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Lipid Binding Properties of Annexin A9

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Abstract: Annexin (ANX) is a family of Ca^{2+}-dependent membrane/lipid binding proteins; altogether 12 types (ANXA1-A11 and A13) have been identified in the mammalian genome. Some of these are important regulators of the cell membrane organization, trafficking, repair, etc. Among them, ANXA9 is the only ANX that does not have the type II Ca^{2+} binding site. ANXs commonly bind to phosphatidylserine (one of the plasma membrane components), via the type II Ca^{2+} binding sites but does not bind to cholesterol. Therefore, ANXA9 appears to have different lipid binding properties and biological functions from other ANXs. In this study, we examined the binding ability of recombinant ANXA9 to cholesterol and its derivatives via the solid-phase assay and characterized the molecular structure of ANXA9 by circular dichroism spectrometry, by determining amino acid sequence motifs, and molecular modeling.

1. Introduction

Annexins (ANXs) are a family of evolutionarily conserved proteins which bind membrane/lipid in a Ca^{2+}-dependent manner. Twelve ANXs have been identified in the mammalian genome. ANXs are involved in several physiological functions related to intracellular membrane trafficking, cell surface membrane repair, and regulation of endocytosis/exocytosis. ANXs interact with Ca^{2+} via the so called type II Ca^{2+} binding sites which are localized to the carboxy-terminal (C-terminus) core domains composed of 4 or 8 repeating, homologous 70-amino acid units (Fig. 4, *shaded*). Although the type II Ca^{2+} binding sites have been considered as a hallmark of the ANX family, ANXA9 is very unusual because it not only the lacks the type II Ca^{2+} binding sites but also shares little amino acid similarity with other ANXs [1]. It is considered that ANXA9 is unable to bind to phosphatidylserine (PS) due to lack of type II sites.

In previous studies, ANXA9 was shown to bind to liposomes prepared from phospholipids derived from bovine brain extracts in a Ca^{2+}-independent manner [2]. ANXA9 was identified as an antigen of the autoantibody of pemphigus vulgaris [3] and as the biomarker of exosomes secreted from colorectal cancer cells [4]. Also, ANXA9 may have functions similar to the membrane proteins because some ANXs have been suggested to be localized in the late endosomes and lysosomes, and may be involved in membrane trafficking and repair [5]. However, detailed lipid binding ability and physiological/pathological functions of ANXA9 have not been elucidated.

In this study, we showed that ANXA9 binds to cholesterol, one of the major membrane lipids besides phospholipids. Membrane cholesterol interacts with phospholipids by its hydrophobic steroid nucleus and regulates the membrane fluidity and permeability. Thus, it is assumed that the cholesterol binding activity of ANXA9 might have relevance to its biological functions. Further, we proposed the possible

cholesterol binding sites of ANXA9 by searching for cholesterol-binding motifs, i.e. CRAC and CARC motifs and hydrophobic/ hydrophilic surface mapping.

2. Material and Methods

Analysis and measurements

A 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) was used to verify the nucleotide sequences. Protein concentration was estimated using a Pierce BCA protein assay kit (Thermo Scientific, Waltham, MA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Laemmli method [6] and the gels were stained using Coomassie Brilliant Blue (CBB). The secondary structures of the proteins were assessed using circular dichroism (CD) spectrometry. Briefly, the protein solutions (200 μg/mL) were prepared in a 10 mM Tris-HCl solution containing 150 mM NaCl, pH 7.5 and were analyzed in the range— 190 to 250 nm using a CD spectrometer (J-820, JASCO, Tokyo, Japan). The optical density (OD) was measured using a microplate reader (Cytation3, BioTek, Winooski, VT).

Expression vector and preparation of recombinant protein

The cDNA encoding the open reading frames of human ANXA9 was ligated into pGEX-6P-1 (GE Healthcare, Chicago, IL). The glutathione S-transferase (GST) fusion proteins were expressed in *Escherichia coli* JM109 cells and affinity-purified using GSH-Sepharose according to the manufacturer's instructions. Recombinant ANXA9 without the GST tag was prepared by digestion with PreScision Protease (GE Healthcare), followed by affinity purification.

Lipid binding assay

Cholesterol, 7-dehydrocholesterol, and phospha -tidylserine (bovine brain) were purchased from Sigma Aldrich. Ergosterol, ergocalciferol, and cholecalciferol were purchased from Tokyo Chemical Industry Co., Ltd. Microtiter plates (Immulon 1B, Thermo Scientific) were coated with lipids (0–1.6 μg/well) by volatilizing ethanol at ambient temperature. Binding assays were carried out in presence of 5 mM $CaCl_2$ or 2 mM EDTA. After washing with TBS, the wells were blocked with 5% BSA in TBS for 1 h. GST-ANXA9 in TBS containing 2.5% BSA was added to the wells (100 μL/well) and incubated for 1 h. Bound proteins were detected by enzyme-linked immunosorbent assay ELISA using rabbit anti-GST tagged antibody (Bethyl Laboratories, Montgomery, TX), HRP-conjugated anti-rabbit antibody (KPL), and *o*-phenylenediamine as a substrate. The absorbance of the colored reaction was measured at 490 nm using a microtiter plate reader.

Molecular modeling and analysis of hydrophobic molecular surfaces

Homology modeling of three-dimensional structure of ANXA9 was constructed using the crystal structure of human ANXA2 (PDB: 1W7B) as a template and surface hydrophobicity/hydrophilicity analysis was performed using the Molecular Operating System (MOE).

3. Results and Discussion

Preparation of recombinant ANXA9

GST-ANXA9 was expressed in an *E. coli* expression system and approximately 16 mg of GST-ANXA9 and 3.5 mg of ANXA9 were obtained from 1 L of culture. Recombinant GST-ANXA9 and ANXA9 resolved as a single band on a CBB stained SDS-PAGE gel at the expected positions— 64 kDa and 38 kDa respectively (Fig. 1A). CD spectrometry of ANXA9 showed a spectrum typical of α-helix structures (Fig. 1B), confirming that ANXA9 is composed of α-helical structures similar to other ANX family proteins [7].

Lipid-binding properties of ANXA9

To test the lipid binding property of ANXA9, solid-phase binding assays using GST-ANXA9 and specific antibodies were carried out. The results showed that ANXA9 significantly binds to cholesterol but not to PS, and Ca^{2+} is not necessary for the interaction between ANXA9 and cholesterol. It was suggested that ANXA9 possesses specific cholesterol

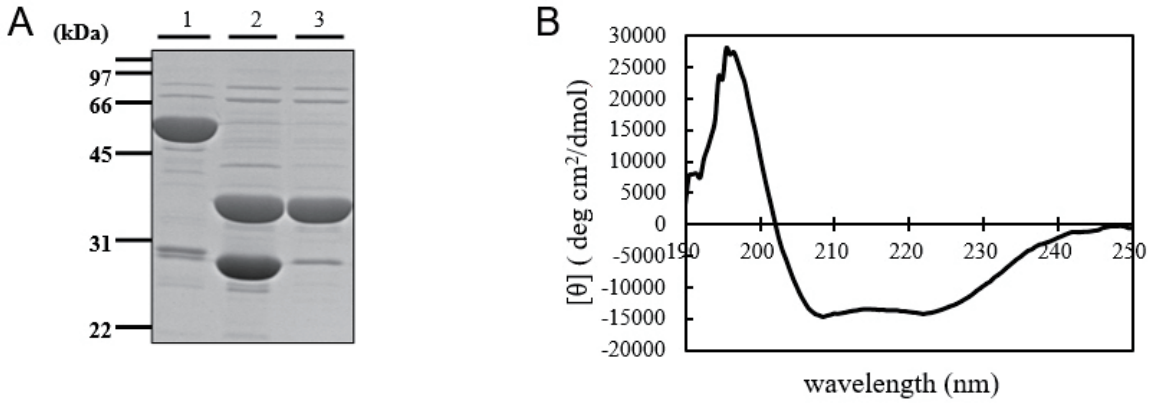


Figure 1. SDS-PAGE and CD spectrometry of recombinant ANXA9.
(A) CBB-stained SDS-PAGE gel loading sequence: purified GST-ANXA9 (lane 1), purified GST-ANXA9 (after PreScission Protease cleavage (lane 2), and purified ANXA9 (lane 3). Molecular mass standards are shown to the left of the image; (B) CD spectrum of purified ANXA9.

binding property, independent of the type II Ca^{2+} binding sites. Next, we tested the binding ability of ANXA9 to steroid compounds (7-dehydrocholesterol and ergosterol) and secosteroid compounds (cholecalciferol and ergocalciferol). The results showed that while ANXA9 binds to cholecalciferol and ergocalciferol to a lesser extent, it does not bind to 7-dehydrocholesterol and ergosterol. Since a hydroxyl group is present in the same position of 7 -dehydrocholesterol and ergosterol relative to cholesterol, it is not considered to be responsible for the ANXA9-cholesterol interaction. On the other hand, unlike cholesterol, 7-dehydrocholesterol and ergosterol have a double bond between C-7 and C-8; therefore ANXA9 may specifically recognize the double bond in the B ring of steroid nucleus. However, the slight interaction of ANXA9 to cholecalciferol and ergocalciferol suggests that ANXA9 also binds to sites other than the B ring structure.

Putative cholesterol binding sites of ANXA9

Cholesterol is relatively abundant in the plasma membrane and endocytic recycling compartment [8] and plays important roles in regulating the cell membrane fluidity and subcellular distribution of transmembrane receptor proteins in the mammalian cells [9]. The membrane-spanning domains of the nicotinic acetylcholine receptor displays a series of cholesterol consensus domains called the cholesterol recognition/ interaction amino acid consensus (CRAC) motif— (L/V)$X_{1\text{-}5}$YX$_{1\text{-}5}$ (R/K). It has been reported that several cholesterol binding membrane receptors also have CRAC motifs and the reverse version, CARC motif— (R/K) $X_{1\text{-}5}$YX$_{1\text{-}5}$ (L/V). Some receptors have a pair of CRAC and CARC motifs in their transmembrane domains and are suggested to interact with two cholesterol molecules in each leaflet of the membrane bilayer [10]. It has been demonstrated that the CRAC/CARC motif binding to cholesterol consist of the following three interactions: the aliphatic (L/V) amino acid residue holds a methyl group, the aromatic (Y) amino acid residue interact by π-π stacking, and the basic (R/K) amino acid residue forms a hydrogen bond with a hydroxyl group of cholesterol [11]. It has also been reported that CRAC/CARC motifs are not paired and exist outside of the transmembrane domain— hMincle [12]. While ANXs do not have a transmembrane domain, they are abundant in α-helices (Fig.1B); therefore we expected that the CRAC/CARC motifs in ANXA9 to be involved in its cholesterol binding. We tried a CRAC/CARC algorithm to find possible cholesterol binding sites of ANXA9. A CRAC motif (V233-R239) and two CARC motifs (K269 -L279 and K315-L324) were identified in ANXA9 in repeats 3 and 4 of the C-terminus core domain (Fig. 3A). These CRAC and CARC regions are shown in the three-dimensional structure of ANXA9 (Fig. 3B). All of them were localized to the concave side of ANXA9.

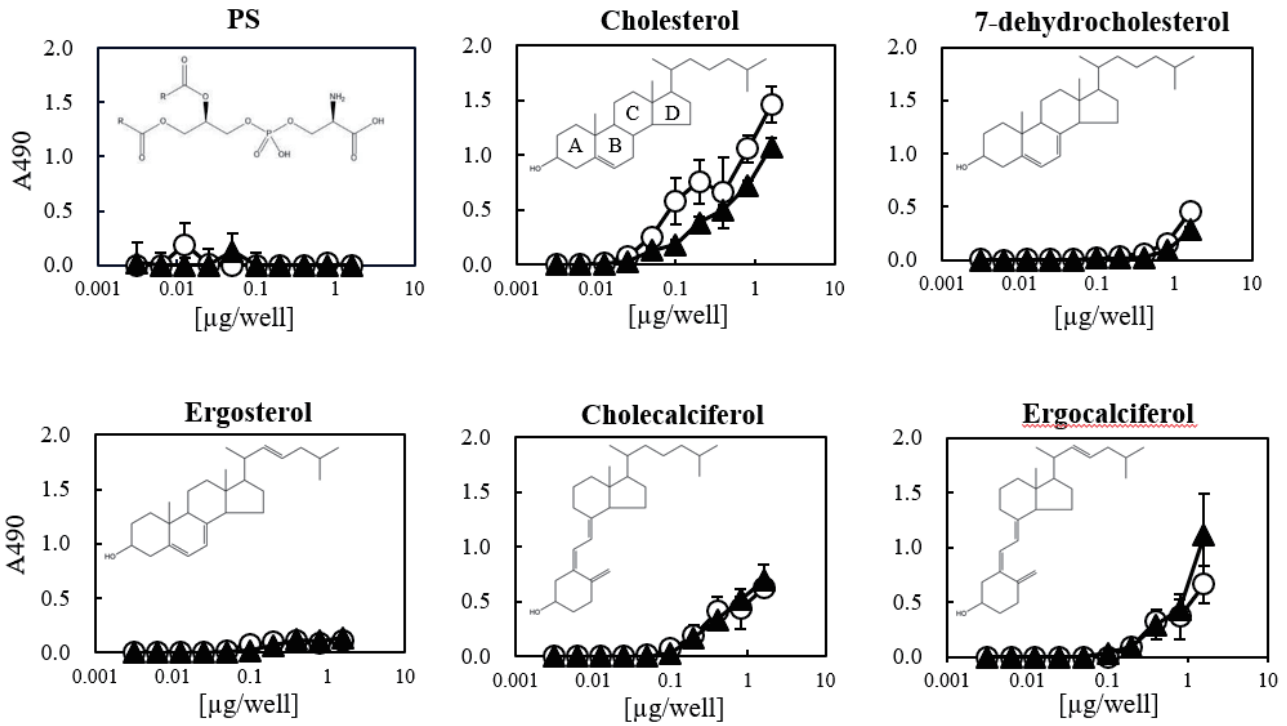
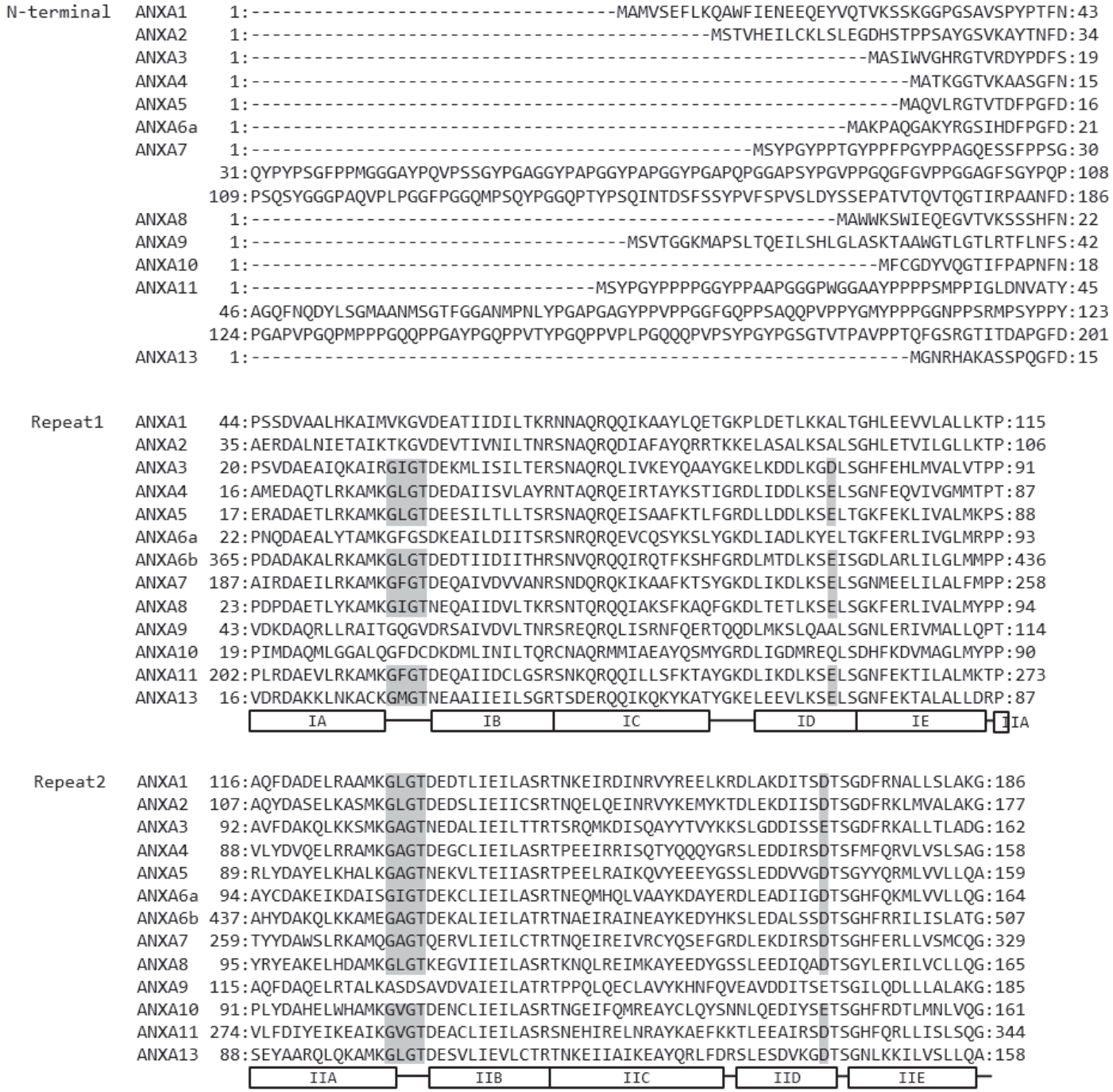


Figure 2. Lipid binding activity of ANXA9.
Microtiter plate wells were lipid coated and incubated with 0.25 μg/mL of GST-ANXA9 in the presence of 5 mM Ca^{2+} (○) or 2 mM EDTA (▲). The bound GST-ANXA9 was detected using anti-GST antibodies and HRP conjugated anti-rabbit IgG. Absorbance was measured at 490 nm on a microtiter plate reader. Data from a triplicate experiment is represented as mean ± standard deviation. Lipid structures are also depicted. Cholesterol, 7-dehydrocholesterol and ergosterol have the steroid nucleus (A-D ring).

Figure 3. CRAC and CARC motifs identified in ANXA9 amino acid sequence.
(A) Amino acid sequence of ANXA9; (B) Putative three-dimensional structure of ANXA9. CRAC and CARC motifs are indicated in *red*.

In addition, some of these motifs in ANXA9 were also found to be in the same regions of other ANXs; ANXA1, A4, A5, A6, A8, A10, A11 (Fig. 4, *boxes*). Whether these ANXs can directly bind to cholesterol remains largely unknown. Previous studies showed that ANXA6 interacts with cholesterol monolayer [14], so ANXA6, as well as ANXA9, might bind to cholesterol via CRAC/CARC motifs. This is only a prediction from the primary structure of the protein and therefore further experimental study is required.

Next, we compared the surface hydrophobic patches of ANXA9 to those of ANXA2 (Fig. 5). It was shown that ANXA9 has a wider hydrophobic surface area than ANXA2. It is suggested that surface hydrophilicity may play a role in the binding of ANXA9 to cholesterol. Patch #7 corresponds to the CARC motif of K315-L324, making it the most probable cholesterol binding site. In future, we plan to



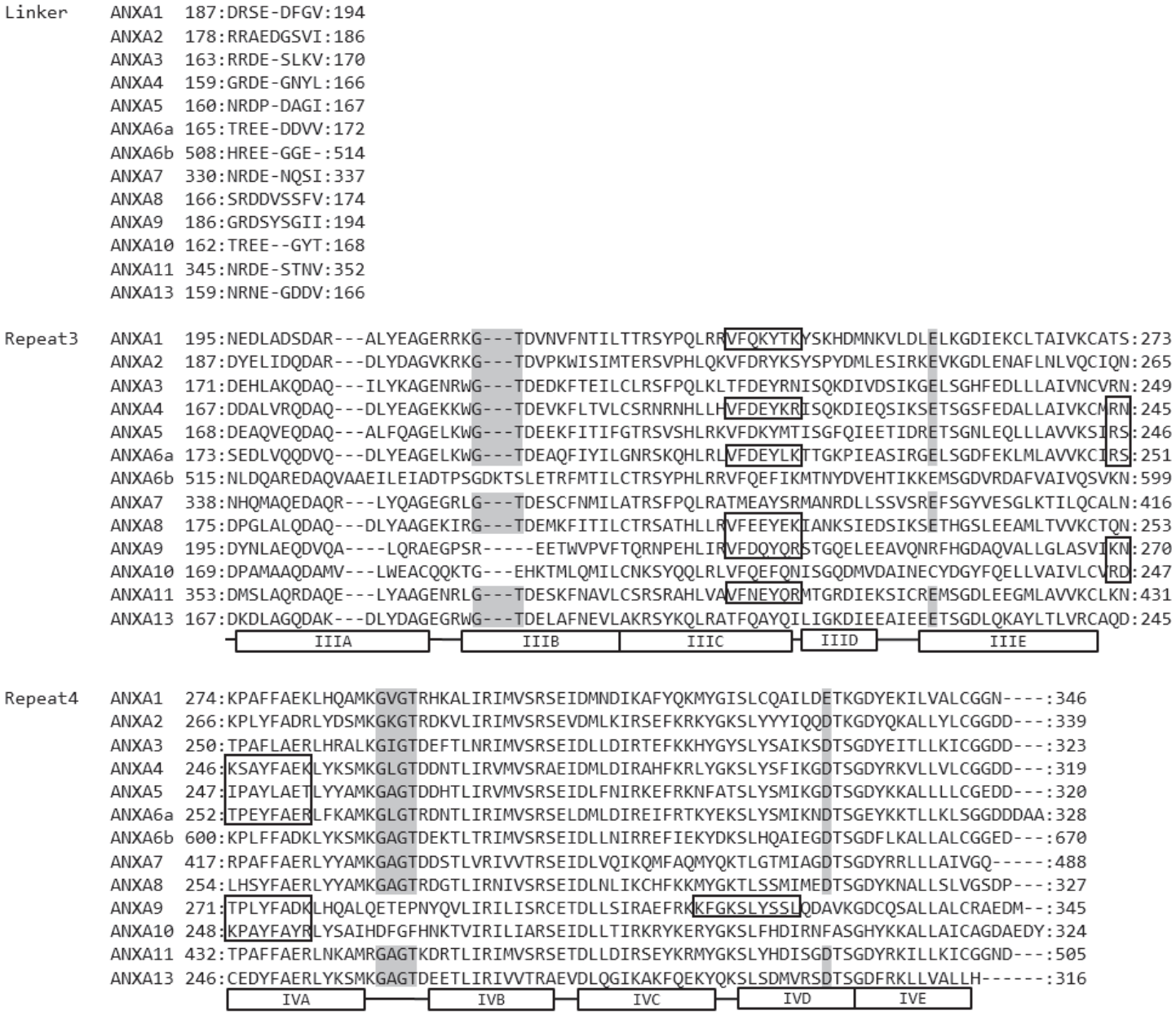
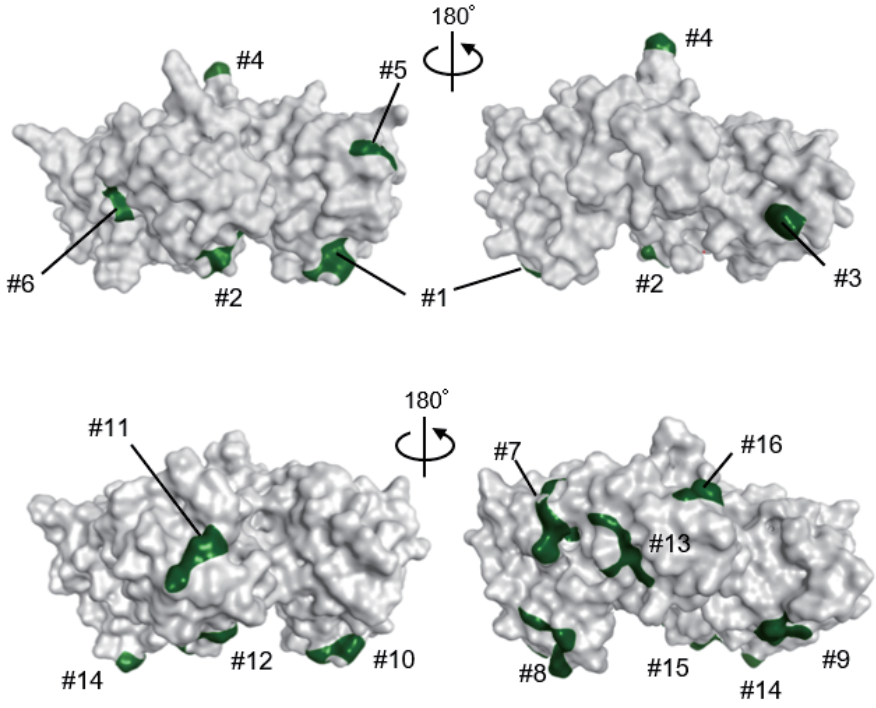


Figure 4. The multiple sequence alignment of all the ANXs repeats.
ANXs repeats (repeat 1-4) and the five helices within each repeat (IA-IE, IIA-IIE, IIIA-IIIE, and IVA-IVE) were referenced by the amino acid sequence alignment described by Huber *et al.*, 1990. Type II Ca^{2+} binding sites—GXGT-(38-40 residues)-D/E (*shaded*) and the identified CRAC/CARC motifs (*boxes*) are highlighted. CRAC/CARC motifs found in the same regions of other ANXs are also highlighted (*boxes*). ANXA6 composed of 8 repeats are split in to two cores— 6a and 6b. All amino acid sequences were referenced from UniProtKB (https://www.uniprot.org/) and the alignment analysis was carried out using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

investigate the cholesterol binding sites of ANXA9 by examining the lipid binding properties of ANXA9 mutants (of potential amino acid residues) and determining the ANXA9 crystal structure.



	Patch No.	Area [Å]
ANXA2	#1	100
	#2	70
	#3	70
	#4	60
	#5	50
	#6	50
	Total	350
ANXA9	#7	220
	#8	120
	#9	100
	#10	80
	#11	70
	#12	60
	#13	60
	#14	50
	#15	50
	#16	50
	Total	860

Figure 5. The hydrophobicity/hydrophilicity analysis of ANXA2 and ANXA9.
The hydrophobic areas (*green*) of the protein surfaces of ANXA2 (*upper*) and ANXA9 (*lower*) were calculated by MOE. Areas for all the patches are summarized in the table.

4. Conclusion

We demonstrated that ANXA9 is an α-helix-rich structure like other ANXs and has the ability to bind to cholesterol in a Ca^{2+}-independent manner. Our results highlight potential cholesterol binding sites of ANXA9: cholesterol binding consensus motifs (CRAC and CARC) and the hydrophobic patches of protein surface.

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