Cholesterol-recognition motifs in membrane proteins

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

Jacques Fantini : INSERM UMR_S 1072, 13015 Marseille, France; Aix-Marseille Université, 13015 Marseille, France (corresponding author : jm.fantini@gmail.com).

Richard M. Epand : Department of Biochemistry and Biomedical Sciences, McMaster University, Health Sciences Centre, Hamilton, Ontario L8S 4K1 Canada.

Francisco J. Barrantes : Laboratory of Molecular Neurobiology, Biomedical Research Institute (BIOMED), UCAï CONICET, Av. Alicia Moreau de Justo 1600, C1107AFF Buenos Aires, Argentina.

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

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 3ôepimer 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 ñraftòphases 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 ñhead-to-head/tail-to-tailò geometry to the CRAC/cholesterol complex (Figure 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 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 ñstacking CH-Pi interactionò[21]. Sometimes, the induced fit mechanism that directs the respective orientation of both rings results in a near perfect geometry, as shown in Figure 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 5 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 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)-$X_{1ï 5}$-(Y/F/W)-$X_{1ï 5}$-(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 (Figure 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 (Figure 4), in agreement with the early views stemming from electron 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 ɔ-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 ɔï 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 ñmirroròtopology 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 (Figure 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 cholesterol binding to CARC or CARC motifs. In particular, the involvement of an aromatic ring that stacks onto the sterane backbone of the sterol seems to be a hallmark of cholesterol-protein interactions in the membrane environment [13]. The particular topology of TMDs together with a universal mechanism of membrane cholesterol binding will probably render possible the prediction of potential cholesterol-binding motifs from sequence databases [16].

2.3. Sterol-sensing domains (SSD)

Unlike CRAC and CARC motifs, that comprise protein segments containing 13 amino acid residues or less, the sterol-sensing domain (SSD) is much larger. It contains approximately 180 residues organized as 5 consecutive transmembrane helices joined by short extramembranous loop regions. Interest in SSDs comes from the fact that they are found in several proteins involved in cholesterol transport, metabolism and storage [39, 40]. An SSD was originally identified in the enzyme 3-hydroxy-3-methylglutaryl coenzyme A-reductase (HMG-CoAR), the enzyme that catalyzes the rate determining step in cholesterol biosynthesis [41]. SSDs have also been found in SCAP (the sterol regulatory element-binding protein-cleavage activating protein). SCAP is an integral membrane protein found in the endoplasmic reticulum that plays a major role in regulating the transcription of genes involved in cholesterol biosynthesis [39, 41].

Other proteins in which SSDs have been identified and which have some relationship to cholesterol include 7-dehydrocholesterol reductase, an enzyme involved in cholesterol biosynthesis, and the Niemann-Pick C1 protein (NCP1), involved in intracellular lipid transport and lipid storage. The most prominent effect of defective NCP1 is the accumulation of unesterified cholesterol in an endosomal/lysosomal compartment. This accumulation occurs because the proteins NCP1 and NCP2 are required for transporting cholesterol out of lysosomes. NCP1 has also been shown to be required for the entry of Ebola virus to the cytoplasm [42, 43]. Other proteins with SSD include Patched (Ptc), a tumor suppressor involved in the signal transduction pathway for Hedgehog, a lipidated protein with covalently-linked cholesterol; Dispatched (DISP), a protein involved in the release of Hedgehog; and PTR, a protein related to Ptc whose function has still not been fully elucidated.

Because of the central role it plays in cholesterol homeostasis, SCAP is the most-studied SSD-containing protein from a mechanistic point of view. It has been shown that the activity of SCAP regulates cholesterol biosynthesis over a low and narrow range of cholesterol concentrations in the endoplasmic reticulum [44], a phenomenon that can be explained by the rapid rise in cholesterol activity over the narrow range of concentrations in which SCAP is activated [44]. Any explanation other than the direct modulation of SCAP activity through the binding of cholesterol to this protein would be hard to justify. However, little is known about the nature of this binding and which region of SCAP is involved in the interaction with cholesterol. There is one study determining the binding of cholesterol to isolated segments of SCAP, suggesting that the cholesterol binding site was in loop 1 [45]. This paper compared the binding of cholesterol and competition with other sterol analogs to loop 1 versus to the entire SSD domain. The study concluded that loop 1 contained the cholesterol binding site, though there are some caveats to this conclusion. In the first place it is not clear how well the protein fragment mimics the structure of this region in the intact protein. Secondly, the binding studies required the addition of a low concentration of detergent and furthermore the binding to loop 1 was done in an all-or-none manner. Given the highly sigmoidal dependence of cholesterol binding, it would be interesting to see a dose-response curve of cholesterol binding to loop 1. It is particularly difficult to determine the specific binding of a lipid, such as cholesterol, to a protein in an insoluble membrane fraction. Other evidence for the involvement of a particular

region of SCAP in binding comes from functional studies in which mutations were introduced in the extra-membranous loop 6 of SCAP containing the sequence MELADL. MELADL is required for the binding of SCAP to Sec23/Sec24. Sec23/Sec24 are proteins on the surface of CopII vesicles that escort SCAP from the endoplasmic reticulum to the Golgi, as the initial step in the pathway for the transcriptional regulation of cholesterol biosynthesis. The nature of the conformational change that results in the loss of exposure of MEDADL when cholesterol binds to SCAP has been recently evaluated by means of the susceptibility of SCAP to proteolytic cleavage [46]. These studies connect cholesterol binding to the functioning of SCAP through a conformational change in the latter that determines the exposure of the MEDADL segment. Cholesterol does appear to bind to loop 1 of SCAP, though additional studies are required to shed more light on this feature. In this context it is interesting to note that one of the juxta-membrane segments of loop 1 is the segment from residue 38 to 46 in human SCAP having the sequence LACCYPLLK. This sequence corresponds to a CRAC motif. However, the cited binding studies were done using the fragment of SCAP comprising residues 46-269 and hence not containing the putative CRAC sequence [45]. The contribution of this CRAC segment to the function of the SSD in SCAP remains to be determined.

There have also been photoaffinity labeling studies using photo-reactive derivatives of cholesterol, demonstrating the importance of the amino-terminal region of SCAP for both cholesterol binding and the functioning of SCAP. The cholesterol affinity probe reacts with a region of SCAP that includes the first transmembrane segment of SCAP [47]. A photoaffinity derivative of 25-hydroxycholesterol does not react with SCAP, showing some specificity for the process. It was demonstrated that the same photolabeling with cholesterol could be performed in whole cells and that reaction with the cholesterol affinity probe blocked the processing of SREBP [47].

Although the functional properties of the SSD-containing protein, SCAP, have been extensively investigated, the structure of an SSD domain is best known for other SSD-containing proteins. NPC1 was first purified and shown to bind to cholesterol and other sterols by Goldstein and his group [48]. The specificity of the cholesterol binding site and the region of the protein to which cholesterol binds was studied [49], leading to the conclusion that the loop 1 region is part of the binding site. Curiously, a Q79A mutation that abolishes the binding of [^{3}H]-

cholesterol and of [^{3}H]-25-hydroxycholesterol to full-length NPC1, was nevertheless able to restore cholesterol transport to NPC1-deficient Chinese hamster ovary cells. Thus, the sterol binding site on luminal loop-1 is not essential for NPC1 function in fibroblasts. It was suggested that this site might be required for cholesterol transport in other cells where NPC1 deficiency produces more complicated lipid abnormalities [49]. Recent X-ray crystallographic studies with NPC1 have yielded a structure at 3.3 Å [50]. In order to obtain a high resolution structure, the full length protein had to be cleaved with a protease that removed a fragment of 313 residues from the amino terminus [51]. This fragment was attached to the remainder of the protein by a polyproline flexible arm [52]that precluded crystallization. The position of the missing N-terminal segment in this crystal structure was shown by cryo-electron microscopy to lie on top of the remainder of the protein in only 45% of the particles, suggesting its flexible linkage to the remainder of the protein [53]. In any case it should be noted that the SSD is not a surface binding site for cholesterol but rather an internal cavity that completely wraps cholesterol (Figure 6).

Some of the proteins with SSD also contain the short sequence YIYF. It has been shown that SCAP [47]and HMG-CoAR [54]require this tetrapeptide fragment to bind to Insig, an anchoring protein of the endoplasmic reticulum. Interestingly, this sequence, YIYF, is also present in other proteins having some interaction with cholesterol [30] ; some of these proteins contain CRAC and/or SSD domains but others do not. The direct role of YIYF in cholesterol binding remains to be fully established.

2.4. Amphipathic helix

There is a recent example of a short peptide segment that is part of an amphipathic helix that controls the cholesterol-mediated turnover of squalene monooxygenase, a rate-limiting enzyme in cholesterol synthesis [55]. Evidence was presented that a 12-residue segment forming part of an amphipathic helix of squalene monooxygenase conveyed cholesterol sensitivity to the binding of the protein to membranes. Although the specific amino acid sequence of this 12-residue fragment may not be required for this cholesterol-dependent function, the general model may have wider applicability. It is known that amphipathic helices have affinity for membranes, but as monomers, they do not insert very deeply into membranes. It is also known that

cholesterol promotes tighter packing of membranes. Hence, it is reasonable to suggest that an amphipathic helix that is weakly bound to a membrane may dissociate from the membrane at higher cholesterol concentrations, as was shown in this case [55]. We thus anticipate that other cholesterol-mediated functions will be discovered in the future that depend on the dissociation of amphipathic helices from membranes in the presence of increased cholesterol concentrations.

3. Role of cholesterol-recognition motifs in binding to GPCR

The possibility that cholesterol could modulate the function of GPCRs has been investigated by numerous authors. In all cases, early studies were faced with the dilemma of being able to decipher what was due to general biophysical effects on the membrane as opposed to specific biochemical effects on receptors. The first account of a direct interaction between cholesterol and a GPCR came from a study on rhodopsin [56]. In these experiments cholestatrienol (a fluorescent sterol) was used to probe interactions between cholesterol and rhodopsin in disk membranes. These interactions were detected by fluorescence energy transfer from protein tryptophan residues to cholestatrienol. The specificity of this interaction was explored by the addition of cholesterol, which inhibited the quenching of fluorescence emission from tryptophan residues of the protein, or ergosterol, which did not. Taken together, these data suggested the existence of a specific cholesterol binding site on rhodopsin [56]. In parallel, other studies were focused on the effect of cholesterol on GPCR function. A pioneering study described the modulatory effect of cholesterol on two GPCRs, the oxytocin receptor and the brain cholecystokinin receptor [57]. Once again, the specificity of cholesterol effects was assessed by comparing its activity with sterol analogues. A major outcome of this study was the demonstration that cholesterol could affect ligand binding to these receptors and subsequent signal transduction [57].

Another way to assess the specificity of cholesterol effects on GPCRs is the use of cholesterol oxidase on native membranes [58]. This enzyme catalyzes the conversion of membrane cholesterol to cholestenone. It turned out that this treatment inhibited the specific binding of agonist and antagonist ligands to the serotonin 5-HT(1A) receptor. Since membrane order was not affected by the enzymatic oxidation of cholesterol to cholestenone, these data

suggested that cholesterol could modulate ligand binding to this GPCR through a specific interaction. The definitive evidence for the existence of cholesterol binding sites on GPCRs came from structural data. For a long time, structural studies of GPCRs have been hampered by the lack of reliable crystallization procedures for integral membrane proteins. The advent of the *in meso* technology (also referred to as the lipid cubic phase) has filled this gap, allowing the production of hundreds of X-ray structures of membrane proteins, with GPCRs representing the highest proportion [59]. Interestingly, the addition of cholesterol in a monoacylglycerol matrix has proved to be critical to the production of structure-grade crystals of most membrane proteins, especially GPCRs [60]. As a consequence, GPCRs are often co-crystallized with cholesterol. Although these data confirmed that GPCRs can bind cholesterol, it has not been possible to determine a unique, consensus profile for the cholesterol binding sites observed in these structures. A canonical motif referred to as CCM was detected as a specific cholesterol binding site in the ß2 adrenergic receptor, but not in other GPCRs sharing the same motif [15, 61]. Moreover, two vicinal cholesterol molecules are bound to this receptor, as shown in Figure 7. Nevertheless, several common features emerged from these structural studies. Consistent with the rules derived from the CRAC/CARC algorithms, branched amino acid residues (Val, Leu, but also Ile) were often involved in cholesterol binding. Stacking interactions mediated by an aromatic residue, including Trp [62], were also frequent. The polar OH group of cholesterol was localized near the water-membrane interface with potential hydrogen bonding to Lys, Arg, but also Asp residues. In fact, it is quite easy to explain the molecular mechanisms of cholesterol-GPCR interactions in the crystal structures obtained by the *in meso* method, but more of these X-ray structures are required before a reliable prediction method for cholesterol-binding sites can be proposed. Meanwhile, identification of CRAC/CARC motifs still represents a valuable strategy to categorize potential points of contact between GPCR TMDs and cholesterol [16].

From a functional point of view, it has been proposed that cholesterol-receptor interactions can exert two complementary effects: i) increasing the compactness of the receptor structure, and ii) improving the conformational stability towards active/ inactive receptor states [63]. These specific modulations of receptor structure and functions are mediated by non-annular sites which, in contrast with annular sites, bind cholesterol both specifically and with high affinity [64].

Experimental data in favor of the co-existence of different types of cholesterol interactions with GPCRs has been recently obtained by means of a nuclear magnetic resonance study of the β2 adrenergic receptor[8]. The authors of this study suggested that a cluster of cholesterol molecules could self-organize around the receptor, certain molecules (non-annular) being in slow exchange and others (annular) in fast exchange, with the former contributing to the specific binding of the latter. In this case, both cholesterol pools could co-operate to facilitate the recruitment of the β2 adrenergic receptor into cholesterol-rich domains and control its oligomerization state [8].

4. Role of cholesterol-recognition motifs in ion channels

Oligomerization is also central to ion channel activity since these membrane proteins consist of individual subunits that are nonfunctional by themselves[65]. Transient receptor potential (TRP) channels, including vanilloid (TRVP), canonical (TRPC), and melastin (TRPM) TRP channels are localized in lipid rafts and are highly sensitive to cholesterol, which controls both their assembly and activity[66]. A thorough study of the effects of cholesterol on ion channel activity has been performed on the inwardly rectifying K^+ channels (Kir) [14, 67, 68]. In these experiments, two stereochemical variants of cholesterol, i.e. ent-cholesterol (the cholesterol enantiomer), and epi-cholesterol (which has the distinct orientation of the OH group) were tested and compared with natural cholesterol in functional studies of ion channel activity. Surprisingly, both cholesterol and its chiral isomer were found to bind to the same site through a non-stereospecific mechanism [68]. However, only natural cholesterol could modulate ion channel activity, indicating that sterol binding alone is not sufficient to regulate the channel. From a molecular point of view, the structural determinant of the cholesterol-binding domains displayed by Kir channels, i.e. a hydrophobic pocket [14] , is consistent with non-stereoselective binding of sterols through poorly discriminant van der Waals interactions. In another study performed on the nicotinic acetylcholine receptor, epicholesterol was able to substitute for cholesterol in terms of its functional effect [69].

On the basis of all these data, it is still difficult to specify exactly how cholesterol binding to ion channels controls subunit assembly and/or channel opening probability [70]. Recent studies of amyloid pore formation in the plasma membrane of brain cells have given some clues on the

molecular mechanisms controlling the assembly of oligomeric Ca^{2+} channels [71, 72]. Amyloid proteins are generally assumed to self-aggregate into fibers that form large plaques in the brain of patients with neurodegenerative disorders such as Alzheimer, Parkinson, or Creutzfeldt-Jakob diseases [73, 74]. However, healthy individuals may also display significant amounts of amyloid plaques in their brain, so that there is no clear-cut correlation between these deposits and neurological symptoms [75]. In fact, amyloid proteins also form a variety of small neurotoxic oligomers, including amyloid pores which are a particular class of Zn^{2+}-sensitive Ca^{2+} channels [76]. These oligomers are considered to be the most toxic species of amyloid proteins and there is growing evidence that they are closely associated with the pathogenesis of neurodegenerative diseases [77]. The oligomerization process that leads to the formation of amyloid pores is a universal two-step mechanism involving successively a ganglioside and cholesterol [78]. The ganglioside ensures the initial adhesion of the amyloid protein to a lipid raft domain [79]. The insertion of the protein within the plasma membrane is then dependent upon cholesterol which interacts with a specific cholesterol-binding domain displayed by the amyloid protein [80]. The cholesterol binding site of amyloid proteins is linear but is not necessarily a CRAC or a CARC domain. The most important feature of this particular class of cholesterol binding domains is that once inserted in the plasma membrane, they adopt a tilted orientation with respect to the main axis of cholesterol [80], just as viral fusion peptides do [81]. In the case of Alzheimerûs β-amyloid peptide, this particular geometry facilitates the oligomerization process which depends on the strict alignment of Lys and Asn residues belonging to vicinal peptide monomers [71, 72]. The assembly of the oligomeric pore is driven by the formation of a hydrogen bond between those Lys and Asn residues [71, 72]. The implication of cholesterol in this process is confirmed by the lack of formation of amyloid pores in cholesterol-depleted cells [78]. Whether cholesterol could play a similar role in larger ion channels remains to be established.

5. Role of cholesterol-recognition motifs in cholesterol trafficking

It was shown earlier on that the enzyme that catalyzed the rate determining step in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A-reductase (HMG-CoAR), contained an SSD. This enzyme is the target for statin drugs. In addition, SCAP (the sterol regulatory

element-binding protein-cleavage activating protein) is a protein of the endoplasmic reticulum that controls the transcription of genes involved in cholesterol biosynthesis, through a feedback mechanism involving the binding of cholesterol to an SSD of SCAP. In addition to controlling the biosynthesis of cholesterol, the intracellular cholesterol trafficking proteins, NCP1 and NCP2, also have SSD domains. In addition to these mechanisms, the transport of cholesterol across the plasma membrane of a cell is another function modulated by the level of cholesterol through the binding to specific sites on these transport proteins.

ATP binding cassette (ABC) transporters are a large family of integral membrane protein transporters with homologous structures comprising 6 or 12 transmembrane helices and one or two ATP binding sites. These transporters are subdivided into 7 subfamilies based on their structural similarities [82]. One of these subfamilies is the ABCG group. Particular attention has been given to ABCG1, which appears to play a prominent role in the export of cholesterol from cells to HDL and is therefore important in ñreverse cholesterol transportò, i.e. the movement of cholesterol from peripheral tissues to the liver. Other members of the ABCG subfamily can also transport cholesterol but may operate by an alternative mechanism, since the other lipids they transport are different from ABCG1 [26]. It has been demonstrated that the final transmembrane segment is important for cholesterol transport [26]. This segment contains several CRAC and CARC segments. In particular, mutational analysis has shown that mutation of the CRAC segment containing Y667 results in loss of cholesterol transport to HDL and loss of stability of the protein in the presence of cholesterol [26]. Another ABC transporter that transports cholesterol is ABCA1. However, this transporter does not have any CRAC segments. It is possible that the specificity of transport of ABCA1 comes about because of its specific binding to HDL [83], which is not required for ABCG1 [26]. It should also be kept in mind that the conformation and activity of ABC cassette proteins are influenced by the surrounding lipid [84], so that cholesterol may modulate the activity of these proteins without directly binding to them.

There has recently been a report suggesting a cholesterol transport role for a family of mammalian proteins that are homologous to the ChUP proteins of *C. elegans* [85]. Evidence is presented that these mammalian SIDT proteins transport cholesterol. Furthermore, they have a CRAC domain, which when mutated prevents FRET between these proteins and the fluorescent

cholesterol analog, dehydroergosterol [85]. Further studies are required to verify whether these proteins are cholesterol transporters in mammals.

6. Role of cholesterol in membrane fusion

Membrane fusion is an important function in many biological systems. Processes such as the exocytosis of endocytic vesicles, sperm-egg fertilization, cell-cell fusion in bone and heart, infection by enveloped viruses and others, all involve the merging of one membrane with another, promoted by specific proteins, among which lipid plays an important role [86]. There are likely to be some common elements among the various types of membrane fusion in terms of how they are modulated by the lipid environment, including the presence of cholesterol.

There are several mechanisms by which cholesterol may affect the rate of fusion. Cholesterol may be required to bind to a fusion protein to stimulate its fusion activity, it may recruit protein components to the site of fusion so that these proteins are more concentrated in a specific domain of a membrane, cholesterol may modify the biophysical properties of the membrane to favor membrane fusion and/or to stabilize regions of high curvature in fusion intermediates. These putative roles of cholesterol are not mutually exclusive, and a specific membrane fusion process may involve more than one of these properties.

SNARE proteins are required for exocytosis, facilitating the fusion between endocytic vesicles and the plasma membrane. Many of the SNARE proteins required for exocytosis contain CRAC or CARC segments [87]. In addition, it is known that cholesterol is required for exocytosis in neurons [88, 89], endocrine [90], neuroendocrine cells [91, 92] as well as cortical vesicles from sea urchins [93, 94]. However, the role of cholesterol in SNARE-dependent exocytosis does not appear to involve the binding of cholesterol to the SNARE protein, but rather is dependent on the changes cholesterol makes in the physical properties of the membrane and its domain organization [3]. Nevertheless, CARC and CRAC motifs are found in some, although not all, SNARE proteins [87], though there is no evidence that when these domains are present they bind cholesterol or facilitate membrane fusion.

Cholesterol also plays an important role in the fusion of enveloped viruses to cell membranes, with Cholesterol-rich domains often serving as the site for such fusion [95], possibly at the

interface between the cholesterol-rich domain and the remainder of the membrane [96], as well as for viral assembly and budding [97, 98].

In addition, some viral fusion proteins contain segments that may interact directly with cholesterol. This includes the membrane proximal region of the GP2 protein of Ebola virus that contains the sequence GXXGXXXA, suggested to interact with cholesterol [99]. The sequence GXXXG is often associated with protein dimerization, but this and similar sequences have been shown to also interact with cholesterol in the amyloid precursor protein [100]. This aspect has not been included in the present review among the sequences associated with binding cholesterol owing to the lack of sufficient examples.

One of the most studied CRAC domains associated with viral fusion is the LWYIK segment found in the membrane proximal domain of the HIV fusion protein gp41 [101]. We have shown that the N-acetyl-LWYIK-amide is able to recruit cholesterol into domains in model membranes, resulting in the deeper penetration of the peptide into the membrane [102]. However, in the gp41 fusion proteins of HIV-2 and SIV, in the location of the LWYIK sequence, one finds the modified sequence LASWIK. This is not a CRAC domain, yet these viruses are still active and can undergo membrane fusion [103]. The peptide N-acetyl-LASWIK-amide has less potency than N-acetyl-LWYIK-amide in forming areas enriched in cholesterol. We suggest that the difference between HIV-1 and HIV-2 glycosphingolipid requirements for determining their tropism is related to the difference in their partitioning to cholesterol-rich domains in biological membranes [103]. We tested the stereochemistry of the induction of cholesterol-rich domains by LWYIK and found that substituting cholesterol with its enantiomer, ent-cholesterol, prevented the LWYIK peptide from sequestering cholesterol. However, the enantiomer of N-acetyl-LWYIK-amide, i.e. the peptide with all D-amino acids, was able to segregate cholesterol, indicating that peptide chirality is not required for interaction with cholesterol-containing membranes. However, a specific chirality of membrane lipids is required for peptide-induced formation of cholesterol-rich domains [6]. Computer modeling studies suggested the nature of the non-covalent interactions between cholesterol and the LWYIK peptide. The modeling studies and fluorescence experiments were supported by single residue mutations in the gp41 protein of HIV-1, in which L 679 is replaced with I. Despite the similarity of the properties of L and I, this single substitution resulted in a marked attenuation of the ability of JC53-BL HeLa-based HIV-1

indicator cells to form syncytia [31], again suggesting a requirement for a CRAC motif. Mutational studies combined with *in silico* predictions and model system studies of cholesterol clustering, supported a specific model for the interaction of LWYIK with cholesterol [104, 105]. X-ray scattering studies were carried out to compare the effects of LWYIK and IWYIK on bilayer thickness. With 50% cholesterol, IWYIK was found to decrease the bilayer repeat distance, while LWYIK increased it [106]. There is evidence that longer peptides containing LWYIK may act as inhibitors of HIV fusion activity [107]. It was found that deletion of LWYIK from the gp41 fusion protein resulted in a fusion inactive virus [108] ; however, this study provided evidence that this segment was needed for the enlargement of fusion pores and for post-fusion activity, rather than for interaction with cholesterol and rafts.

7. Conclusions

In this review we examine the complex structural requirements that define cholesterol-recognition motifs in membrane proteins. The initial overview section introduces the reader to the subjects of affinity, specificity and stereoselectivity of the interactions of the lipid with membrane proteins, and the implications of these properties on binding to transmembrane proteins. The next sections provide a detailed dissection of the molecular aspects currently used to identify cholesterol recognition sites in membrane proteins: CRAC, CARC, SSD and amphipathic helix motifs.

The functional implications of cholesterol-recognition motifs are covered next, using two important and paradigmatic superfamilies of membrane proteins: the G-protein coupled receptors (GPCR) and ion channels, which together represent the largest collection of membrane proteins having key roles in signal recognition and signal transduction. The possible involvement of cholesterol dysfunctional conditions in neurological disorders such as Alzheimer, Parkinson or Creutzfeldt-Jakobs diseases is also discussed. This is followed by the analysis of cholesterol recognition motifs in cholesterol trafficking from the plasmalemma to intracellular compartments and the discussion of cholesterol-recognition motifs in membrane fusion, including that of virus with eukaryotic cells, HIV fusion proteins and synaptic SNARE proteins. Without attempting to provide a comprehensive coverage of cholesterol interactions with membrane proteins, the review provides a state-of-the-art overview of the key molecular aspects

of the molecular partners, i.e. cholesterol and amino acid motifs in membrane-embedded regions of membrane proteins that define the physiologically relevant crosstalk between the two. This is an ongoing and continually evolving process that in future years may lead to additional novel cholesterol binding motifs that will bind cholesterol and affect protein function.

Reference list

1. Kimura T, Jennings W, Epand RM: **Roles of specific lipid species in the cell and their molecular mechanism.** *Prog Lipid Res* 2016, **62:**75-92.
2. Fantini J, Garmy N, Mahfoud R, Yahi N: **Lipid rafts: structure, function and role in HIV, Alzheimer's and prion diseases.** *Expert Rev Mol Med* 2002, **4:**1-22.
3. Yang ST, Kreutzberger AJB, Lee J, Kiessling V, Tamm LK: **The role of cholesterol in membrane fusion.** *Chem Phys Lipids* 2016, **199:**136-143.
4. Howe V, Sharpe LJ, Alexopoulos SJ, Kunze SV, Chua NK, Li D, Brown AJ: **Cholesterol homeostasis: How do cells sense sterol excess?** *Chem Phys Lipids* 2016, **199:**170-178.
5. Jafurulla M, Chattopadhyay A: **Structural Stringency of Cholesterol for Membrane Protein Function Utilizing Stereoisomers as Novel Tools: A Review.** In *Cholesterol Homeostasis: Methods and Protocols.* Edited by Gelissen IC, Brown AJ. New York, NY: Springer New York; 2017: 21-39
6. Epand RM, Rychnovsky SD, Belani JD, Epand RF: **Role of chirality in peptide-induced formation of cholesterol-rich domains.** *Biochem J* 2005, **390:**541-548.
7. Sodt AJ, Sandar ML, Gawrisch K, Pastor RW, Lyman E: **The molecular structure of the liquid-ordered phase of lipid bilayers.** *J Am Chem Soc* 2014, **136:**725-732.
8. Gater DL, Saurel O, Iordanov I, Liu W, Cherezov V, Milon A: **Two classes of cholesterol binding sites for the beta2AR revealed by thermostability and NMR.** *Biophys J* 2014, **107:**2305-2312.
9. Rouviere E, Arnarez C, Yang L, Lyman E: **Identification of Two New Cholesterol Interaction Sites on the A2A Adenosine Receptor.** *Biophys J* 2017, **113:**2415-2424.
10. Byrne EFX, Sircar R, Miller PS, Hedger G, Luchetti G, Nachtergaele S, Tully MD, Mydock-McGrane L, Covey DF, Rambo RP, et al: **Structural basis of Smoothened regulation by its extracellular domains.** *Nature* 2016, **535:**517-522.
11. Posada IM, Fantini J, Contreras FX, Barrantes F, Alonso A, Goni FM: **A cholesterol recognition motif in human phospholipid scramblase 1.** *Biophys J* 2014, **107:**1383-1392.
12. Fantini J, Di Scala C, Baier CJ, Barrantes FJ: **Molecular mechanisms of protein-cholesterol interactions in plasma membranes: Functional distinction between topological (tilted) and consensus (CARC/CRAC) domains.** *Chem Phys Lipids* 2016, **199:**52-60.
13. Fantini J, Barrantes FJ: **How cholesterol interacts with membrane proteins: an exploration of cholesterol-binding sites including CRAC, CARC, and tilted domains.** *Front Physiol* 2013, **4:**31.
14. Rosenhouse-Dantsker A, Noskov S, Durdagi S, Logothetis DE, Levitan I: **Identification of novel cholesterol-binding regions in Kir2 channels.** *J Biol Chem* 2013, **288:**31154-31164.
15. Hanson MA, Cherezov V, Griffith MT, Roth CB, Jaakola VP, Chien EY, Velasquez J, Kuhn P, Stevens RC: **A specific cholesterol binding site is established by the 2.8 A structure of the human beta2-adrenergic receptor.** *Structure* 2008, **16:**897-905.
16. Fantini J, Di Scala C, Evans LS, Williamson PT, Barrantes FJ: **A mirror code for protein-cholesterol interactions in the two leaflets of biological membranes.** *Sci Rep* 2016, **6:**21907.
17. Jaremko L, Jaremko M, Giller K, Becker S, Zweckstetter M: **Structure of the mitochondrial translocator protein in complex with a diagnostic ligand.** *Science* 2014, **343:**1363-1366.

18. Li H, Papadopoulos V: **Peripheral-type benzodiazepine receptor function in cholesterol transport. Identification of a putative cholesterol recognition/interaction amino acid sequence and consensus pattern.** *Endocrinology* 1998, **139:**4991-4997.
19. Baier CJ, Fantini J, Barrantes FJ: **Disclosure of cholesterol recognition motifs in transmembrane domains of the human nicotinic acetylcholine receptor.** *Sci Rep* 2011, **1:**69.
20. Palmer M: **Cholesterol and the activity of bacterial toxins.** *FEMS Microbiol Lett* 2004, **238:**281-289.
21. Nishio M, Umezawa Y, Fantini J, Weiss MS, Chakrabarti P: **CH-pi hydrogen bonds in biological macromolecules.** *Phys Chem Chem Phys* 2014, **16:**12648-12683.
22. Lee AG: **Lipid-protein interactions in biological membranes: a structural perspective.** *Biochim Biophys Acta* 2003, **1612:**1-40.
23. Fantini J, Yahi N: *Brain Lipids in Synaptic Function and Neurological Disease. Clues to Innovative Therapeutic Strategies for Brain Disorders.* San Francisco: Elsevier; 2015.
24. Fantini J, Barrantes FJ: **How membrane lipids control the 3D structure and function of receptors.** *AIMS Biophysics* 2018, **5:**22-35.
25. Pydi SP, Jafurulla M, Wai L, Bhullar RP, Chelikani P, Chattopadhyay A: **Cholesterol modulates bitter taste receptor function.** *Biochim Biophys Acta* 2016, **1858:**2081-2087.
26. Sharpe LJ, Rao G, Jones PM, Glancey E, Aleidi SM, George AM, Brown AJ, Gelissen IC: **Cholesterol sensing by the ABCG1 lipid transporter: Requirement of a CRAC motif in the final transmembrane domain.** *Biochim Biophys Acta* 2015, **1851:**956-964.
27. Robinson LE, Shridar M, Smith P, Murrell-Lagnado RD: **Plasma membrane cholesterol as a regulator of human and rodent P2X7 receptor activation and sensitization.** *J Biol Chem* 2014, **289:**31983-31994.
28. Singh AK, McMillan J, Bukiya AN, Burton B, Parrill AL, Dopico AM: **Multiple cholesterol recognition/interaction amino acid consensus (CRAC) motifs in cytosolic C tail of Slo1 subunit determine cholesterol sensitivity of Ca2+- and voltage-gated K+ (BK) channels.** *J Biol Chem* 2012, **287:**20509-20521.
29. Jamin N, Neumann JM, Ostuni MA, Vu TK, Yao ZX, Murail S, Robert JC, Giatzakis C, Papadopoulos V, Lacapere JJ: **Characterization of the cholesterol recognition amino acid consensus sequence of the peripheral-type benzodiazepine receptor.** *Mol Endocrinol* 2005, **19:**588-594.
30. Epand RM: **Cholesterol and the interaction of proteins with membrane domains.** *Prog Lipid Res* 2006, **45:**279-294.
31. Epand RF, Thomas A, Brasseur R, Vishwanathan SA, Hunter E, Epand RM: **Juxtamembrane protein segments that contribute to recruitment of cholesterol into domains.** *Biochemistry* 2006, **45:**6105-6114.
32. Ulmschneider MB, Sansom MS: **Amino acid distributions in integral membrane protein structures.** *Biochim Biophys Acta* 2001, **1512:**1-14.
33. Ferraro M, Masetti M, Recanatini M, Cavalli A, Bottegoni G: **Mapping Cholesterol Interaction Sites on Serotonin Transporter through Coarse-Grained Molecular Dynamics.** *PLoS One* 2016, **11:**e0166196.
34. Morrill GA, Kostellow AB, Gupta RK: **The role of receptor topology in the vitamin D3 uptake and Ca(2+) response systems.** *Biochem Biophys Res Commun* 2016, **477:**834-840.
35. Morrill GA, Kostellow AB, Gupta RK: **Computational analysis of the extracellular domain of the Ca(2)(+)-sensing receptor: an alternate model for the Ca(2)(+) sensing region.** *Biochem Biophys Res Commun* 2015, **459:**36-41.
36. Marsh D, Barrantes FJ: **Immobilized lipid in acetylcholine receptor-rich membranes from Torpedo marmorata.** *Proc Natl Acad Sci U S A* 1978, **75:**4329-4333.
37. Barrantes FJ: **Structural basis for lipid modulation of nicotinic acetylcholine receptor function.** *Brain Res Brain Res Rev* 2004, **47:**71-95.
38. Song Y, Kenworthy AK, Sanders CR: **Cholesterol as a co-solvent and a ligand for membrane proteins.** *Protein Sci* 2014, **23:**1-22.

39. Goldstein JL, DeBose-Boyd RA, Brown MS: **Protein sensors for membrane sterols.** *Cell* 2006, **124:**35-46.
40. Kuwabara PE, Labouesse M: **The sterol-sensing domain: multiple families, a unique role?** *Trends Genet* 2002, **18:**193-201.
41. Goldstein JL, Brown MS: **Regulation of the mevalonate pathway.** *Nature* 1990, **343:**425-430.
42. Carette JE, Raaben M, Wong AC, Herbert AS, Obernosterer G, Mulherkar N, Kuehne AI, Kranzusch PJ, Griffin AM, Ruthel G, et al: **Ebola virus entry requires the cholesterol transporter Niemann-Pick C1.** *Nature* 2011, **477:**340-343.
43. Cote M, Misasi J, Ren T, Bruchez A, Lee K, Filone CM, Hensley L, Li Q, Ory D, Chandran K, Cunningham J: **Small molecule inhibitors reveal Niemann-Pick C1 is essential for Ebola virus infection.** *Nature* 2011, **477:**344-348.
44. Gay A, Rye D, Radhakrishnan A: **Switch-like responses of two cholesterol sensors do not require protein oligomerization in membranes.** *Biophys J* 2015, **108:**1459-1469.
45. Motamed M, Zhang Y, Wang ML, Seemann J, Kwon HJ, Goldstein JL, Brown MS: **Identification of luminal Loop 1 of Scap protein as the sterol sensor that maintains cholesterol homeostasis.** *J Biol Chem* 2011, **286:**18002-18012.
46. Gao Y, Zhou Y, Goldstein JL, Brown MS, Radhakrishnan A: **Cholesterol-induced conformational changes in the sterol-sensing domain of the Scap protein suggest feedback mechanism to control cholesterol synthesis.** *J Biol Chem* 2017, **292:**8729-8737.
47. Adams CM, Reitz J, De Brabander JK, Feramisco JD, Li L, Brown MS, Goldstein JL: **Cholesterol and 25-hydroxycholesterol inhibit activation of SREBPs by different mechanisms, both involving SCAP and Insigs.** *J Biol Chem* 2004, **279:**52772-52780.
48. Infante RE, Abi-Mosleh L, Radhakrishnan A, Dale JD, Brown MS, Goldstein JL: **Purified NPC1 protein. I. Binding of cholesterol and oxysterols to a 1278-amino acid membrane protein.** *J Biol Chem* 2008, **283:**1052-1063.
49. Infante RE, Radhakrishnan A, Abi-Mosleh L, Kinch LN, Wang ML, Grishin NV, Goldstein JL, Brown MS: **Purified NPC1 protein: II. Localization of sterol binding to a 240-amino acid soluble luminal loop.** *J Biol Chem* 2008, **283:**1064-1075.
50. Li X, Lu F, Trinh MN, Schmiege P, Seemann J, Wang J, Blobel G: **3.3 A structure of Niemann-Pick C1 protein reveals insights into the function of the C-terminal luminal domain in cholesterol transport.** *Proc Natl Acad Sci U S A* 2017, **114:**9116-9121.
51. Li X, Wang J, Coutavas E, Shi H, Hao Q, Blobel G: **Structure of human Niemann-Pick C1 protein.** *Proc Natl Acad Sci U S A* 2016, **113:**8212-8217.
52. Kwon HJ, Abi-Mosleh L, Wang ML, Deisenhofer J, Goldstein JL, Brown MS, Infante RE: **Structure of N-terminal domain of NPC1 reveals distinct subdomains for binding and transfer of cholesterol.** *Cell* 2009, **137:**1213-1224.
53. Gong X, Qian H, Zhou X, Wu J, Wan T, Cao P, Huang W, Zhao X, Wang X, Wang P, et al: **Structural Insights into the Niemann-Pick C1 (NPC1)-Mediated Cholesterol Transfer and Ebola Infection.** *Cell* 2016, **165:**1467-1478.
54. Sever N, Song BL, Yabe D, Goldstein JL, Brown MS, DeBose-Boyd RA: **Insig-dependent ubiquitination and degradation of mammalian 3-hydroxy-3-methylglutaryl-CoA reductase stimulated by sterols and geranylgeraniol.** *J Biol Chem* 2003, **278:**52479-52490.
55. Chua NK, Howe V, Jatana N, Thukral L, Brown AJ: **A conserved degron containing an amphipathic helix regulates the cholesterol-mediated turnover of human squalene monooxygenase, a rate-limiting enzyme in cholesterol synthesis.** *J Biol Chem* 2017, **292:**19959-19973.
56. Albert AD, Young JE, Yeagle PL: **Rhodopsin-cholesterol interactions in bovine rod outer segment disk membranes.** *Biochim Biophys Acta* 1996, **1285:**47-55.
57. Gimpl G, Burger K, Fahrenholz F: **Cholesterol as modulator of receptor function.** *Biochemistry* 1997, **36:**10959-10974.

58. Pucadyil TJ, Shrivastava S, Chattopadhyay A: **Membrane cholesterol oxidation inhibits ligand binding function of hippocampal serotonin(1A) receptors.** *Biochem Biophys Res Commun* 2005, **331:**422-427.
59. Caffrey M: **A comprehensive review of the lipid cubic phase or in meso method for crystallizing membrane and soluble proteins and complexes.** *Acta Crystallogr F Struct Biol Commun* 2015, **71:**3-18.
60. Caffrey M, Li D, Dukkipati A: **Membrane protein structure determination using crystallography and lipidic mesophases: recent advances and successes.** *Biochemistry* 2012, **51:**6266-6288.
61. Paila YD, Chattopadhyay A: **Membrane cholesterol in the function and organization of G-protein coupled receptors.** *Subcell Biochem* 2010, **51:**439-466.
62. Hua T, Vemuri K, Nikas SP, Laprairie RB, Wu Y, Qu L, Pu M, Korde A, Jiang S, Ho JH, et al: **Crystal structures of agonist-bound human cannabinoid receptor CB1.** *Nature* 2017, **547:**468-471.
63. Gimpl G: **Interaction of G protein coupled receptors and cholesterol.** *Chem Phys Lipids* 2016, **199:**61-73.
64. Paila YD, Tiwari S, Chattopadhyay A: **Are specific nonannular cholesterol binding sites present in G-protein coupled receptors?** *Biochim Biophys Acta* 2009, **1788:**295-302.
65. Clarke OB, Gulbis JM: **Oligomerization at the membrane: potassium channel structure and function.** *Adv Exp Med Biol* 2012, **747:**122-136.
66. Levitan I, Fang Y, Rosenhouse-Dantsker A, Romanenko V: **Cholesterol and ion channels.** *Subcell Biochem* 2010, **51:**509-549.
67. Rosenhouse-Dantsker A, Noskov S, Logothetis DE, Levitan I: **Cholesterol sensitivity of KIR2.1 depends on functional inter-links between the N and C termini.** *Channels (Austin)* 2013, **7:**303-312.
68. Barbera N, Ayee MAA, Akpa BS, Levitan I: **Differential Effects of Sterols on Ion Channels: Stereospecific Binding vs Stereospecific Response.** *Curr Top Membr* 2017, **80:**25-50.
69. Addona GH, Sandermann H, Jr., Kloczewiak MA, Husain SS, Miller KW: **Where does cholesterol act during activation of the nicotinic acetylcholine receptor?** *Biochim Biophys Acta* 1998, **1370:**299-309.
70. Bukiya AN, Osborn CV, Kuntamallappanavar G, Toth PT, Baki L, Kowalsky G, Oh MJ, Dopico AM, Levitan I, Rosenhouse-Dantsker A: **Cholesterol increases the open probability of cardiac KACh currents.** *Biochim Biophys Acta* 2015, **1848:**2406-2413.
71. Di Scala C, Chahinian H, Yahi N, Garmy N, Fantini J: **Interaction of Alzheimer's beta-amyloid peptides with cholesterol: mechanistic insights into amyloid pore formation.** *Biochemistry* 2014, **53:**4489-4502.
72. Di Scala C, Troadec JD, Lelievre C, Garmy N, Fantini J, Chahinian H: **Mechanism of cholesterol-assisted oligomeric channel formation by a short Alzheimer beta-amyloid peptide.** *J Neurochem* 2014, **128:**186-195.
73. Irvine GB, El-Agnaf OM, Shankar GM, Walsh DM: **Protein aggregation in the brain: the molecular basis for Alzheimer's and Parkinson's diseases.** *Mol Med* 2008, **14:**451-464.
74. Harrison RS, Sharpe PC, Singh Y, Fairlie DP: **Amyloid peptides and proteins in review.** *Rev Physiol Biochem Pharmacol* 2007, **159:**1-77.
75. Esparza TJ, Zhao H, Cirrito JR, Cairns NJ, Bateman RJ, Holtzman DM, Brody DL: **Amyloid-beta oligomerization in Alzheimer dementia versus high-pathology controls.** *Ann Neurol* 2013, **73:**104-119.
76. Quist A, Doudevski I, Lin H, Azimova R, Ng D, Frangione B, Kagan B, Ghiso J, Lal R: **Amyloid ion channels: a common structural link for protein-misfolding disease.** *Proc Natl Acad Sci U S A* 2005, **102:**10427-10432.
77. Jang H, Connelly L, Arce FT, Ramachandran S, Lal R, Kagan BL, Nussinov R: **Alzheimer's disease: which type of amyloid-preventing drug agents to employ?** *Phys Chem Chem Phys* 2013, **15:**8868-8877.

78. Di Scala C, Yahi N, Boutemeur S, Flores A, Rodriguez L, Chahinian H, Fantini J: **Common molecular mechanism of amyloid pore formation by Alzheimer's beta-amyloid peptide and alpha-synuclein.** *Sci Rep* 2016, **6:**28781.
79. Yahi N, Fantini J: **Deciphering the glycolipid code of Alzheimer's and Parkinson's amyloid proteins allowed the creation of a universal ganglioside-binding peptide.** *PLoS One* 2014, **9:**e104751.
80. Fantini J, Carlus D, Yahi N: **The fusogenic tilted peptide (67-78) of alpha-synuclein is a cholesterol binding domain.** *Biochim Biophys Acta* 2011, **1808:**2343-2351.
81. Charloteaux B, Lorin A, Brasseur R, Lins L: **The "Tilted Peptide Theory" links membrane insertion properties and fusogenicity of viral fusion peptides.** *Protein Pept Lett* 2009, **16:**718-725.
82. Kerr ID, Haider AJ, Gelissen IC: **The ABCG family of membrane-associated transporters: you don't have to be big to be mighty.** *Br J Pharmacol* 2011, **164:**1767-1779.
83. Vedhachalam C, Duong PT, Nickel M, Nguyen D, Dhanasekaran P, Saito H, Rothblat GH, Lund-Katz S, Phillips MC: **Mechanism of ATP-binding cassette transporter A1-mediated cellular lipid efflux to apolipoprotein A-I and formation of high density lipoprotein particles.** *J Biol Chem* 2007, **282:**25123-25130.
84. Neumann J, Rose-Sperling D, Hellmich UA: **Diverse relations between ABC transporters and lipids: An overview.** *Biochim Biophys Acta* 2017, **1859:**605-618.
85. Mendez-Acevedo KM, Valdes VJ, Asanov A, Vaca L: **A novel family of mammalian transmembrane proteins involved in cholesterol transport.** *Sci Rep* 2017, **7:**7450.
86. Risselada HJ: **Membrane Fusion Stalks and Lipid Rafts: A Love-Hate Relationship.** *Biophys J* 2017, **112:**2475-2478.
87. Enrich C, Rentero C, Hierro A, Grewal T: **Role of cholesterol in SNARE-mediated trafficking on intracellular membranes.** *J Cell Sci* 2015, **128:**1071-1081.
88. Wasser CR, Ertunc M, Liu X, Kavalali ET: **Cholesterol-dependent balance between evoked and spontaneous synaptic vesicle recycling.** *J Physiol* 2007, **579:**413-429.
89. Linetti A, Fratangeli A, Taverna E, Valnegri P, Francolini M, Cappello V, Matteoli M, Passafaro M, Rosa P: **Cholesterol reduction impairs exocytosis of synaptic vesicles.** *J Cell Sci* 2010, **123:**595-605.
90. Hao M, Bogan JS: **Cholesterol regulates glucose-stimulated insulin secretion through phosphatidylinositol 4,5-bisphosphate.** *J Biol Chem* 2009, **284:**29489-29498.
91. Koseoglu S, Love SA, Haynes CL: **Cholesterol effects on vesicle pools in chromaffin cells revealed by carbon-fiber microelectrode amperometry.** *Anal Bioanal Chem* 2011, **400:**2963-2971.
92. Zhang J, Xue R, Ong WY, Chen P: **Roles of cholesterol in vesicle fusion and motion.** *Biophys J* 2009, **97:**1371-1380.
93. Churchward MA, Rogasevskaia T, Brandman DM, Khosravani H, Nava P, Atkinson JK, Coorssen JR: **Specific lipids supply critical negative spontaneous curvature--an essential component of native Ca2+-triggered membrane fusion.** *Biophys J* 2008, **94:**3976-3986.
94. Churchward MA, Rogasevskaia T, Hofgen J, Bau J, Coorssen JR: **Cholesterol facilitates the native mechanism of Ca2+-triggered membrane fusion.** *J Cell Sci* 2005, **118:**4833-4848.
95. Manes S, del Real G, Martinez AC: **Pathogens: raft hijackers.** *Nat Rev Immunol* 2003, **3:**557-568.
96. Yang ST, Kiessling V, Simmons JA, White JM, Tamm LK: **HIV gp41-mediated membrane fusion occurs at edges of cholesterol-rich lipid domains.** *Nat Chem Biol* 2015, **11:**424-431.
97. Scheiffele P, Roth MG, Simons K: **Interaction of influenza virus haemagglutinin with sphingolipid-cholesterol membrane domains via its transmembrane domain.** *Embo j* 1997, **16:**5501-5508.
98. Freed EO: **HIV-1 assembly, release and maturation.** *Nat Rev Microbiol* 2015, **13:**484-496.

99. Lee J, Nyenhuis DA, Nelson EA, Cafiso DS, White JM, Tamm LK: **Structure of the Ebola virus envelope protein MPER/TM domain and its interaction with the fusion loop explains their fusion activity.** *Proc Natl Acad Sci U S A* 2017, **114:**E7987-e7996.
100. Barrett PJ, Song Y, Van Horn WD, Hustedt EJ, Schafer JM, Hadziselimovic A, Beel AJ, Sanders CR: **The amyloid precursor protein has a flexible transmembrane domain and binds cholesterol.** *Science* 2012, **336:**1168-1171.
101. Klug YA, Rotem E, Schwarzer R, Shai Y: **Mapping out the intricate relationship of the HIV envelope protein and the membrane environment.** *Biochim Biophys Acta* 2017, **1859:**550-560.
102. Epand RM, Sayer BG, Epand RF: **Peptide-induced formation of cholesterol-rich domains.** *Biochemistry* 2003, **42:**14677-14689.
103. Epand RF, Sayer BG, Epand RM: **The tryptophan-rich region of HIV gp41 and the promotion of cholesterol-rich domains.** *Biochemistry* 2005, **44:**5525-5531.
104. Vishwanathan SA, Thomas A, Brasseur R, Epand RF, Hunter E, Epand RM: **Large changes in the CRAC segment of gp41 of HIV do not destroy fusion activity if the segment interacts with cholesterol.** *Biochemistry* 2008, **47:**11869-11876.
105. Vishwanathan SA, Thomas A, Brasseur R, Epand RF, Hunter E, Epand RM: **Hydrophobic substitutions in the first residue of the CRAC segment of the gp41 protein of HIV.** *Biochemistry* 2008, **47:**124-130.
106. Greenwood AI, Pan J, Mills TT, Nagle JF, Epand RM, Tristram-Nagle S: **CRAC motif peptide of the HIV-1 gp41 protein thins SOPC membranes and interacts with cholesterol.** *Biochim Biophys Acta* 2008, **1778:**1120-1130.
107. Carravilla P, Cruz A, Martin-Ugarte I, Oar-Arteta IR, Torralba J, Apellaniz B, Perez-Gil J, Requejo-Isidro J, Huarte N, Nieva JL: **Effects of HIV-1 gp41-Derived Virucidal Peptides on Virus-like Lipid Membranes.** *Biophys J* 2017, **113:**1301-1310.
108. Chen SS, Yang P, Ke PY, Li HF, Chan WE, Chang DK, Chuang CK, Tsai Y, Huang SC: **Identification of the LWYIK motif located in the human immunodeficiency virus type 1 transmembrane gp41 protein as a distinct determinant for viral infection.** *J Virol* 2009, **83:**870-883.

Figure Legends

Figure 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.

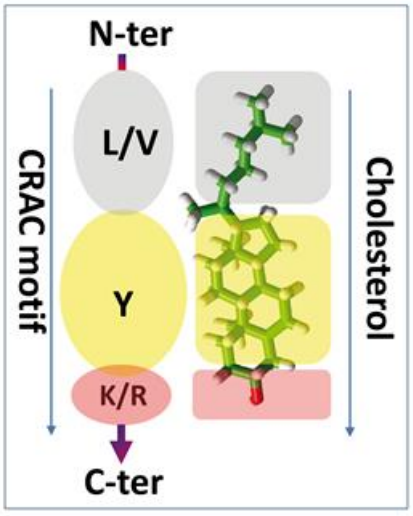

Figure 2. CH-π stacking interaction in the CRAC/cholesterol complex. Three distinct views of cholesterol (in yellow) (A, B, and C) bound to the CRAC domain of the human delta-type opioid receptor are shown. The near perfect superposition of the aromatic ring of Tyr-77 onto the second ring of cholesterol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

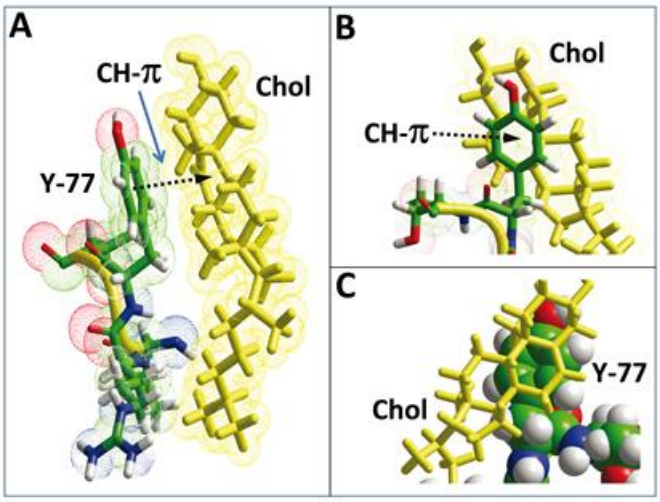

Figure 3. The CARC motif of Class I bitopic membrane proteins is located in the outer leaflet of the plasma membrane. The CARC motif and cholesterol are represented with the same color as in Figure 1. A. Topology of the CARC-cholesterol complex. B. Membrane localization of the CARC-cholesterol complex.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

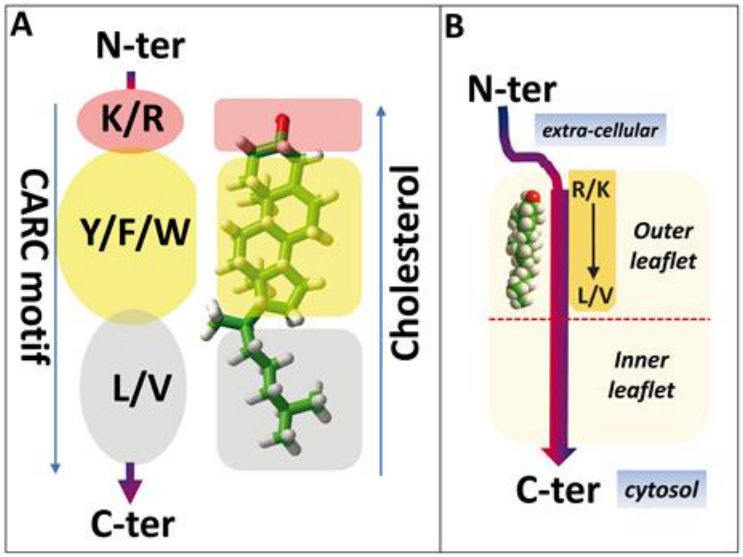

Figure 4. Docking of 15 cholesterol molecules onto the nicotinic acetylcholine receptor.
Three cholesterol molecules bound to the γ subunit of the acetylcholine receptor are shown at a different scale. The picture on the right shows the cholesterol molecule bound to the CARC motif in the 4th TMD of the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

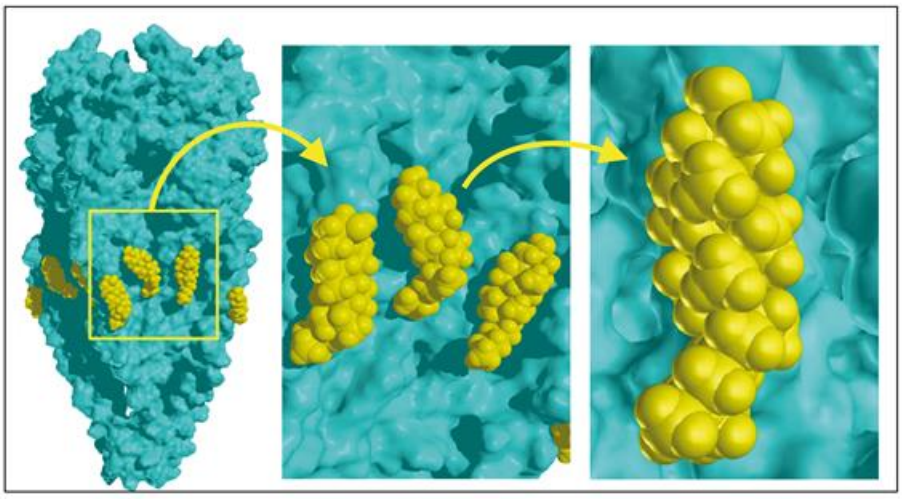

Figure 5. Mirror topology of CARC/CRAC motifs within the same TMD. Three distinct views of the complex are shown. Cholesterol in yellow is bound to CARC, and cholesterol in red is bound to CRAC. The TMD shown is the 7th TMD of the human adenosine receptor A1.

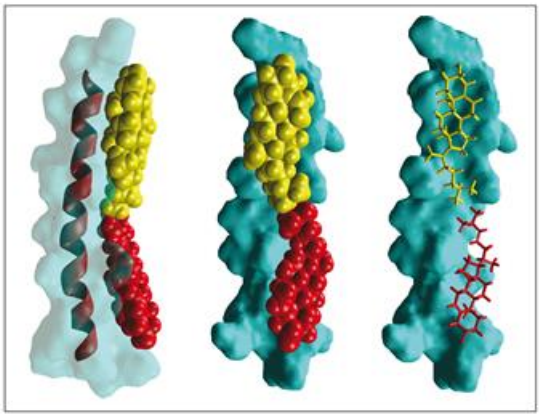

Figure 6. The SSD of NPC1 totally wraps cholesterol. The protein is represented as a ribbon diagram (A) or with a surface rendition (B). Alpha helices are in red, beta strands in blue and cholesterol in yellow. The structure of the cholesterol-NPC1 complex is retrieved from PDB file # 3GKI.

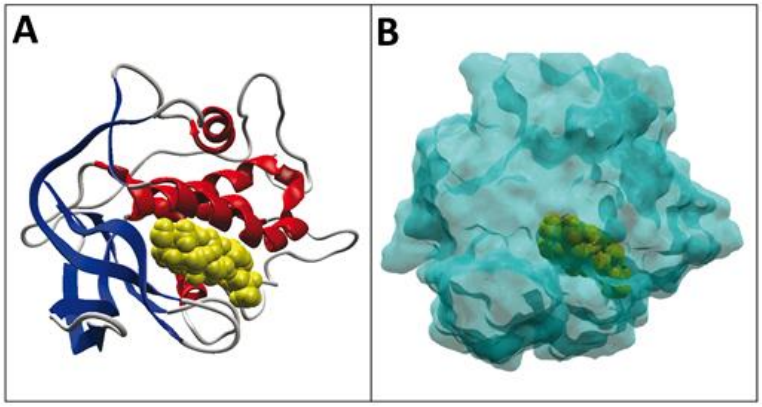

Figure 7. Two cholesterol molecules bound to the human β2 adrenergic receptor (retrieved from PDB file # 3D4S). Three distinct views of the complex (A, B and C) are shown. One cholesterol is in green, the other one in yellow.