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Direct Mechanisms in Cholesterol Modulation of Protein Function

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Editors

Direct Mechanisms in Cholesterol Modulation of Protein Function

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Preface

Cholesterol is a major lipid component of the plasma membrane where it constitutes up to ~50 mol% of the total membrane lipids. As such, it is not surprising that cholesterol effects on protein function have been historically attributed to its effect on the physical properties of lipid bilayers. First indications that cholesterol may bind directly to proteins started to emerge in the mid-1970s in studies on the eukaryotic sugar transport system [1], the Folch-Lees proteolipid protein, the major protein component of myelin [2], the Na^+/K^+-ATPase [3], and the band 3 protein that constitutes the main integral protein of the human erythrocyte membrane [4]. These studies opened a floodgate, and since then, cholesterol has been shown to play a direct role in the regulation of an ever-growing number of proteins.

In 1998, the first amino acid consensus sequence for cholesterol binding, the cholesterol recognition amino acid consensus (CRAC) motif, was proposed in the context of the peripheral-type benzodiazepine receptor, a transmembrane protein that mediates the translocation of cholesterol [5]. While the CRAC motif has been identified in multiple proteins since then, several other cholesterol binding motifs followed, and the characteristics of cholesterol-binding sites in proteins have continued to be defined.

The first structural evidence that cholesterol can bind directly to proteins emerged in mid-2002 with the determination of the structure of the cryptogein-cholesterol complex via X-ray crystallography at a 1.45 Å resolution [6]. Despite its small size, cryptogein, a fungal elicitor, displayed a large inner hydrophobic cavity that harbored the cholesterol molecule. The same year, a structure of the ligand-binding domain of the retinoic acid orphan receptor α was determined at 1.63 Å resolution in complex with cholesterol [7]. This was followed by a structure of the cholesterol-bound oxysterol-binding protein Osh4 at a 1.6 Å resolution in 2005 [8].

In 2007, a structure of the β2 adrenergic G-protein-coupled receptor was crystallized at a 2.4 Å resolution in complex with cholesterol [9]. In this structure, cholesterol mediated receptor-receptor interactions improving the stability of the receptor. This was another milestone in the quest to uncover the direct roles of cholesterol-protein interactions in protein function.

Over the course of several decades, numerous functional, structural, and computational studies have continued to shape our understanding of cholesterol-protein interactions, unraveling the growing number of roles that they play in cellular function. These range from cholesterol transport and storage to protein stability, folding, and localization. While many questions regarding the underlying molecular mechanisms remain unresolved, significant advances in our understanding of direct cholesterol-protein interactions have been made in recent years, and are the topic of this volume.

This is the second in a sequel of two volumes on the mechanisms of cholesterol modulation of protein function. The first volume (1115 in the Advances in Experimental Medicine and Biology Series) focused on sterol specificity as a means to distinguish between direct and indirect effects of cholesterol as well as on indirect mechanisms that impact protein function in response to variations in cholesterol level. The current volume complements the picture by focusing on protein targeting via direct interactions of the cholesterol molecule with sterol-sensing protein sites.

The first part of this volume introduces the reader to the general characteristics of cholesterol binding sites. This part starts with a survey of the different cholesterol-binding motifs that have been proposed over the years followed by an overview of the major classes of proteins that bind steroids and the insights gained from their study using X-ray crystallography. It then continues to two studies that utilize the growing number of structures of cholesterol-bound proteins available in the Protein Data Bank to present new insights into the molecular and structural characteristics of cholesterol-binding sites. The second part of this volume delves into more specific cases of cholesterol binding to G-protein-coupled receptors, ion channels, and cholesterol transporters that have been studied using combinations of experimental and computational approaches.

The editors are grateful to all the authors who contributed to this project aimed at portraying the intricate interactions between a variety of proteins and cholesterol. The editors are also thankful to senior mentors, collaborators, and colleagues for stimulating discussions, and for fostering a supportive environment for the completion of this diverse collection of contributions to the field.

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Contents

Part I General Characteristics of Cholesterol Binding Sites

Cholesterol-Recognition Motifs in Membrane Proteins 3
Jacques Fantini, Richard M. Epand, and Francisco J. Barrantes

Crystallographic Studies of Steroid-Protein Interactions 27
Arthur F. Monzingo

Molecular Determinants of Cholesterol Binding to Soluble and Transmembrane Protein Domains 47
Jessica Ounjian, Anna N. Bukiya, and Avia Rosenhouse-Dantsker

Modes of Cholesterol Binding in Membrane Proteins: A Joint Analysis of 73 Crystal Structures 67
Cong Wang, Arthur Ralko, Zhong Ren, Avia Rosenhouse-Dantsker, and Xiaojing Yang

Part II Cholesterol Binding Sites in Proteins: Case Studies

Effects of Cholesterol on GPCR Function: Insights from Computational and Experimental Studies 89
Sofia Kiriakidi, Antonios Kolocouris, George Liapakis, Saima Ikram, Serdar Durdagi, and Thomas Mavromoustakos

Cholesterol as a Key Molecule That Regulates TRPV1 Channel Function 105
Sara L. Morales-Lázaro and Tamara Rosenbaum

Cholesterol Binding Sites in Inwardly Rectifying Potassium Channels . . 119
Avia Rosenhouse-Dantsker

Insights into the Molecular Mechanisms of Cholesterol Binding to the NPC1 and NPC2 Proteins 139
Stephanie M. Cologna and Avia Rosenhouse-Dantsker

Index 161

Part I
General Characteristics of Cholesterol Binding Sites

Cholesterol-Recognition Motifs in Membrane Proteins

Jacques Fantini, Richard M. Epand, and Francisco J. Barrantes

Abstract The impact of cholesterol on the structure and function of membrane proteins was recognized several decades ago, but the molecular mechanisms underlying these effects have remained elusive. There appear to be multiple mechanisms by which cholesterol interacts with proteins. A complete understanding of cholesterol-sensing motifs is still undergoing refinement. Initially, cholesterol was thought to exert only non-specific effects on membrane fluidity. It was later shown that this lipid could specifically interact with membrane proteins and affect both their structure and function. In this article, we have summarized and critically analyzed our evolving understanding of the affinity, specificity and stereoselectivity of the interactions of cholesterol with membrane proteins. We review the different computational approaches that are currently used to identify cholesterol binding sites in membrane proteins and the biochemical logic that governs each type of site, including CRAC, CARC, SSD and amphipathic helix motifs. There are physiological implications of these cholesterol-recognition motifs for G-protein coupled receptors (GPCR) and ion channels, in membrane trafficking and membrane fusion (SNARE) proteins. There are also pathological implications of cholesterol binding to proteins involved in neurological disorders (Alzheimer, Parkinson, Creutzfeldt-Jakob) and HIV fusion. In each case, our discussion is focused on the key molecular aspects of the cholesterol and amino acid motifs in membrane-embedded regions of membrane proteins that define the physiologically relevant crosstalk between the two.

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Our understanding of the factors that determine if these motifs are functional in cholesterol binding will allow us enhanced predictive capabilities.

Keywords Cholesterol · Binding site · Membrane protein · Membrane fusion · Virus fusion · Neurological disease

1 Overview of Lipid Recognition Motifs in Proteins: Range of Specificity and Affinity

Quantifying binding affinities in interactions between membrane components or between a membrane component and a water-soluble molecule can be far from straightforward, since the membrane components may be in a different, 2-dimensional phase, meaning that their binding cannot be dealt with by applying the same methods as those used in solution thermodynamics. Qualitative binding behavior, however, can be more easily assessed. Binding specificity for membrane lipid components often depends on interaction with the lipid headgroup. For example, phosphatidylinositol and its several phosphorylated derivatives have very different binding affinities for certain proteins determined by the number and position of phosphate groups on the inositol ring. This type of headgroup structure, with its capacity to form hydrogen and electrostatic bonds, does not exist for sterols. Cholesterol, for example, has only a single OH group as its polar moiety. In addition to the headgroup, however, binding can also occur at the hydrocarbon portion of the lipid, accounting for the observation that both the headgroup and hydrocarbon regions of lipids determine their biological function [1].

In addition to the direct binding of proteins to cholesterol, cholesterol can also induce the binding of proteins to membranes by affecting membrane physical properties. Cholesterol plays important roles in the formation of domains in biological membranes [2], as well as in modulating membrane physical properties [3]. Because of the importance of cholesterol in determining membrane properties, there are multiple mechanisms involving cholesterol binding to proteins, to maintain cholesterol homeostasis [4]. This regulation of the metabolism and transport of cholesterol is dependent on the specific cholesterol binding sites on proteins. The specificity of protein binding to cholesterol will likely include interactions with both the hydroxyl group and with portions of the hydrocarbon region. The degree of specificity can be assessed by comparing the binding to cholesterol with binding to ergosterol, a closely related sterol from yeast. Stereochemical isomers of cholesterol can also test specificity [5]. The sterol analogs include epicholesterol, the 3′ epimer of cholesterol and *ent*-cholesterol, the enantiomer of cholesterol. *Ent*-cholesterol is the closest analog, but its use requires the total synthesis of the sterol. With epicholesterol the hydroxyl group protrudes from the sterol ring system at an angle in contrast with cholesterol in which the sterol ring system will be in the same plane as the hydroxyl group. Hence, it is not likely that a protein binding site for cholesterol would also bind epicholesterol. The situation is different with *ent*-cholesterol, the enantiomer or mirror image of cholesterol. Lipids

generally have few chiral sites, so that the interactions of cholesterol and *ent*-cholesterol with phospholipids in bilayer membranes are generally identical. However, in the presence of peptides or proteins, there are chiral sites at every amino acid residue, with the result that there is usually a difference between the binding of cholesterol vs. *ent*-cholesterol [6], though there are examples of proteins that can bind equally well to cholesterol and *ent*-cholesterol. Differences in the binding affinity of these two enantiomorphs can therefore be used as evidence of the presence of a cholesterol binding site in proteins, whereas if the binding affinities are the same one may conclude that the cholesterol binding site in the protein is not stereospecific. There has been limited use of this tool since *ent*-cholesterol is not commercially available and its synthesis is complex.

Another factor affecting protein binding to a lipid in a membrane is the distribution in the plane of the membrane and the formation of domains. This is particularly true for cholesterol, which can promote the formation of phases showing liquid-liquid immiscibility. The liquid-ordered, L_o phase has a higher cholesterol concentration [7]. Such cholesterol-enriched phases have been suggested to represent putative "raft" phases that occur naturally in biological membranes. Thus, another factor potentially affecting protein binding to cholesterol in membranes containing liquid-ordered domains, is whether or not the protein sequesters into these domains. Because the mol fraction of cholesterol is higher in these domains, proteins will not require such a high affinity to bind cholesterol.

In many cases, the interaction of cholesterol with proteins may be even more complicated than a single uniform binding site, as described above. For example, an NMR study of the interaction of cholesterol with the β_2 adrenergic receptor showed that there were two classes of cholesterol binding to this protein. One class corresponded to a limited number of high affinity sites having sub-nanomolar affinity for this lipid. However, there was a second class of cholesterol binding in fast exchange with unbound cholesterol and with an affinity that was lower by several orders of magnitude. It was suggested that these represented transient cholesterol clusters around high affinity cholesterol binding sites [8]. There has also been a recent molecular dynamics study demonstrating distinct cholesterol binding sites in the A_{2A} adenosine receptor [9].

2 Cholesterol-Recognition Motifs

Studying a lipid-protein binding process calls for an understanding of the basic principles of this interaction. In the case of cholesterol and membrane proteins, the problem may look simple at first glance, but as we will see, it can be far more complex than expected. Schematically, the binding reaction involves two partners: a cholesterol molecule and a membrane protein. Since the lipid bilayer of the biological membrane is the natural medium for the cholesterol molecule, several simplifications can reasonably be applied to the system. Firstly, only protein domains that cross the lipid bilayer are involved. Although this may be considered

patently obvious, exceptions to this rule have been reported, as for the human oncoprotein Smoothened (SMO), which displays a functional cholesterol binding site in the extracellular domain, i.e. outside the membrane bilayer boundaries [10]. In the case of human phospholipid scramblase 1, cholesterol binds to a specific domain that includes both a membrane-embedded and an extracellular coil [11]. Apart from these rare cases, most cholesterol binding sites of integral membrane proteins lie within their α-helical transmembrane domains (TMDs) that totally cross the lipid bilayer. Several cholesterol-binding sites have been found in TMDs [12, 13]. Some of these sites are clearly three-dimensional [14, 15], whereas others follow linear motifs [16, 17]. Among these motifs, the linear CRAC domain (Cholesterol Recognition/interaction Amino acid Consensus sequence) [18] and its reverse formulation CARC [19] have received considerable attention.

2.1 CRAC Motif

The CRAC motif is defined by the consensus (L/V)-$X_{1–5}$-(Y)-$X_{1–5}$-(K/R) from the N-terminus to C-terminus direction [18]. This motif can be considered a chemical fingerprint of cholesterol. Each of the three amino acid residues that define the CRAC motif has a specific function in cholesterol recognition. The N-terminal branched residue (valine or leucine) binds the iso-octyl chain of cholesterol through van der Waals interactions. At the opposite end, the C-terminal polar residue (lysine or arginine) faces the OH group of cholesterol, allowing the establishment of a hydrogen bond. In addition, the CRAC motif is vectorial, imposing a parallel "head-to-head/tail-to-tail" geometry to the CRAC/cholesterol complex (Fig. 1). This, in turn, facilitates the aromatic structure of tyrosine stacking onto one of the four rings of sterane. It should be noted that the position of tyrosine is determined by the length of a couple of $X_{1–5}$ linkers that separate the aromatic residue of CRAC from the ends of the motif. The presence of such variable segments, which differ in both length and composition, has been viewed as a serious weakness by some authors [20]. But in fact, this

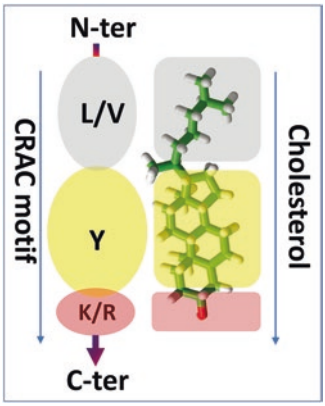

Fig. 1 Geometry of the CRAC/cholesterol complex. The motif is oriented in the N-ter (top) to C-ter (bottom) direction. It displays three distinct zones (apolar in blue, aromatic in yellow, cationic in purple) that fit with the chemical structure of cholesterol

variability reveals a hallmark of cholesterol binding sites found in most cholesterol-TMD complexes: the essential contribution of CH-Pi stacking interactions [21].

When an aromatic ring faces an aliphatic cycle, it adjusts its orientation so that the Pi electron cloud attracts the hydrogen atoms linked to the aliphatic cycle, resulting in a coordinated network of favorable interactions. This particular case of attraction between the C-H groups of a saturated cyclic hydrocarbon and an aromatic ring is referred to as the "stacking CH-Pi interaction" [21]. Sometimes, the induced fit mechanism that directs the respective orientation of both rings results in a near perfect geometry, as shown in Fig. 2. In the case of the CRAC-cholesterol complex, the establishment of such an optimal geometry requires that the aromatic ring of Tyr is parallel to sterane. Obviously, it is the distance between Tyr and the ends of the motif that determines which of the four rings of cholesterol is selected for the establishment of the stacking CH-Pi system. Thus, the length of the linkers (from one to five amino acid residues) allows several possible stacking interactions. In other words, thanks to both linkers, the Tyr residue can be viewed as a cursor able to occupy any possible position in the motif [12], and this unique feature would not be possible if the linkers had a fixed length. The total length of the CRAC motif ranges from five amino acid residues (both linkers with only one residue) to 13 residues (both linkers with five residues). The maximal size of CRAC motifs is by no means a coincidence. Indeed, an α-helix stretch of 13 amino acid residues has approximately the same size as cholesterol, i.e. 20 Å [22, 23]. The fact that the linkers have no sequence requirements confirms that only their length matters, which is remarkably consistent with the biochemical mechanisms underlying the formation of a CRAC-cholesterol complex.

In membrane areas where cholesterol is present in both leaflets of the plasma membrane, the same TM domain can theoretically interact with two cholesterol molecules (one in each leaflet). However, the vectorial nature of the CRAC motif is compatible with only one of these possibilities, depending on the orientation of the TMD. If the TMD crosses the bilayer in the N-terminus to C-terminus direction, the CRAC domain may interact with a cholesterol molecule located in the cytoplasmic

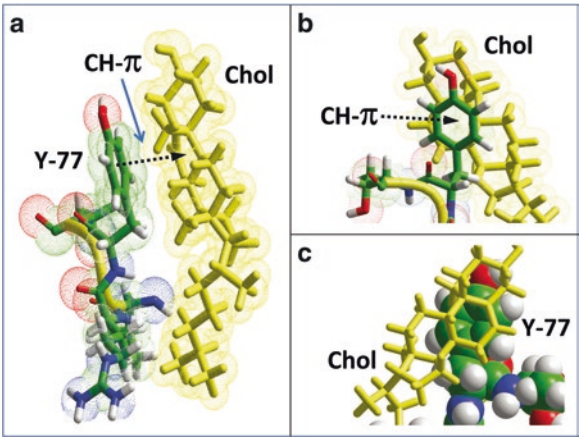

Fig. 2 CH-π stacking interaction in the CRAC/cholesterol complex. Three distinct views of cholesterol (in yellow) (**a**–**c**) bound to the CRAC domain of the human delta-type opioid receptor are shown. The near perfect superposition of the aromatic ring of Tyr-77 onto the second ring of cholesterol is particularly well illustrated in **b** and **c**

leaflet of the membrane, but not in the extracellular leaflet [23]. Therefore, a CRAC domain in the unique TMD of a bitopic membrane protein will interact with cholesterol in the inner leaflet. Similarly, TMDs I, III, V, and VII of G-protein coupled receptors (GPCRs) displaying a CRAC motif will also select cholesterol in the inner leaflet [24]. Conversely, the interaction of CRAC with cholesterol in the exofacial leaflet requires that the TMD crosses the bilayer in the C- terminus to N-terminus direction. This kind of situation applies for type II bitopic membrane proteins and GPCRs (TMDs II, IV, and VI).

The CRAC motif has been found in various proteins known to bind cholesterol and in many cases the interaction between cholesterol and CRAC has been confirmed by various physicochemical and/or functional approaches [12, 24–28]. Moreover, single mutations in the CRAC domain have been found to markedly decrease or even abolish the interaction. In this respect, it should be noted that in most instances, the Tyr residue cannot be replaced by Phe or Trp [29–31]. Nevertheless, a thorough analysis of CRAC domains through molecular docking studies suggests that, at least in some cases, the aromatic residue may not be directly involved in cholesterol recognition [13]. In other cases, the aromatic ring of Phe could sustain CH-Pi stacking interactions when Tyr is not present in the motif [16]. Future studies will likely lead to a refinement of the definition of the CRAC domain, especially for membrane proteins.

2.2 CARC Motif

The impossibility of the CRAC motif to interact with cholesterol in the exofacial domain of a large number of TMDs implied the possible existence of another specific cholesterol-binding motif. Indeed, the discovery of a new motif, referred to as CARC, was primarily due to the fact that no CRAC motifs were found in the TMDs of the nicotinic acetylcholine receptor protein; instead, CARC motifs were found [19]. Basically, CARC is an inverted and slightly modified version of the CRAC motif: (K/R)-$X_{1–5}$-(Y/F/W)-$X_{1–5}$-(L/V). The CARC domain displays remarkably specific features that take into account the membrane environment. Firstly, the central residue is still aromatic, but unlike CRAC which, in theory, has a specific requirement for Tyr, the CARC motif can accept Tyr, Phe, or Trp, consistent with the presence of all these residues in TMDs of various membrane proteins [32]. Secondly, the basic amino acid of CARC is located at the N-terminus. This distinctive feature explains why the CARC domain of class I membrane proteins (the most abundant bitopic proteins) can form a complex with cholesterol in the exofacial leaflet (Fig. 3). The same is true for TMDs I, III, V, and VII of GPCRs.

The biochemical rules that apply to the CRAC-cholesterol interactions also apply for CARC, since both motifs share a similar organization, i.e. a triad of mandatory amino acids with a central aromatic residue flanked by a basic and a branched apolar residue at each end. In both cases, spacers consisting of one to five unspecified amino acids ensure that the aromatic ring in the central position of the cholesterol-binding motif can optimally stack onto one of the sterane rings.

The CARC domain has been detected in a wide range of membrane proteins, including neurotransmitter receptors and transporters, ion channels and GPCRs [12, 13, 15, 16, 24, 33–35]. The nicotinic acetylcholine receptor displays 15 cholesterol binding sites (3 per subunit) that fulfill the CARC algorithm [19]. Docking studies have led to the proposal of a crown-like distribution of those cholesterol molecules around the receptor (Fig. 4), in agreement with the early views stemming from elec-

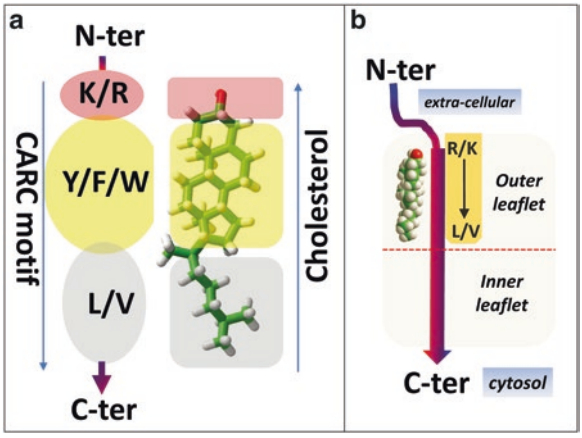

Fig. 3 The CARC motif of Class I bitopic membrane proteins is located in the outer leaflet of the plasma membrane. The CARC motif and cholesterol are represented with the same color as in Fig. 1. (**a**) Topology of the CARC-cholesterol complex. (**b**) Membrane localization of the CARC-cholesterol complex. The border between the outer and inner membrane leaflets is indicated by a dashed line

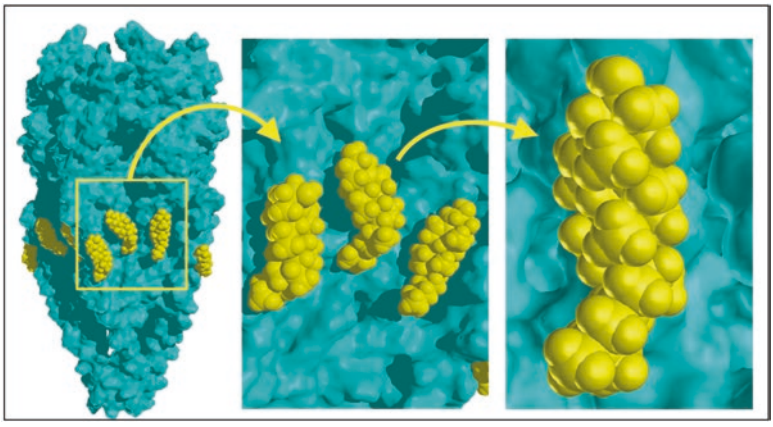

Fig. 4 Docking of 15 cholesterol molecules onto the nicotinic acetylcholine receptor. Three cholesterol molecules bound to the γ subunit of the acetylcholine receptor are shown at a different scale. The picture on the right shows the cholesterol molecule bound to the CARC motif in the fourth TMD of the γ subunit

tron spin resonance studies [36], as reviewed in ref. [37]. Biophysical studies with synthetic peptides encompassing a CARC domain have provided experimental support to the cholesterol-binding activity of the motif. A deuterium NMR spectrum of the CARC motif of the *Torpedo* nicotinic acetylcholine receptor γ-TM4 showed that the presence of cholesterol within the bilayer induced a reduction in the rotational motion of the peptide within the bilayer, a change consistent with cholesterol promoting the oligomerization of the γ–TM4 segment [16]. Moreover, mutational studies of this domain confirmed the prominent role of its central Phe residue. Indeed, the interaction with a cholesterol-containing monolayer was dramatically decreased by a single Phe→Ala mutation, whereas it was not significantly affected by the conservative Phe→Trp substitution [16]. Consistent with these experimental data, molecular docking studies indicated that the central aromatic residue of this CARC domain (Phe-452) is the most important energetic contributor of the complex.

A TMD has generally 22–26 amino acid residues [16]. Since CARC and CRAC motifs comprise between 5 and 13 amino acid residues, it is theoretically possible for a TMD to possess both motifs. An analysis of sequence databases has recently confirmed that such a "mirror" topology actually exists in various types of membrane proteins, including ion channels, neurotransmitter receptors, ABC transporters and GPCRs [16]. In all these cases, molecular dynamics simulations indicated that mirror TMDs could perfectly well accommodate two cholesterol molecules in a typical tail-to-tail orientation, one bound to CARC and the other to CRAC (Fig. 5). Future studies will be necessary to evaluate the functional impact of two symmetric cholesterol molecules on membrane proteins.

A common criticism of the definition of CRAC and CARC is that the consensus sequence defining the two motifs is too general to have any predictive value with respect to cholesterol binding [20]. Indeed, available crystal structures of membrane proteins complexed with cholesterol have made it possible to identify 3D pockets rather than linear binding sites [38]. Interestingly, the biochemical rules controlling cholesterol binding to these 3D sites are basically the same as those that apply for

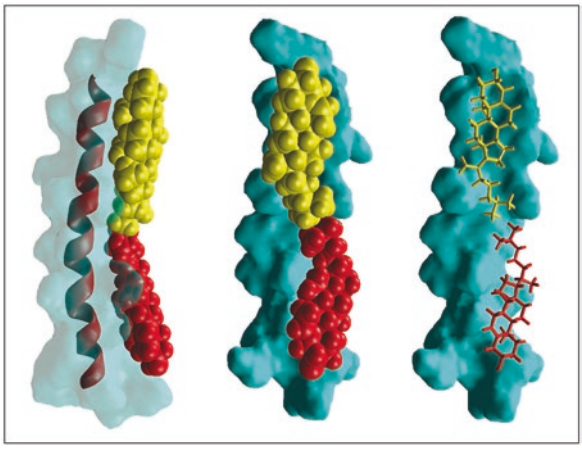

Fig. 5 Mirror topology of CARC/CRAC motifs within the same TMD. Three distinct views of the complex are shown. Cholesterol in yellow is bound to CARC, and cholesterol in red is bound to CRAC. The TMD shown is the seventh TMD of the human adenosine receptor A1