



HAL
open science

Investigations of the Structure, Topology, and Interactions of the Transmembrane Domain of the Lipid-Sorting Protein p24 Being Highly Selective for Sphingomyelin-C18

Christopher Aisenbrey, Patricia Kemayo-Koumkoua, Evgeniy Salnikov, Elise Glattard, Burkhard Bechinger

► To cite this version:

Christopher Aisenbrey, Patricia Kemayo-Koumkoua, Evgeniy Salnikov, Elise Glattard, Burkhard Bechinger. Investigations of the Structure, Topology, and Interactions of the Transmembrane Domain of the Lipid-Sorting Protein p24 Being Highly Selective for Sphingomyelin-C18. *Biochemistry*, 2019, 58 (24), pp.2782-2795. <10.1021/acs.biochem.9b00375>. <hal-02323783>

HAL Id: hal-02323783

<https://hal.science/hal-02323783v1>

Submitted on 17 Nov 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

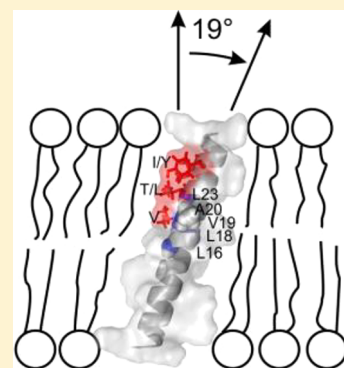
Investigations of the Structure, Topology, and Interactions of the Transmembrane Domain of the Lipid-Sorting Protein p24 Being Highly Selective for Sphingomyelin-C18

Christopher Aisenbrey,[†] Patricia Kemayo-Koumkoa,[†] Evgeniy S. Salnikov, Elise Glattard, and Burkhard Bechinger*[✉]

Université de Strasbourg/CNRS, UMR7177, Institut de Chimie, 4, rue Blaise Pascal, 67070 Strasbourg, France

S Supporting Information

ABSTRACT: The p24 proteins play an important role in the secretory pathway where they selectively connect various cargo to other proteins, thereby being involved in the controlled assembly and disassembly of the coat protein complexes and lipid sorting. Recently, a highly selective lipid interaction motif has been identified within the p24 transmembrane domain (TMD) that recognizes the combination of the sphingomyelin headgroup and the exact length of the C18 fatty acyl chain (SM-C18). Here, we present investigations of the structure, dynamics, and sphingomyelin interactions of the p24 transmembrane region using circular dichroism, tryptophan fluorescence, and solid-state nuclear magnetic resonance (NMR) spectroscopies of the polypeptides and the surrounding lipids. Membrane insertion and/or conformation of the TMD is strongly dependent on the membrane lipid composition where the transmembrane helical insertion is strongest in the presence of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and SM-C18. By analyzing solid-state NMR angular restraints from a large number of labeled sites, we have found a tilt angle of 19° for the transmembrane helical domain at a peptide-to-lipid ratio of 1 mol %. Only minor changes in the solid-state NMR spectra are observed due to the presence of SM-C18; the only visible alterations are associated with the SM-C18 recognition motif close to the carboxy-terminal part of the hydrophobic transmembrane region in the proximity of the SM headgroup. Finally, the deuterium order parameters of POPC-*d*₃₁ were nearly unaffected by the presence of SM-C18 or the polypeptide alone but decreased noticeably when the sphingomyelin and the polypeptide were added in combination.



The secretory pathway is essential in the synthesis, transport, and processing of proteins and bioactive molecules and plays a key role in intercellular communication.¹ Thereby, it is vitally important and can be linked to a variety of diseases. A well-functioning secretory pathway assures the controlled transport and sorting of proteins and lipids along subcompartments of the endoplasmic reticulum, the Golgi apparatus, and the plasma membrane. During the early secretory pathway, the combination of anterograde and retrograde transport by vesicles covered by coat protein I and II (COPI- and COPII-coated transport vesicles, respectively) and sorting ensure a controlled and tunable system assuring the correct amount, processing, and destination of proteins and lipids in each compartment.^{1,2}

COPI vesicles ensure the retrograde transport of the early secretory pathway by shuttling from the early Golgi back to the endoplasmic reticulum.¹ Interestingly, these vesicles are depleted of cholesterol and sphingomelin (SM) except for one SM species carrying a C18 fatty acyl chain providing evidence for an early segregation and sorting of these lipids.³ The vesicles are made of an outer heptameric coatomer showing similarities to the clathrin coat, the p24 family of proteins that connect to various cargos in a selective manner, and a number of proteins

that mediate contact between these layers and are involved in the controlled assembly and disassembly of the complexes.^{1,2,4,5}

The p24 proteins exist in mammals, yeast, and plants and are involved in cellular signaling, hormone secretion, and the occurrence of Alzheimer's disease.⁵ They have also been shown to be important during the development of mice embryos.⁶ Lipid cross-linking experiments in cells using a radioactive sphingolipid precursor and immunoprecipitation revealed a strong interaction of the sphingolipid with p24 but not with p23, another member of the family.⁷ In follow-up experiments, the transmembrane domains of p23 and p24 were prepared as fusions with maltose binding proteins and reconstituted into model membranes and their interactions with pentaenoyl-sphingomyelins were investigated by FRET.^{8,9} Distinct FRET was observed for the Trp4 residue of the p24 transmembrane domain with SM-C18:5 but not for p23. Furthermore, the FRET signal was considerably weaker in the presence of sphingomyelins with different chain lengths or for the corresponding phosphatidylcholine (PC-C18:5) indicating a highly specific

Received: April 26, 2019

Revised: May 22, 2019

Published: May 23, 2019

69 interaction of the p24TMD and SM-C18. An alanine scanning
70 experiment revealed a number of residues that represent a
71 structural motif for lipid selectivity. These are all positioned at
72 the carboxy-terminal end of the TMD and on the same face of
73 the helix. MD simulations indeed show transient interactions of
74 SM-C18 with p24 involving these amino acid residues with a
75 lifetime of the complex in the range of 250 ns.⁸

76 Whereas the modulation of protein activity by sphingolipids
77 and their binding to proteins has previously been character-
78 ized,¹⁰ the selection of a molecular species based on headgroup
79 and fatty acyl chain composition is rather unique.⁸ It has been
80 shown that sphingomyelin-C18 modulates the inactive mono-
81 mer to active oligomer equilibrium of p24,⁸ thereby regulating
82 its interactions with cargo and/or other proteins of the COPI
83 complex.² An allosteric mechanism of regulation has been
84 suggested in which polar residues involved in dimer formation
85 are localized opposite the SM-C18 molecular recognition
86 motif.¹⁰ A bioinformatics analysis identified related shingomye-
87 lin recognition motifs of the type [V/I/T/L]XX[V/I/T/L][V/
88 I/T/L]XX[V/I/T/L][F/W/Y] in a wide variety of membrane
89 proteins, including the major histocompatibility complex II Q α -
90 chain or GPCRs, in particular within helix 6.¹¹ The bulky β -
91 branched residues and the aromatic residues of the motif form a
92 crevice recognized by SM-C18.

93 To gain better insight into the lipid interactions of p24 here,
94 we used circular dichroism, fluorescence, and oriented solid-
95 state NMR spectroscopies to investigate the structure, topology,
96 and dynamics of the p24 transmembrane domain in
97 phospholipid bilayers. We also tested for conformational and/
98 or topological changes that may occur due to interactions with
99 SM-C18.

100 Solid-state NMR spectroscopy of uniaxially oriented lipid
101 bilayers is a well-established technique for the investigation of
102 the topology of membrane-associated polypeptides.^{12–15} When
103 several orientation-dependent parameters have been measured,
104 accurate helix topologies have been determined.^{12,14,16–18} In
105 particular, the combination of oriented solid-state NMR spectra
106 of ¹⁵N-labeled peptide bonds with methyl-deuterated alanines
107 provides a sensitive measure of the topology of the membrane-
108 associated helices where changes in the tilt and rotational pitch
109 angles of as little as 1° become apparent.^{19–21} Furthermore, the
110 line shapes of the backbone and side chains are indicators of
111 motional averaging and structural heterogeneity.^{22–24}

112 The peptides were reconstituted in various phospholipid
113 model membranes, including complex mixtures that represent
114 well the lipid composition of the Golgi membranes.⁸ Finally, the
115 changes in the lipid alignment and fatty acyl chain order
116 parameters due to the presence of p24TMD and/or the SM have
117 been monitored using ³¹P and ²H solid-state NMR spectroscop-
118 y;^{25–27} thus, a complete picture of the p24–lipid supra-
119 molecular arrangement is obtained.

120 By combining several biophysical approaches and a good
121 number of isotopic labeling schemes, the work presented here
122 reveals important structural details such as the secondary
123 structure, helix tilt angle, side chain dynamics, and protein–lipid
124 interactions, and only minor changes in the p24TMD structure
125 and dynamics were observed upon addition of SM-C18 to
126 supported phosphatidylcholine bilayers.

127 ■ MATERIALS AND METHODS

128 Organic solvents were purchased from Sigma-Aldrich (St. Louis,
129 MO) with a purity of 99%; cholesterol and phospholipids,
130 including *N*-octadecanoyl-D-erythro-sphingosylphosphorylcho-

line (SM-C18), were purchased from Avanti Polar Lipids 131
(Birmingham, AL). Fmoc amino acids were from NovaBio- 132
chem/Merck KGaA (Darmstadt, Germany), and isotope- 133
labeled amino acids from Cortecnet (Voisins les Bretonneux, 134
France) or Aldrich (St. Louis, MO). TentaGel-R-RAM resin was 135
from Rapp Polymer GmbH (Tübingen, Germany). 136

Peptide Synthesis. The human p24TMD peptides with the 137
sequence KKTNS RVVLW SFFEA LVLVA MTLGQ IYYLK R- 138
CONH₂ (UniProt entry Q15363 TMED2_HUMAN, residues 139
165–193 and two additional lysines at the amino terminus) 140
were prepared by solid phase peptide synthesis using an 141
automatic Millipore 9050 synthesizer, TentaGel-R-RAM resins 142
(Rapp Polymer GmbH), and a 4-fold excess of Fmoc-protected 143
amino acid (NovaBiochem/Merck KGaA). Isotope-labeled 144
peptides were prepared using ¹⁵N- and ²H-labeled amino acids 145
(Cortecnet) incorporated at chosen positions. After cleavage 146
and deprotection in a trifluoroacetic acid (TFA)/water/ 147
triethylsilane mixture [28/1.5/0.3 (v/v/v)], the peptide in 148
solution was taken up in toluene [50/50 (v/v)] and the solvent 149
evaporated. The peptide was washed three times in cold ether at 150
4 °C. The peptide was purified by semipreparative reverse phase 151
HPLC using ProntoSIL 300-6-C4 5.0 μ m (Bischoff, Leonberg, 152
Germany) or Luna 100-C18 5.0 μ m columns (Phenomenex) 153
and an acetonitrile/water gradient. The purity of p24TMD was 154
tested by analytical HPLC, and the identity was verified by 155
matrix-assisted laser desorption ionization mass (MALDI) 156
spectrometry. The final yields of the pure peptide were typically 157
10% with a loss of ~83% during the purification step. The dry 158
peptide pellet was solubilized in 4% acetic acid to exchange the 159
counterions and lyophilized. 160

The peptides with the following isotopic labels were 161
synthesized: ¹⁵N-Leu23 and ²H₈-Val19, ¹⁵N-Val19 and ²H₁₀- 162
Leu23, ¹⁵N-Leu23 and ²H₃-Ala20, ¹⁵N-Ala20, ¹⁵N-Leu18 and 163
²H₃-Ala20, ¹⁵N-Val19 and ²H₃-Ala15, ¹⁵N-Leu18 and ²H₃- 164
Ala15, and ¹⁵N-Leu16 and ²H₃-Ala15. Notably, here the 165
numbering of residues includes the full peptide and thereby 166
adds +6 when compared to the numbering of ref 8. The 167
corresponding positions of the full-length protein are obtained 168
by adding +162 to the numbering used here. 169

Reconstitution in Liposomes. On the basis of extensive 170
reconstitution assays,^{28,29} proteoliposomes were prepared in the 171
following manner. Aliquots of 30 μ M peptide in water were 172
prepared from a 1 mg/mL stock solution, and the water was 173
removed by lyophilization; 100 μ L of a 10 mM stock solution of 174
lipid in hexafluoroisopropanol (HFIP) was added to the 175
lyophilized peptide, and the mixture completed with solvent 176
to give a final volume of 1 mL. After vortexing, the solvents were 177
evaporated under a stream of nitrogen gas. The remaining traces 178
of solvent were removed by high vacuum overnight. The 179
resulting film was hydrated in 10 mM phosphate buffer (pH 7). 180
Small unilamellar vesicles (SUVs) were obtained by tip 181
sonication at 4 °C (Sonoplus HD 200; Bandelin, Berlin, 182
Germany). Aggregates and titanium debris from the tip were 183
removed at room temperature by centrifugation for 30 min at 184
11000g. 185

Reconstitution in Detergent. A stock solution of peptide 186
was prepared in water; samples were diluted to a final 187
concentration of 30 μ M, and the water was removed by 188
lyophilization overnight. The peptide was dissolved again in 189
HFIP, and the solvent was evaporated under a stream of 190
nitrogen gas and under vacuum overnight. Thereafter, it was 191
resuspended in 10 mM phosphate buffer (pH 7). Detergent 192
stock solutions of *n*-dodecylphosphocholine (DPC) and SDS 193

194 were prepared at final concentrations of 10 and 100 mM,
195 respectively, and diluted to different concentrations ranging
196 from 0.1 to 16 mM during peptide titrations.

197 **Circular Dichroism.** CD spectra were recorded from 30 μ M
198 peptide solutions (and 1 mM lipid were applicable) in 300 μ L
199 cuvettes (Hellma Analytics, Mühlheim, Germany) on a Jasco
200 (Tokyo, Japan) J-510 spectropolarimeter using a scan speed of
201 50 nm/min, a bandwidth of 3 nm, and five scans collected
202 covering the range from 250 to 190 nm using a quartz cell with a
203 1 mm path length, at 25 °C. The spectra were processed using
204 the spectra manager software of the instrument, and the solvent
205 contributions subtracted. Secondary structures were analyzed
206 with the CDpro Web server using the CONTINLL algorithm.³⁰

207 **Tryptophan Fluorescence.** The intrinsic fluorescence of
208 p24TMD due to the presence of a tryptophan was measured
209 using a FluoroLog spectrophotometer (HORIBA, Ltd., Kyoto,
210 Japan) with the polarization filters always at the magic angle.
211 Fluorescence emission spectra were recorded from 295 to 455
212 nm at an excitation wavelength of 280 nm. The excitation slit
213 width was 1 nm; the emission slit width was 4 nm. The peptide
214 was prepared in 10 mM phosphate buffer (pH 7.2) at a final
215 concentration of 30 μ M. A series of emission spectra were
216 recorded at 25 °C while samples were being stirred.

217 **Preparation of Oriented Samples for Solid-State NMR**
218 **Spectroscopy.** Selectively labeled ¹⁵N and/or ²H p24TMD
219 was first dissolved in HFIP, and the solvent was removed under a
220 stream of nitrogen. The peptide was then dissolved in a 50/50
221 (v/v) HFIP/water mixture and added in a stepwise manner to
222 ~150 mg of POPC in HFIP with a final peptide-to-lipid (P/L)
223 molar ratio of 1%. Thereafter, the solvent was gently evaporated
224 under a stream of nitrogen to decrease its total volume to ~0.5
225 mL. To avoid peptide aggregation, small volumes of HFIP were
226 added; thus, the water content remained low during the
227 process.²⁸ The resulting clear and viscous sample that was
228 deposited onto ~20 ultrathin glass plates (6 mm \times 11 mm or 8
229 mm \times 22 mm, thickness 00, i.e., ~80 μ m, Paul Marienfeld,
230 Lauda-Königshofen, Germany) slowly dried in air, and the
231 residual solvent evaporated under high vacuum. After the sample
232 had equilibrated at 93% humidity for 2–3 days at room
233 temperature, the glass plates were stacked on top of each other.
234 The stack was stabilized by Teflon tape, and the sample sealed in
235 plastic wrapping to avoid dehydration.

236 **Preparation of Non-Oriented Samples.** Non-oriented
237 samples were prepared by dissolving 3 mg of deuterated POPC
238 (POPC-*d*₃₁) and 0.14 mg of p24TMD (1 mol %; pH adjusted to
239 7) and/or 0.15 mg of SM-C18 (5 mol %) in HFIP. The solvent
240 was evaporated under a stream of nitrogen and high vacuum
241 overnight; thus, a film forms on the walls of the small glass tube
242 (6 mm outer diameter). The sample was then resuspended in 15
243 μ L of 10 mM phosphate buffer (pH 7.1) by vortexing and
244 sonication in a water bath followed by five freeze/thaw cycles. In
245 some cases, stable vesicles were obtained only after a drying and
246 resuspension step. For NMR spectral acquisition, the glass tube
247 with the sample was directly inserted into the solenoidal coil of a
248 static solid-state NMR probe.

249 **Solid-State NMR Experiments.** Solid-state NMR spectra
250 were recorded on a Bruker Avance NMR spectrometer
251 operating at 9.4 T (some spectra were acquired at 7.05 T).
252 The oriented samples were inserted into commercial E-free flat
253 coil NMR probes (Bruker, Rheinstetten, Germany) with the
254 normal parallel to the magnetic field.³¹ All spectra were recorded
255 at 295 K.

Proton-decoupled ¹⁵N solid-state NMR spectra were
256 recorded at 40.54 MHz on a Bruker Avance NMR 400 MHz
257 spectrometer using a cross-polarization (CP) pulse sequence.
258 The CP contact time was 800 μ s, the repetition time 3 s, the ¹H
259 B₁ field 31 kHz, and the spectral width 38 kHz, and the
260 acquisition times ranged from 6 to 20 ms. The spectra were
261 calibrated relative to external ammonium chloride (¹⁵NH₄Cl) at
262 40 ppm.³² An exponential apodization function with a line
263 broadening of 200 Hz was applied prior to Fourier trans-
264 formation. 265

Proton-decoupled ³¹P solid-state NMR spectra were recorded
266 at 161.937 MHz on a Bruker Avance 400 MHz NMR
267 spectrometer using a Hahn-echo pulse sequence,³³ an echo
268 time of 40 μ s, a repetition time of 3 s, a ³¹P B₁ field of 60–80
269 kHz, and a spectral width of 40–120 kHz. The spectra were
270 referenced relative to 85% phosphoric acid (H₃PO₄) at 0 ppm.
271 An exponential line broadening (LB) of 150 Hz was applied
272 prior to Fourier transformation. 273

Deuterium solid-state NMR spectra of the ²H₃-alanine-
274 labeled peptide reconstituted in lipid bilayers were recorded at
275 61.4 MHz on a Bruker Avance 400 MHz NMR spectrometer
276 using a quadrupole-echo sequence.³⁴ A dwell time of 1 μ s was
277 chosen to allow a precise adjustment of the echo by left-shifting
278 the FID after the acquisition (corresponding to a spectral
279 window of 500 kHz); a B₁ field of 30–50 kHz, an acquisition
280 time of 8 ms, interpulse delays of 30–50 μ s, and a recycling time
281 of 1.5 s were used. For processing, the FID was left-shifted to
282 the top of the echo. An exponential multiplication corresponding to
283 a LB of 500 Hz was applied before Fourier transformation. All
284 ²H solid-state NMR spectra were referenced relative to external
285 ²H₂O at 0 ppm. 286

For ²H solid-state NMR spectra of deuterated lipids, a
287 repetition delay of 0.3 s, an echo time of 100 μ s, a dwell time of
288 0.5 μ s, and a B₁ field of 40 kHz were used. The processing
289 included an exponential apodization with a line broadening of
290 100 Hz. The temperature was set to 37 °C. 291

Calculation of Angular Restraints from Experimental
292 **Solid-State NMR Spectra.** To determine the peptide
293 orientations that agree with the experimental spectra, a
294 coordinate system was defined. Within the α -helix, the tilt is
295 defined as the angle between the long axis of the helix and the
296 membrane normal, and the pitch angle as that between the
297 membrane normal and the line within an arbitrary plane of the
298 peptide helical wheel projection. Protein Data Bank (PDB)
299 coordinates of an ideal α -helix were generated in MOLMOL.³⁵
300 The coordinates of the labeled ¹⁵N atom, the corresponding
301 amide proton, and the C atom of the previous residue were
302 extracted and used to calculate the ¹⁵N chemical shift tensor in
303 the same reference frame using the main ¹⁵N chemical shift
304 tensors reported previously (55.8, 81.4, and 228.6 ppm).³⁶ By
305 successively rotating the peptide molecule around the pitch and
306 the tilt angle, we systematically screened the three-dimensional
307 orientational space in 50 \times 50 steps using a program written in
308 MATHEMATICA 3.0 (Wolfram Research, Champaign, 309
310 IL).^{19,37} Contour plots mark the angular pairs that agree with
311 the experimental results. The simulation of the ²H solid-state
312 NMR spectra was performed on the same principles by
313 extracting the coordinates of the C $_{\alpha}$ and C $_{\beta}$ atoms in the
314 oriented PDB files. The contour plots of several ¹⁵N- and ²H-
315 labeled sites were superimposed in a single plot. 315

Calculation of the Lipid Order Parameters. To
316 investigate the lipid fatty acyl chain packing and dynamics in
317 the presence of p24, the TMD was reconstituted into liposomes 318

319 where the palmitoyl chain of POPC lipids is deuterated
320 throughout. Therefore, the deuterium solid-state NMR spectra
321 from such samples represent several overlapping quadrupolar
322 splittings, each providing information about the order parameter
323 of the deuterated CD₂ and CD₃ sites (see Figure 8). The
324 deuterium order parameters (S_{CD}) of each CD₂ and CD₃ group
325 are extracted according to the equation $S_{CD}^i = \frac{4}{3} \frac{h}{e^2 q Q} \Delta^i \nu$, where
326 $e^2 q Q/h$ is the static quadrupole coupling constant (167 kHz) for
327 a C–D bond.³⁸

328 ■ RESULTS

329 **Preparation of the Synthetic Peptides.** To perform
330 structural investigations of the p24 transmembrane domain, a
331 peptide encompassing the hydrophobic membrane anchor and
332 the SM-C18 recognition motif was prepared by solid phase
333 peptide synthesis. Eight additional amino acids of the native
334 human p24 sequence were included as well as two lysines at the
335 amino terminus added to make the peptide more water-soluble
336 (see the sequence shown in Table 1). The yield after purification

spectra indicate that the environment of the p24 tryptophan 356
becomes less polar upon addition of detergents. When DPC is 357
added, the intensity changes are pronounced (2.5-fold) until the 358
CMC is reached while the fluorescence maximum remains at 359
331 nm (Figure 1B,G). The changes upon titration with SDS are 360
more complex. Whereas the intensity at 331 nm increases by 361
only ~30%, additional spectral intensities appear between 300 362
and 325 nm (Figure 1D,G). Whereas the use of these detergents 363
offers a first view of the protein structure in membranelike 364
environments, many details about the conformation and lipid 365
interactions require investigations in liquid crystalline phospho- 366
lipid bilayers.⁴⁰ 367

Therefore, in a next step, the peptides were reconstituted into 368
bilayers of different lipid compositions using a previously 369
developed reconstitution protocol.²⁸ Liquid crystalline bilayers 370
made of zwitterionic phosphatidylcholines provide simple 371
model systems for eukaryotic membranes where this lipid 372
headgroup is abundant. The hydrophobic thickness of the 373
DMPC, POPC, and POPE membranes corresponds to that of 374
natural eukaryotic membranes; the latter represents well the 375
mixture of saturated and unsaturated fatty acyl chain, by being 376
made from a single component correlating biophysical analysis 377
to the specific characteristics of phospholipids. For example, 378
whereas the cylindrical shape of POPC results in stable bilayer 379
arrangements, the small headgroup of POPE results in negative 380
curvature strain.^{41,42} 381

The CD and fluorescence spectra of p24TMD in POPC or 382
DMPC (Figure 1E,F) are indicative of a helical peptide in a 383
hydrophobic environment similar to the spectra obtained at 384
intermediate DPC concentrations (Figure 1A,B). A $\Theta_{222}/\Theta_{209}$ 385
ratio of >1 is suggestive of significant oligomerization of the 386
peptide in membranes,⁴³ where the Trp of p24TMD in the 387
saturated DMPC exhibits a somewhat more polar environment. 388

When 5 mol % SM is included in the POPC bilayers, the 389
helical features and the hydrophobic environment of p24TMD 390
are much increased (Figure 1E,F) and the spectra resemble 391
those of the highest DPC concentrations investigated (Figure 392
1A,B). In contrast, when samples are reconstituted into POPE 393
membranes, the CD spectra are featureless and the fluorescence 394
spectra are indicative of a rather polar environment with a low 395
quantum yield and an intensity maximum at 302 nm (Figure 396
1E,F). Interestingly, when a POPC membrane encompassing 10 397
mol % POPE and 5 mol % SM is investigated, the PE offsets the 398
effects of the SM. As a consequence, the CD and fluorescence 399
spectra are intermediate with respect to those obtained in the 400
presence of DMPC or POPC (Figure 1E,F). 401

When ¹⁵N-labeled peptides are reconstituted in uniaxially 402
oriented samples and investigated with the membrane normal 403
parallel to the magnetic field of the NMR spectrometer, the ¹⁵N 404
chemical shifts provide a direct indicator of the approximate tilt 405
angle.⁴⁴ Whereas transmembrane helical domains exhibit ¹⁵N 406
chemical shifts around 200 ppm, helices that align parallel to the 407
bilayer surface are characterized by chemical shifts of <100 ppm. 408
An accurate determination of the tilt and rotational pitch angles 409
of a polypeptide domain is obtained by combining information 410
from the ²H quadrupolar splitting of ²H₃-labeled alanines and 411
the ¹⁵N chemical shift of labeled peptide bonds using doubly 412
labeled peptides prepared by chemical peptide synthesis.^{20,21,37,45} 413
414

Because POPC showed the best results when membrane 415
insertion and structural formation of the p24TMD were tested 416
under a variety of conditions^{28,29} and in optical analysis (Figure 417
1E,F), this lipid was used as a reference to extensively investigate 418

Table 1. ¹⁵N Chemical Shift Measurements of the p24TMD Reconstituted into Oriented Phospholipid Bilayers of either POPC or the 95/5 POPC/SM Mixture at a Lipid-to-Peptide Ratio of 100^a

KKTNS RVVLW SFEEA LVLVA MTLGQ IY ^Y LK R-CONH ₂		
¹⁵ N label position	¹⁵ N chemical shift in POPC (ppm)	¹⁵ N chemical shift in 95/5 POPC/SM (ppm)
L23	218 ± 4	221 ± 3
A20	224 ± 4	224 ± 4
V19	217 ± 4	218 ± 7
L18	206 ± 9	205 ± 8
L16	216 ± 5	216 ± 4
² H ₃ label position	² H ₃ quadrupolar splitting in POPC (kHz)	² H ₃ quadrupolar splitting in 95/5 POPC/SM (kHz)
A15	20 ± 3	20 ± 3

^aThe sequence is indicated with the recognition motif in red, the labeled sites in bold, and the TMD underlined. The indicated range of chemical shifts and quadrupolar splittings represents the line width at 80% maximal intensity and was used for the restriction analysis. The chemical shift maxima can be defined with higher precision (cf. Figure 4A).

337 by semipreparative HPLC using an acetonitrile/water gradient
338 was ~10%, where most of the decrease in yield was associated
339 with the purification step. The purity of the final product was
340 estimated to be ≥90% as determined by analytical HPLC, and its
341 composition was verified by MALDI mass spectrometry
342 (Figures S1 and S2).

343 **p24TMD Secondary Structure in Micelles and Lipid**
344 **Bilayers.** To monitor the secondary structure of the p24TMD
345 in membrane environments, CD spectra of 30 μM p24TMD
346 were recorded upon addition of *n*-dodecylphosphocholine
347 (DPC) or sodium dodecyl sulfate (SDS). Whereas in the
348 absence of a detergent the dichroic spectra were featureless as is
349 often observed for aggregated peptides, minima at 209 and 222
350 nm quickly appeared upon addition of a detergent (Figure
351 1A,C). In the case of DPC, a sudden transition occurs between
352 1.2 and 2 mM, i.e., once the CMC of this detergent is reached
353 (Figure 1A,G).³⁹ Upon addition of SDS, the helical features
354 appear more slowly in agreement with the higher CMC of this
355 detergent (Figure 1C,G). The corresponding fluorescence

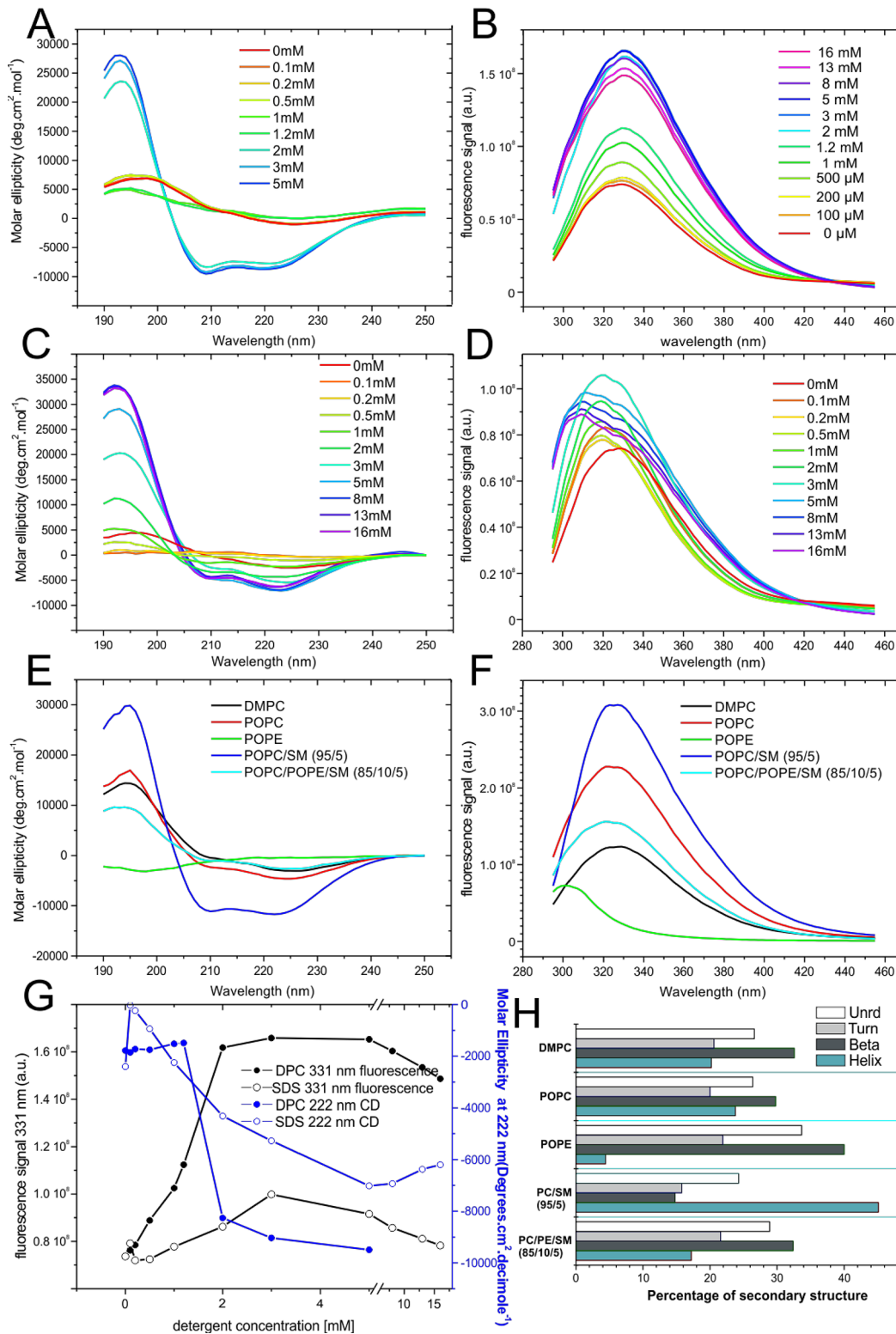


Figure 1. CD and fluorescence spectra of p24TMD in membrane environments. (A, C, and E) CD and (B, D, and F) tryptophan fluorescence spectra of 30 μM p24TMD in 10 mM phosphate buffer (pH 7) in the presence of increasing concentrations of DPC (A and B) or SDS (C and D) or in the presence of small unilamellar vesicles made from the phospholipids indicated at a peptide-to-lipid ratio of 3 mol % (E and F). (G) The fluorescence intensity at 331 nm (black traces) and the molar ellipticity at 222 nm (blue traces) are shown as a function of DPC (filled circles) and SDS (open circles) concentrations (data from panels A–D). (H) Analysis of the secondary structure composition from the spectra shown in panel E using CDPro.³⁰ The data were recorded at 25 °C except for the spectra in the presence of DMPC (37 °C).

419 the domain by solid-state NMR spectroscopy. As discussed
 420 above, POPC forms stable lipid bilayers and provides a suitable
 421 model system for eukaryotic membranes. Several p24TMD

peptides were prepared carrying ¹⁵N- and ²H₃-alanine isotopic 422
 labels at specific sites (Table 1), reconstituted into oriented 423
 POPC membranes at a peptide-to-lipid ratio of 1/100, and 424

425 investigated by ^{15}N solid-state NMR spectroscopy (Figure 2A–
426 E). Here, we focused on the carboxy-terminal end of the TMD

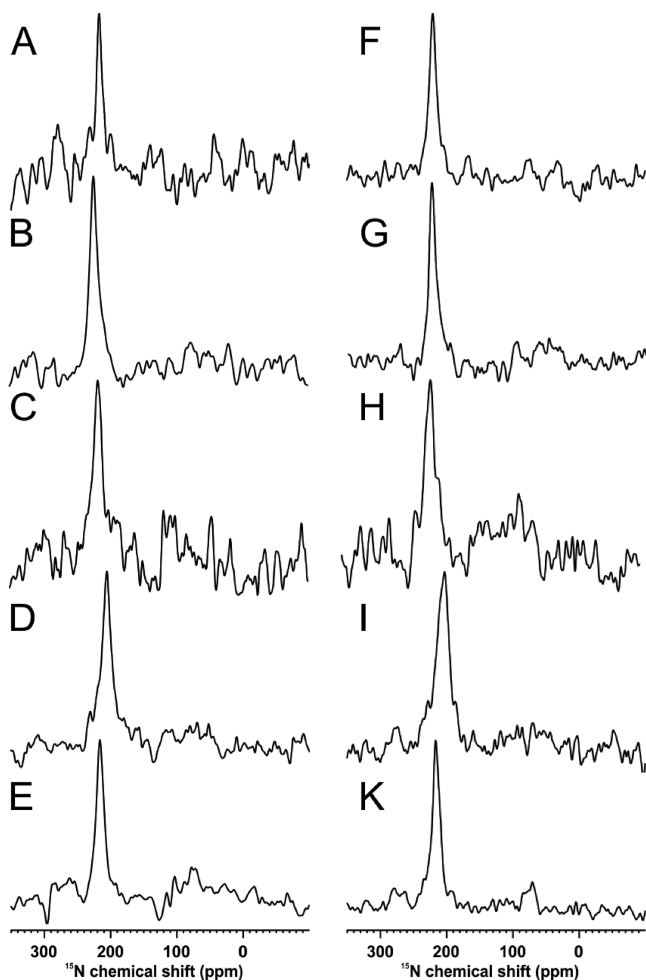


Figure 2. Proton-decoupled ^{15}N solid-state NMR spectra of p24TMD reconstituted into (A–E) oriented POPC or (F–I and K) 95/5 POPC/SM-C18 bilayers at peptide-to-lipid ratios of 1 mol %. The sample was uniaxially aligned with the normal parallel to B_0 . The labeled positions are (A and F) ^{15}N -L23 and $^2\text{H}_3$ -A20, (B and G) ^{15}N -A20, (C and H) ^{15}N -V19 and $^2\text{H}_{10}$ -L13, (D and I) ^{15}N -L18 and $^2\text{H}_3$ -A15, and (E and K) ^{15}N -L16 and $^2\text{H}_3$ -A20. The measurements were performed at ambient temperature.

427 because Val19, Thr22, Leu23, I26, and Tyr27 have been found
428 to be important for the interaction of the TMD with the SM-C18
429 lipid.⁸ The ^{15}N chemical shifts of a number of labeled residues,
430 including sites that are part of the recognition motif or close by,
431 have been determined in successive experiments and are listed in
432 Table 1. The spectra exhibit well-oriented line shapes with
433 chemical shifts between 205 ± 2 and 224 ± 4 ppm, a range that is
434 associated with transmembrane helical alignments (Figure 2A–
435 E). The corresponding ^{31}P NMR spectra show major intensities
436 around 30 ppm indicative of liquid crystalline PC aligned with
437 the normal parallel to the magnetic field. Additional intensities
438 extending to -15 ppm are from lipids that exhibit different
439 headgroup conformations and/or are oriented at other angles,
440 including contributions from the non-oriented sample (representative spectra are shown in Figure 3D–F).²⁵ In view of the
442 good alignment of the polypeptides as judged from the ^{15}N
443 solid-state NMR spectra, the ^{31}P intensity distribution probably

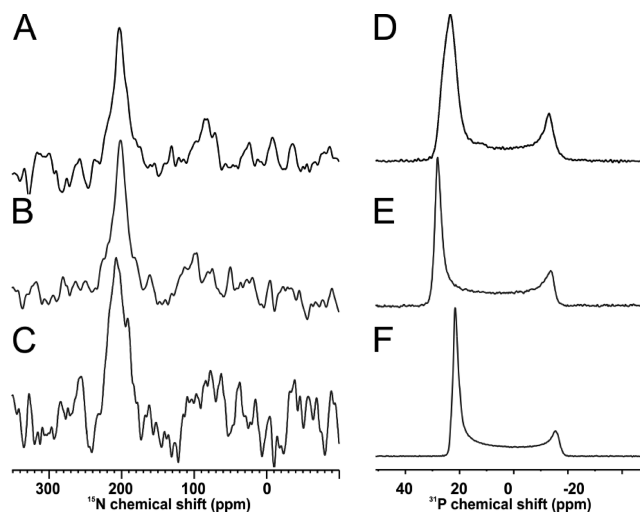


Figure 3. Proton-decoupled ^{15}N solid-state NMR spectra of [^{15}N -L18, $^2\text{H}_3$ -A20]p24TMD reconstituted into (A) oriented POPC, (B) 95/5 POPC/SM-C18, or (C) 90/10 POPC/SM-C18 bilayers at a peptide-to-lipid ratio of 1 mol %. The samples were uniaxially oriented with the normal parallel to B_0 . The corresponding ^{31}P solid-state NMR spectra of the same samples are shown in panels D–F, respectively. The measurements were performed at ambient temperature.

reflects an inherent property arising from the TMD–lipid
interactions (Figures 2 and 3).

To test for the effect of the sphingolipid, 5 mol % SM was
added to the phosphatidylcholine membranes. Closely related
 ^{15}N spectral line shapes and chemical shifts were observed
Only the L23 position exhibits a
visible chemical shift difference. To further investigate the effect
of SM-C18, its content was increased to 10 mol % and the ^{15}N -
Leu18 TMD investigated. Indeed, a small increase in the
chemical shift maximum by ~ 5 ppm and a line broadening effect
were observed for the 10% SM sample (Figure 3A–C). The
broader line shape indicates a more heterogeneous population of
the p24 peptide in these membranes. Interestingly, despite the
weak tendency of the ^{15}N label to shift to higher values and/or to
cause somewhat broader lines, the ^{31}P chemical shift
anisotropies of the SM18-containing samples remain constant
or even tend to decrease (Figure 3D–F).

The peptide carrying a ^{15}N -labeled L23 was also reconstituted
into an oriented lipid bilayer of a composition that more closely
mimics that of the membrane of the Golgi apparatus. The
spectrum closely matches that obtained in the presence of a 95/5
POPC/SM mixture (Figure 4A). To compare more closely the
effects of SM-C18, Figure 4 shows expansions of the ^{15}N solid-
state NMR spectra obtained for the L23, A20, and L16 sites in
the presence of POPC or a 95/5 POPC/SM mixture. Whereas
the spectra of A20 and L16 superimpose in the absence or
presence of SM-C18 (Figure 4B,C), the L23 site shows a small
difference in the chemical shift maximum when 5 or 8 mol % SM
is present (Figure 4A).

^2H Quadrupolar Splittings of Alanines. Whereas the ^{15}N
solid-state spectra shown in Figures 2–4 provide a direct
indicator of the transmembrane orientation of the p24TMD
helix, more accurate topologies are obtained when samples are
analyzed in combination with ^2H solid-state NMR spectra of
alanines that carry deuterons at their methyl group and are
located within the same domain.¹⁹ The resulting quadrupolar
splitting reports on the alignment of the C_α – C_β bond relative to

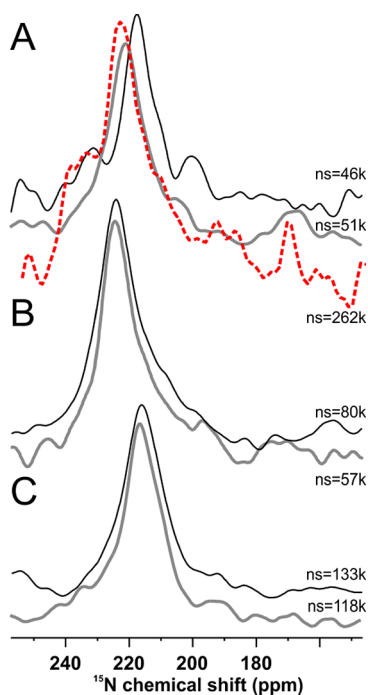


Figure 4. Proton-decoupled ^{15}N solid-state NMR spectra of p24TMD reconstituted into oriented POPC (black lines), 95/5 POPC/SM-C18 (gray lines), or 52/19/5/16/8 POPC/POPE/POPS/cholesterol/SM-C18 bilayers (red hatched line) at a peptide-to-lipid ratio of 1 mol %. The samples were uniaxially aligned with the normal parallel to B_0 . The labeled positions are (A) ^{15}N -L23 and $^2\text{H}_3$ -A20, (B) ^{15}N -A20, and (C) ^{15}N -L16 and $^2\text{H}_3$ -A15. The measurements were performed at ambient temperature. The number of scans is indicated for each experiment.

panels D–F of Figure 5, respectively. The alanine side chain exhibits an intensity distribution of quadrupolar splittings; however, the overall intensity is relatively low. Therefore, it is difficult to analyze these spectra in further detail, and the simulations in Figure 5D–F are shown to illustrate how even small changes in angles affect the spectral line shapes. In the presence of 5 mol % SM, a single quadrupolar splitting of ~ 20 kHz (Figure 5E) appears to be suggestive of a more homogeneous alignment under these conditions, although overall the intensity distribution is quite similar to the spectra in the absence or presence of 10% SM-C18 (Figure 5D,F).

Restriction Analysis. The orientational restraints were used to calculate the topology of an ideal helix encompassing the residues listed in Table 1. Whereas one NMR parameter can be used to provide semiquantitative information about the alignment of the domain, they all have to agree with a single tilt and pitch angular pair of a polypeptide domain. To analyze the data in a quantitative manner, all possible alignments of the p24 helix are tested by rotating the TMD in a stepwise manner around the helix axis as well as a perpendicular director. For each alignment thus obtained, the ^{15}N chemical shifts and the ^2H quadrupolar splitting are calculated and compared with the experimental values, including their dispersion that has been extracted from the line width at 80% maximal intensity (Table 1). When agreement is obtained with a measurement, the angular pair is labeled on the restriction plot shown in Figure 6A, where the black trace represents the ^2H quadrupolar splitting of Ala15 and the colored traces represent the ^{15}N chemical shifts of Leu16 (blue), Leu18 (red), Val19 (green), Ala20 (pink), and Leu23 (turquoise) when reconstituted into uniaxially oriented 95/5 POPC/SM membranes (Table 1). The circle highlights the tilt and pitch angular pair where most measurements coincide. Only the ^{15}N chemical shift of alanine 20 misses this intersection by a few degrees. The corresponding tilt and pitch angles of 19° and 235° , respectively, are represented by the helical structure shown in Figure 6B. Figure 6A also reveals the highly complementary nature of ^{15}N and ^2H solid-state NMR measurements.

To further explore the structure and dynamics of the p24TMD side chains, a peptide deuterated at the Leu23 side chain was prepared and investigated. The Leu23 residue is part of the SM-C18 recognition motif⁸ (Table 1). The labeled leucine side chain encompasses 10 deuterons, two CD_3 groups, two CD groups at the α - and γ -positions, and a CD_2 at the β -position. Whereas the three deuterons of the methyl group of alanine result in a single quadrupolar splitting that reflects the alignment of the C_α – C_β bond and thus the backbone topology, the situation for most of the leucine deuterons, in particular the six deuterons of the CD_3 groups, is different because of the additional degrees of freedom that arise from combined rotations around the C_α – C_β and C_β – C_γ bonds.^{51,52} Therefore, at room temperature, relatively narrow spectral lines are obtained (Figure 7A,B). Slowing these motions at low temperatures results in a much broader intensity distribution ranging over 37 kHz with a different line shape (Figure 7C,D). Under those conditions, the presence of 5 mol % SM results in an $\sim 10\%$ increase in the maximal quadrupolar splitting.

Investigation of p24TMD in a Golgi-like Membrane. As the sorting of SM-C18 in the COPI vesicles involves p24TMD when localized in the Golgi cellular compartment,³ the polypeptide was also investigated in POPC/POPE/POPS/cholesterol/SM bilayers at a 52/19/5/16/8 molar ratio, thereby more closely mimicking the lipid composition of the Golgi

the magnetic field direction as well as the mosaic spread of this angle.¹⁹ When the p24TMD labeled with $^2\text{H}_3$ at the alanine 15 position was reconstituted into POPC or 95/5 POPC/SM oriented membranes, the ^2H solid-state spectra exhibit within experimental error closely related quadrupolar splittings of $\sim 20 \pm 1.5$ kHz (Figure 5A,B). These values represent a C_α – C_β bond alignment relative to the magnetic field direction (membrane normal) of $\sim 44^\circ$ or $\sim 67^\circ$ (depending on whether the splitting has a positive or negative sign). Spectral line shape analysis indicates that the mosaicity of the samples is 7 – 8° (Figure 5A,B, red lines). When the 95/5 POPC/SM sample is measured at 4 $^\circ\text{C}$, much of the intensity is lost, suggesting exchange processes that occur at intermediate time scales close to the gel-to-liquid crystalline phase transition of the membrane (not shown). The phase transition temperature of pure SM-C18 is 45 $^\circ\text{C}$,⁴⁶ and that of POPC -2 $^\circ\text{C}$,⁴⁷ where mixtures of phospholipids often show a broad transition in the intermediate range.^{48,49}

When the sample is investigated at -20 $^\circ\text{C}$, a spectrum representing a large range of C_α – C_β alignments is obtained (Figure 5C). A predominant transmembrane alignment with little additional intensity between 40 and 100 ppm is observed in the corresponding ^{15}N solid-state NMR spectrum of ^{15}N -Ala15 in POPC.²⁹ Thus, the peptide remains globally aligned also when the bilayers are frozen (see, e.g., ref 50). The data suggest that the alanine 15 side chain scans a considerable orientational space. Whereas this dispersion is averaged at ambient temperature, it becomes evident at -20 $^\circ\text{C}$.

The ^2H solid-state spectra of $^2\text{H}_3$ -alanine 20 after reconstitution into oriented membranes made from POPC, 95/5 POPC/SM, and 90/10 POPC/SM bilayers are shown in

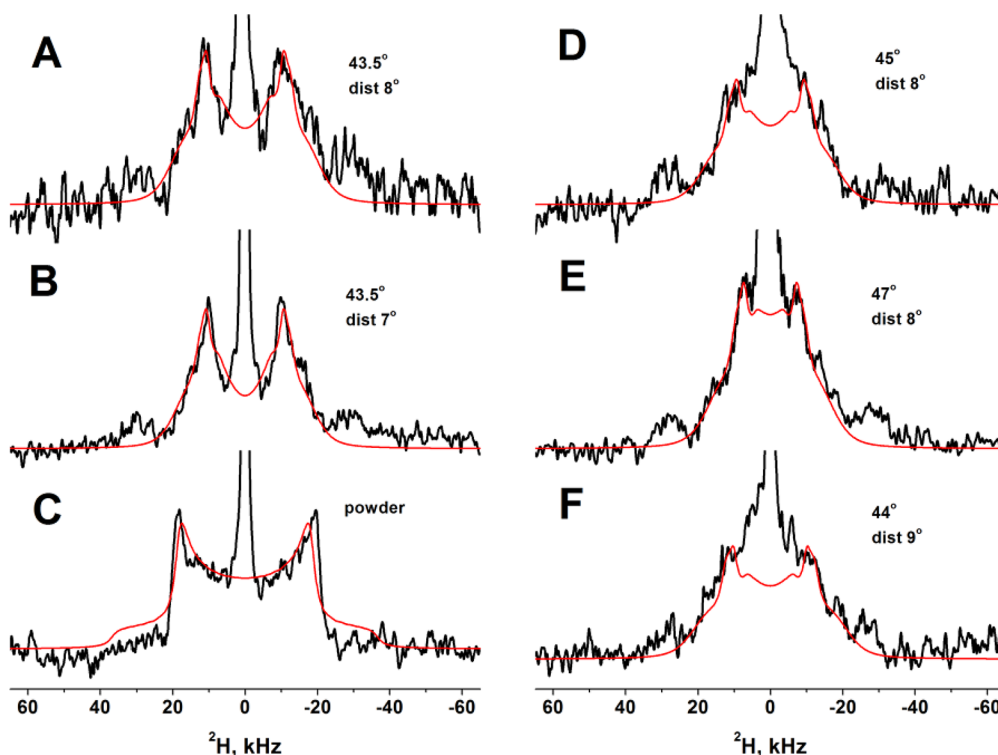


Figure 5. ^2H solid-state NMR spectra of p24TMD labeled with $^2\text{H}_3$ -alanine at the (A–C) A15 or (D–F) A20 site and reconstituted into (A and D) oriented POPC, (B, C, and E) 95/5 POPC/SM-C18, or (F) 90/10 POPC/SM-C18 bilayers at peptide-to-lipid ratios of 1 mol %. The sample was uniaxially oriented with the normal parallel to B_0 . The measurements were performed at ambient temperature (A, B, and D–F) or at -20°C (C). The central peaks are from residual HDO. The interpulse delay in the solid-echo sequence was $50\ \mu\text{s}$ (A–C). The spectra were acquired with 11K (C and D) to 27K (F) scans. The red lines in panels A, B, and D–F show simulated spectra assuming an average alignment relative to the membrane normal (B_0 field) with a Gaussian distribution of the C_α – C_β bond. The corresponding angles (in the range of 43.5 – 47°) and distribution (SDs around 8°) are shown to the right of the spectra. Panel C compares the experimental spectrum to a powder pattern line shape.

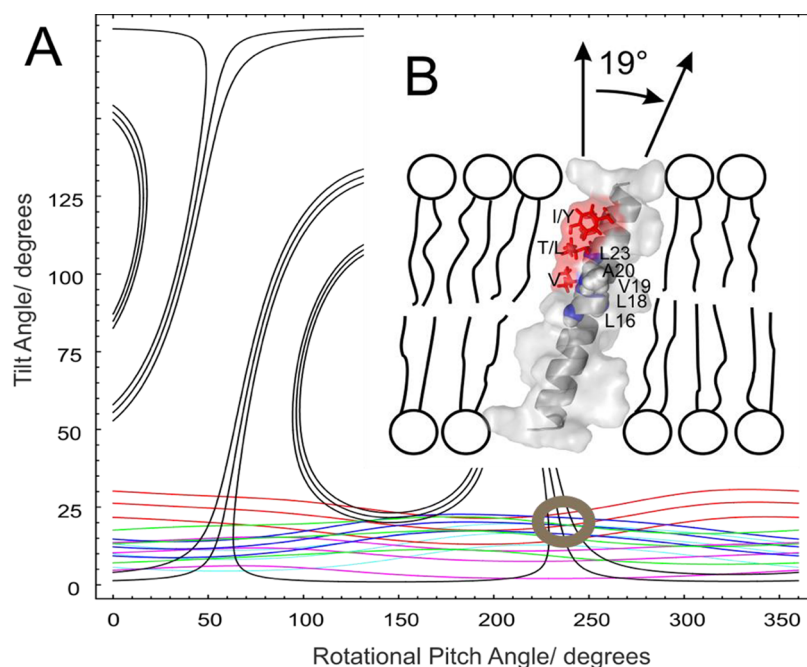


Figure 6. (A) Restriction analysis for the p24TMD topology based on the solid-state NMR measurements shown in panels F–I and K of Figure 2 and Table 1. The orientational restraints from the $^2\text{H}_3$ -Ala20-labeled site (black) as well as from the ^{15}N measurements of Leu16 (blue), Leu18 (red), Val19 (green), Ala20 (pink), and Leu23 (turquoise) are shown. The circle highlights the topologies around the tilt and pitch angular pair (19° and 235° , respectively) common to all but one orientational restraint. (B) Structural model of p24TMD in a lipid bilayer representing the topology from the restriction analysis of solid-state NMR measurements.

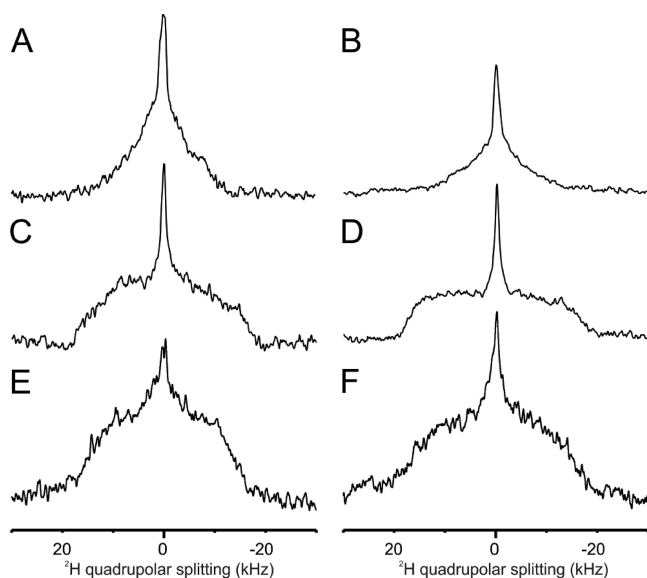


Figure 7. ^2H solid-state NMR spectra of p24TMD labeled with (A–D) $^2\text{H}_{10}$ -leucine at position L23 or (E and F) $^2\text{H}_8$ -valine at position V19 reconstituted into oriented POPC (A and C), 95/5 POPC/SM-C18 (B and D), 60/19/5/16 POPC/POPE/POPS/Chol (E), and 52/19/5/16/8 POPC/POPE/POPS/Chol/SM-C18 (F) at peptide-to-lipid molar ratios of 1 mol %. The sample was uniaxially oriented with the normal parallel to B_0 . The measurements were performed at ambient temperature (A and B), $-20\text{ }^\circ\text{C}$ (C and D), or $40\text{ }^\circ\text{C}$ (E and F). The interpulse delay in the solid-echo sequence was $30\text{ }\mu\text{s}$.

environment and does not change much by freezing the sample 588 to $-20\text{ }^\circ\text{C}^{29}$ (not shown). When 8 mol % SM-C18 of this 589 complex mixture is replaced with POPC, the width of the 590 intensity distribution decreases by $\sim 10\%$ at $40\text{ }^\circ\text{C}$ (Figure 7E) 591 but remains largely unaffected at lower temperatures²⁹ (not 592 shown). 593

Investigation of the POPC- d_{31} Order Parameters in the Presence of SM and p24TMD. To investigate the lipid fatty 595 acyl chain packing and dynamics in the presence of SM-C18 596 and/or p24TMD, liposomes were prepared with POPC lipids 597 deuterated throughout their palmitoyl chain. The deuterium 598 solid-state NMR spectra from such samples encompass several 599 overlapping characteristic quadrupolar splittings, each providing 600 information about the order parameter of the deuterated CD_2 601 and CD_3 sites (Figure 8A). The deuterium order parameters 602 (S_{CD}) of each C–D bond are extracted directly from these 603 spectra in a position-dependent manner (Figure 8B). 604

The ^2H solid-state NMR spectra of POPC- d_{31} and 95/5 605 POPC- d_{31} /SM lipid vesicles in the absence of peptides are 606 shown in Figure 8A. The largest quadrupolar splitting that is 607 assigned to the relatively rigid CD_2 groups closest to the glycerol 608 backbone decreases from 25.2 to 24.9 kHz in the presence of 5 609 mol % SM, while the smallest quadrupolar splitting assigned to 610 the methyl group at the end of the acyl chains within the bilayer 611 core stays unchanged at 2.3 kHz. Thereby, the palmitoyl S_{CD} 612 relative profiles exhibit an only minor effect due to the presence 613 of 5 mol % SM-C18. 614

When 1 mol % p24TMD was added to POPC- d_{31} membranes, 615 only small disturbances of the ^2H quadrupolar splittings 616 comparable with the effect of SM-C18 at 5 mol % are observed 617 (Figure 8A). The largest and smallest quadrupolar splitting are 618 24.8 and 2.4 kHz, respectively, which correspond to relative 619 order parameters of 0.98 and 1.03, respectively, when 620 considering the ratio with the corresponding order parameters 621 of pure POPC- d_{31} (Figure 8B). In contrast, the addition of 622 p24TMD to 95/5 POPC- d_{31} /SM membranes at 1 mol % has a 623 pronounced influence on the ^2H quadrupolar splittings. The 624 outermost quadrupolar splitting decreases to 24.6 kHz, and that 625 of the methyl to 2.16 kHz (Figure 8A). The order parameter 626 profile decreases by $\sim 7\%$ when the palmitoyl CD_2 segments 627 more deeply buried inside the bilayer are considered (Figure 628 8B). 629

574 membrane.^{8,53} The ^{15}N solid-state NMR spectra of the peptide 575 labeled at position 23 exhibit a transmembrane chemical shift of 576 223 ppm (Figure 4A). However, considerable intensities 577 corresponding to different peptide alignments, conformations, 578 or the non-oriented sample were also observed, and the intensity 579 of the signal of the TM peak correspondingly decreased (not 580 shown). The ^2H solid-state NMR spectrum of the deuterated 581 valine- d_8 side chain of p24TMD position 19 reconstituted into 582 this more complex membrane is shown in Figure 7F. The spectra 583 are dominated by six deuterons associated with two methyl 584 groups next to two CD groups at the α - and β -positions. The 585 broad spectral line ranging over $\sim 30\text{ kHz}$ even at higher 586 temperatures is thus indicative of a relatively restricted 587 environment of the valine 19 side chains in the hydrophobic

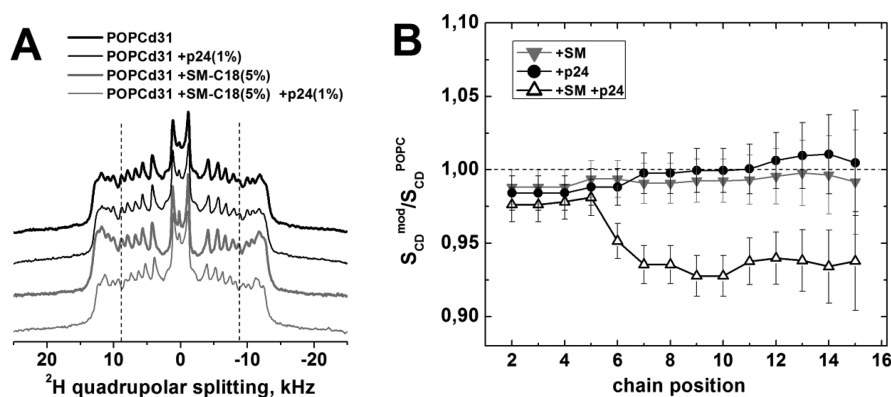


Figure 8. (A) ^2H solid-state NMR spectra of POPC- d_{31} (black lines) or 95/5 POPC- d_{31} /SM-C18 vesicles (gray lines) in the absence (thick lines) or presence of 1 mol % p24TMD (thin lines) recorded at $37\text{ }^\circ\text{C}$. (B) The position-dependent relative order parameters of the pure lipids are shown for POPC in the presence of 1 mol % p24TMD (●) and 95/5 POPC/SM bilayers in the absence (▼) or presence of 1 mol % p24TMD (△). The error bars in panel B are propagated by reading out the quadrupolar splitting uncertainties of $\pm 150\text{ Hz}$.

630 ■ DISCUSSION

631 The members of the p24 family of proteins play important roles
632 in the early secretory pathway, i.e., the controlled transport and
633 processing of proteins and lipids within and between the
634 endoplasmic reticulum and the Golgi apparatus.⁵ They have
635 been proposed to function as cargo receptors, to provide
636 additional control mechanisms for transport within these
637 intracellular compartments,^{1,2} and to be important regulators
638 of transport.⁵ Within the p24 transmembrane domain, an amino
639 acid motif that is responsible for very specific interactions with
640 SM carrying a C18 fatty acyl chain has been identified.⁸ To the
641 best of our knowledge, this is the only example in which a highly
642 selective protein–lipid interaction has been characterized
643 involving both the headgroup and the fatty acyl chain of lipids.
644 The amino acid motif that assures such preferential interactions
645 with SM-C18 over sphingomyelins carrying shorter or longer
646 fatty acyl chains has been identified to be VXXTLXXIY^{8,9} (cf.
647 Table 1). Here we present the first biophysical and structural
648 investigations of p24TMD in phospholipid bilayers and of its
649 lipid interactions.

650 CD and fluorescence spectroscopic investigations show that
651 after reconstitution into phospholipid bilayers²⁸ p24TMD
652 adopts a largely helical conformation and its Trp4 is localized
653 in a hydrophobic environment (Figure 1). Whereas the
654 spectroscopic changes correlate with the CMC of detergent
655 micelles (Figure 1A–D,G), the behavior of p24TMD in lipid
656 bilayers is considerably more complex (Figure 1E,F). The helical
657 contributions and the fluorescence intensities are quite
658 pronounced upon reconstitution in POPC bilayers, and this
659 lipid has served as a reference for further comparison. In the
660 presence of DMPC, the helical content was slightly different,
661 although the fluorescence intensity of tryptophan, being at the
662 fourth position of the transmembrane domain, is much
663 decreased. Possibly, due to the location of this residue close to
664 the amino-terminal end of the hydrophobic TMD, the
665 somewhat thinner DMPC membranes expose more of this
666 amino acid to the aqueous environment. A much more
667 pronounced difference is observed when the bilayers were
668 made of POPE carrying a smaller headgroup. Both CD and
669 fluorescence spectroscopy show a different mode of interaction,
670 the peptide possibly tending to oligomerize into larger structures
671 in this environment. Interestingly, the addition of 5 mol % SM
672 considerably increases both the CD helical content of the
673 p24TMD and its fluorescence intensity. The presence of 10%
674 POPE and the presence of 5% SM-C18 largely compensate for
675 each other; thus, the optical parameters are again close to those
676 of pure PC membranes (Figure 1E,F). Thus, the dependence of
677 CD and fluorescence spectra on the lipid headgroup, the
678 membrane thickness, and/or the presence of SM possibly
679 reflects the structural properties of the SM-C18 recognition
680 motif of p24TMD.^{8,10} It is noteworthy that the SM headgroup
681 resembles that of PC whereas both SM-C18 and the oleoyl fatty
682 acyl chains of POPC extend over 18 C atoms. Via comparison of
683 the different lipids, both the headgroup and the fatty acyl chain
684 turn out to be important for the p24 transmembrane insertion
685 (Figure 1E,F).

686 As a next step, the helix topology was determined using solid-
687 state NMR of p24TMD reconstituted into membranes oriented
688 with their long axes parallel to the magnetic field direction of the
689 NMR spectrometer. Whereas under these conditions a set of ¹⁵N
690 chemical shifts around 210 ppm (Figures 2–4) are an indicator
691 of transmembrane helix alignments,⁴⁴ the highly complementary

information obtained from methyl-deuterated alanines (Figure
5) provides detailed information about the helical tilt and pitch
angles.^{12,21} Thus, a tilt angle of 19° was determined for p24TMD
(Figure 6). The same analysis provides a pitch angle of 235°, but
the numerical value is dependent on how the corresponding
coordinate system was initially defined within the p24 helix.
Therefore, Figure 6B also shows the positioning of the labeled
amino acids relative to the membrane normal. It should be noted
that the ¹⁵N chemical shift of alanine 20 misses the common
intersection point by a few degrees. However, here an ideal
helical conformation encompassing residues 16–23 was
assumed. In previous investigations, small adjustments in the
helical conformation⁵⁴ and/or the introduction of wobbling and
rocking motions have been taken into account to reach
coincidence.²⁰ Indeed, the ²H solid-state NMR spectra recorded
from the methyl groups of alanine 20 and alanine 15 (Figure 5)
indicate an angular distribution of these C_α–C_β bonds and thus
in the topology and conformation of the sites. With regard to the
accuracy of the tilt and pitch angles determined in this manner,
we refer to previous investigations in which these angles shifted
by a few degrees when motions were taken into consideration or
slightly different helical conformations.^{20,54} This previous work
also presents a detailed analysis of how experimental and
systematic errors influence the tilt and pitch angle analysis.

Notably, when the spectra in the absence and presence of SM-
C18 are compared to each other (Table 1 and Figures 2–5),
there is only little variation in the ¹⁵N chemical shifts or ²H₃-
alanine deuterium quadrupolar splittings, indicating that the
backbone topology and conformation of p24TMD seem hardly
affected by the presence of the sphingomyelin (Figures 2–5 and
Table 1). Of the six labeled amino acid residues, two are part of
the SM-C18 recognition motif (V19 and L23) whereas others
are close by or on the backside of the helix (A15, L16, L18, and
A20). The latter have been suggested to be involved in the
dimerization of the TMDs.¹⁰ In the presence of 5 or 8 mol %
SM-C18, the L23 amide shows some small ¹⁵N chemical shift
changes (Figure 4A), which represent differences in the angle
between the corresponding amide ¹⁵NH vector and the B₀ field/
membrane normal of ~2°. ⁴⁴ Notably, L23 is at the very C-
terminal end of the hydrophobic domain, whereas all other
residues are more deeply inserted in the low-dielectric part of the
lipid bilayer, which is expected to enhance the stability of the H-
bonding network.⁵⁵ Furthermore, small changes in the dynamics
of the Leu23 and Val19 side chains are observed, which were
somewhat more restricted in the presence of SM.

The leucine 23-*d*₁₀ spectra are dominated by the six deuterons
associated with the two methyl groups not only because of their
large number but also because the interpulse delays reduce the
signal intensities of the less mobile deuterons of the C_α and C_β
positions. Indeed, similar ²H NMR line shapes have been
observed when the TM segment of phospholamban was
investigated.⁵¹ In this prior work, methyl group rotation about
the C_γ–C_δ bond and at 0 °C additional librational motions
about the C_α–C_β and C_β–C_γ bonds were used to simulate such
line shapes. At –25 °C, the quadrupolar splittings of the three
leucines in phospholamban were 30–36 kHz, while at room
temperature, additional 2-fold jumps between predominant
leucine rotamers, fast side chain reorientation, or off axis
motions result in further averaging.⁵¹ Although the spectra
observed for p24TMD were obtained from macroscopically
oriented samples, the degrees of freedom around the C_α–C_β and
C_β–C_γ bonds result in an angular distribution of methyl groups
approaching that of a powder. Similar to the observations with

755 the phospholambdan leucines, the ^2H NMR spectra of p24TMD
756 leucine 23- d_{10} are suggestive of methyl group rotation and some
757 librational motions, which are more restricted in the presence of
758 SM-C18.

759 Also, in the case of deuterated valine, comparison with earlier
760 work is of interest. Rotation around the $\text{C}_\beta\text{--C}_\gamma$ bond results in
761 quadrupolar splittings of ~ 35 kHz for both valine- d_6 model
762 compounds or of purple membranes containing γ - d_6 -valines over a
763 similar range of temperatures.^{56,57} The broad spectral line
764 shapes in these early publications were simulated by fast
765 rotational averaging about the $\text{C}_\beta\text{--C}_\gamma$ bond. Additional fast
766 averaging about the $\text{C}_\alpha\text{--C}_\beta$ bond results in quadrupolar
767 splittings of 14 kHz. The width of the ^2H spectra suggests that
768 similar motional regimes are present in p24TMD where the
769 presence of SM-C18 has an only minor effect on the spectral line
770 shapes (Figure 7E,F).

771 Considering that SM-C18–p24TMD interactions have been
772 shown to be highly specific, are associated with a shift in
773 monomer–dimer equilibria of p24 proteins, and are important
774 on a functional level,^{5,8,10} one may have expected a more
775 pronounced change in the structural features of p24TMD upon
776 addition of SM-C18. Previously, it was shown that 1 mol %
777 brominated SM-C18 quenches the tryptophan fluorescence of
778 p24-TMD and 0.2 mol % nonbrominated quencher is sufficient
779 to re-establish 50% of the fluorescence (Figure 9 of ref 8),
780 suggesting that there is an excess of SM-C18 in the 5% samples.
781 Of all backbone and side chain spectra investigated here, only
782 the most carboxy-terminal residue L23 exhibited some
783 noticeable changes in both its ^{15}N and ^2H solid-state NMR
784 spectra. First, of the labeled sites, only V19 and L23 are part of
785 the motif that has been identified experimentally and simulated
786 to be in contact with the sphingolipid.⁸ Whereas L23 may
787 undergo conformational changes due to the proximity of the SM
788 headgroup, V19 is positioned deeper in the membrane where
789 the fatty acyl chains of PC and SM dominate the environment.
790 Second, previous studies have measured a p24 concentration
791 within the cell membranes in the range of 2 ng/nmol lipid
792 concomitant with changes in the monomer–dimer equilibrium
793 when different organelles are compared to each other.⁵⁸ This
794 corresponds to a polypeptide-to-lipid ratio of $\sim 1/10000$, which
795 is 100-fold lower when compared to the experimental conditions
796 of our NMR samples. It is thus likely that the p24TMD in the
797 NMR samples is already in its dimeric state even in the absence
798 of SM-C18. Thus, whereas in the cell SM-C18–p24 interactions
799 result in changes in conformation and oligomerization
800 concomitant with an allosteric regulation of activity,¹⁰ the
801 comparatively high P/L ratio in our experiments could promote
802 the dimeric state even in the absence of SM-C18. Indeed, when
803 investigated by SDS–PAGE, the peptides showed a tendency to
804 form oligomers in the absence and presence of POPC or 9S/5
805 POPC/SM bilayers at peptide-to-lipid ratios of 1/100 (Figure
806 S3). Helical tilt angles in a related range have also been observed
807 for dimers formed by the TMD of glycophorin or the amyloid
808 precursor protein.^{22,59,60} Unfortunately, it is difficult to further
809 reduce the polypeptide content of the NMR samples because the
810 NMR spectra shown typically required a day or more to acquire.
811 Third, the possibility that the lipid properties may change upon
812 addition of SM-C18, which by itself has a gel-to-liquid crystalline
813 phase transition (45 °C)⁴⁶ above room temperature that was
814 used in the experiments presented here and much higher than
815 that of POPC (–2 °C), must be considered.⁶¹ At low SM
816 concentrations, the POPC/SM mixtures have been shown to
817 remain in a liquid crystalline phase at ambient temperature.⁴⁸

When spectra have been recorded at low temperatures, 818
considerably broadened liquid crystalline–gel phase transitions 819
have to be considered where the phase transition temperature 820
often represents a weighted average.^{48,49} The phase behavior of 821
the complex Golgi lipid mixture investigated here has to the best 822
of our knowledge not yet been investigated. The modest changes 823
in the side chain heterogeneity of A15 and A20 as well as the 824
decrease in the side chain (and backbone) dynamics of V19, 825
A20, and L23 could reflect a small decrease in membrane fluidity 826
upon addition of SM-C18, point to direct molecular 827
interactions, or both. 828

Therefore, it is of interest to also take into consideration the 829
changes in the lipid properties that have been investigated by 830
measuring the ^{31}P and ^2H solid-state NMR spectra of the 831
phospholipids. The lipid order parameter provides information 832
about the alignment and dynamics of the deuterated segments 833
and has been used previously to study polypeptide–lipid 834
interactions.^{62,63} After deconvolution of the ^2H solid-state NMR 835
spectra, the order parameter for each segment is obtained with 836
the highest order parameters being associated with the CD_2 837
groups close to the glycerol backbone of the phospholipid.⁶⁴ 838
The order parameter of the palmitoyl fatty acyl chain of POPC is 839
hardly affected by the presence of either 1% p24TMD or 5% SM 840
(Figure 8) in accordance with an only slight increase in the 841
POPC- d_{31} order parameter in the presence of 50 mol % SM- 842
C16.^{65,66} However, when the peptide is added to a POPC/SM 843
bilayer, a noticeable decrease in order parameter is observed, 844
similar to the behavior of polypeptides that insert into the 845
membrane interface.^{62,63,67} Similar observations have been 846
made with TM sequences of the MHC class II receptor.⁶⁸ 847
These observations are suggestive that the somewhat tilted 848
p24TMD shingomyelin interacts with SM-C18 in such a manner 849
to exert positive curvature strain in the POPC membrane. 850

851 CONCLUSIONS

In the work presented here, the transmembrane domain of p24 852
was investigated by CD, fluorescence, and solid-state NMR 853
spectroscopies. The degree of membrane insertion is heavily 854
dependent on the exact phospholipid composition where in 855
agreement with prior investigations⁸ both the sphingomyelin 856
headgroup and a fatty acyl chain length of 18 C atoms were 857
found to be important (Figure 1E,F). In POPC or in mixed 858
POPC/SM-C18 bilayers, the polypeptide adopts a helical 859
structure (Figure 1E) that is oriented at a tilt angle of $\sim 19^\circ$ 860
(Figure 6). Whereas the oriented ^{15}N chemical shift and ^2H 861
solid-state NMR spectra of most residues remain unaffected, 862
small alterations are observed for the L23 and V18 sites (Figures 863
2–5 and 7), suggesting that only minor changes in 864
conformation, topology, and dynamics occur due to the 865
presence of 5 or 10 mol % SM-C18. It is likely that under 866
conditions of this investigation (peptide-to-lipid ratio of 1% in 867
POPC) the peptide occurs predominantly as a dimer. When the 868
deuterium order parameters of POPC- d_{31} were investigated, the 869
presence of SM-C18 or the polypeptide alone had little 870
influence, but a considerable disordering effect was observed 871
when they were added in combination. 872

873 ASSOCIATED CONTENT

874 Supporting Information

The Supporting Information is available free of charge on the 875
ACS Publications website at DOI: 10.1021/acs.bio- 876
chem.9b00375. 877

878 Analytical data after peptide preparation and SDS–PAGE
879 data of p24 in POPC membranes (PDF)

880 Accession Codes

881 p24 protein, UniProt entry Q15363 TMED2_HUMAN,
882 residues 165–193.

883 AUTHOR INFORMATION

884 Corresponding Author

885 *Faculté de chimie, Institut le Bel, 4, rue Blaise Pascal, 67070
886 Strasbourg, France. Telephone: +33 3 68 85 13 03. E-mail:
887 bechinge@unistra.fr.

888 ORCID

889 Burkhard Bechinger: 0000-0001-5719-6073

890 Author Contributions

891 †C.A. and P.K.-K. contributed equally to this work.

892 Funding

893 The financial contributions of the Agence Nationale de la
894 Recherche (Projects ProLipIn 10-BLAN-731, membraneDNP
895 12-BSVS-0012, MemPepSyn 14-CE34-0001-01, InMembrane
896 15-CE11-0017-01, and the LabEx Chemistry of Complex
897 Systems 10-LABX-0026_CSC), the University of Strasbourg,
898 the CNRS, the Région Grand-Est (Alsace), and the RTRA
899 International Center of Frontier Research in Chemistry are
900 gratefully acknowledged.

901 Notes

902 The authors declare no competing financial interest.

903 ACKNOWLEDGMENTS

904 The authors are grateful to Britta Brügger, Felix Wieland, and
905 their teams for most valuable discussions and for making data
906 available prior to publication. Delphine Hatey helped with
907 peptide synthesis. B.B. is grateful to the Institut Universitaire de
908 France for providing additional time to be dedicated to research.

909 ABBREVIATIONS

910 CD, circular dichroism; CMC, critical micelle concentration;
911 COPI, coat protein I; DMPC, 1,2-myristoyl-*sn*-glycero-3-
912 phosphocholine; DPC, dodecylphosphocholine; FID, free
913 induction decay; Fmoc, fluorenylmethoxycarbonyl; FRET,
914 Förster resonance energy transfer; GPCR, G-protein-coupled
915 receptor; HFIP, hexafluoroisopropanol; HPLC, high-perform-
916 ance liquid chromatography; LWHH, line width at half-height;
917 MALDI, matrix-assisted laser desorption ionization; MD,
918 molecular dynamics; NMR, nuclear magnetic resonance; P/L,
919 peptide-to-lipid; PC, phosphatidylcholine; POPC, 1-palmitoyl-
920 2-oleoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-
921 oleoyl-*sn*-glycero-3-phosphoethanolamine; POPS, 1-palmitoyl-
922 2-oleoyl-*sn*-glycero-3-phospho-L-serine; SDS, sodium dodecyl
923 sulfate; PAGE, polyacrylamide gel electrophoresis; SM,
924 sphingomyelin; SM-C18, *N*-octadecanoyl-D-erythro-sphingo-
925 sylphosphorylcholine; TFA, trifluoroacetic acid; TMD, trans-
926 membrane domain.

927 REFERENCES

928 (1) Strating, J. R., and Martens, G. J. (2009) The p24 family and
929 selective transport processes at the ER-Golgi interface. *Biol. Cell* 101,
930 495–509.
931 (2) Popoff, V., Adolf, F., Brugger, B., and Wieland, F. (2011) COPI
932 budding within the Golgi stack. *Cold Spring Harbor Perspect. Biol.* 3,
933 No. a005231.
934 (3) Brugger, B., Sandhoff, R., Wegehingel, S., Gorgas, K., Malsam, J.,
935 Helms, J. B., Lehmann, W. D., Nickel, W., and Wieland, F. T. (2000)

Evidence for segregation of sphingomyelin and cholesterol during
formation of COPI-coated vesicles. *J. Cell Biol.* 151, 507–518. 936
937
(4) Dodonova, S. O., Diestelkoetter-Bachert, P., von Appen, A.,
Hagen, W. J., Beck, R., Beck, M., Wieland, F., and Briggs, J. A. (2015)
VESICULAR TRANSPORT. A structure of the COPI coat and the role
of coat proteins in membrane vesicle assembly. *Science* 349, 195–198. 940
941
(5) Pastor-Cantizano, N., Montesinos, J. C., Bernat-Silvestre, C.,
Marcote, M. J., and Aniento, F. (2016) p24 family proteins: key players
in the regulation of trafficking along the secretory pathway. *Protoplasma*
253, 967–985. 944
945
(6) Denzel, A., Otto, F., Girod, A., Pepperkok, R., Watson, R.,
Rosewell, I., Bergeron, J. J., Solarie, R. C., and Owen, M. J. (2000) The
p24 family member p23 is required for early embryonic development.
Curr. Biol. 10, 55–58. 949
950
(7) Haberkant, P., Schmitt, O., Contreras, F. X., Thiele, C., Hanada,
K., Sprong, H., Reinhard, C., Wieland, F. T., and Brugger, B. (2008)
Protein-sphingolipid interactions within cellular membranes. *J. Lipid*
Res. 49, 251–262. 952
953
(8) Contreras, F. X., Ernst, A. M., Haberkant, P., Björkholm, P.,
Lindahl, E., Gönen, B., Tischer, C., Elofsson, A., von Heijne, G., Thiele,
C., Pepperkok, R., Wieland, F., and Brügger, B. (2012) Molecular
recognition of a single sphingolipid species by a protein's trans-
membrane domain. *Nature* 481, 525–529. 958
959
(9) Ernst, A. M., Contreras, F. X., Thiele, C., Wieland, F., and Brugger,
B. (2012) Mutual recognition of sphingolipid molecular species in
membranes. *Biochim. Biophys. Acta, Biomembr.* 1818, 2616–2622. 960
961
(10) Ernst, A. M., and Brugger, B. (2014) Sphingolipids as modulators
of membrane proteins. *Biochim. Biophys. Acta, Mol. Cell Biol. Lipids*
1841, 665–670. 964
965
(11) Björkholm, P., Ernst, A. M., Hacke, M., Wieland, F., Brugger, B.,
and von Heijne, G. (2014) Identification of novel sphingolipid-binding
motifs in mammalian membrane proteins. *Biochim. Biophys. Acta,*
Biomembr. 1838, 2066–2070. 968
969
(12) Bechinger, B., Resende, J. M., and Aisenbrey, C. (2011) The
structural and topological analysis of membrane-associated polypep-
tides by oriented solid-state NMR spectroscopy: Established concepts
and novel developments. *Biophys. Chem.* 153, 115–125. 972
973
(13) Naito, A., Matsumori, N., and Ramamoorthy, A. (2018)
Dynamic membrane interactions of antibacterial and antifungal
biomolecules, and amyloid peptides, revealed by solid-state NMR
spectroscopy. *Biochim. Biophys. Acta, Gen. Subj.* 1862, 307–323. 976
977
(14) Gopinath, T., Mote, K. R., and Veglia, G. (2015) Simultaneous
acquisition of 2D and 3D solid-state NMR experiments for sequential
assignment of oriented membrane protein samples. *J. Biomol. NMR* 62,
53–61. 980
981
(15) Cross, T. A., Ekanayake, V., Paulino, J., and Wright, A. (2014)
Solid state NMR: The essential technology for helical membrane
protein structural characterization. *J. Magn. Reson.* 239, 100–109. 983
984
(16) Van Der Wel, P. C., Strandberg, E., Killian, J. A., and Koeppe, R.
E. (2002) Geometry and intrinsic tilt of a tryptophan-anchored
transmembrane alpha-helix determined by (2)H NMR. *Biophys. J.* 83,
1479–1488. 987
988
(17) Ramamoorthy, A., Wei, Y., and Lee, D. (2004) PISEMA Solid-
State NMR Spectroscopy. *Annu. Rep. NMR Spectrosc.* 52, 1–52. 989
990
(18) Salnikov, E. S., Aisenbrey, C., Aussenac, F., Ouari, O., Sarrouj, H.,
Reiter, C., Tordo, P., Engelke, F., and Bechinger, B. (2016) Membrane
topologies of the PGLa antimicrobial peptide and a transmembrane
anchor sequence by Dynamic Nuclear Polarization/solid-state NMR
spectroscopy. *Sci. Rep.* 6, 20895. 994
995
(19) Aisenbrey, C., and Bechinger, B. (2004) Tilt and rotational pitch
angles of membrane-inserted polypeptides from combined 15N and 2H
solid-state NMR spectroscopy. *Biochemistry* 43, 10502–10512. 996
997
(20) Michalek, M., Salnikov, E., and Bechinger, B. (2013) Structure
and topology of the huntingtin 1–17 membrane anchor by a combined
solution and solid-state NMR approach. *Biophys. J.* 105, 699–710. 1000
1001
(21) Resende, J. M., Verly, R. M., Aisenbrey, C., Cesar, A., Bertani, P.,
Pilo-Veloso, D., and Bechinger, B. (2014) Membrane interactions of
Phylloseptin-1, -2, and -3 peptides by oriented solid-state NMR
spectroscopy. *Biophys. J.* 107, 901–911. 1004

- (22) Itkin, A., Salnikov, E. S., Aisenbrey, C., Raya, J., Glattard, E., Raussens, V., Ruysschaert, J. M., and Bechinger, B. (2017) Evidence for heterogeneous conformations of the gamma cleavage site within the amyloid precursor proteins transmembrane domain. *ACS Omega* 2, 6525–6534.
- (23) Chu, S., Coey, A. T., and Lorigan, G. A. (2010) Solid-state (2)H and (15)N NMR studies of side-chain and backbone dynamics of phospholamban in lipid bilayers: investigation of the N27A mutation. *Biochim. Biophys. Acta, Biomembr.* 1798, 210–215.
- (24) Aisenbrey, C., and Bechinger, B. (2004) Investigations of peptide rotational diffusion in aligned membranes by 2H and 15N solid-state NMR spectroscopy. *J. Am. Chem. Soc.* 126, 16676–16683.
- (25) Bechinger, B., and Salnikov, E. S. (2012) The membrane interactions of antimicrobial peptides revealed by solid-state NMR spectroscopy. *Chem. Phys. Lipids* 165, 282–301.
- (26) Henzler-Wildman, K. A., Martinez, G. V., Brown, M. F., and Ramamoorthy, A. (2004) Perturbation of the hydrophobic core of lipid bilayers by the human antimicrobial peptide LL-37. *Biochemistry* 43, 8459–8469.
- (27) Kim, C., Spano, J., Park, E. K., and Wi, S. (2009) Evidence of 1024 pores and thinned lipid bilayers induced in oriented lipid membranes 1025 interacting with the antimicrobial peptides, magainin-2 and aurein-3.3. 1026 *Biochim. Biophys. Acta, Biomembr.* 1788, 1482–1496.
- (28) Kemayo Koumkoua, P., Aisenbrey, C., Salnikov, E. S., Rifi, O., 1027 and Bechinger, B. (2014) On the design of supramolecular assemblies 1028 made of peptides and lipid bilayers. *J. Pept. Sci.* 20, 526–536.
- (29) Kemayo-Koumkoua, P. (2015) Structural characterization of 1029 highly specific membrane protein-lipid interactions involved in cellular 1030 function. Ph.D. Thesis, University of Strasbourg, Strasbourg, France.
- (30) Sreerama, N., and Woody, R. W. (2000) Estimation of protein 1031 secondary structure from circular dichroism spectra: comparison of 1032 CONTIN, SELCON, and CDSSTR methods with an expanded 1033 reference set. *Anal. Biochem.* 287, 252–260.
- (31) Bechinger, B., and Opella, S. J. (1991) Flat-Coil Probe for NMR 1034 Spectroscopy of Oriented Membrane Samples. *J. Magn. Reson.* 95, 585– 1035 588.
- (32) Bertani, P., Raya, J., and Bechinger, B. (2014) 15N chemical shift 1036 referencing in solid state NMR. *Solid State Nucl. Magn. Reson.* 61–62, 1037 15–18.
- (33) Rance, M., and Byrd, R. A. (1983) Obtaining High-Fidelity Spin- 1038 1/2 Powder Spectra in Anisotropic Media: Phase-Cycled Hahn Echo 1039 Spectroscopy. *J. Magn. Reson.* 52, 221–240.
- (34) Davis, J. H., Jeffrey, K. R., Bloom, M., Valic, M. I., and Higgs, T. P. 1040 (1976) Quadrupolar Echo Deuteron Magnetic Resonance Spectros- 1041 copy in Ordered Hydrocarbon Chains. *Chem. Phys. Lett.* 42, 390–394.
- (35) Koradi, R., Billeter, M., and Wüthrich, K. (1996) MOLMOL: a 1042 program for display and analysis of macromolecular structures. *J. Mol.* 1043 *Graphics* 14, 51–55.
- (36) Salnikov, E., Bertani, P., Raap, J., and Bechinger, B. (2009) 1044 Analysis of the amide ¹⁵N chemical shift tensor of the C(alpha) 1045 tetrasubstituted constituent of membrane-active peptaibols, the alpha- 1046 aminoisobutyric acid residue, compared to those of di- and tri- 1047 substituted proteinogenic amino acid residues. *J. Biomol. NMR* 45, 1048 373–387.
- (37) Aisenbrey, C., Sizun, C., Koch, J., Herget, M., Abele, U., 1049 Bechinger, B., and Tampe, R. (2006) Structure and dynamics of 1050 membrane-associated ICP47, a viral inhibitor of the MHC I antigen- 1051 processing machinery. *J. Biol. Chem.* 281, 30365–30372.
- (38) Batchelder, L. S., Niu, H., and Torchia, D. A. (1983) Methyl 1052 reorientation in polycrystalline amino acids and peptides: A 2H NMR 1053 spin lattice relaxation study. *J. Am. Chem. Soc.* 105, 2228–2231.
- (39) Aisenbrey, C., Michalek, M., Salnikov, E. S., and Bechinger, B. 1054 (2013) Solid-state NMR approaches to study protein structure and 1055 protein-lipid interactions. In *Lipid-Protein Interactions: Methods and* 1056 *Protocols* (Kleinschmidt, J. H., Ed.) pp 357–387, Springer, New York.
- (40) Chipot, C., Dehez, F., Schnell, J. R., Zitzmann, N., Pebay- 1057 Peyroula, E., Catoire, L. J., Miroux, B., Kunji, E. R. S., Veglia, G., Cross, 1058 T. A., and Schanda, P. (2018) Perturbations of Native Membrane 1059 Protein Structure in Alkyl Phosphocholine Detergents: A Critical 1060 Assessment of NMR and Biophysical Studies. *Chem. Rev.* 118, 3559– 1061 3607.
- (41) Aisenbrey, C., Marquette, A., and Bechinger, B. (2019) The 1062 Mechanisms of Action of Cationic Antimicrobial Peptides Refined by 1063 Novel Concepts from Biophysical Investigations. In *Antimicrobial* 1064 *Peptides, Advances in Experimental Medicine and Biology* (Matsuzaki, K., 1065 Ed.) Springer Nature, Singapore.
- (42) Kollmitzer, B., Heftberger, P., Rappolt, M., and Pabst, G. (2013) 1066 Monolayer spontaneous curvature of raft-forming membrane lipids. 1067 *Soft Matter* 9, 10877–10884.
- (43) Khemtouri, L., Buchoux, S., Aussenac, F., and Dufourc, E. J. 1068 (2007) Dimerization of Neu/Erb2 transmembrane domain is 1069 controlled by membrane curvature. *Eur. Biophys. J.* 36, 107–112.
- (44) Bechinger, B., and Sizun, C. (2003) Alignment and structural 1070 analysis of membrane polypeptides by 15N and 31P solid-state NMR 1071 spectroscopy. *Concepts Magn. Reson.* 18A, 130–145.
- (45) Resende, J. M., Moraes, C. M., Munhoz, V. H. D. O., Aisenbrey, 1072 C., Verly, R. M., Bertani, P., Cesar, A., Pilo-Veloso, D., and Bechinger, B. 1073 (2009) Membrane structure and conformational changes of the 1074 antibiotic heterodimeric peptide distinctin by solid-state NMR 1075 spectroscopy. *Proc. Natl. Acad. Sci. U. S. A.* 106, 16639–16644.
- (46) Maulik, P. R., and Shipley, G. G. (1996) Interactions of N- 1076 stearyl sphingomyelin with cholesterol and dipalmitoylphosphatidyl- 1077 choline in bilayer membranes. *Biophys. J.* 70, 2256–2265.
- (47) Silvius, J. R. (1982) *Thermotropic Phase Transitions of Pure Lipids* 1078 *in Model Membranes and Their Modifications by Membrane Proteins*, John 1079 Wiley & Sons, New York.
- (48) de Almeida, R. F., Fedorov, A., and Prieto, M. (2003) 1080 Sphingomyelin/phosphatidylcholine/cholesterol phase diagram: 1081 boundaries and composition of lipid rafts. *Biophys. J.* 85, 2406–2416.
- (49) Pozo Navas, B., Lohner, K., Deutsch, G., Sevcik, S., Riske, K. A., 1082 Dimova, R., Garidel, P., and Pabst, G. (2005) Composition dependence 1083 of vesicle morphology and mixing properties in a bacterial model 1084 membrane system. *Biochim. Biophys. Acta, Biomembr.* 1716, 40–48.
- (50) Salnikov, E., Rosay, M., Pawsey, S., Ouari, O., Tordo, P., and 1085 Bechinger, B. (2010) Solid-state NMR spectroscopy of oriented 1086 membrane polypeptides at 100 K with signal enhancement by dynamic 1087 nuclear polarization. *J. Am. Chem. Soc.* 132, 5940–5941.
- (51) Tiburu, E. K., Karp, E. S., Dave, P. C., Damodaran, K., and 1088 Lorigan, G. A. (2004) Investigating the dynamic properties of the 1089 transmembrane segment of phospholamban incorporated into 1090 phospholipid bilayers utilizing 2H and 15N solid-state NMR 1091 spectroscopy. *Biochemistry* 43, 13899–13909.
- (52) Ferreira, H. E., and Drobny, G. P. (2017) Solid state deuterium 1092 NMR study of LKalpha14 peptide aggregation in biosilica. 1093 *Biointerphases* 12, 02D418.
- (53) van Meer, G., Voelker, D. R., and Feigenson, G. W. (2008) 1094 Membrane lipids: where they are and how they behave. *Nat. Rev. Mol.* 1095 *Cell Biol.* 9, 112–124.
- (54) Salnikov, E. S., Anantharamaiah, G. M., and Bechinger, B. (2018) 1096 Supramolecular Organization of Apolipoprotein-A-I-Derived Peptides 1097 within Disc-like Arrangements. *Biophys. J.* 115, 467–477.
- (55) Partridge, A. W., Therien, A. G., and Deber, C. M. (2002) Polar 1098 mutations in membrane proteins as a biophysical basis for disease. 1099 *Biopolymers* 66, 350–358.
- (56) Kinsey, R. A., Kintanar, A., Tsai, M. D., Smith, R. L., Janes, N., 1100 and Oldfield, E. (1981) First observation of amino acid side chain 1101 dynamics in membrane proteins using high field deuterium nuclear 1102 magnetic resonance spectroscopy. *J. Biol. Chem.* 256, 4146–4149.
- (57) Keniry, M. A., Kintanar, A., Smith, R. L., Gutowsky, H. S., and 1103 Oldfield, E. (1984) Nuclear magnetic resonance studies of amino acids 1104 and proteins. Deuterium nuclear magnetic resonance relaxation of 1105 deuteriomethyl-labeled amino acids in crystals and in Halobacterium 1106 halobium and Escherichia coli cell membranes. *Biochemistry* 23, 288– 1107 298.
- (58) Jenne, N., Frey, K., Brugger, B., and Wieland, F. T. (2002) 1108 Oligomeric state and stoichiometry of p24 proteins in the early 1109 secretory pathway. *J. Biol. Chem.* 277, 46504–46511.

- 1142 (59) Sato, T., Tang, T. C., Reubins, G., Fei, J. Z., Fujimoto, T.,
1143 Kienlen-Campard, P., Constantinescu, S. N., Octave, J. N., Aimoto, S.,
1144 and Smith, S. O. (2009) A helix-to-coil transition at the epsilon-cut site
1145 in the transmembrane dimer of the amyloid precursor protein is
1146 required for proteolysis. *Proc. Natl. Acad. Sci. U. S. A.* 106, 1421–1426.
- 1147 (60) Smith, S. O., Eilers, M., Song, D., Crocker, E., Ying, W.,
1148 Groesbeek, M., Metz, G., Ziliox, M., and Aimoto, S. (2002)
1149 Implications of threonine hydrogen bonding in the glycophorin A
1150 transmembrane helix dimer. *Biophys. J.* 82, 2476–2486.
- 1151 (61) Harzer, U., and Bechinger, B. (2000) The alignment of lysine-
1152 anchored membrane peptides under conditions of hydrophobic
1153 mismatch: A CD, 15 N and 31 P solid-state NMR spectroscopy
1154 investigation. *Biochemistry* 39, 13106–13114.
- 1155 (62) Thennarasu, S., Tan, A., Penumatchu, R., Shelburne, C. E., Heyl,
1156 D. L., and Ramamoorthy, A. (2010) Antimicrobial and membrane
1157 disrupting activities of a peptide derived from the human cathelicidin
1158 antimicrobial peptide LL37. *Biophys. J.* 98, 248–257.
- 1159 (63) Salnikov, E. S., Mason, A. J., and Bechinger, B. (2009) Membrane
1160 order perturbation in the presence of antimicrobial peptides by 2H
1161 solid-state NMR spectroscopy. *Biochimie* 91, 734–743.
- 1162 (64) Seelig, J. (1977) Deuterium magnetic resonance: theory and
1163 application to lipid membranes. *Q. Rev. Biophys.* 10, 353–418.
- 1164 (65) Bunge, A., Muller, P., Stockl, M., Herrmann, A., and Huster, D.
1165 (2008) Characterization of the ternary mixture of sphingomyelin,
1166 POPC, and cholesterol: support for an inhomogeneous lipid
1167 distribution at high temperatures. *Biophys. J.* 94, 2680–2690.
- 1168 (66) Bartels, T., Lankalapalli, R. S., Bittman, R., Beyer, K., and Brown,
1169 M. F. (2008) Raftlike mixtures of sphingomyelin and cholesterol
1170 investigated by solid-state 2H NMR spectroscopy. *J. Am. Chem. Soc.*
1171 130, 14521–14532.
- 1172 (67) Michalek, M., Salnikov, E. S., Werten, S., and Bechinger, B.
1173 (2013) Membrane interactions of the amphipathic amino-terminus of
1174 huntingtin. *Biochemistry* 52, 847–858.
- 1175 (68) Aisenbrey, C., Salnikov, E., and Bechinger, B. (2019) Solid-state
1176 NMR investigations of the MHC II transmembrane domains –
1177 topological equilibria and lipid interactions. Submitted for publication.