ligand binding mode of Mincle. We describe steps for preparing labeled Mincle ectodomain, data acquisition, and analysis of nuclear magnetic resonance experiments using non-detergent sulfobetaine-195. This protocol can be applied to other protein-ligand interactions that have aggregation problems for complex formation.

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

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## Protocol

## Protocol to identify the ligand binding site of Mincle using NMR spectroscopy

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## SUMMARY

**Mincle (macrophage-inducible C-type lectin, CLEC4E) is a C-type lectin immune-stimulatory receptor that can be targeted for inducing potent adjuvant effects. Mincle can recognize trehalose dimycolate and related glycolipids. Here, we present a protocol to identify the ligand binding mode of Mincle. We describe steps for preparing labeled Mincle ectodomain, data acquisition, and analysis of nuclear magnetic resonance experiments using non-detergent sulfobetaine-195. This protocol can be applied to other protein-ligand interactions that have aggregation problems for complex formation. For complete details on the use and execution of this protocol, please refer to Furukawa et al.<sup>1</sup>**

## BEFORE YOU BEGIN

The protocol describes here is the detail steps for determining glycolipid binding sites of Mincle using solution NMR spectroscopy. Notably, this protocol using NDSB-195 can be used for the analysis of interactions between proteins and other small molecules, especially, if interactions cause aggregation.

In this protocol, we first described the procedure for expressing and purifying <sup>13</sup>C/<sup>15</sup>N uniformly labeled Mincle protein. We further described the data acquisition and analysis of NMR spectra. We also described the procedure to perform backbone assignments of Mincle, to trace cross-peak movements in ligand titration experiments for determining interacting surfaces, and to map the binding site onto 3D structure of Mincle.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Competent cells BL21-Gold (DE3)	Stratagene	CAT# 230132, Lot# 0770198
Rosetta2 (DE3)	Novagen	Cat# 71397

*(Continued on next page)*

<b>Continued</b>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Chemicals, peptides, and recombinant proteins</b>		
Triton X-100	Nacalai Tesque	Cat# 35501-15
CHL medium (X10) ( <sup>15</sup> N: –99%)	SI Science	N/A
CHL medium (X10) ( <sup>13</sup> C, <sup>15</sup> N: –97%)	SI Science	N/A
Guanidine-HCl	FUJIFILM Wako	Product # 074-05005, Lot # CAL1442
2-(N-morpholino)ethanesulfonic acid (MES)	Dojindo Laboratories	Product # GB12, Lot # JU070
NaCl	FUJIFILM Wako	Product # 197-01667, Lot# DLR1126
EDTA	Dojindo Laboratories	Product #N001, Lot # LB153
Dithiothreitol	FUJIFILM Wako	Product # 042-29222, Lot # LKQ2881
2-Amino-2-hydroxymethyl-1, 3-propanediol (Tris-HCl)	FUJIFILM Wako	Product # 203-06277, Lot # SKF7764
L-arginine	FUJIFILM Wako	Product # 280-56551, Lot # LEQ1994
CaCl <sub>2</sub>	FUJIFILM Wako	Product # 039-00475, Lot# HWH7483
D(+)-Glucose	FUJIFILM Wako	Product # 049-31165, Lot# DCN6677
<sup>15</sup> NH <sub>4</sub> Cl	Sigma-Aldrich	Product # 299251 Lot# TA1296V
MnCl <sub>2</sub> ·4H <sub>2</sub> O	Nacalai Tesque	Product # 21211-45, Lot# M9E4837
MgSO <sub>4</sub> ·7H <sub>2</sub> O	FUJIFILM Wako	Product # 131-00405, Lot# SER2697
FeCl <sub>3</sub> ·6H <sub>2</sub> O	FUJIFILM Wako	Product # 091-00872, Lot# WKH5575
D-biotin	MP Biomedicals	Product # 101023, Lot# 3570J
Thiamine	FUJIFILM Wako	Product # 203-00851, Lot# PDN2668
Cytidine	Tokyo Chemical Industry	Product # 2C0522, Lot # 5PVOG-K0
Guanosine	FUJIFILM Wako	Product # 079-01111, Lot # DCE1994
Adenosine	Nacalai Tesque	Product # 01994-54, Lot# M2K1414
Thymidine	Nacalai Tesque	Product # 07147-61, Lot #M2E8546
KH <sub>2</sub> PO <sub>4</sub>	FUJIFILM Wako	Product # 169-04245, Lot# SAF5755
Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O	FUJIFILM Wako	Product # 196-02835, Lot# SKF4981
Cystamine	FUJIFILM Wako	Product # 030-17632, Lot # PTF0078
Cysteamine	FUJIFILM Wako	Product # 032-19392, Lot # CAH4591
HEPES	Dojindo Laboratories	Product # GB10, Lot # JJ052
NDSB-195	FUJIFILM Wako	Product # 148-08831, Lot # WDM6790
Tetracycline	FUJIFILM Wako	Product # 205-08591, Lot# TSF0373
Ampicillin	FUJIFILM Wako	Product # 014-23302, Lot# PTG5411
Trehalose-C10	Dojindo Laboratories	Product #T460, Lot# EX006
Trehalose	FUJIFILM Wako	Product # 209-14165, Lot# STH0357
Glycerol	FUJIFILM Wako	Product # 075-00616, Lot# SKF4281
D <sub>2</sub> O	ISOTEC	Product # 151882, Lot# TA2195V
<b>Deposited data</b>		
Backbone resonances of I99K Mincle	This study	BMRB:50217
<b>Recombinant DNA</b>		
pET22b-Mincle I99K	Furukawa et al. <sup>2</sup>	N/A
<b>Software and algorithms</b>		
NMRpipe	Delaglio et al. <sup>3</sup>	N/A
Sparky	N/A	<a href="https://www.cgl.ucsf.edu/home/sparky/">https://www.cgl.ucsf.edu/home/sparky/</a>
PyMOL	N/A	<a href="http://www.pymol.org/pymol">http://www.pymol.org/pymol</a>
<b>Other</b>		
HiLoad 26/60 Superdex 75 pg	Cytiva (GE Healthcare)	Product # 17-1070-01
Superdex 10/300 GL	Cytiva (GE Healthcare)	Product # 17-5174-01
5-mm microcell NMR tube	Shigemi	Product # BMS-005B
5 mm thin wall precision NMR sample tube 8" L, 600 MHz	Wilmad LabGlass	Product #1050-54611
Pasteur pipette	Hilgenberg	Product # 31-501-66
Diaphragm pump	Iwaki	Product # FTP-20A
Sonicator (SONIFIER 250 and SONIFIER 450)	Branson	N/A

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Amicon Ultra Ultracel-3 regenerated cellulose membrane, 4 mL sample volume	Merck	Product # UFC800324
0.22 $\mu\text{m}$ filter	Merck	Product # SLGVR33RB
Incubator shaker	Eppendorf	Product # M1335-0015

## MATERIALS AND EQUIPMENT

### General stock solution

- 1 M Tris-HCl (pH 8.0) buffer: add 121.14 g of Tris-HCl in 900 mL ddH<sub>2</sub>O, adjust the pH to 8.0, and finally fill up to 1 L. The buffer can be stored at 25°C for 1 month.
- 5 M NaCl: add 292.5 g of NaCl in 900 mL ddH<sub>2</sub>O, dissolve completely, and then, fill up to 1 L. The buffer can be stored at 25°C for one year.
- 1 M MES (pH 6.5) buffer: add 19.5 g of 2-(N-morpholino)ethanesulfonic acid (MES) in 90 mL ddH<sub>2</sub>O, adjust the pH to 6.5 and finally fill up to 100 mL. The buffer should be stored with the protection from the light and can be kept at 25°C for one year.
- 0.5 M EDTA (pH 8.0): add 93.06 g of Ethylenediamine-*N,N,N',N'*-tetraacetic acid (EDTA) in 400 mL ddH<sub>2</sub>O, adjust the pH to 8.0 and finally fill up to 500 mL. The buffer can be kept at 25°C for one year.
- 1 M CaCl<sub>2</sub>: add 110.98 g of NaCl in 900 mL ddH<sub>2</sub>O, dissolve completely, and then, fill up to 1 L. The buffer can be stored at 25°C for one year.
- 50 mM CaCl<sub>2</sub>: Mix 5 mL of 1 M CaCl<sub>2</sub> buffer and 95 mL ddH<sub>2</sub>O. The buffer can be stored at 25°C for one year.
- 10 mM FeCl<sub>3</sub>: add 270 mg of FeCl<sub>3</sub>·6H<sub>2</sub>O in 90 mL ddH<sub>2</sub>O, dissolve completely, and then, fill up to 100 mL. The buffer can be stored at 25°C for one year.
- 1 M MgSO<sub>4</sub>: add 24.65 g of NaCl in 90 mL ddH<sub>2</sub>O, dissolve completely, and then, fill up to 100 mL. The buffer can be stored at 25°C for one year.
- 50 mM MnCl<sub>2</sub>: add 990 mg of NaCl in 90 mL ddH<sub>2</sub>O, dissolve completely, and then, fill up to 100 mL. The buffer can be stored at 25°C for one year.

### M9 medium

Prepare M9 medium by the mixture of 100 mL of Solution A, 880 mL of Solution B, 20 mL of Solution C, 2 mL of 50 mM CaCl<sub>2</sub> solution and 1 mL of glycerol. The M9 minimal medium can be stored at 4°C for 1 week. Tap water contains trace amounts of minerals, such as metal ions. Therefore, tap water in Solutions A and C can be used to enhance *E. coli* growth in minimum medium.

#### [Solution A] The buffer should be autoclaved after the preparation

Reagent	Final concentration	Amount
KH <sub>2</sub> PO <sub>4</sub>	22 mM	3 g
Na <sub>2</sub> HPO <sub>4</sub>	49 mM	6 g
NaCl	8.6 mM	0.5 g
Tap water		Up to 100 mL
<b>Total</b>		<b>100 mL</b>

#### [Solution B] The buffer should be autoclaved after the preparation

Reagent	Final concentration	Amount
10 mM FeCl <sub>3</sub>	56 $\mu\text{M}$	5.0 mL
1 M MgSO <sub>4</sub>	2.3 mM	2.0 mL
50 mM MnCl <sub>2</sub>	56 $\mu\text{M}$	1.0 mL

(Continued on next page)

**Continued**

Reagent	Final concentration	Amount
Thiamine Hydrochloride	270 $\mu$ M	80 mg
Adenosine	8.5 $\mu$ M	2 mg
Thiamine	8.6 $\mu$ M	2 mg
Guanosine	8.0 $\mu$ M	2 mg
Cytosine	20 $\mu$ M	2 mg
D-biotin	9.3 $\mu$ M	2 mg
Deionized Water		872 mL
<b>Total</b>		<b>880 mL</b>

**[Solution C] The buffer should be filtered with 0.2  $\mu$ m filter after the preparation**

Reagent	Final concentration	Amount
$^{15}\text{NH}_4\text{Cl}$ or $^{14}\text{NH}_4\text{Cl}$	1.8–1.9 M	2 g
$^{13}\text{C}$ Glucose or $^{12}\text{C}$ Glucose	2.8 M	10 g
Tap Water		Up to 20 mL
<b>Total</b>		<b>20 mL</b>

**[Suspension buffer] This buffer can be stored at 25°C for one month**

Reagent	Final concentration	Amount
1 M Tris-buffer (pH8.0)	50 mM	50 mL
5 M NaCl	100 mM	20 mL
Deionized Water		930 mL
<b>Total</b>		<b>1000 mL</b>

**[Triton buffer] This buffer can be stored at 25°C for one month**

Reagent	Final concentration	Amount
1 M Tris-buffer (pH 8.0)	50 mM	50 mL
5 M NaCl	100 mM	20 mL
Triton X-100	0.5%	5 mL
Deionized Water		925 mL
<b>Total</b>		<b>1000 mL</b>

**[Solubilization buffer] The buffer should be stored with protection from light and kept at 25°C for one year**

Reagent	Final concentration	Amount
Guanidine	6 M	57.3 g
1 M MES (pH 6.5)	50 mM	5 mL
5 M NaCl	100 mM	2 mL
0.5 M EDTA (pH 8.0)	10 mM	2 mL

Adjust the pH of the buffer to 6.5 at 25°C. Add Milli-Q water up to 100 mL.

**[Refolding buffer] This buffer should be used immediately after the preparation**

Reagent	Final concentration	Amount
1 M tris-buffer (pH8.0)	100 mM	20 mL
L-arginine	1 M	174.2 g
1 M $\text{CaCl}_2$ buffer	20 mM	20 mL
Cystamine	3.73 mM	840 mg
Cysteamine	6.73 mM	720 mg
Deionized Water		Mess up to 1000 mL
<b>Total</b>		<b>1000 mL</b>

**[Gel filtration buffer] This buffer can be stored at 25°C for one month**

Reagent	Final concentration	Amount
1 M tris-buffer (pH8.0)	20 mM	20 mL
5 M NaCl	100 mM	20 mL
1 M CaCl <sub>2</sub> buffer	20 mM	20 mL
Deionized Water		940 mL
<b>Total</b>		<b>1000 mL</b>

## STEP-BY-STEP METHOD DETAILS

### Production of the Mincle protein for NMR measurements

⌚ Timing: 1–5 days

1. Transform *Escherichia coli* using the pET22b plasmid encoding the human Mincle ectodomain (residues 74-219).
  - a. Grow Transformed Rosetta2 (DE3) strain chemically competent cells on LB agar plates containing both 100 µg/mL ampicillin and 20 µg/mL chloramphenicol.
  - b. Grow BL21-Gold (DE3) strain and grown on LB agar plates containing both 100 µg/mL ampicillin.
2. Inoculate some colonies into 10 mL of 2xYT medium containing 100 µg/mL ampicillin and 20 µg/mL chloramphenicol (only in case of Rosetta2 (DE3)) at 37°C with shaking at 200 rpm, 14–18 h.
3. Pour Rosetta2 (DE3) strain culture medium into 1 L of CHL of <sup>15</sup>N, or <sup>15</sup>N and <sup>13</sup>C labeled medium containing 100 µg/mL ampicillin and 20 µg/mL chloramphenicol or BL21-Gold (DE3) into M9 medium containing 100 µg/mL ampicillin in a 2 L plastic Flask and continue growing cells in an Incubator Shaker at 37°C with shaking at 200 rpm until the optical density of the culture medium reaches from 0.4 to 0.6 at an absorbance of 600 nm.
4. Add 1 mL of 1.0 M Isopropyl β-d-1-thiogalactopyranoside (IPTG) and incubate at 37°C with shaking for 4–6 h.
5. Harvest cells by centrifugation at 5,000 rpm (4,424 × g) at 4°C for 10 min and discard supernatant.

⏸ Pause point: The *E. coli* pellet can be stored at –80°C for at least one year.

6. Purification of inclusion body.
  - a. Suspend *E. coli* pellet with 30 mL of the suspension buffer and sonicate it using SONIFIER 250 (Branson) with output control as 5 and duty cycle of 50% for 20 min.
  - b. Centrifuge the sonicated solution with 8590 × g at 4°C for 15 min.
  - c. Wash the pellet with the triton buffer.
  - d. Further, wash the pellet with the Triton buffer three times.
7. Solubilization of the denature Mincle protein.
  - a. Add 6 mL of the solubilization buffer to the pellet obtained at step 6 and rotate overnight.
  - b. On the following day, centrifuge the solution with 8590 × g at 4°C for 15 min. Preserve the supernatant as the inclusion body solubilized solution.

⏸ Pause point: The inclusion body can be stored at –80°C for at least one year.

8. Refolding and purification of Mincle protein.
  - a. One hour after the addition of dithiothreitol (10 mM), 100 mg of the solubilized proteins were slowly diluted into 1000 mL of a refolding buffer solution.
  - b. The refolding mixture was purified by gel filtration chromatography with HiLoad 26/60 Superdex 75 pg and subsequently with Superdex 10/300 GL (Cytiva, Tokyo, Japan). Finally, 9.6 mg of the Mincle protein was obtained.

9. Change the buffer of Mincle protein at a concentration to 10 mM HEPES-NaOH (pH 7.0) containing 20 mM CaCl<sub>2</sub>, 0.5 or 0 M NDSB-195 by amicon Ultra.
10. Adjust the volume of protein to 360 μL and add 40 μL of D<sub>2</sub>O (final D<sub>2</sub>O concentration becomes to 10%).
11. Transfer protein solution to a 5-mm microcell NMR tube (Shigemi, Tokyo, Japan).
12. Degas dissolved air in the tube using the diaphragm pump (IWAKI GLASS, FTP-20A).
13. Insert the inner tube and remove air bubbles from the sample portion.

### NMR measurement using topspin

⌚ Timing: 6–7 days

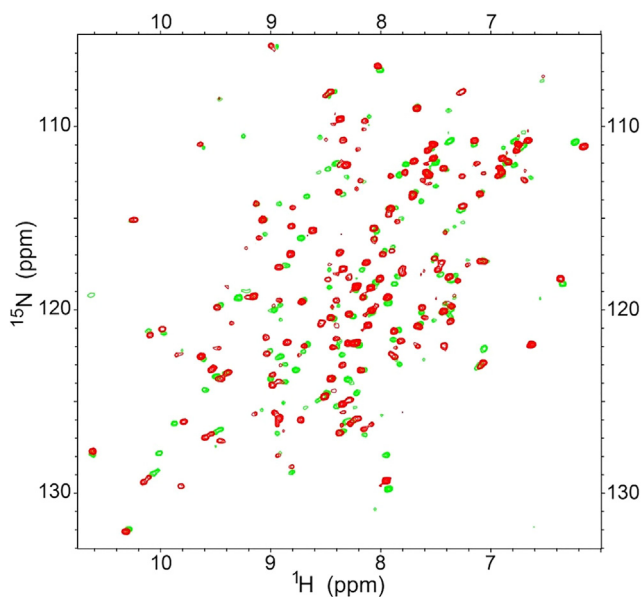
14. Here, a Bruker 800 MHz AVANCE NEO NMR system equipped with a cryogenic triple resonance probe, CPTCI was used.
15. Click topspin icon on NMR instrument and open the topspin-gui.
16. Enter 'ej' command in topspin-gui terminal to float the NMR sample tube with sample-holder and enter the 'ij' to load the NMR sample tube on to the probe.
17. Create a new experimental directory using the command 'new' and load the experimental parameter, ZGPR. Set up the sample temperature of the sample to 298 K using edte panel (command: edte) on topspin-gui and equilibrate the temperature of the sample for 5 min.
18. Use the commands "lock H2O + D2O", "atma" and "topshim rga shigemi full" for frequency lock, tune and shimming, respectively. Notes: Details are described in the TopSpin manuals.
19. Calibrate the 90° pulse width for 1 H (90° hard pulse) for the sample using a command 'pulsecal'.
20. Start the measurement by command 'zgefz'. The obtained spectrum confirms the suppression of the water signal.
21. Create a new experiment directory to be carried over for this calibrated experiment. (using the command 'new' with check the 'Use current experiment').
22. Load the Bruker pulse sequences for 2D sofast HN-HMQC experiments using the following commands, 'sofast'.
23. Notes: The NMR instrument used introduced the sofast pulse program packages described in the following paper. Therefore, The 'sofast' command automatically optimizes the parameters required for a sofast experiment from the calibrated experiment.<sup>4</sup>
24. Set the number of integration times (Number of scan) by command 'ns'.
25. Execute the measurement by command 'zg'.
26. After the measurement is completed, use the 'xfb' command to perform a 2D Fourier transform to check the spectrum.

### NMR data processing

⌚ Timing: 6–7 days

Prerequisite: NMRPipe and NMRdraw,<sup>3</sup> and Sparky.<sup>5</sup>

27. Perform Fourier transform by command 'convft.com'.
28. Open the NMRdraw program by command 'nmrDaw'.
29. Open file. File name is should be "xxx.ft".
30. Command 'h' and one dimensional spectrum will be appeared. Change the value of p0 to make the downward signal disappear. Check the p0 value and close NMRdraw.
31. Command 'gedit convft.com' under the directory where [convft.com](http://convft.com) locates.
32. Change the -p0 value of -fn sol (For example, if p0 value above is -65 and original p0 value is -153, it should be changed to -218).
33. Command 'wq'.
34. Perform Fourier transform again by command 'convft.com'.



**Figure 1. The HSQC spectrum of Mincle without (green peaks) and with NDSB-195 (red peaks)**

35. Execute the NMRdraw by command 'nmrdraw'.
36. Open file.
37. Check the phase.
38. Close NMRdRaw.
39. Command 'pipe2ucsf xxx.ft yyy.ucsf' to convert from NMRdraw format spectrum file to Sparky format file.

### Backbone assignment

⌚ Timing: 8–14 days

40. Execute the Sparky by command 'sparky'.
41. The basic Sparky commands were referred to the following web page <https://www.cgl.ucsf.edu/home/sparky/manual/index.html>.
42. Open all ucsf files (Figure 1).
43. Synchronize the N axis of [1H-15N] HSQC with the N axis of CBCA(CO)NH, HNCACB, and HNCA by command yt. Similarly, the 1H axis is also synchronized.
44. Tentative numbers are assigned to the signals observed in [1H-15N] HSQC. Here, MCn (n is a unique number) is used.
45. Peak pick for the entire [1H-15N] HSQC spectrum.
46. Select each peak from the peak list and assign a tentative assignment, where 'Group' is MCn for both the 15N and 1H axes, and 'Atom' is N and HN, respectively.
47. Perform assignment on the signals observed in the HNCA that are derived from each of the tentatively assigned [1H-15N] HSQC signals.
48. The 'Group' name on the 13C axis is MCn and the Atom name is CA. If a signal derived from one previous residue is observed, the group name is attributed as MCn-1.
49. Next, we perform the attribution operation for CBCA(CO)NH, where the Group name for the 13C axis is MCn-1, and if the signal is from Cβ, the Atom name is CB.
50. Finally, the attribution operation is performed for HNCACB, where the group name on the 13C axis is MCn, but if it is from one residue earlier, MCn-1 is used.
51. Search for the combination of MCn and MCn-1 linked by the obtained chemical shift value information of CA.



52. If there are multiple candidate linkages, the chemical shift values of CB are also used to determine the linkage combination.
53. Combining multiple chain combinations, a cluster of chains is constructed.
54. For each signal that constitutes a cluster, search the amino acid sequence for the part of the sequence that is consistent with the chemical shift values of CA and CB derived from each residue.
55. If the corresponding amino acid sequence part is the only one, change the tentative assignment to the original assignment. For example, if it is Lys131, the Group is renamed to K131.

### Titration analysis of ligands and observation of chemical shift perturbation (CSP)

⌚ Timing: 15–18 days

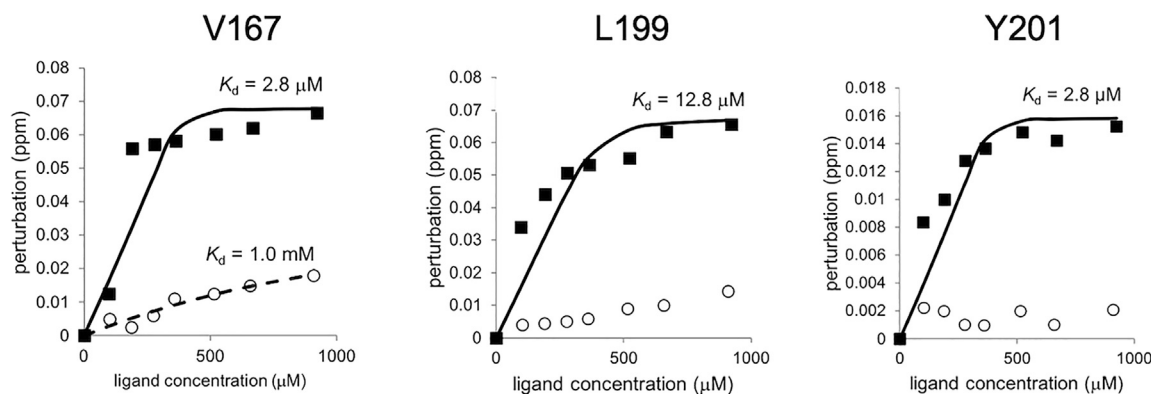
56. Change the buffer of Mincle protein to 10 mM HEPES-NaOH (pH 7.0) containing 20 mM CaCl<sub>2</sub>, 0.5 or 0 M NDSB-195 by amicon Ultra.
57. Adjust the volume of protein to 360 μL.
58. Add 40 μL of D<sub>2</sub>O (final D<sub>2</sub>O concentration becomes to 10%).
59. Transfer protein solution to 5 mm Thin Wall Precision NMR Sample Tube 8" L, 600 MHz (Wilmad LabGlass).
60. Prepare a 10-fold higher concentration of ligands, such as trehalose C10 and trehalose against Mincle protein. Here, we used trehalose C10 and trehalose as the examples. If the protein concentration is 0.45 mM, the concentration of trehalose C10 should be 4.5 mM.
61. Measure NMR spectrum in the same procedure as the section of "NMR measurement using top spin" (This spectrum is the spectrum of the ligand concentration of zero equivalent).
62. Transfer the protein solution to a 1.5 mL tube using a Pasteur pipette. Add 10 μL of trehalose C10 and trehalose to the protein solution and mix well (This solution is equivalent to 0.25 equivalent). Return the mixture to NMR tube and start the acquisition.
63. After the acquisition, transfer the protein solution to a 1.5 mL tube using a Pasteur pipette. Add 10 μL of trehalose C10 and trehalose to the protein solution and mix well (This solution is equivalent to 0.5 equivalent). Return the mixture to NMR tube and start the acquisition.
64. Continue step 56 until the chemical shift perturbation is not observed.
65. Apply each step of the "NMR Data processing" section to obtained HSQC spectrum.
66. In Sparky, open the obtained spectrums.
67. Command 'Ct' to set the counter level. Set the counter level (such as 4.0e+05) to decrease noises and clear peaks from amino acid residues. Set the level as "20" and click the "Apply" button.
68. Overlay spectrums of each titration.
69. Produce the peak lists containing the <sup>1</sup>H and <sup>15</sup>N coordinates of individual peaks of the spectra.
70. Calculate the observed CSPs in Mincle at each protein/ligand ratio relative to free state using the following equation,

$$\Delta\delta_{\text{obs}} = \sqrt{(\Delta\delta_{1\text{HN}})^2 + \left(\frac{1}{6}\Delta\delta_{15\text{N}}\right)^2}$$

where  $\Delta\delta_{\text{NH}}$  and  $\Delta\delta_{\text{N}}$  represent the chemical shift perturbations in the <sup>1</sup>H and <sup>15</sup>N dimensions, respectively.

71. To determine the dissociation constant ( $K_D$ ), titration curves were fitted to the following equation, valid for 1:1 complex formation in fast exchange on the NMR chemical shift time-scale:

$$\Delta\delta_{\text{obs}} = \Delta\delta_{\text{max}} \frac{(K_D + [L] + [P]) - \sqrt{(K_D + [L] + [P])^2 - 4[P][L]}}{2[P]}$$



**Figure 2.** The chemical perturbation change of Q149, L199, and Y201 of human Mincle by the titration of trehalose C10 and trehalose. The chemical shift perturbation of Q149, L199, and Y201 upon the titration of trehalose C10 (black-filled squares) and trehalose (open circles) were shown.  $K_D$  was determined by fitting the equation in step 65 against the titration curve of trehalose C10 and trehalose titration to Mincle.

where [L] and [P] represent the ligand and protein concentrations, respectively.  $\Delta\delta_{(obs)}$  is the observed chemical shift perturbation at the ligand concentration [L].  $\Delta\delta_{max}$  is the chemical shift perturbation at the saturation of ligand.<sup>6</sup> The non-linear curve fitting using the Microsoft Excel 2010 Solver (Washington, USA) was applied for the determination of  $\Delta\delta_{max}$  and  $K_D$  (Figure 2).<sup>7</sup>

72. In detail,  $\Delta\delta_{max}$  and  $K_D$  are variable parameters. In solver,  $\Delta\delta_{max}$  and  $K_D$  are changed and finally determined to decrease the difference between the calculated and observed  $\Delta\delta_{obs}$  and  $\Delta\delta_{obs}$ . Map amino acids that showed chemical shift perturbation to Mincle protein structure using Pymol.

## EXPECTED OUTCOMES

By the whole experiment, it is expected to identify the ligand binding site in Mincle and determine the  $K_D$  value of the interaction with ligands, such as glycolipid<sup>1</sup> and glycerolipid.<sup>8</sup> This method is also suitable for the identification of the binding site of a drug without the co-crystallization of a protein and a drug. In our study, 1 L cell culture grown in M9 media yields more than 80 mg of Mincle.

## LIMITATIONS

In Mincle protein, NMR signals of some amino acids near the presumable glycolipids binding site were unobserved due to their flexibility. These unobserved peaks prevented the complete understanding of the ligand binding mechanism. However, in some proteins, peaks are appeared after the ligand binding because the flexibility is decreased.

## TROUBLESHOOTING

### Problem 1

The yield of the inclusion body of the labeled protein is low (step 7).

### Potential solution

- Test other *E. coli* strains. For example, when we used BL21(DE3) with M9 medium, the expression of Mincle from 1 L culture was 3.1 mg, however, using BL21-Gold (DE3) with M9 medium, the expression of Mincle protein was 83.1 mg.
- Use a codon-optimized expression vector.

### Problem 2

The final yield (refolding efficiency) is low (step 8).

**Potential solution**

- Check the purity of inclusion bodies.
- Modify the ratio of cytamine and cysteamine.
- Add EDTA (such as 0.5 mM), dithiothreitol (such as 1 mM) and/or CHAPS (such as 10 mM)

**Problem 3**

Observable peaks are less than the number of amino acid residues (step 51).

**Potential solution**

- Decrease the temperature to suppress the flexibility of a protein.
- Add the ligand to form the inflexible conformation of a protein.
- Change the pH of the buffer and the concentration of salt.

**Problem 4**

Doublet peaks are observed (step 51).

**Potential solution**

- Check if the dimer is formed by gel filtration chromatography.
- Add triton (such as 0.05%) and/or dithiothreitol (such as 1 mM) to prevent aggregation.
- The mixture of Cis and Trans formation of prolines might cause the appearance of double peaks. Make proline to alanine mutant and perform steps from 1 to 38 and check the HSQC spectrum if the mixture of Cis and Trans formation produced the duplication.

**Problem 5**

Line broadening and disappearance of the peaks were observed during titration. (step 53).

**Potential solution**

Perform experiments in NDSB-195 containing buffer or detergent-containing buffer, such as 0.1% Triton X-100 or 1% tween-20.

**RESOURCE AVAILABILITY****Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Katsumi Maenaka ([maenaka@pharm.hokudai.ac.jp](mailto:maenaka@pharm.hokudai.ac.jp)).

**Technical contact**

Questions about the technical specifics of performing the protocol should be directed to and will be answered by the technical contact, Katsumi Maenaka ([maenaka@pharm.hokudai.ac.jp](mailto:maenaka@pharm.hokudai.ac.jp)).

**Materials availability**

All reagents generated in this study will be made available on reasonable request.

**Data and code availability**

In this protocol, original data and code were not provided.

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### AUTHOR CONTRIBUTIONS

Conceptualization, A.F. and K.M.; methodology, A.F., H.K., T.S., and K.M.; investigation, A.F., H.K., T.S., and K.M.; writing – original draft, A.F., H.K., T.S., and K.M.; writing – review and editing, A.F., H.K., T.S., and K.M.; funding acquisition, A.F. and K.M.; resources, A.F., H.K., T.S., and K.M.; supervision, K.M.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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