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The dynamic binding of cholesterol to the multiple sites of C99: as revealed by coarse-grained and all-atom simulations[†]

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It is generally believed that the etiology of Alzheimer's disease (AD) is closely related to the amyloid- β polypeptides, produced from γ -secretase cleavage of C99. There is preliminary evidence that cholesterol directly activates γ -secretase cleavage of C99 through mechanisms that have not been understood so far. In this article, coarse-grained (CG) and all-atom (AT) simulations were employed to investigate the association between C99 and cholesterol, which is essential for our understanding of the role of cholesterol in the amyloidogenic pathway. Firstly, we find that both the N-terminus and the C-terminus of the C99 transmembrane domain (TMD) show interactions with cholesterol. Secondly, a multi-site dynamic cholesterol binding model was captured from the simulations, where 6 binding sites in the C99 TMD were presented. The analyses of the binding energies show that cholesterol prefers the site no. 1, 2, 4 and 5 over others. The most favorable binding energy of nearly -58.857 kJ mol⁻¹ is from site 1, the repeat GxxxG motif. There are two pathways and two binding states of cholesterol binding to this site. Ser₆₉₇ and Phe₆₉₀ contribute most to the stabilization of the tightly binding state and the loosely binding state, respectively. The other binding sites described may also be potential drug targets. Thirdly, the residues GAVILMTKF, especially IVKF play a key role in this association. The C99 model appears to suggest a new mechanism for cholesterol binding. Finally, the multiple-site dynamic cholesterol binding model better explains the hypotheses that cholesterol promotes the amyloidogenic ABPP route. The GxxxA motif in the middle of the C99 transmembrane domain is completely exposed without cholesterol sheltering, which might help γ -secretase identify the cleavage sites and then promote γ -cleavage. Our results provide a detailed picture of dynamic cholesterol binding, which is crucial to our recognition of the potential influence of cholesterol on the C99 process and the etiology of AD.

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Introduction

The amyloid- β (A β) peptide is thought to play a key pathogenic role in Alzheimer's disease (AD), a disease showing progressive cognitive decline. The aggregation of amyloid- β (A β) peptides constitutes the main ingredients of neuritic plaques, which are pathologically characterized within the gray matter of the brain of AD patients.¹⁻⁴

A β is cleaved by γ -secretase from C99, the 99 residues in the C-terminal domain of the amyloid precursor protein (APP),

which is generated upon the cleavage of APP by β -secretase. A large body of evidence has shown that elevated levels of cholesterol (CHOL) promote the amyloidogenic pathway (the process of the formation of C99 from APP, mediated by β -secretase), resulting in increased levels of amyloid- β production and inhibition of the competing nonamyloidogenic cleavage pathway (in this route, APP is cleaved by α -secretase, then produces C83, which precludes the formation of C99 and A β) (Scheme 1).⁵⁻¹⁴ Additionally, studies with purified γ -secretase have shown a 2- to 4-fold enhancement in the rates of production of both A β 40 and A β 42 in the presence of cholesterol with optimal levels of 5–20 mol%, depending on vesicle composition.¹⁵

With regard to the possible association between C99 and CHOL, this phenomenon can be explained by two hypotheses: (1) the specific interactions between C99 and cholesterol tend to allocate C99 and APP into cholesterol-rich membrane domains known as lipid rafts,^{16,17} with which β -secretase and γ -secretase preferentially associate.^{18–23} (2) The association between C99 and

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cholesterol directly promotes the amyloidogenesis by helping C99 bind to γ -secretase.²⁴

In the research by Barrett and his colleagues,^{16,24} they put forward a cholesterol binding C99 model where cholesterol forms a 1:1 binary complex with monomeric C99 at the repeat GxxxG motif, and this complex competes with the C99 dimer. Mutation of any Gly residue or Phe₆₉₁ or Glu₆₉₃ to Ala substantially diminished cholesterol binding. This surprising observation strongly supports the above hypotheses, and hints at a significant functional effect of cholesterol on C99 dimerization and y-cleavage. Subsequently, Lukasz Nierzwicki et al. accurately described the structure and energetics of the 1:1 complex identified by Barrett's NMR experiments, which suggests the existence of two different binding states at the GxxxG motif.²⁵ Recently, Straub et al. revealed that binding of cholesterol to the C99 GxxxG motif is a sensitive function of the pH, and is heavily dependent on the charge states of Glu₆₉₃ and Asp_{694} .

Straub and Nierzwicki et al. have both simulated the 1:1 complex identified by Barrett's NMR experiments as the initial structure, since they were mainly aimed at the study of cholesterol binding at the GxxxG motif of C99. Nevertheless, the obtained results are significantly affected by the initial structure if the time scale of the simulation is limited. It raises the question of how the cholesterol binding occurs and if there are other binding sites that were neglected.

To observe the broader landscape of the association between C99 and cholesterol, in this study, a multi-scale computational approach was employed combining coarse-grained (CG) and all-atom (AT) simulations of the C99 monomer (based on the PDB structure 2LP1) in the DPPC: CHOL = 4:1 bilayer.

To assess the longtime dynamics and the role of fluctuations in the protein and lipid conformational ensemble,26-28 CG simulations were performed for $10 \times 3 \mu s$ using the MARTINI force field²⁹⁻³¹ to obtain more sampling, while AT simulation was performed for 1 µs using the CHARMM36 force field to obtain more accurate forecasts for the association between C99 and cholesterol.

Models and methodology

The experimentally derived NMR structure (PDB 2LP1¹⁶) was employed as the initial structure of the C99 monomer. Here, the protein sequence of C99683-728 is VHHQKLVFFAEDVGSNK GAIIGLMVGGVVIATVIVITLVMLKKKQW, in which V₆₈₃HHQKL VFFAEDVGSNK₆₉₉ was defined as the juxtamembrane region, G700AIIGLMVGGVV711 was defined as the N terminus of C99 TMD, and I712ATVIVITLVML723 was defined as the C terminus of C99 TMD. The GxxxG motif here refers to the $G_{696}xxxG_{700}xxxG_{704}xxxG_{708}$ zipper region.

CG model simulations

The Martinize.py script was used to create protein topology information, and the CG simulations were performed under the MARTINI 2.2 force field.²⁹ The insane.py script³² was employed to build the DPPC : CHOL = 4 : 1 CG lipid systems (size = $8 \times 8 \text{ nm}^2$), which contain 20% cholesterol levels (where mol% cholesterol = 100[moles cholesterol/(moles DPPC + moles cholesterol)]) to match the optimal concentration in Song' experiments.²⁴ The filling of cholesterol was consistent in the upper and lower layers. The monomer was then placed within the pre-equilibrated

lipid systems. The CG bilayer system consisted of C99 monomer, 120 DPPC lipids, 30 cholesterol molecules, 3863 water particles, and 3 Cl^- ions to neutralize the system.

All CG systems experienced the following three steps: energy minimization, *NVT* and *NPT* equilibration, and the molecular dynamics balance. The energy of each system was repeatedly minimized followed by 3 ns position-restrained simulation for better packing of the lipid molecules around the TM (transmembrane) helices. 10 replicas of 3 μ s CG simulations were performed on each system in consideration of sample stability. Multiple independent dynamical trajectories were initiated from the experimentally determined 2LP1 PDB structure.

The temperature of the systems was set to 300 K using the Berendsen weak coupling method³³ with a relaxation time of 0.1 ps. The pressure was set to 1 bar using a semi-isotropic coupling for a bilayer using the Berendsen algorithm. An integration time step of 20 fs was used in all simulations. Nonbonded interactions were truncated using shift functions (between 0.9 and 1.2 nm for Lennard-Jones interactions and between 0 and 1.2 nm for electrostatics).³⁰

Restraint CG simulations

To explore a possible cholesterol binding model of C99, restrained simulations of an equilibrated C99 conformation, which was obtained from CG simulations and reached the platform period of RMSD (Fig. S2, ESI†), were performed in DPPC: CHOL = 4:1; where the position of protein atoms was limited by the harmonic potential with the force constant of 1000 kJ mol⁻¹ nm⁻² on each atom of the whole protein in the *X*, *Y* and *Z* directions. During the simulations, the protein is almost stationary with tiny harmonic vibration of the side chains. The other physiological conditions and simulation parameters of the protein restrained simulations are the same as the above CG model simulations.

AT model simulations

The AT model was constructed using the CHARMM-GUI Membrane Builder.³⁴ The final system is composed of a C99 fragment (Val683-Tyr728), 120 DPPC molecules, 30 cholesterol molecules, 5090 water molecules, 15 $K^{\!\!+}$ and 18 Cl^{-} ions (0.15 M). Proteins and lipids were presented using the CHARMM36 force field,³⁵ and a TIP3P model³⁶ was used for water. The NMR structure (PDB 2LP1¹⁶) was employed as the initial configuration of C99. The AT simulation system experienced the following three steps: energy minimization, NVT and NPT equilibration, and the molecular dynamics simulation. The temperature was set to 300 K by means of Langevin dynamics and the pressure was maintained at 1 bar using the Langevin piston method.³⁷ Periodic boundary conditions were employed, and the particle mesh Ewald algorithm was applied in full electrostatics with a real-space cut-off of 10 Å.³⁸ van der Waals interactions were truncated using shift functions between 10 Å and 12 Å. All covalent bonds including hydrogen were restrained by the LINCS method.³⁹ An integration time step of 2 fs was used in 1 µs AT simulation using the velocity Verlet algorithm.40

Binding energy calculation

g_mmpbsa^{41,42} was used to evaluate binding energies as well as estimate the energy contribution of each residue to the binding energy. The binding energy is calculated based on the equation: $\Delta G_{\text{bind}} = \Delta E_{\text{MM}} + \Delta G_{\text{psolv}} + \Delta G_{\text{npsolv}} - T\Delta S$. ΔE_{MM} is the sum of van der Waals and electrostatic interactions. The entropy contribution ($T\Delta S$) is not calculated in g_mmpbsa, so this binding energy is the relative binding energy rather than the absolute binding energy.⁴³ All energy components of binding energies for the binding complex were calculated at every 500 ps interval from the 10 ns production trajectory (cut out from the 1 µs AT simulations) where the binding conformation is relatively stable. In this study, ΔE_{MM} contribution of each residue was employed to evaluate the residue contribution, as it is comparable to the binding energy.

All simulations were carried out using GROMACS (v4.6.1), and the analysis was performed using the GROMACS package, CG tailored scripts using python and CG analysis libraries.^{44–48} Images were generated using VMD.⁴⁹ Detailed simulations are listed in Table S1 (ESI[†]).

Results and discussion

The C-terminus and the N-terminus of C99 both have short time interactions with cholesterols

Tracking each cholesterol molecule near C99 monomer in all independent dynamical trajectories and analyzing the COM distance between the cholesterol and C99 monomer, no cholesterol molecule was found to keep a stable distance with the C99 monomer below 1 nm throughout the trajectory (Fig. 1A), which indicates that C99 cannot form 1:1 stable binding with cholesterol. But multiple COM distances within 1 nm in a short time about the 10–100 ns level were observed (Fig. 1A); this suggests that C99 might have short time interactions with cholesterol. In the CG trajectories, C99 is just like a flower with multiple bees of cholesterol molecules dynamically circling around, binding to and flying away from time to time. The flip-flop of cholesterol molecules over the upper and lower layers occurs infrequently during the simulations.

The cholesterol density distribution presents statistical aggregations of cholesterol molecules around the protein with a radial distance of about 0.5 nm in the upper and lower leaflets (Fig. 1B). This result clearly suggests that both the C-terminus and the N-terminus of C99 have interactions with cholesterol. And it is similar to Anupam Prakash's result about ebrB2,⁵⁰ which also presents a cholesterol surrounding model but with a more symmetrical distribution compared with C99 (Fig. S1, ESI†). The more pronounced aggregation of cholesterol in the C-terminus of C99 TMD reflects that the C-terminus might have more abundant interaction with cholesterol than the N-terminus, which can also be reflected in the cholesterol surrounding model (Fig. 2A).

However, we can hardly tell the cholesterol binding sites from this surrounding model due to the dynamics of the protein. In order to obtain the possible binding sites of C99, an equilibrated



Fig. 1 (A) The COM (center of mass) distance between a cholesterol and the N-terminus or C-terminus of the C99 TMD as a function of time from a representative example. (B) The cholesterol axial-radial number density distribution, calculated from 10 \times 3 μs CG trajectories, where the C99 protein was fitted onto a unique reference structure to maintain the frame of the reference. The X-axis represents the axial orientation of the C99 TMD. The Y-axis represents the radial distance to the TMD. At the radial distance below 0.5 nm is the corresponding residue distribution. The colored scale at the bottom defines the cholesterol number density.

2LP1 structure derived from the CG trajectory was employed in protein position-restrained CG simulation, and the results which were based upon the selected stable C99 structure were then further validated by the AT simulations where the flexibility of the protein and the C99-cholesterol interactions can be described more accurately.

A multi-site cholesterol binding model was captured in the simulations

The isosurface of cholesterol in protein-restrained CG simulations (Fig. 2B) depicts 6 possible binding sites in the C99 TMD (transmembrane domain): two of which are in the N-terminus including the GxxxG motif, and the other four in the C-terminus (Fig. 2B and C). All binding sites located in the groove between two contact surfaces are concave for the anchorage of cholesterol (Fig. 2B and C). Cholesterol adjusts conformations to fit the different shapes of the binding sites. The cholesterol binding is constantly changing in forms of multiple binding, singular binding and no binding.

The results above are instructive, since all the binding sites found in restrained CG simulations have been screened out in the AT trajectories (Fig. 2E), according to the following



Fig. 2 (A and B) The images of the cholesterol isosurface (green).⁴⁴ C99 protein is shown in white, the phosphocholine group in cyan and Gly residues in red. (A) calculated from $10 \times 3 \ \mu s$ CG trajectories, where the C99 was fitted onto a unique reference structure. (B) calculated from $10 \times 3 \ \mu s$ protein restraint CG simulation. (C and D) The schematic diagram of cholesterol binding. From left to right and top to bottom, the binding sites are described accordingly as regions no. 1–6. (C) for CG simulation depicted according to (B). (D) For AT simulation depicted according to the observation of AT trajectories. (E) From left to right, the typical binding conformations from AT simulation, respectively, correspond to no. 1–6 sites of C99. The residues that contribute most at each binding site are shown in color, with detailed descriptions in Fig. S3–S9 (ESI†).

standards: (1) cholesterol is closely attached to the protein surface; (2) cholesterol binds in the same area, which can be identified with residues as the scale; and (3) the combination time reaches the level of 10 ns. This suggests that those binding sites found in restrained CG simulations do exist. And it is feasible that protein restrained CG simulations can be performed as a preliminary search scheme of binding sites, complementary to detailed AT simulations.

Additionally, site 0 which is not fully reflected in the cholesterol isosurface (Fig. 2B) has been found, and it may be an intermediate state for cholesterol binding to site 1 (Fig. 3E). It is worth mentioning that the backbone conformation of the C99 TM in CG or AT simulation is consistent, which provides a theoretical basis for the similarities of the cholesterol binding sites in the two simulations. However, the binding positions of cholesterol at sites 1, 3 and 6 are slightly different from those in



Fig. 3 The snapshots of different binding states in the GxxxG motif from AT trajectories. The protein is shown in cyan, cholesterol in green, the repeat GxxxG motif in yellow, Lys in purple, Phe in red, Asp₆₉₄ in blue, Glu₆₉₃ in orange and Ser₆₉₇ in white.

the restrained CG model (Fig. 2C and D), as the protein structure in AT simulations is more dynamic and flexible compared to that in the semi-rigid protein restrained CG simulations, which result in shrinkage or deformation of the groove. Moreover, in CG simulations, the juxtamembrane region of C99 swings all the way, and the orientation of juxtamembrane along the membrane surface constantly changes. While, this orientation are relatively fixed in the AT simulations. This difference may be due to the more intense dynamics of the CG simulations.

In order to analyse the binding sites in detail, mmpbsa binding energies were obtained for each binding site based on the AT trajectories and show that cholesterol prefers site no. 1, 2, 4 and 5 over others (Table 1). This is consistent with the cholesterol binding time at each site: 10 ns level for site 3 and site 6, while 100 ns level for site no. 1, 2, 4 and 5. More detailed analysis of the energy terms showed that van der Waals interactions, electrostatic interaction and non-polar solvation energy

contribute negatively to the binding energy which polar solvation energy contributes positively to. And van der Waals interaction contributes most in the terms of negative contribution for all the cases. This suggests that van der Waals interaction, electrostatic interaction and non-polar solvation energy together contribute to the stability of the C99–cholesterol binding complex.

The dynamic process of cholesterol binding to the GxxxG motif

The most favorable binding energy of nearly -58.857 kJ mol⁻¹ (Table 1) is from site 1, the GxxxG motif, which has been identified by Barrett *et al.* using NMR-controlled titration experiments.⁵¹ It should be noted that cholesterol binding to this site is more complicated than to other sites since there are two pathways for cholesterol binding to this site which can be occupied by two different binding states.

The formation of site 1, which strongly depends on the appropriate conformation of the C99 N-loop, needs the collaboration of

Table 1	The average binding energy of cholesterol at each binding site of C99, calculated using g_mmpbsa. ²	^{1,42} We extracted the 10 ns trajectories in
which ch	: holesterol binds to a specific site from the 1 μs simulation, and then performed the mmpbsa calculation (c	riginal data are provided in Fig. S3–S9, ESI)

Bing sites	van der Waals (kJ mol ⁻¹)	Electrostatic (kJ mol ⁻¹)	Polar solvation $(kJ mol^{-1})$	Non-polar solvation (kJ mol ⁻¹)	Binding energy (kJ mol ⁻¹)
NO. 0	-27.241 ± 4.200	-19.923 ± 4.690	35.962 ± 4.474	-4.797 ± 0.562	-15.999 ± 4.686
NO. 1	-71.330 ± 9.957	-6.040 ± 5.083	31.633 ± 4.365	-11.227 ± 1.206	-56.964 ± 10.392
NO. 1	-75.938 ± 6.144	-11.955 ± 2.991	41.007 ± 4.636	-11.971 ± 0.908	-58.857 ± 7.168
NO. 2	-55.697 ± 14.379	-8.496 ± 4.230	23.237 ± 17.196	-9.223 ± 1.298	-50.180 ± 22.613
NO. 3	-34.322 ± 8.212	-0.581 ± 2.130	9.211 ± 2.992	-7.136 ± 1.298	-32.828 ± 8.230
NO. 4	-57.073 ± 16.038	-12.339 ± 12.81	23.437 ± 25.997	-10.484 ± 2.073	-56.459 ± 15.571
NO. 5	-54.493 ± 10.298	-1.932 ± 4.335	11.594 ± 8.770	-9.894 ± 1.249	-54.725 ± 1.818
NO. 6	-20.594 ± 6.614	-3.710 ± 1.410	0.047 ± 3.242	-4.786 ± 1.084	-29.043 ± 6.774

Note: NO. 1 is for the tightly binding state, and NO. 1 is for the loosely binding state at site 1.

Paper

two steps: firstly, juxtamembrane regions of C99 stretch and take the pistol-like Phe₆₉₀ away from the GxxxG motif, leaving a pocket space for the cholesterol to anchor (Fig. 3A and B). In the meantime, the cholesterol cruises into the pocket, and the juxtamembrane region of C99 retracts with the side chain of Phe₆₉₀ clamping the cholesterol tightly. Thus, cholesterol binding at the GxxxG motif occurs (Fig. 3C and D), where the head of cholesterol faces the Phe₆₉₀, forming a hydrogen bond throughout the binding (Fig. 3D and Table S2, ESI†), and the cholesterol methyl ring binds closely to the broad GxxG motif (Fig. 3D and E). Phe₆₉₀, Gly₇₀₄, Ala₇₀₁, Leu₇₀₅, Gly₇₀₈, Val₇₁₁, Ile₇₁₂ and Val₇₁₅ contribute most to this loosely binding state with a binding energy of ~ -56.964 (Fig. S4, ESI†).

While Glu₆₉₃ and Phe₆₉₁, the key residues in the 1:1 binary complex proposed by Barrett,⁵¹ have little contact with cholesterol in our results where Phe₆₉₀ is prominent instead (Fig. S4, ESI†). In fact, Straub *et al.* have explained that Barrett's NMR experimental data were collected at pH 4.5, which results in the protonation of Glu₆₉₃ and Asp₆₉₄ (natural pK_a values of Glu and Asp side chains are 4.4 and 4.0 respectively), and leads to the insertion of Glu₆₉₃ and Asp₆₉₄ in the membrane interior together with Phe₆₉₁. Under a neutral pH, Glu₆₉₃, Asp₆₉₄ and Phe₆₉₁ shift toward the membrane exterior; thus, no significant contacts were observed between cholesterol and these three residues.⁵² This description by Straub is in agreement with our observation.

It is interesting to point out that the escape of the cholesterol from the GxxxG binding site is also very difficult. When the cholesterol fights to escape from the pocket of site 1, it has to trek through the bulge of Phe₆₉₀. Even if it succeeds, cholesterol will quickly encounter the second barrier Lys₆₈₇ which will strongly attract it via the hydrogen bond (Fig. 3E and F and Table S2, ESI[†]), and the flexible tail of cholesterol can hardly resist the temptation of the hydrophobic residues Ile712 and Val₇₁₁. The binding state at site 0 with binding energy \sim -15.999 kJ mol⁻¹ (Fig. 3F and Table 1), which the residues Lys₆₈₇-Phe₆₉₀ contribute most to (Fig. S3, ESI[†]), is the most appropriate for the escape of the cholesterol. Otherwise, the Phe₆₉₀ turns sideways and the juxtamembrane region of C99 retracts close to the GxxxG motif in the meantime. In this way, cholesterol will be sent back to the GxxxG motif and experiences the loosely binding state again (Fig. 3F and G). Therefore, there are two pathways by which cholesterol binds to the GxxxG motif: from Fig. 3B to C, or Fig. 3F to G. The difficult process of cholesterol binding to or disassociation from the GxxxG motif may suggest site 1 to be a kinetically unfavorable site for cholesterol binding, while the relatively strong binding energy may suggest it to be a thermodynamically favorable site. In a general reaction process, the molecule needs to overcome an energy barrier to reach the favorable energy binding sites; while a greater energy barrier should be overcome in the reverse dissociation reaction.

It is surprising that the structure of C99 could be further optimized in the loosely binding state: the N-loop expands, allowing cholesterol to access the bottom of the pocket, where Ser_{697} forms a strong hydrogen bond with the cholesterol head (Fig. 3G and H and Table S2, ESI[†]). The residues Ser_{697} ,

Ala₇₀₁, Val₇₁₁, and Ile₇₁₂ and all the four Gly residues of $G_{696}xxxG_{700}xxxG_{704}xxxG_{708}$ zipper motifs contribute most to this tightly binding state (Fig. S4, ESI[†]). It is more dynamically stable than the loosely binding state: ~-58.857 binding for the tightly binding state and ~-56.964 for the loosely binding state.

The above observation is in agreement with the description by Łukasz Nierzwicki *et al.* that there exist two binding states at the GxxxG motif, whose free energies are almost equal. The interactions between the GxxxG motif and the cholesterol rings are not sufficient for binding.²⁵ In the tightly binding state, the cholesterol methyl ring associates intimately with the GxxxG motifs of C99. The loosely binding state is stabilized primarily by the interactions between the 3'-hydroxyl group and the A ring of cholesterol with the juxtamembrane of C99.²⁵ Additionally, more details were revealed in our simulations. In the tightly binding state, the cholesterol is largely stabilized by Ser₆₉₇ in the juxtamembrane of C99; while in the loosely binding state, Phe₆₉₀ plays a key role in the stabilization of cholesterol binding (Fig. S4, ESI†).

The dynamic process of cholesterol binding to other sites

As regards site 5, whose binding energy is nearly -54.725 kJ mol⁻¹ (Table 1 and Fig. S8, ESI[†]), the dynamic process is relatively simple. The groove between two contact surfaces has been an empty seat for cholesterol before its arrival. Cholesterols adhere to the protein surface like flies with the protein dynamics, as if protein shivers with itching but cannot shake off the cholesterol. The long side chain of Lys₇₂₅₋₇₂₇ residues is cross-arranged like the lotus base holding the head of cholesterol (Fig. S8 and Table S2, ESI[†]), which has a similar role to that in the complex: cholesterol-binding A β .⁵³ The residues Val₇₁₀, Ile₇₁₂, Ala₇₁₃, Ile₇₁₆, Val₇₁₇, Leu₇₂₀ and Lys₇₂₆ contribute most to the binding (Fig. S8, ESI[†]).

The processes of cholesterol binding to other sites are roughly similar to that at site 5 but with different binding strengths (Table 1 and Fig. S3-S9, ESI[†]). In fact, there are also more superficial and shorter time contacts of cholesterol with protein. Even when cholesterol binds to these relatively stable sites above, it is not completely fixed; it adopts a corresponding fine-tuning in the binding area together with the dynamic movement of the protein. The types of cholesterol binding can be divided into two categories: the β surface binding (the apposed α face is smooth, while the β surface is relatively rough) and side surface binding. "The β surface of cholesterol has two methyl groups (C18 and C19) protruding out of the plane of the sterol ring. These methyls can serve as knobs to fit in grooves or holes in the protein surface".54 On the other hand, the binding sites can also be divided into two kinds according to the binding position: the groove binding and the ridge binding. The groove binding was observed at sites 1, 2, 4 and 5; (Fig. 4 and Fig. S3-S9, ESI⁺), where cholesterol tends to bind in the groove space with the β surface and switch to side surface binding when the groove space shrinks (Fig. 4). In contrast, cholesterol often binds the ridge with the side surface at site 3 and site 6 (Fig. S6 and S9, ESI[†]). In particular, in our 1 µs AT simulations, no matter in a loosely binding state or tightly binding state at the GxxxG motif,

Fig. 4 The trajectory snapshots of different binding states in site 2. The protein is shown in cyan and cholesterol in green. The left is β surface binding, the right is side surface binding, and the middle presents the groove of site 2.

cholesterol mostly interacts with the GXXXG motif in side surface binding. This is different with Straub's observation that cholesterol prefers α surface binding with the GxxxG motif,⁵² which may be related to the initial location of the cholesterol in the 1:1 binary complex. The binding conformations, hydrogen bonds and residue contribution at each site are also provided in the ESI† (Fig. S3–S9 and Table S2).

The amino acid GAVILMTKF, especially VIKF plays a key role in this association

The summary of residue contributions to the Δ MM energy (Fig. 5) shows that the high contribution residues are, respectively, Phe₆₉₀, Lys₆₉₉, Ile₇₀₂–Met₇₀₆, Gly₇₀₈, Val₇₁₀–Ile₇₁₈ and Lys₇₂₅; in which Phe₆₉₀, Lys₆₉₉, Ile₇₁₂, Val₇₁₇ and Lys₇₂₅ are particularly outstanding. While Leu, Gly and Ala are essential to some binding sites, a detailed description of residue contribution to each binding sites is provided in the ESI.[†] Given the cholesterol's hydrophobicity, the result is of no surprise as these residues are all aliphatic amino acids except Phe. Branched residues such as Ile, Val, Leu and Met can be used to form a groove in the protein surface to accommodate cholesterol. The glycine zipper, Ala and Thr with an empty space is conducive to the parking of the cholesterol methyl ring. While the hydrogen bond is usually

formed between Lys or Phe or Ser and the cholesterol head, the π - π interaction is often formed between rings of Phe and cholesterol.

In fact, cholesterol profoundly impacts trafficking or sorting of membrane proteins by the formation of raft-like membrane domains.^{18,55,56} Many proteins, both soluble and membranebound, bind cholesterol to form saturable complexes.^{57–60} Research by Hulce has identified that 250 candidate proteins are associated with cholesterol in HeLa cells.⁶¹ Among the 20 high-resolution protein structures available in the Protein Data Bank, Charles Sander and his colleague analyzed 19 crystal structures and found that the hydrophobic residues Ile, Leu, and Phe are three of the most highly occurred residues at cholesterol binding sites. The most common residues that interact with the isooctyl tail of cholesterol are Ala and residues with branched side chains such as Leu and Val,⁵⁴ which are in line with our finding.

The consensus motif of cholesterol binding

Much effort has been devoted to seeking consensus on cholesterol binding sequence motifs.^{62,63} The first proposal of the "cholesterol recognition amino acid consensus (CRAC)" domain (L/V)-X₁₋₅-Y-X₁₋₅-(R/K) was suggested to form a cholesterol binding motif in the C-terminus of the peripheral benzodiazepine receptor (PBR).^{64,65} Later, a reversed-CRAC motif called "CARC" (K/R)-X₁₋₅-Y-X₁₋₅-(L/V) was proposed to be associated with cholesterol binding. Besides the reverse orientation, CARC is distinct from CRAC in that the central aromatic amino acid can be either Tyr or Phe.⁶⁶ Additionally, the "cholesterol consensus motif" (CCM) (R/K)-X₂₋₆-(I/V/L)-X₃-(W/Y) has been proposed based on a crystal structure of the b2-adrenergic receptor.⁶⁷

With regard to C99, Barrett *et al.* previously identified the $G_{XXX}G$ motif as a binding site.¹⁶ Later, Sanders *et al.* noted that C99 has a CARC motif: -K₆₈₇-X3-F₆₉₁-X3-V₆₉₅-, but suggested that the presence of this common motif in C99 is essentially unrelated to cholesterol binding.⁵⁸ To date, no other binding sites of C99 have been identified.



Fig. 5 Summary of residue contributions to the Δ MM energy of all the binding sites

Paper

In our results, six binding sites were presented, where the residue IVLGAMTKF plays a key role. The sequences of these binding sites bear great similarity to the consensus above, especially for the binding sites containing the Lys residue. Differently, these binding sites do not involve the aromatic residue. In fact, in the study regarding the CRAC of the TM5 domain of human type 3 somatostatin receptor, Jacques Fantin et al. explained that the central Y-226 residue is not involved in the interaction.⁷³ Additionally, fusogenic tilted peptides can interact with cholesterol, although they do not contain the central residues.⁶⁸ On the other hand, Fantin et al. have illustrated that the TM domain, which contains both a CRAC and a CARC sequence, can allow the simultaneous binding of two cholesterol molecules.⁷³ Moreover, the energetic pattern of the cholesterol binding site, 221-VICLCYLLIVVKK-232 (note: residues involved in cholesterol binding are underlined and in bold), do not overlap with any of the two CRAC motifs (LCYLLIVVK or VICLCYLLIVVK).⁷³ These results support the establishment of multiple binding in the same sequence.

The C99 model appears to suggest a new interpretation for cholesterol binding. It is believed that cholesterol binding is not only dependent upon simple residue effects but also greatly upon the appropriate structural domains with which cholesterol chooses a flexible conformation to fit. Another reason for cholesterol binding, to a larger extent is due to hydrogen bonding between specific amino acids and cholesterol's hydroxyl head groups. For integral membrane proteins, the hydrogen bonding has, so far, always been located near the watermembrane interface.⁵⁴ It is plausible that the essence of cholesterol binding is to bind the head of cholesterol by the hydrogen bond with residues Lys or Phe and bind the methyl ring and isooctyl tail of cholesterol by van der Waals interactions with an appropriate structural domain consisting of aliphatic amino acids (Gly, Ala, Val, Ile, Leu, Met and Thr). For the monomer-cholesterol interfaces, entropy should be considered. The association between the cholesterol and the protein is favored in terms of entropy, since the formation of the rigid and flat surfaces by cholesterol binding somehow dampened the protein dynamic motions, their surfaces would be less solvated in a fluid phase membrane.69,70

Overall, cholesterol has a wide association with a variety of proteins, and that there is a consensus of sequences in association with cholesterol. The specific model depending on protein structures might be significant for the execution of physiological functions.^{71,72} Although a cholesterol recognition amino acid consensus has been postulated, the specific binding sites, binding number, binding strength and the effect of cholesterol binding on protein physiological functions require further exploration.

The interpretation of the 1:1 C99-cholesterol binary complex

Because of the important roles of C99 and cholesterol in the amyloidogenic pathway, it has become a research hotspot. Previously, Barrett *et al.* identified a 1:1 binary complex (cholesterol binding C99 at the GxxxG motif) using NMR-controlled titration experiments.¹⁶ Recently, Łukasz Nierzwicki²⁵ and Straub's⁵² simulated the 1:1 binary complex identified by

Barrett's NMR experiments,¹⁶ to describe the dynamics of cholesterol binding at the GxxxG motif of C99. However, the 1:1 binary complex concluded by Barrett et al. is a fitting result of the overall titration, which does not necessarily mean that C99 has only one binding site for cholesterol. In order to explore more possibilities for cholesterol binding to C99, we first expanded the sampling with 10 \times 3 μ s CG simulations and then further validated the results by AT simulation with more accurate description of protein flexibility and the C99-cholesterol interactions. The most stable binding site GxxxG motif characterized by the CG/AT simulations is in line with the above finding. And our results bear a strong agreement with Nierzwicki's description that the existence of two different binding states at the GxxxG motif, with more details captured. Moreover, our result is mutually supported by Straub's finding that the position of Glu₆₉₃ and Asp₆₉₄ related to membrane is the key to the formation of strong binding. At the neutral pH, there is no significant contact of cholesterol with Phe₆₉₁, Glu₆₉₃ and Asp₆₉₄.

The other binding sites presented in our study are in conjunction with the previously proposed consensus motif of cholesterol binding, and may also be potential drug targets. In any case, multiple site dynamic binding will result in significantly different consequences of the C99 process compared with one stable binding.

The influence of cholesterol on C99 γ-cleavage

Studies have shown that elevated cholesterol levels directly activate the amyloidogenic pathway.^{5–14} There is also preliminary evidence that cholesterol directly activates γ -secretase cleavage of C99 through mechanisms that have not been understood so far.¹⁵

Our results further validate the existence of cholesterol interaction with C99 in multiple binding modes, with the cholesterol molecules dynamically binding to and flying away from time to time, which results in a scenario in which cholesterols surrounding C99. There is evidence that shows that two cholesterol molecules can form a tail-to-tail or a face-to-face complex. In the latter case, the self-recognition properties of cholesterol can induce the dimerization of membrane receptors.⁶³ In this sense, a similar inducer role may be charged with cholesterol in the interaction between C99 and γ -secretase $(\beta$ - and γ -secretases preferentially associate with the lipid raft), where the dynamic cholesterol binding will allow cholesterols to move away and not be a steric hindrance. Compared with a single cholesterol stable binding model, the multiple-site dynamic cholesterol binding model better explains that elevated cholesterol promotes the partition of APP and C99 into raft-like membrane domains where it is more likely to encounter the β - and γ -secretases and less likely to come across the α -secretase.^{16–18} It is interesting to note that $G_{709}xxxA_{713}$ is just located in the middle of the upper and lower layers. Only when cholesterol flipflops over the bilayer, it will go through this region. Therefore, the backbones of the $G_{709}xxxA_{713}$ motif (γ -secretase cleavage sites of C9973-75) are completely exposed without cholesterol shelter (Fig. 2A–D), this might help γ -secretase identify the cleavage sites and prompt the cleavage.

In summary, the association between cholesterol and C99 is a dynamic and multi-site interaction characterized by molecular simulation, and it plays a special role in the amyloidogenic pathway. There are still many issues, which need to be studied in further detail.

Conclusions

Both the N-terminus and the C-terminus of C99 show interactions with cholesterol, which leads to multiple binding modes rather than the one stable binding mode. The multi-site dynamic cholesterol binding model was captured based on an equilibrated C99 conformation and has been further validated by AT simulation. In this model, there are six regions of cholesterol binding in the C99 TMD (transmembrane domain): two of which are in the N-terminus including the repeat GxxxG motif, and the other four in the C-terminus. The analysis of binding energy shows that cholesterol prefers the sites: no. 1, 2, 4 and 5 over others. The most favorable binding energy of nearly -58.857 kJ mol⁻¹ is from site 1, the repeat GxxxG motif, and there are two pathways and two states of cholesterol binding to this site. Ser₆₉₇ and the Phe₆₉₀ contribute the most in the stabilization of a tightly binding state and a loosely binding state, respectively. The type of cholesterol binding can be divided into two categories: the β surface binding and side surface binding. On the other hand, the binding sites can also be divided into two kinds according to the binding position: the groove and convex binding sites. The association of cholesterol is commonly found within a variety of proteins, and the residue GAVILMTKF, particularly VIKF, plays a key role in this association. The C99 model appears to suggest a new mechanism for cholesterol binding. It is plausible that the essence of cholesterol binding is to bind the head of cholesterol by hydrogen bond with the residue Phe or Lys and bind the isooctyl chain and the methyl ring of cholesterol by van der Waals interactions with an appropriate structural domain consisting of aliphatic amino acids (GAVILMT). Compared with a single cholesterol stable binding model, the multi-site dynamic cholesterol binding model of C99 better explains the hypotheses that cholesterol promotes the amyloidogenic ABPP route. The backbones of the G709XXXA713 motif are completely exposed without a cholesterol shelter, which might help γ -secretase identify the cleavage sites and then promote γ -cleavage.

Competing financial interest

The authors declare no competing financial interest.

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