A Plug Release Mechanism for Membrane Permeation by MLKL

Supplementary Figures.

Figure S1. The extra residues in MLKL(2-178) do not alter the overall structure of MLKL(2-154). **A.** Superposition of ^{1}H-^{15}N HSQC spectra of MLKL(2-154) (black) and MLKL(2-178) (red). The spectra are the same shown in Figure 1 but were plotted at lower contour levels to enable observation of the weaker cross-peaks from MLKL(2-178). Selected cross-peaks that are markedly shifted and or broadened when comparing the two spectra are labeled with the corresponding residue number. The inset shows a ribbon diagram of the structure of MLKL(2-154) described here (in blue) with the residues labeled on the ^{1}H-^{15}N HSQC spectra colored in yellow. Note that all these residues are near the C-terminus (labeled C). **B.** ^{1}H-^{15}N HSQC spectrum of MLKL(2-154) showing assignment of selected well-resolved cross-peaks. Related to Figure 1.

Figure S2. Liposome co-floatation assays with MLKL(2-154). The assays were performed with liposomes containing different percentages of cardiolipin (CL) as indicated above the lanes. Samples were analyzed by SDS PAGE and coomassie blue staining. The positions of molecular weight markers (kDa) are indicated on the right. Related to Figure 2.

Figure S3. MLKL(2-154) does not cause membrane fusion. The bar diagrams illustrate dynamic light scattering data obtained for liposomes under the conditions of the leakiness assays

described in Figure 3, in the absence (**A**) and presence (**B**) of 150 nM MLKL(2-154). The average radii (R) and polydispersity (%Pd) yielded by the data are indicated. Related to Figure 3.

Figure S4. Sequence alignment of the MLKL N-terminal domains. An alignment of the sequences of the N-terminal domains of MLKL from different species is shown, with selected residue numbers corresponding to the human sequence above the alignment. Residues are color-coded as follows: red, small plus hydrophobic (excluding Tyr); blue, acidic; magenta, basic plus His; green, polar plus Gly. The alignment was prepared with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Related to Figure 4.

Figure S5. Effects of mutagenesis and NBD labeling on the liposome leakage activity of MLKL(2-154). **A.** CD spectra of 2 μM MLKL(2-154) in 20 mM sodium phosphate pH 7.4, 100 mM NaCl, in the absence (black) and presence (red) of liposomes (1 mM lipids) with the same lipid composition used for leakiness assays. **B.** Liposome leakage assays performed as in Figure 3 with 2 μM MLKL(2-154) in the absence (black) and presence (red) of 1 mM DTT. **C,D.** Liposome leakage assays in the presence of WT MLKL(2-154) (black curve), and the indicated NBD labeled mutants. Proteins were added after 200 s. The data were normalized with the fluorescence observed after adding detergent, and setting at zero the fluorescence right before protein addition. Related to Figure 5.

Figure S6. Complementary analysis of packing between helices in the MLKL N-terminal region. **A**. Helices H2 and H5 in the crystal structure of MLKL (Murphy et al., 2013). The atoms of the side chains of basic residues are colored in blue and those of acidic side chains are colored in

red. In other side chains and the backbone, carbon atoms are in green, oxygen atoms are in red, nitrogen atoms are in blue and sulfur atoms are in yellow. The helices are indicated at the bottom. Note the poor packing between the two helices. **B.** Two-dimensional ^{1}H-^{1}H strip from a three-dimensional ^{1}H-^{13}C NOESY-HSQC spectrum of MLKL(2-154) taken at the ^{13}C chemical shift corresponding to W109 HH2. Cross-peak assignments are indicated on the right. **C**. Ribbon and stick model of the region containing the W109 side chain in the solution structure of MLKL(2-154). Residues with protons near the W109 HH2 proton are labeled. Related to Figure 6.

Figure S7. A. Superposition of ^{1}H-^{15}N HSQC spectra of MLKL(2-154) (black) and MLKL(2-123) (red). **B**. Liposome co-floatation assays with MLKL(2-154), MLKL(2-123), MLKL(2-154)K26E,R30E and MLKL(2-154)K22Q,K25Q. The assays were performed with liposomes containing 15% cardiolipin (CL). Samples were analyzed by SDS PAGE and coomassie blue staining. The positions of molecular weight markers (kDa) are indicated on the right. **C.** Ribbon diagram showing the region where part of helix H6 packs against helix H4. Side chains are shown as stick models with carbon atoms in green, oxygen atoms in red, nitrogen atoms in blue and sulfur atoms in yellow. The side chains of C86 from helix H4 and F148 from helix H6 are labeled. Related to Figure 7.

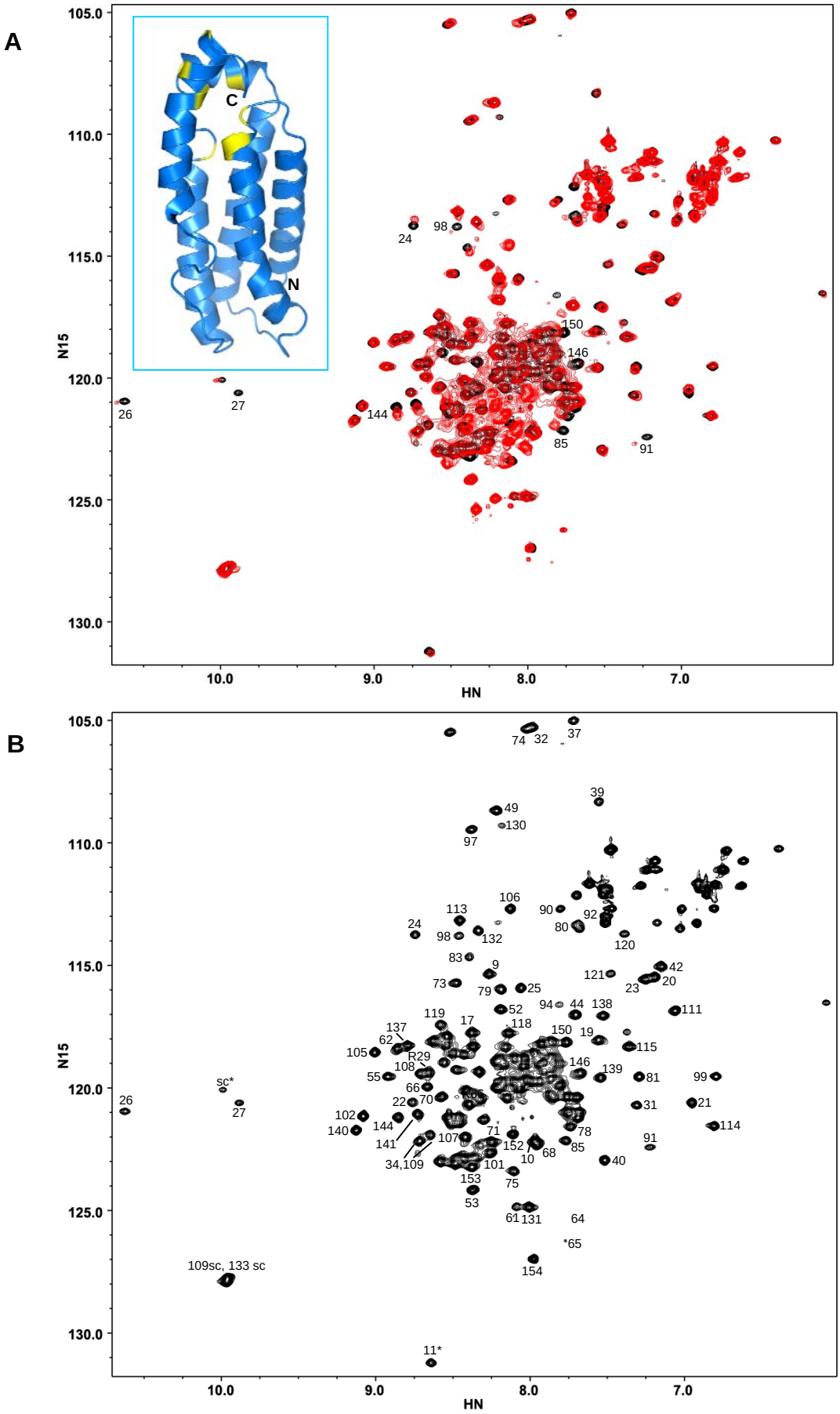

Figure S1
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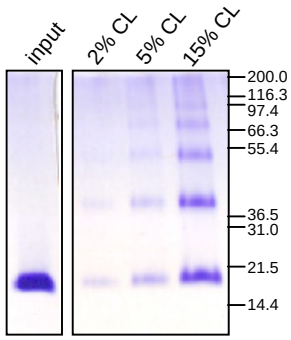

Figure S2
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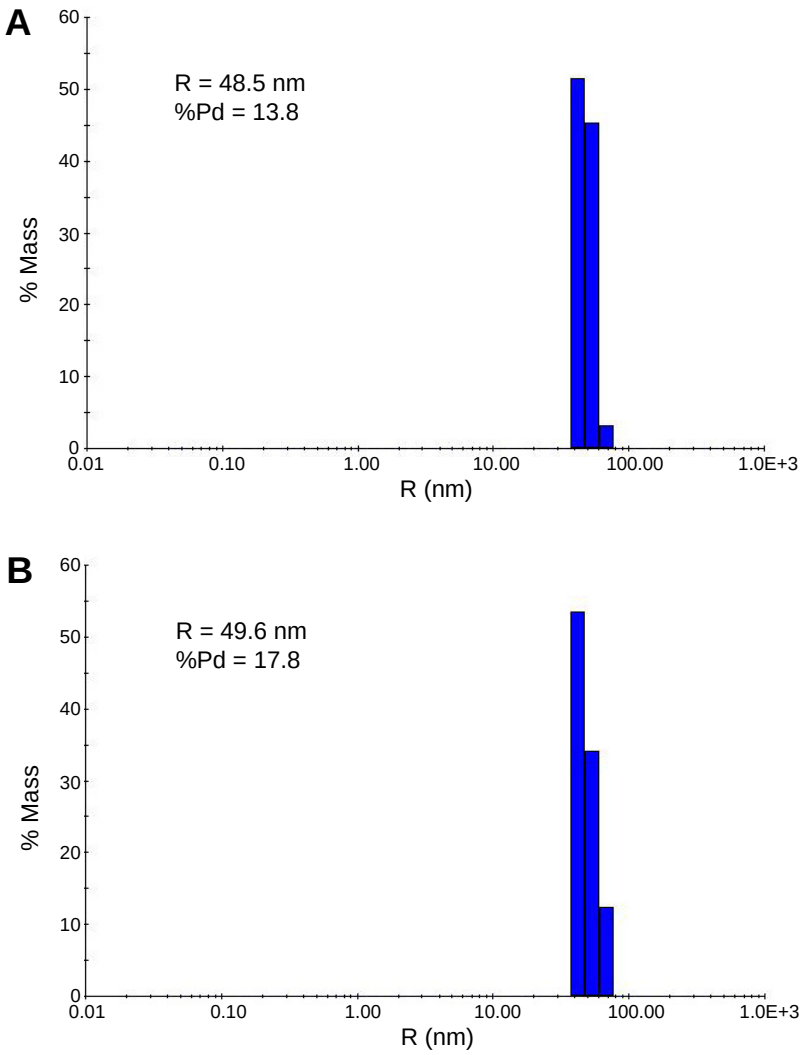

Figure S3
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A

Figure S4
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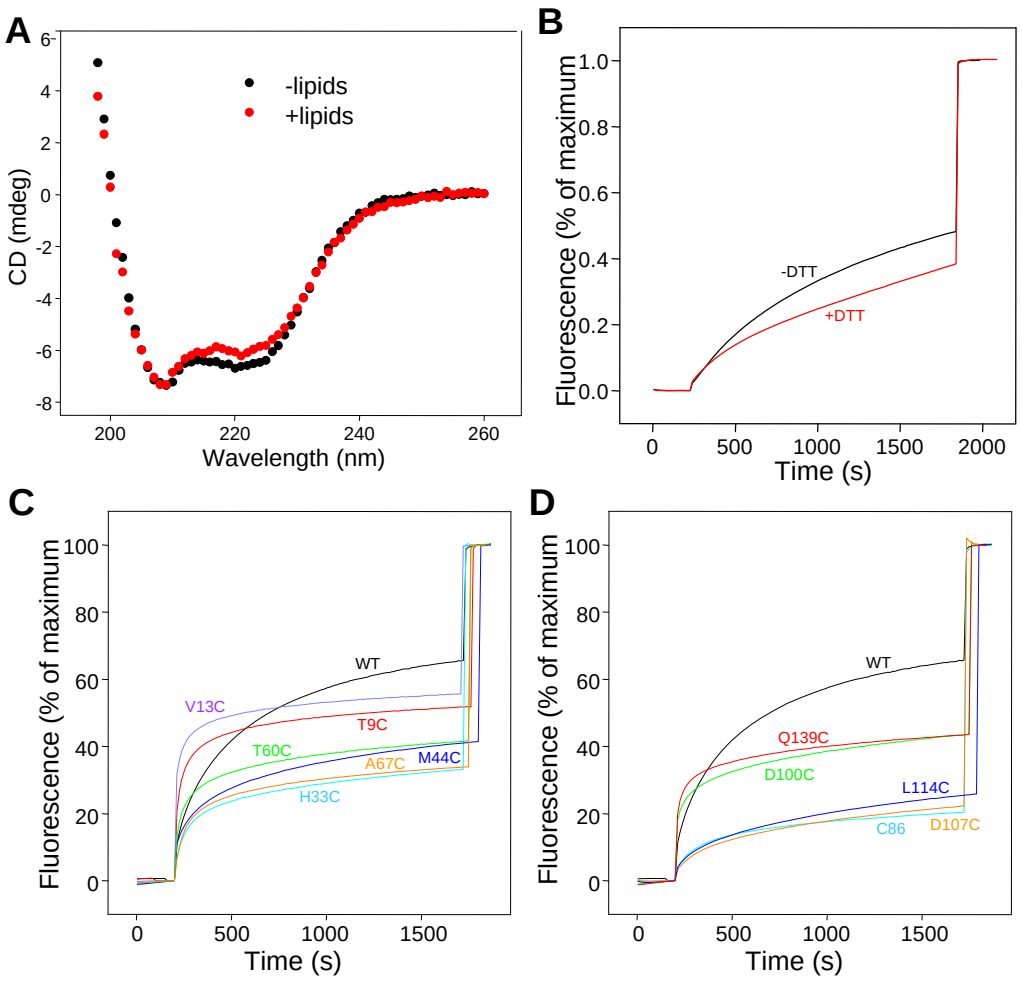

Figure S5
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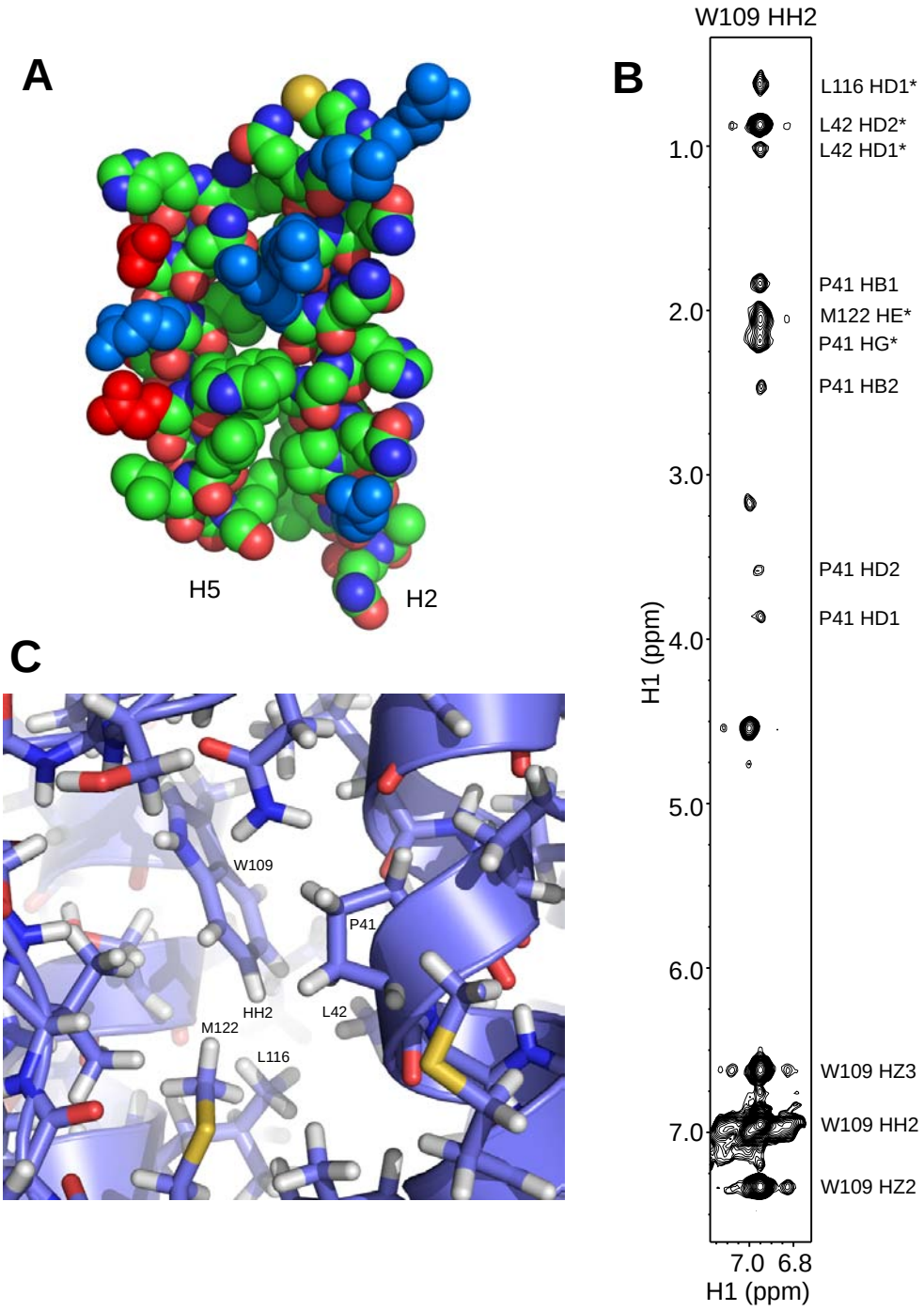

Figure S6
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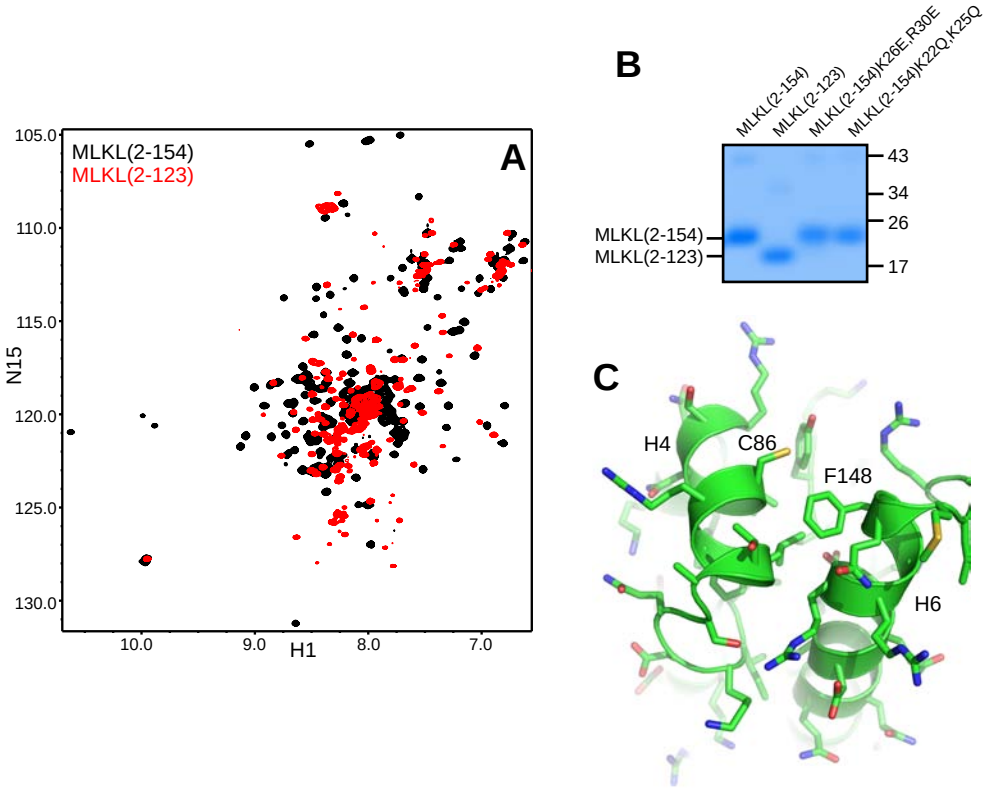

Figure S7
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Supplementary Experimental Procedures

NMR spectroscopy

Initial ^{1}H-^{15}N HSQC spectra of MLKL(2-123) (50 μM), MLKL(2-154) (200 μM) and MLKL (2-178) (200 μM) were acquired at 600 MHz on INOVA or DD2 spectrometers equipped with cold probes (Agilent), using samples dissolved in 20 mM HEPES (pH 7.0), 150 mM NaCl. For analysis of binding to nanodiscs and liposomes, ^{1}H-^{15}N HSQC spectra were acquired at 800 MHz on an INOVA800 spectrometer equipped with a cold probe (Agilent) with samples dissolved in 20 mM HEPES (pH 7.4), 100 mM NaCl. The protein concentrations were 20-50 μM and the total lipid concentrations were 1 mM. Nanodiscs of 12 nm diameter containing 45% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 30% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 10% L-α-phosphatidylinositol (PI) and 15% cardiolipin were prepared as described (Brewer et al., 2011). Liposomes for NMR experiments contained the same lipid composition and were prepared as described below for the leakage assays.

Liposome co-flotation assays

Liposomes with lipid composition of 58% POPC, 30% POPE, 10% PI and 2% cardiolipin; 55% POPC, 30% POPE, 10% PI and 5% cardiolipin; or 45% POPC, 30% POPE, 10% PI and 15% cardiolipin, were prepared as described above for the dye leakage assays. Liposome samples containing 2 mM lipids were first incubated with 5 μM MLKL(2-154) at room temperature for 1 hour. The proteins bound to the liposomes were separated from the unbound proteins by floatation on a Histodenz density gradient (40%:35%:30%) as described (Guan et al., 2008).

After centrifugation, the proteins bound to the liposomes that migrated to the top of the gradient were taken (30 µl) and analyzed by SDS-PAGE and Coomassie blue staining.

NBD fluorescence experiments

To label MLKL (2-154) at specific places with NBD, the four endogenous cysteines (C18, C24, C28 and C86) were first mutated to serines. Single cysteine mutations were then introduced into the six different helices of MLKL(2-154): T9C, V13C, H33C, M44C, T60C, A67C, D100C, D107C, L114C and Q139C. The C86 MLKL(2-154) mutant was made by mutating only the other three native cysteines of MLKL(2-154) (C18, C24 and C28). For labeling with NBD, 100 µM solutions of the mutants were incubated with 1 mM NBD-maleimide (IANBD amide; Invitrogen) in a buffer containing 20 mM HEPES pH7.2, 100 mM NaCl, 200 µM TCEP at room temperature for 2 hours. The samples were then incubated with 20 mM DTT at room temperature for 15 min to quench the free NBD-maleimide. The NBD-labeled protein samples were then separated from the free NBD-maleimide by gel filtration using Superdex 200 10/30 with a buffer containing 20 mM HEPES pH7.4, 100 mM NaCl.

Liposomes (5 mM) with lipid composition of 45% POPC, 30% POPE, 10% PI and 15% cardiolipin were prepared as described above for the leakage assays but without sulforhodamine B. To study the insertion of the NBD probes of the different mutants into the membrane, NBD fluorescence emission (500-650 nm) spectra were acquired with samples containing 0.2 µM NBD-labeled protein samples without or with 300 µM lipid vesicles in buffer containing 20 mM HEPES (pH 7.4) and 100 mM NaCl. The experiments were performed at room temperature on a PTI spectrofluorimeter with excitation at 485 nm.

Supplementary References

Guan,R., Dai,H., and Rizo,J. (2008). Binding of the Munc13-1 MUN Domain to Membrane-Anchored SNARE Complexes. Biochemistry *47*, 1474-1481.