Cholesterol-binding site of the influenza M2 protein in lipid bilayers from solid-state NMR

Matthew R. Elkinsa, Jonathan K. Williamsa, Martin D. Gelentera, Peng Dai, Byungsu Kwonb, Ivan V. Sergeyevb, Bradley L. Pentelutec, and Mei Hongd,1

*Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139; and bBruker Biospin, Billerica, MA 01821

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The influenza M2 protein not only forms a proton channel but also mediates membrane scission in a cholesterol-dependent manner to cause virus budding and release. The atomic interaction of cholesterol with M2, as with most eukaryotic membrane proteins, has long been elusive. We have now determined the cholesterol-binding site of the M2 protein in phospholipid bilayers using solid-state NMR spectroscopy. Chain-fluorinated cholesterol was used to measure cholesterol proximity to M2 while sterol-deuterated cholesterol was used to measure bound-cholesterol orientation in lipid bilayers. Carbon–fluorine distance measurements show that at a cholesterol concentration of 17 mol%, two cholesterol molecules bind each M2 tetramer. Cholesterol binds the C-terminal transmembrane (TM) residues, near an amphipathic helix, without requiring a cholesterol recognition sequence motif. Deuterium NMR spectra indicate that bound cholesterol is approximately parallel to the bilayer normal, with the rough face of the sterol rings opposed to methyl-rich TM residues. The distance- and orientation-restrained cholesterol-binding site structure shows that cholesterol is stabilized by hydrophobic interactions with the TM helix and polar and aromatic interactions with neighboring amphipathic helices. At the 1:2 binding stoichiometry, lipid–19F spectra show an isotropic peak indicative of high membrane curvature. This M2–cholesterol complex structure, together with previously observed M2 localization at phase boundaries, suggests that cholesterol mediates M2 clustering to the neck of the budding virus to cause the necessary curvature for membrane scission. The solid-state NMR approach developed here is generally applicable for elucidating the structural basis of cholesterol’s effects on membrane protein function.

Significance

Cholesterol is important for membrane protein function, but cholesterol-binding structures of membrane proteins are difficult to determine by X-ray crystallography and electron microscopy due to the small size and dynamic nature of cholesterol. We have developed a solid-state NMR approach to determine the cholesterol-binding structure of membrane proteins in lipid bilayers. Applied to the influenza M2 protein, the measured interatomic distances and cholesterol orientational angles indicate that cholesterol binds M2 in a substoichiometric fashion, flanking methyl-rich transmembrane (TM) residues near an amphipathic helix, without requiring a cholesterol recognition sequence motif, and this substoichiometric binding uniquely correlates with membrane curvature generation. These results give unprecedented insights into how cholesterol clusters M2 to the neck of the budding virus to mediate membrane scission.

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1To whom correspondence should be addressed. Email: meihong@mit.edu.

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*To whom correspondence should be addressed. Email: meihong@mit.edu.
17 mol% cholesterol, a composition that has been shown by confocal and electron microscopy to permit membrane budding (8). By measuring protein–cholesterol distances, we show that cholesterol binds at a unique site of M2. Comparing M2 sequences with and without the amphipathic helix, and with and without the CRAC motif, we show that cholesterol binding requires the amphipathic helix but not the CRAC motif. Furthermore, we used 1H NMR to measure the orientation of bound cholesterol. The resulting structural model of the cholesterol-binding site of M2 gives molecular insight into how M2 co-opts cholesterol to carry out membrane scission and mediate virus release.

Results

Protein Sequence Requirements for Cholesterol Binding. To measure cholesterol–M2 proximity, we combined chain-heptafluorinated cholesterol (F7-cholesterol, Fig. 1 B) with 13C-labeled protein and conducted 13C–1H rotational-echo–double-resonance (REDOR) distance measurements (17–19) (Fig. S1). F7-cholesterol has the same interfacial area, molecular orientation, and phospholipid interaction as hydrogenated cholesterol based on pressure-area isotherm data (20). Six residues on the lipid-facing surface and helix-helix interface of the TM domain were 13C-labeled for the distance experiments (Fig. 1 C and Table S1). M2 peptides (residues 22–61) corresponding to the CRAC-containing Weybridge strain (M2W) and the CRAC-absent Udorn strain (M2U) were synthesized. The 13C chemical shifts of the labeled residues are well resolved (Fig. 2), thus allowing Cα distances to F7-cholesterol to be measured and compared. In both M2W and M2U TM-AH peptides, the I35, L36, I39, and L40 Cα atoms whose distances to cholesterol were measured are shown as balls, and the putative CRAC residues are shown as sticks.

Fig. 1. Strategy for determining the cholesterol-binding structure of M2. (A) Amino acid sequences of Weybridge and Udorn M2, showing the CRAC motif residues (red) in M2W and its loss in M2U (blue). (B) Cholesterol labeled with 19F, 13C, and 2H for determining cholesterol binding to M2. (C) SSNMR structure of Udorn M2(22–62) (PDB ID code 2L0J). Cα atoms whose distances to cholesterol were measured are shown as balls, and the putative CRAC residues are shown as sticks.

To determine cholesterol-binding distances, we conducted 13C-dephased spectra (5) of 13C-labeled M2W and M2U peptides. The resulting spectra of 13C-labeled M2W and M2U peptides correspond to the CRAC-containing Weybridge strain (M2W) and the CRAC-absent Udorn strain (M2U) were synthesized. The corresponding distances to cholesterol were measured as shown in Fig. 2 C and Table S1. In both M2W and M2UTM-AH peptides, the I35, L36, I39, and L40 Cα atoms whose distances to cholesterol were measured are shown as balls, and the putative CRAC residues are shown as sticks.

Control experiments on 5- 19F-tryptophan gave the expected REDOR dephasing to ~0.5 to reflect the 1:2 binding stoichiometry. This analysis yielded distances of 7–10 Å for the four Cα atoms of I35, L36, I39, and L40, while V28 and A29 showed distances longer than 11 Å. Further, when a carbon atom is more than ~6 Å away from the center of the two methyl groups, the REDOR dephasing mainly depends on the distance between the carbon relative to the pF vector (Fig. S2). In the first step, we simulated the REDOR dephasing curves of one Cα coupled to two CF3 groups to a single pseudofluorine (pF) atom, since the dipolar coupling of a 13C atom to a fast-rotating CF3 group can be calculated based on the motional symmetry (Methods). This simplified the consideration of a 13C spin coupled to two CF3 groups to a three-spin system containing one 13C and two pF atoms. Furthermore, when a carbon atom is more than ~6 Å away from the center of the two methyl groups, the REDOR dephasing mainly depends on the distance Rα (Fig. 3 B) and not on the incident angle of the carbon relative to the pF–pF vector (Fig. S2). In the first step, we simulated the REDOR dephasing curves of one Cα coupled to two CF3 groups to a single cholesterol molecule, and scaled the resulting curves by 0.5 to reflect the 1:2 binding stoichiometry. This analysis yielded distances of 7–10 Å for the four Cα atoms of I35, L36, I39, and L40, while V28 and A29 showed distances longer than 11 Å.

In the second step, we searched a (50 Å)3 cube surrounding the TM–AH structure (PDB ID code 2L0J) at 0.5 Å resolution to find cholesterol tail positions that agree with the four Cα–(pF)2 distances to within ±1 Å. This search restricted the cholesterol isocyanate tail to the exterior of the four-helix bundle, adjacent to the TM helices, while locations inside the pore were readily ruled out by the REDOR data (Fig. S3). Since two cholesterol molecules bind each tetramer (Fig. 3 B), we considered simultaneous dephasing of each Cα by four pF atoms. The two cholesterol molecules can bind the tetramer in either a proximal or a diagonal configuration (Fig. 3 C and D). Since the measured 13C signals are the average of four carbons for each residue, we simulated the REDOR curves of all four Cα carbons of each labeled site and averaged these to obtain the final calculated REDOR curves, which were compared with the experimental data. These calculations yielded closest-approach Cα–pF distances of 7.0–7.1 Å for L36 and I39, while I35 and L40 Cα–pF distances of 3.6 Å show closest distances of the Cα–pF vectors, respectively (Fig. 3 A and Table S2). The proximal and diagonal binding modes gave indistinguishable REDOR curves and are thus both possible from these data.

These protein–cholesterol distances were corroborated by 2D 13C–13C correlation spectra of 25, 26, 27, 13C-labeled cholesterol...
(Fig. 1B) in M2U-containing membranes. At a mixing time of 1.0 s and with 38-fold sensitivity enhancement by dynamic nuclear polarization (23), the I39 Cα and Cγ2 atoms exhibit cross-peaks with 13C-labeled cholesterol C26 and C27 (Fig. S4). These cross-peaks were absent at short mixing times and absent in samples with unlabeled cholesterol, confirming the assignment. The I39-cholesterol cross-peaks have ~70% of the intensities of an intramolecular G34–I39 cross-peak, which corresponds to a 9.0-Å distance. Given the 12 binding stoichiometry of cholesterol to M2, the cross-peak intensities suggest ~8-Å distances between cholesterol 26,27–13C and I39 carbons. G34 and A29 do not exhibit cross-peaks to cholesterol, consistent with the 13CJ-P REDOR data. The agreement between the 13C–19F REDOR data and the 13C–1P REDOR data also confirms that fluorination does not affect cholesterol binding to M2. Additionally, at the protein monomer:lipid:cholesterol (P:L:C) molar ratio of 1:10:2, the average distance between the edges of two TM tetramers is ~20 Å; thus, the cholesterol distances of 7–9 Å to TM residues is not due to confinement of cholesterol to insufficiently solvated M2 tetramers.

**Determination of M2-Bound Cholesterol Orientation.** In addition to the distance constraints, we measured the sterol orientation of bound cholesterol using 2H NMR line shapes of peptide-containing and peptide-free membranes indicate that bound and free cholesterol adopt similar orientations at the concentration of 17–44 mol% in the membrane. For peptide-free membranes, the best-fit cholesterol orientation has β = 11° and γ = 8° (Table S3). However, the quadrupolar couplings are slightly larger in the presence of M2 than in its absence. This difference is indicated by the molecular order parameter S0mol, which describes cholesterol wobbling from the bilayer normal. The S0mol value is 0.85 for the peptide-free sample, in good agreement with the literature (25), and increases to 0.89 tor M2-containing membranes, indicating that the degree of cholesterol wobbling is restricted by the protein. This is supported by the d8-cholesterol 2H spectra (Fig. 4B), which show that the 25-2H quadrupolar coupling is broadened by M2W and increased by M2U compared with the peptide-free spectrum. Thus, M2 partially immobilizes both the sterol rings and the isocytostyl tail.

**Distance- and Orientation-Constrained Cholesterol Docking Structure to M2.** To obtain energetically reasonable structures of the M2–cholesterol complex that incorporate these distance and orientational constraints, we docked cholesterol in vacuo onto the Udorn M2 tetramer using Autodock (26). The REDOR distance constraints were implemented by restricting the cholesterol C25 within a steep Gaussian potential imposed about a central point calculated from the (50 Å)3 grid for each helix. The side-chain χ2 angles of I39, I42, L43, L46, and F47 were allowed to freely rotate during docking. The docking results were screened based on the calculated (β, γ) angles of the sterol rings; only results that were closest to the experimentally measured orientation were selected.

Each docked cholesterol molecule binds at the vertex between two subunits (Fig. 5A and C), along the C-terminal half of the TM helix. The sterol plane is roughly tangential to the TM helix, with the methyl-rich rough β face apposing the methyl-rich Ile and Leu side chains from residues 35–43, forming favorable van der Waals interactions, while the smooth α face points to membrane phospholipids (Fig. 5B). Many docking results can be ruled out due to sterol plane orientations that are incompatible with the 2H NMR
data (Fig. 5C). The CF₃ groups lie at the depth of L36 while the hydroxyl group lies near the amphipathic helix, in close proximity to the F47 ring, thus explaining a previously observed cross-peak between cholesterol C3 and a Phe side chain (11). Each cholesterol molecule contacts the same subunit’s TM residues and the beginning of the amphipathic helix, but the sterol head may also be stabilized by polar and aromatic interactions with the neighboring subunit’s R61, and the aromatic residue 57. The side-chain conformations of AH residues are currently unknown, but rotameric flexibility and backbone structural adjustments may facilitate protein interactions with the sterol head. These multiple stabilizing interactions with two neighboring amphipathic helices explain the requirement of the AH for M2–cholesterol interaction and the loss of cholesterol binding to the TM peptide. The bound cholesterol is far from Y52 and residue 54, which is Arg in Weybridge M2 and Phe in Udorn M2, consistent with the CRAC independence of cholesterol binding. The similarity of bound- and free-cholesterol orientations suggests that M2 co-opts the natural tendency of cholesterol, but uses two AHs and the surface of one TM helix to sequester the ligand.

Discussion

The above data show that, with 17 mol% cholesterol in the membrane, cholesterol and M2 form a 1:2 complex. This sub-stoichiometric binding provides crucial insights into how cholesterol promotes membrane scission by M2. For a 1 cholesterol:2 protein complex, the proximal binding configuration is statistically twice as likely as diagonal. The higher probability of the proximal configuration attracts the M2–cholesterol complex to the edge of the budding virus, because the virus lipid envelope is far more enriched in cholesterol than the host membrane (27); moreover, the inner leaflet of the host membrane is deficient in cholesterol compared with the outer leaflet while the virus envelope has similar cholesterol levels in both leaflets (28). Thus, in a host membrane with emerging viruses, a significant lateral and longitudinal cholesterol concentration gradient exists, and the proximal M2–cholesterol complex would cluster the M2 tetramers to the...
Fig. 5. Structure of the cholesterol-binding site of M2. (A) Distance- and orientation-constrained docked cholesterol structure to M2. The proximal binding model is shown. (B) Cholesterol contacts methyl-rich TM residues and is stabilized by aromatic and polar interactions with amphipathic helix residues. (C) Representative cholesterol orientations from docking analysis and their (β, γ) angles. The (β, γ) = (24°, 17°) solution agrees with the 2H NMR data, while the other two orientations are incompatible with the 2H NMR data. (D) Proposed model of how the M2–cholesterol complex promotes membrane curvature and scission. M2 tetramers cluster to the neck of the cholesterol-rich budding virus because the proximal complex has a twofold higher statistical probability than the diagonal complex. (E) Static 31P-NMR spectra of POPC:POPG:cholesterol membranes with and without M2 at varying P:L:C ratios. A strong isotopic peak indicative of high curvature is observed in the 17% cholesterol membrane but not in the 44% cholesterol membrane.

boundary between the host membrane and the budding virus (29) (Fig. 5D). Concentrated at this interface, M2 tetramers induce membrane curvature because the wedge-shaped four-helix bundle excludes different amounts of volume in the two lipid leaflets (30), the insertion of the amphipathic helix into the cytoplasmic leaflet of the host membrane can induce stacking defects (31), and cholesterol binding to the inner leaflet on the side of the amphipathic helix can amplify the preexisting line tension at the phase boundary (31, 32) (Fig. S6). Confocal microscopy data of fluorescently tagged GUVs containing sphingomyelin, phosphocholine, and cholesterol showed that M2 clusters at the interface between the cholesterol-rich liquid-ordered (Lo) and cholesterol-poor liquid-disordered (Ld) phases (8). The current data indicate that this interfacial location of M2 is promoted by cholesterol binding to the tetramers in a proximal manner. At the edge of the budding virus, the multiple curvature-inducing effects can act in concert to excise the cholesterol-rich phase from the host membrane, in excellent agreement with the observed outward budding of the Lo phase from GUVs (8, 33).

The 1:2 binding stoichiometry found at 17 mol% membrane cholesterol, where virus budding is active, reflects the equilibrium of cholesterol binding to M2, with an estimated dissociation constant (Kd) of ~13 mol%. When the cholesterol concentration increased to 44 mol%, a level at which M2 no longer causes budding (8), the REDOR intensity ratio decreased to ~0.45 (Fig. 2D), indicating a higher binding stoichiometry. This suggests that, when M2 resides in the cholesterol-rich virus envelope, the population of the 1:1 complex increases, whose symmetry would then turn off the curvature-inducing function. To verify that the M2–cholesterol complex indeed causes curvature, we measured static 31P-NMR spectra of the POPC:POPG membranes. Consistent with previous results on virus-mimetic lipid membranes, the spectra show a distinct isotropic peak, which is indicative of high membrane curvature, for the 17% cholesterol membrane (Fig. 5E and Fig. S6), but the isotropic peak is suppressed in the 44% cholesterol membrane. Thus, low cholesterol levels and substoichiometric cholesterol binding to M2 correlate with curvature generation, while high cholesterol levels and increased binding to M2 inhibit curvature induction.

Our data show that cholesterol binding to M2 requires a specific 3D fold instead of a specific primary sequence. The binding-competent 3D fold is an L-shaped structure formed by the TM helix and the amphipathic helix of the same sub unit. The distance- and orientation-constrained structure of the M2–cholesterol complex explains the CRAC independence of cholesterol binding: the aromatic Y52 and the cationic residue 54, which are part of the CRAC motif, are far from cholesterol (Fig. 5). This cholesterol-binding site structure differs from the other cholesterol-docked protein structures associated with the CCM, CRAC, and CARC motifs. While M2 utilizes a TM and a peripheral helix to form the binding pocket, the CCM motif in G-protein–coupled receptors employs multiple TM helices (5), and the CRAC and CARC motifs are proposed for single TM helices. The vertical orientation of M2-bound cholesterol is also qualitatively different from the ~45° tilted cholesterol proposed to bind α-symuclein (27). Finally, the amyloid precursor protein C99 uses a GXXG motif to bind cholesterol, in contrast to the large hydrophobic sidechains that constitute the M2–cholesterol interface (5).

M2 is known to have pronounced conformational plasticity in response to pH, drug, and membrane properties (34, 35), and cholesterol is no exception. Cholesterol stabilizes M2 against drug-induced conformational changes (11), and in cholesterol-containing membranes the amphipathic helix adopts two conformations, as shown by double electron–electron resonance EPR measurements (36). Thus, cholesterol affects M2 structure and dynamics in a complex fashion. The SSNMR structure of the TM–AH peptide in cholesterol-free DOPC/DOPE bilayers (22) shows the tetramer as a C4-symmetric bundle. In the presence of cholesterol, the C4 symmetry will likely break, and the protein structure should depend on whether the complex contains proximally or diagonally bound cholesterol. This hypothesized symmetry
breaking by substoichiometric cholesterol binding, which is im-
portant for virus budding, is unrelated to the symmetry breaking
of M2 seen in cholesterol-free diphytanoylphosphocholine mem-
branes (37). Since none of the existing M2 structures were solved in
the presence of cholesterol, determining the high-resolution
structure of M2 in cholesterol-containing membranes should
allow the distinction of the proximal and diagonal binding modes
and further elucidate the molecular basis of influenza virus
budding.

Methods
M2 peptides were synthesized using custom-designed fast-flow synthesizers and
Fmoc solid-phase protocols (38, 39), and were purified using reverse-phase
HPLC. Peptide purity and mass were verified using liquid chromatography
(LC-MS). Peptides for 13C–1H REDOR experiments were dialyzed against a 1% acetic
acid solution to remove trifluoroacetate ions. Site-specifically 13C,15N-
labeled peptides were reconstituted into POPC/POPG (4:1) membranes with var-
iations in concentrations of 15N,1H or 13C,1H-labeled cholesterol (Table 51). Carbon-
fluorine distance experiments and 1H NMR experiments were mainly conducted
on a 400-MHz spectrometer, while dynamic nuclear polarization (DNP) spectra
were measured on a 600-MHz spectrometer. Methyl-rotation averaged 13C,1H-
REDOR curves were simulated using the SIMPSON software and analyzed in
MATLAB, while orientation calculations were carried out in MATLAB. Choles-
terol docking to M2 was performed using Autodock 4.2. Additional details of
the experiments and data analysis are given in SI Methods.

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