Relevance of CARC and CRAC Cholesterol-Recognition Motifs in the Nicotinic Acetylcholine Receptor and Other Membrane-Bound Receptors

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Contents

1. Introduction 2
2. Experimental Validation of the CARC Motif 4
3. Coexistence of CARC and CRAC Sequences Within the Same TM Domain 8
4. Reliability of the CARC and CRAC Algorithms 12
5. Cholesterol and Its Key Role in Liquid-Ordered Lipid Domains 13
6. Evolutionary Conservation of Sterol-Recognition Motifs 16
Acknowledgments 17
References 17

Abstract

Cholesterol is a ubiquitous neutral lipid, which finely tunes the activity of a wide range of membrane proteins, including neurotransmitter and hormone receptors and ion channels. Given the scarcity of available X-ray crystallographic structures and the even fewer in which cholesterol sites have been directly visualized, application of in silico computational methods remains a valid alternative for the detection and thermodynamic characterization of cholesterol-specific sites in functionally important membrane proteins. The membrane-embedded segments of the paradigm neurotransmitter receptor for acetylcholine display a series of cholesterol consensus domains (which we have coined "CARC"). The CARC motif exhibits a preference for the outer membrane leaflet and its mirror motif, CRAC, for the inner one. Some membrane proteins possess the double

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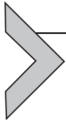
CARC–CRAC sequences within the same transmembrane domain. In addition to in silico molecular modeling, the affinity, concentration dependence, and specificity of the cholesterol-recognition motif–protein interaction have recently found experimental validation in other biophysical approaches like monolayer techniques and nuclear magnetic resonance spectroscopy. From the combined studies, it becomes apparent that the CARC motif is now more firmly established as a high-affinity cholesterol-binding domain for membrane-bound receptors and remarkably conserved along phylogenetic evolution.



1. INTRODUCTION

Cholesterol modulates the activity of a wide range of membrane receptors and ion channels in multiple ways, i.e., via general effects on the bulk bilayer lipid, altering membrane fluidity (el Battari, Ah-Kye, Muller, Sari, & Marvaldi, 1985; Lazar & Medzihradsky, 1992; Maguire & Druse, 1989) or curvature (Lee, 2004; Yesylevskyy, Demchenko, Kraszewski, & Ramseyer, 2013) or through direct binding to these proteins (Baier, Fantini, & Barrantes, 2011; Barrantes, 2004; Dopico & Bukiya, 2014; Fantini & Barrantes, 2013; Levitan, Singh, & Rosenhouse-Dantsker, 2014; Picazo-Juarez et al., 2011; Popot, Demel, Sobel, van Deenen, & Changeux, 1977; Posada et al., 2014; Singh, Shentu, Enkvetchakul, & Levitan, 2011; Rosenhouse-Dantsker, Noskov, Durdagi, Logothetis, & Levitan, 2013; Singh et al., 2012). The availability of the crystal structures of the β2-adrenergic receptor represented an important milestone in the identification of direct interactions between a paradigmatic transmembrane (TM) protein and member of the most abundant and functionally important superfamily of receptors in eukaryotic cells, i.e., the G-protein-coupled receptors (GPCRs) on the one hand and cholesterol (Cherezov et al., 2007; Rosenbaum et al., 2007) on the other. The X-ray data of the β2-adrenergic receptor rapidly catalyzed a cascade reaction in the crystallography of membrane-embedded proteins, which led to the identification of cholesterol-binding sites in various other GPCRs like the β1-adrenergic receptor (Warne et al., 2008) and several other members of the GPCRs (reviewed, e.g., in Vaidehi, Bhattacharya, & Larsen, 2014). Cholesterol–β-adrenergic receptor interactions have been reported to increase the compactness of the receptor structure and to enhance the conformational stability toward active or inactive receptor states (Gimpl, 2016). The available crystal structures of these macromolecules represent, however, only a minor fraction of the genome coding for functionally relevant

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membrane protein targets. Viable alternative methodologies for identifying cholesterol-recognition motifs in hormone or neurotransmitter receptors and ion channels are therefore currently being sought in membrane biology. Various groups, including ours, have resorted to computational methods to explore sequences in protein data banks and detect the presence of putative cholesterol-binding linear domains in the TM regions of proteins, with special emphasis on neurotransmitter receptors such as GPCRs (Baier et al., 2011; Fantini, Di Scala, Evans, Williamson, & Barrantes, 2016; Jafurulla, Tiwari, & Chattopadhyay, 2011), ion channels (Fantini, Di Scala, Baier, & Barrantes, 2016), and transporters (Clay, Lu, & Sharom, 2015; Gal et al., 2015; Sharpe et al., 2015). This approach resulted in the definition of consensus motifs with predictive value, which can be further applied for identifying cholesterol-binding linear domains (Baier et al., 2011; Epand, 2008; Epand, Thomas, Brasseur, & Epand, 2010; Epand et al., 2006; Jamin et al., 2005). The first such consensus motif to be identified was defined by the sequence array (L/V)-$X_{1–5}$-(Y)-$X_{1–5}$-(K/R) and coined "cholesterol-recognition amino acid consensus" (CRAC) (Jamin et al., 2005; Li & Papadopoulos, 1998). This motif was readily found in several proteins known to bind cholesterol, including both viral and host membrane proteins (Epand, 2006, 2008; Epand et al., 2010, 2006). We subsequently introduced the linear sequence (K/R)-$X_{1–5}$-(Y/F)-$X_{1–5}$-(L/V), which is essentially the reverse or mirror version of the CRAC algorithm, and hence referred to it as the "CARC" consensus motif (Baier et al., 2011).

The CARC motif was originally explored in greater detail using the nicotinic acetylcholine receptor (nAChR), the paradigm of the pentameric ligand-gated ion channels (Barrantes, 2015). The free energy of interaction between cholesterol molecules and the nAChR is about $-510/-530$ kJ mol^{-1}, i.e., more than -100 kJ mol^{-1} per subunit. The particularly favorable fit between the "CARC-like" γTM4 segment from human nAChR (428RVCFLAML435) and cholesterol is noteworthy, with an energy of interaction of about -60 kJ mol^{-1}, i.e., ~60% of the total energy of interaction of the entire γ subunit, which exhibits the highest affinity for cholesterol among all nAChR subunits (cf. Table 1).

Thermodynamic analyses of the energy of interaction of cholesterol with an assortment of membrane proteins (Fig. 4) revealed that the CARC motif generally exhibits more affinity for cholesterol than the CRAC motif (Fantini & Barrantes, 2013). We provided physicochemical arguments to account for the difference in the predictive value of the two linear algorithms, i.e., the snorkeling effect of Lys/Arg residues (the apolar part of the amino acid chain buried in the membrane and cationic group outside)

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Table 1 Energetics of Interaction of Cholesterol and the CARC and CARC-Like Motifs in the Transmembrane Domains of Human Muscle-Type AChR

AChR TM Domain	Energy of Interaction ($kJ\ mol^{-1}$)
αTM1	−35.129
αTM3	−31.729
αTM4	−27.903
Total α subunit	−94.761
βTM1	−52.332
βTM3	−20.808
βTM4	−26.241
Total β subunit	−99.453
γTM1	−30.542
γTM3	−37.066
γTM4	−59.961
Total γ subunit	−127.569
δTM1	−46.184
δTM3	−33.083
δTM4	−29.197
Total δ subunit	−108.464
εTM1	−44.438
εTM3	−24.421
εTM4	−44.050
Total ε subunit	−112.909
Embryonic AChR[a] ($\alpha_2\beta\gamma\delta$)	−525.008
Adult AChR[a] ($\alpha_2\beta\varepsilon\delta$)	−510.348

[a]The stoichiometric contribution of two α subunits is taken into account in the estimation of the total energy of interaction of the AChR pentamer with cholesterol molecules.
From Baier, C. J., Fantini, J., & Barrantes, F. J. (2011). Disclosure of cholesterol recognition motifs in transmembrane domains of the human nicotinic acetylcholine receptor. Scientific Reports, 1, 69.

(Strandberg & Killian, 2003) and cholesterol structure (Fantini & Barrantes, 2009; as reviewed in Fantini & Barrantes, 2013; Fantini, Di Scala, Baier, et al., 2016).

2. EXPERIMENTAL VALIDATION OF THE CARC MOTIF

A series of sequence analyses and molecular dynamics studies describing the occurrence of the CRAC mirror sequence ("CARC") in several membrane (Baier et al., 2011; Fantini & Barrantes, 2013) and soluble (Morrill & Kostellow, 2016; Morrill, Kostellow, & Gupta, 2014, 2016)

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proteins have provided substantial evidence for the possible functional relevance of these linear amino acid sequences in cholesterol–protein interactions. However, direct experimental demonstration of physical interactions between the two partners was lacking for proteins other than GPCRs. In a recent work the working hypothesis was subjected to experimental test by studying the interaction of a prototype CARC domain with cholesterol employing lipid monolayer strategies and nuclear magnetic resonance (NMR) spectroscopy.

Selection of a representative CARC domain for these experimental validations was based on previous photolabeling studies of the *Torpedo* nAChR with the cholesterol analogue probe [^{3}H]azicholesterol, which led to the identification of a (predominant) cholesterol-binding domain in the fourth transmembrane domain (TM4) of the human nAChR γ subunit (Hamouda, Chiara, Sauls, Cohen, & Blanton, 2006). Subsequent in silico computational approaches (Baier et al., 2011) led us to identify a typical CARC motif: 455-**R**VC**F**LAM**L**-462 (the characteristic Arg, Phe, and Leu amino acid residues outlined bold and underlined) in the human γTM4 that incorporated most of the label in the photoaffinity studies. Furthermore, the molecular modeling simulations disclosed that this TM segment displayed the highest energy of interaction (in the order of -60 kJ mol^{-1}) with cholesterol when compared to all other subunits of the nAChR in *Homo sapiens* and other species (Baier et al., 2011). The homologous γTM4 segment in the *Torpedo* nAChR possesses a CARC motif similar to the human form: 449-**K**AC**F**WIA**L**-456. In *Torpedo* TM4 the highest [^{3}H]azicholesterol labeling is observed in Asp448, the second residue after Lys449, which is the first amino acid of the N-term CARC motif.

To further refine the correlation between the [^{3}H]azicholesterol photolabeling data with the in silico calculations, additional molecular dynamics studies were carried out on the cholesterol derivative-*Torpedo* γTM4 segment (445–460), that is, comprising the CARC domain plus a few upstream and downstream amino acid residues. We found that azicholesterol interacted with the amino acid residues defined by the CARC algorithm, namely Lys449, Phe452, and Leu456, and that the azi-group was at a distance of only 2 Å from the side chain of Asp448. The CARC motif present within the γTM4 exhibited a tight cholesterol binding, although it seemed unlikely that the strength of the interaction sufficed to account for the reduction in mobility observed in the molecular simulations. This led us to propose that the reduction in mobility arises from the cholesterol-induced oligomerization of γTM4, which is

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consistent with previous fluorescence studies from our laboratory showing the cholesterol-dependent oligomerization of γTM4 in POPC bilayers (de Almeida et al., 2004). The molecular dynamics studies further revealed that the cholesterol contact with the CARC motif within the γTM4 was established with the β-face, leaving the α-face exposed, suggesting that the cholesterol-mediated oligomerization of the peptide is dictated by cholesterol–cholesterol interactions rather than by protein–protein interactions (Fantini & Barrantes, 2009). When we compared the interaction between cholesterol and either wild-type or mutant TM4 peptides, the WT CARC motif was found to exhibit a high affinity for cholesterol. All three amino acids defining the CARC domain were found to interact with cholesterol, especially the central Phe452 residue. Replacement of this aromatic residue with alanine (F-452/A mutant) resulted in a significant loss of affinity. This important result was fully confirmed by physicochemical lipid monolayer studies (Di Scala, Chahinian, Yahi, Garmy, & Fantini, 2014) of CARC–cholesterol interactions (Fig. 1). In these experiments, a lipid monolayer is prepared at the air–water interface at a controlled surface pressure (π_0), after which the peptide is injected in the aqueous phase. The interaction of the peptide with the lipid is assessed by surface pressure measurements (basically a surface pressure increase $\Delta\pi$ measured in $\mathrm{mN\,m^{-1}}$) (Di Scala et al., 2014). The conservative F-452/W mutation had no effect, indicating that it is the aromatic nature of Phe452, and not its specific structure, that is required for optimal binding. This is in line with previous studies, suggesting that CARC motifs could contain any of the three aromatic residues, i.e., Phe, Trp, or Tyr (Baier et al., 2011; Fantini & Barrantes, 2013). The other major outcomes of our physicochemical studies are the demonstration of lipid specificity (CARC recognized cholesterol but not phosphatidylcholine) and the concentration dependency of the binding (saturation was reached for peptide concentrations $<10\ \mu M$) (Fig. 1).

The second line of experimental validation stemmed from NMR studies (MAS triple resonance magic-angle spinning deuterium NMR using deuterated Ala471) (Fantini, Di Scala, Evans, et al., 2016). Inclusion of cholesterol to phospholipid bilayers containing a synthetic $^{13}C/^{15}N$-labeled peptide corresponding to Asp464 to Val492 in the intact *Torpedo* γTM4 peptide caused a reduction in the rotational motion of the peptide within the bilayer, a result consistent with the cholesterol-mediated peptide oligomerization, as discussed in the preceding paragraph. The functional significance of this in the intact receptor remains to be elucidated, but the location of the

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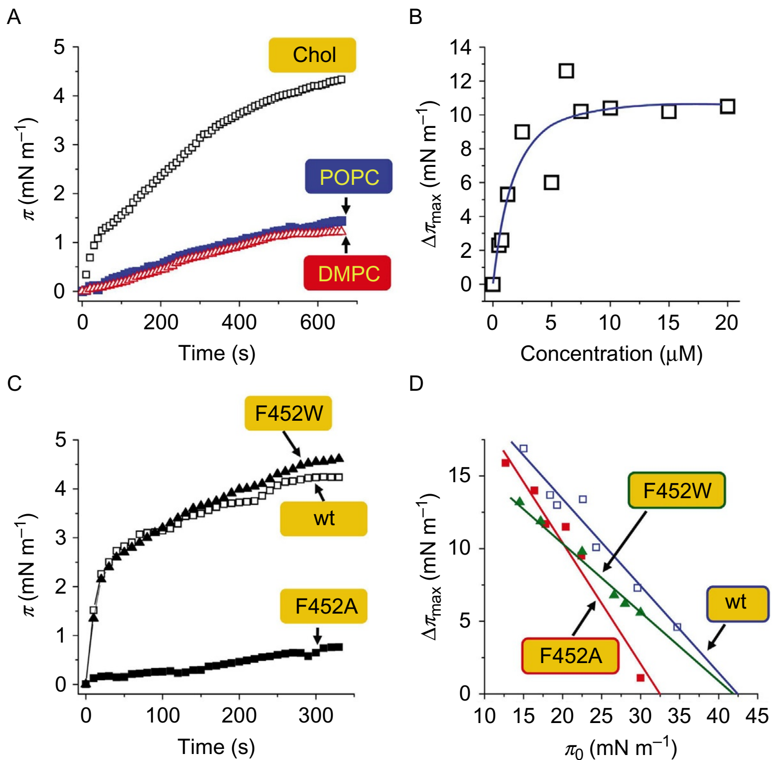

Fig. 1 Lipid monolayer studies of CARC–cholesterol interactions. (A) Kinetics of interaction of a synthetic γTM4 peptide corresponding to fragment 445–460 of the *Torpedo* nAChR γ subunit with a monolayer of cholesterol (Chol) or phosphatidylcholine (palmitoyl-oleoyl phosphatidylcholine, POPC or dimyristoyl phosphatidylcholine, DMPC). The peptide is injected in the aqueous phase underneath the lipid monolayer, and the interaction is measured by the surface pressure increase (π) induced by the peptide. (B) Effect of peptide concentration on the interaction between nAChR γTM4 and cholesterol (surface pressure increase $\Delta\pi_{max}$ induced by the peptide after 30 min of incubation). (C) Kinetics of interaction of wild-type (wt) and mutant peptides (F-452/A; F-452/W) derived from γTM4 with cholesterol monolayers ($\pi_0 = 30 \pm 3.5$ mN m^{-1}). (D) Maximal surface pressure increase ($\Delta\pi_{max}$) induced by wild-type and mutant peptides on cholesterol monolayers at various π_0 values. *From Fantini, J., Di Scala, C., Evans, L. S., Williamson, P. T. F., & Barrantes, F. J. (2016). A mirror code for protein-cholesterol interactions in the two leaflets of biological membranes.* Scientific Reports*, 6, 21907.*

CARC domain on the lipid-facing surface of the helix may play a role in cholesterol-mediated clustering of the receptor. In summary, the experimental approaches combining lipid monolayer data, NMR spectroscopy, and in silico molecular modeling simulations provided much stronger

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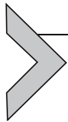
evidence for the direct physical interaction of cholesterol with a CARC cholesterol-recognition motif. The interaction was found to be of high affinity, lipid specific, and saturable (Fantini, Di Scala, Evans, et al., 2016).



3. COEXISTENCE OF CARC AND CRAC SEQUENCES WITHIN THE SAME TM DOMAIN

We have recently conducted a search for CARC/CRAC cholesterol-recognition motifs over a large series of membrane proteins (listed in Fig. 4) in combination with molecular dynamics simulations of the whole TM regions of various membrane-embedded proteins in order to determine whether two cholesterol molecules could actually be docked onto these domains (Fantini, Di Scala, Evans, et al., 2016). This was indeed the case. As shown in Fig. 2, the CRAC and CARC motifs are vectorial ("apolar" Leu/Val→ "basic" Lys/Arg for CRAC and "basic" Lys/Arg→ "apolar" Leu/Val for CARC, from the N-terminus to the C-terminus sequence).

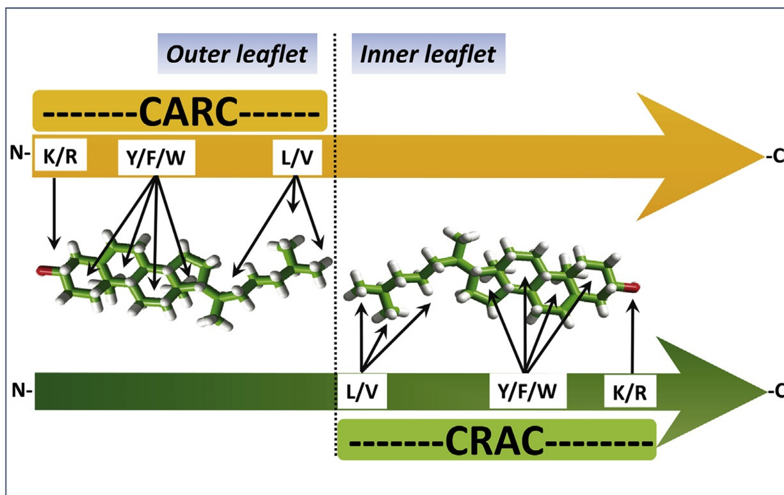


Fig. 2 The vectorial arrangement of CARC and CRAC motifs. For proteins whose N-terminus is extracellular, the CARC domain is located in the outer leaflet and the CRAC domain is in the inner one. This topology applies for type-1 membrane proteins as well as for TM domains 1, 3, 5, and 7 of G-protein-coupled receptors (GPCRs) with seven transmembrane domains. For type-2 membrane proteins (extracellular C-terminus) and domains 2, 4, and 6 of GPCRs, the algorithms still apply, but CARC is located in the inner leaflet and CRAC in the outer one. *From Fantini, J., Di Scala, C., Evans, L. S., Williamson, P. T. F., & Barrantes, F. J. (2016). A mirror code for protein-cholesterol interactions in the two leaflets of biological membranes.* Scientific Reports*, 6, 21907.*

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The CARC sequence starts with a basic residue (Arg or Lys), and this feature makes the CARC motif ideally suited for interaction with cholesterol in the outer leaflet of biological membranes. Indeed, the N-terminal domain of type I membrane proteins is extracellular, such that the carbon chain enters the membrane bilayer in the N- to C-terminus direction (Fig. 2). This is also the case for TM domains 1, 3, 5, and 7 in the GPCRs (see below).

The three main amino acid residues defining the CARC and CRAC motifs were always involved in the interaction. In addition, in both cases the central aromatic residue could be either Phe or Tyr (and even Trp in the case of CARC). This finding is consistent with the nature of the interaction between cholesterol and aromatic rings, i.e., the CH–π stacking interaction (Nishio, Umezawa, Fantini, Weiss, & Chakrabarti, 2014). The branched aliphatic residues (Leu/Val) are well suited to accommodate the protruding methyl groups of cholesterol. Furthermore, the terminal basic residue of the motif often forms a hydrogen bond with the oxygen atom of the –OH group of cholesterol (Fantini & Barrantes, 2013). Thus, the selection of CARC and CRAC as cholesterol-binding motifs is justified by robust physicochemical rules. These rules can be put into practice via a combination of London, CH–π stacking, and hydrogen bonding that cooperate to control protein–cholesterol interactions in the membrane environment (Fantini & Barrantes, 2013).

Since the consensus CRAC sequence starts with an aliphatic residue (Leu or Val), its N-terminal is expected to interact with the apolar groups of cholesterol (sterane, methyl, and isooctyl) in the inner leaflet (Fig. 2). In other words, the sequential chaining of CARC and CRAC motifs in the amino acid sequence of a TM domain, starting from the N-terminus, is consistent with the binding of a cholesterol molecule in each leaflet.

The coexisting presence of CARC and CRAC motifs, one in each leaflet of the membrane, has an important consequence: the host TM protein segment can accommodate two opposite (tail-to-tail) cholesterol molecules. This ensures that the polar amino acid residues of the motif (Lys/Arg) face the intra- and extracellular milieu, whereas the apolar ends of the motifs (Leu/Val) are deeply buried in the most hydrophobic region of the lipid bilayer (Fig. 2).

This information has provided a new twist in the interpretation of the mode of action of the mitochondrial translocator protein, TSPO, and its interaction with cholesterol. In a recent work, Jaremko, Jaremko, Giller, Becker, and Zweckstetter (2014) had speculated that the binding of

cholesterol at the outside of the TSPO structure and the ability of cholesterol to dimerize were the two factors determining the oligomeric state of the transporter. Our studies showed that TSPO possesses not only a CRAC domain but also a CARC motif in the same TM region (Fig. 3). Furthermore, the CARC motif has an exceptionally high energy of interaction with cholesterol, in the order of -62 kJ mol^{-1} (Fantini, Di Scala, Evans, et al., 2016). The additional cholesterol site on the same membrane-embedded surface provides further energetic grounds for the cholesterol-mediated oligomerization hypothesis.

If one searches protein sequence databases for the occurrence of both CARC and CARC motifs in the same TM domain, one finds that for type I membrane proteins and for domains 1, 3, 5, and 7 of GPCRs, the CARC motif is always located in the outer leaflet, whereas the CRAC sequence is found in the inner leaflet (Fig. 4). The examples include signaling membrane receptors like the somatostatin, GABA, serotonin, adenosine, VIP, and cannabinoid receptors, as well as the voltage-dependent TRVP1 channel. Overall, the mean energy of interaction was in the order of -58 kJ mol^{-1}

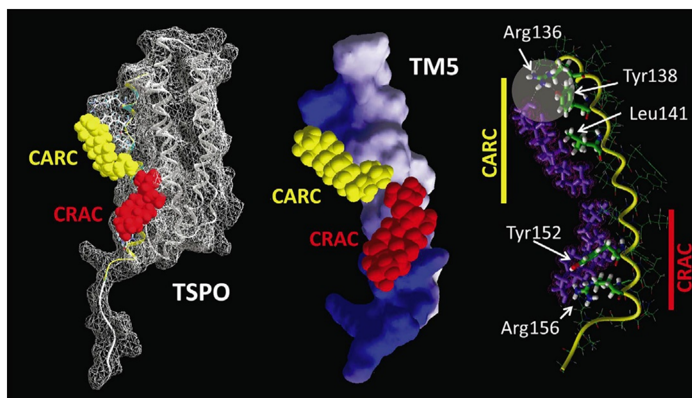

Fig. 3 Coexistence of both CARC and CRAC motifs in the transmembrane domain of the mitochondrial translocator protein TSPO. On the *left*, the docking of cholesterol on the CARC motif (cholesterol in *yellow*) and the CRAC motif (cholesterol in *red*). The 3D structure of cholesterol was retrieved from PDB entry 1MT5. The model in the *middle* represents the fifth transmembrane domain (TM5) of TSPO with cholesterol bound to both CARC and CRAC motifs. The model on the *right* shows the molecular interactions between cholesterol (in *purple*) and each cholesterol-binding motif of TM5. The hydrogen bond network stabilizing the cholesterol–CARC interaction is indicated by a *disk*. *From Fantini, J., Di Scala, C., Evans, L. S., Williamson, P. T. F., & Barrantes, F. J. (2016). A mirror code for protein-cholesterol interactions in the two leaflets of biological membranes.* Scientific Reports*, 6, 21907.*

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N-ter/Extracellular→CARC---CRAC→C-ter/Intracellular

Type 3 somatostatin receptor Human #P32745
TM5 203- RAGFIIYTAALGFFGPLLVICLCYLLIVVK-232
Energy: -54.0 -43.6

Mitochondrial translocator protein Mouse #P50637
TM5 135- RLLYPYLAWLAFATVLNYYVWR-156
Energy: -62.4 -44.4

Neuropeptide FF receptor 2 Human #Q9Y5X5
TM5 318-RKIYTTVLFANIYLAPLSLIVIMYGR-343
Energy: -32.7 -52.5

Metabotropic glutamate receptor 5 Human #P41594
TM7 798-KIITMCFSVSLSATVALGCMFVPK-821
Energy: -60.6 -39.0

GABA type B receptor subunit 2 Human #O75899
TM1 475-RKISLPLYSILSALTILGMIMASAFLFFNIK-555
Energy: -64.1 -31.3

Cannabinoid receptor 1 Human #P21554
TM3 186-RNVFLFKLGGVTASFTASVGSLFLTAIDR-214
Energy: -56.7 -60.6
TM7 375-KTVFAFCSMLCLLNSTVNPIIYALR-400
Energy: -53.5 -55.3

5-hydroxytryptamine receptor 7 Human #P34969
TM7 367-RTFLWLGYANSLINPFIYAFFNRDLR-392
Energy: -78.1 -53.2

Adenosine receptor A1 #P30542
TM7 265-KPSILTYIAIFLTHGNSAMNPIVYAFRIQK-294
Energy: -54.9 -45.9

VIP receptor 1 Human #P32241
TM1 143-KTGYTIGYGLSLATLLVATAILSLFRK-169
Energy: -54.2 -46.4

Prolactin-releasing peptide receptor Human #P49683
TM5 221-RQLYAWGLLLVTYLLPLLVILLSYVR-247
Energy: -55.3 -27.3

Oxytocin receptor Human #P30559
TM5 198-KAYITWITLAVYIVPVIVLAACYGLISFK-226
Energy: -50.1 -46.4

TRPV1 Human #Q8NER1
TM2 468-KTGDYFRVTGEILSVLGGVYFFFRGIQYFLQRR-500
Energy: -83.2 -63.2

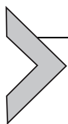
Corticotropin-releasing factor receptor 1 Human #P34998
TM1 111-KSKVHYHVAVIINYLGHCISLVALLVAFVLFLRLR-145
Energy: -52.6 -60.8

Mean energy:	CARC – 58.03 ± 12.10 kJ. mol^{-1}
	CRAC – 47.85 ± 10.68 kJ. mol^{-1}

Fig. 4 Energetics of cholesterol binding to TM domains displaying *both* CARC and CRAC motifs. The CARC motif is framed in *yellow*, and the CRAC motif in *green*. The calculated energy of interaction (in kJ mol^{-1}) is indicated under each motif. The UniProt entry is indicated for each protein after the # symbol. *From Fantini, J., Di Scala, C., Evans, L. S., Williamson, P. T. F., & Barrantes, F. J. (2016). A mirror code for protein-cholesterol interactions in the two leaflets of biological membranes.* Scientific Reports, *6, 21907.*

for CARC and $-48\ \mathrm{kJ\,mol^{-1}}$ for CRAC, indicating that the CARC domain generally exhibits more affinity for cholesterol than a CRAC domain (Baier et al., 2011; Fantini & Barrantes, 2013; Fantini, Di Scala, Evans, et al., 2016). Exceptions to this rule can be found, e.g., for neuropeptide FF and corticotrophin-releasing factor receptors (Fig. 4).

In assessing the possible biological implications of the coexisting CARC–CRAC motifs within the same membrane-embedded peptide domain, the heterologous protein expression in the yeast system poses a singular case, since the functional expression of, e.g., human receptors is not always sustained (Opekarova & Tanner, 2003). In this context, it is interesting to note that yeasts have an essential requirement for ergosterol for cell growth, and cholesterol is not a valid substitute for the former sterol. However, ligand binding to the human μ-opioid receptor was found to increase in transfected *Saccharomyces* cells when ergosterol was replaced by cholesterol (Lagane et al., 2000). We have speculated (Fantini, Di Scala, Evans, et al., 2016) that human CARC/CRAC domains might exhibit species specificity for cholesterol and cannot mediate functional ergosterol binding in yeast, perhaps due to subtle conformational differences between the two sterol molecules, cholesterol being more flexible due to the presence of several extra-double bonds in ergosterol (Czub & Baginski, 2006; Baginski et al., 1989).



4. RELIABILITY OF THE CARC AND CRAC ALGORITHMS

Despite the robust biochemical rules that explain why CARC and CRAC domains exhibit specific cholesterol-binding properties (Fantini & Barrantes, 2013), it remains the case that the algorithms used for the detection of these domains are very general (basically a vectorial triad of key amino acid residues). As a matter of fact, multiple copies of CARC and/or CRAC may be detected in the same protein (Palmer, 2004). However, this drawback should not be regarded as insurmountable. In the specific case of membrane proteins, which have been extensively studied (Fantini & Yahi, 2015), additional criteria might be considered to determine whether the presence of a consensus CARC/CRAC motif is likely to constitute a functional cholesterol-binding domain. From a molecular point of view, the reliability of the CARC/CRAC algorithm is excellent. This statement is based on the experimental demonstration that (i) all synthetic peptides derived from CARC/CRAC motifs tested so far display specific cholesterol-binding properties, and (ii) mutations affecting the motif, especially the central aromatic residue, always

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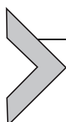
decrease the binding of cholesterol (Fantini, Di Scala, Evans, et al., 2016). These data of course only prove that these consensus motifs are able to bind cholesterol, not that the protein displaying the motifs actually interacts with this lipid. In addition, the motif has to be located in a TM domain (Fantini, Di Scala, Evans, et al., 2016). The bioavailability of cholesterol in the membrane area surrounding the TM domain displaying a CARC/CRAC motif will determine whether cholesterol interacts with this protein, and when it does so. Combining all these criteria led us to propose a step-by-step method for identifying linear cholesterol-binding motifs in membrane proteins (Fantini, Di Scala, Evans, et al., 2016).

Finally, one should note that besides the CARC and CRAC motifs, cholesterol can bind to three-dimensional pockets that combine several TM domains and thus might remain undetected by the CARC/CRAC algorithms. Specifically, it was shown that Kir2 channels have a functionally important cholesterol-binding pocket with residues that do not contain any of the previously identified cholesterol-recognition motifs (Levitan et al., 2014; Rosenhouse-Dantsker et al., 2013). Nevertheless, the key amino acid residues that define the CARC/CRAC motif (e.g., an aromatic one) are generally present in those three-dimensional motifs, as is the case for instance with the cholesterol consensus motif (CCM) (Hanson et al., 2008). In any case, it appears that the interaction of a TM domain with cholesterol is controlled by general biochemical rules that determine a series of fully predictable van der Waals interactions and hydrogen bonding (Fantini & Yahi, 2015).



5. CHOLESTEROL AND ITS KEY ROLE IN LIQUID-ORDERED LIPID DOMAINS

Cholesterol occurs in the inner or outer leaflet of the membrane bilayer, or in both leaflets. In the inner leaflet, it may interact with phosphatidylserine, whereas in the outer leaflet it associates predominantly with sphingomyelins. In general, the latter possess a more rigid apolar surface than glycerophospholipids, and this facilitates the preferential interaction with cholesterol. The cholesterol–sphingomyelin association is further enriched in glycerophospholipids with saturated fatty acyl chains (relative to the average saturation in the rest of the bilayer). The lipid raft hypothesis proposes that these ternary complexes formed by specific self-associated lipid species constitute microdomains or platforms that can intervene in protein partition, signaling, and other functional events in cell physiology (Anderson & Jacobson, 2002; Lingwood & Simons, 2010; Simons & Ikonen, 1997;

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Simons & van Meer, 1988). The physicochemical basis for the formation of these domains probably stems mostly from the peculiar and still not fully understood thermodynamic properties of biological membranes: favorable and unfavorable lipid–lipid interactions result in transient lateral heterogeneities that join or segregate their constituent molecules, respectively. Above/below certain critical concentrations and/or temperatures, these lateral heterogeneities generate transiently separated lipid phases, the two most prominent of which are the liquid-disordered (Ld) and liquid-ordered (Lo) phases (Marsh, 1991; Yeagle, 1989). The temperature and compositional range over which these lateral separations into liquid phases occur is rather large (Veatch & Keller, 2002, 2003a, 2003b; Veatch, Polozov, Gawrisch, & Keller, 2004). The domains enriched in cholesterol–sphingomyelin–saturated glycerophospholipids constitute the Lo phase, a more condensed, rigid, and thicker fraction of the membrane. Outside these Lo domains, cholesterol associates with other glycerophospholipids (mainly phosphatidylcholines) in a rather loose manner, and at relatively lower concentrations (Fig. 5).

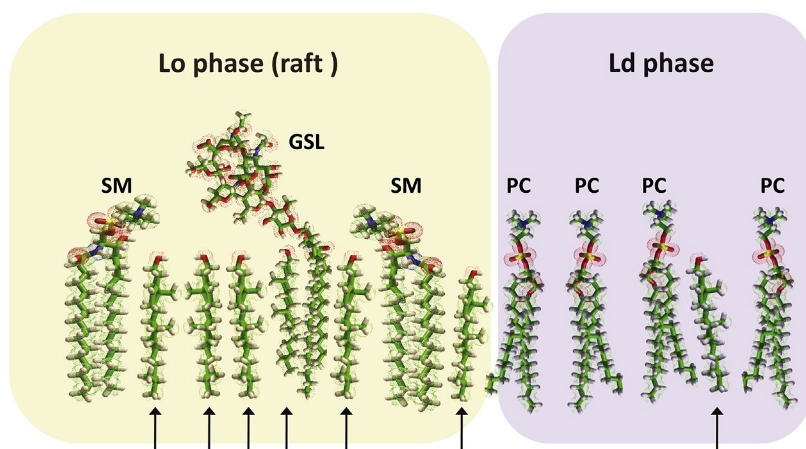

Fig. 5 Cholesterol accessibility in Lo and Ld phases. In the Lo phase (cholesterol–sphingomyelin enriched, "raft" domains), cholesterol (*arrows*) is masked by sphingolipids such as sphingomyelin (SM) or glycosphingolipids (GSL). In the Ld phase, cholesterol molecules (*arrows*) are more sparsely distributed among glycerophospholipids such as phosphatidylcholine (PC). In this case the polar –OH group of cholesterol is accessible to extracellular ligands. *From Fantini, J., Di Scala, C., Baier, C. J., & Barrantes, F. J. (2016). Molecular mechanisms of protein-cholesterol interactions in plasma membranes: Functional distinction between topological (tilted) and consensus (CARC/CRAC) domains.* Chemistry and Physics of Lipids, 199, *52–60.*

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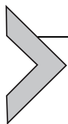
Lipid domains apparently cover a wide range of sizes, from assemblies with <5 nm radius ("ultrananodomains"; Pathak & London, 2015), comprising a couple of hundred lipid molecules per bilayer, to micron-sized platforms with thousands of molecules readily observable by conventional wide-field light microscopy (Griffie, Burn, & Owen, 2015; Maxfield, 2002; Rao & Mayor, 2014; van Zanten & Mayor, 2015). Functionally, lipid domains play important roles in the cell by way of the lateral separation of chemical species in the plane of the membrane. The chemical analysis of the postsynaptic apparatus in the peripheral nervous system shows that cholesterol is a very abundant component (see review in Barrantes, 1989) of this specialized membrane. This sterol is an essential partner of the nAChR, affecting its distribution and several of its functional properties in the peripheral synapse, the neuromuscular junction (Barrantes, 2010, 2012). The lateral heterogeneity of lipids in the postsynaptic membranes of the *Torpedo* electrocyte was an early biophysical finding: protein-associated lipids were shown to be immobilized with respect to bulk membrane lipid (Marsh & Barrantes, 1978), and subsequent work has shown that cholesterol-like molecules form part of this protein-immobilized pool (Barrantes, 2007). The functional implications of this finding became apparent when it was demonstrated that cholesterol is an essential component for maintaining nAChR agonist-dependent state transitions in the postsynaptic membrane (Criado, Eibl, & Barrantes, 1982). It has been proposed that there are two cholesterol populations in nAChR-rich membranes from *Torpedo*: an easily extractable fraction that influences the bulk fluidity of the membrane and a tightly bound receptor-associated fraction (Leibel, Firestone, Legler, Braswell, & Miller, 1987).

In muscle cells, cholesterol was found to influence the formation of micron-sized nAChR clusters induced by agrin (Campagna & Fallon, 2006). Signaling via the agrin/MuSK complex and interaction between the receptor and rapsyn appears to involve lipid platforms (Zhu, Xiong, & Mei, 2006). Using Laurdan two-photon fluorescence microscopy (Stetzkowski-Marden, Gaus, Recouvreur, Cartaud, & Cartaud, 2006), it was concluded that nAChR clusters reside in Lo membrane domains. Another study (Willmann et al., 2006) proposed that these cholesterol-rich lipid microdomains and Src-family kinases both contribute to stabilizing nAChRs and the postsynaptic apparatus. We often resort to an experimental clonal cell line, CHO-K1/A5, which is devoid of nAChR-clustering proteins such as rapsyn and tyrosine kinases, and therefore, homophilic protein–protein interactions, heterophilic protein–lipid interactions, and links with the actin cytoskeleton are more likely candidates for maintaining the

nAChR nanocluster assemblies. Membrane-embedded proteins with preferential affinities for Lo or Ld domains could influence both the lifetime and size of the domains in which they are located by selecting their local lipid environment. A fraction of nAChRs has indeed been found in Lo domains in mammalian cells (Bruses, Chauvet, & Rutishauser, 2001; Campagna & Fallon, 2006; Marchand, Devillers-Thiery, Pons, Changeux, & Cartaud, 2002; Stetzkowski-Marden et al., 2006; Willmann et al., 2006; Zhu et al., 2006). On the other hand, when reconstituted in a sphingomyelin–cholesterol–POPC (1:1:1) model system, purified nAChR protein from *Torpedo* appears not to exhibit any preference for Lo domains in vitro (Bermudez, Antollini, Fernandez Nievas, Aveldano, & Barrantes, 2010). However, inclusion of some sphingomyelin molecular species (brain sphingomyelins, 16:0, 18:0, or 24:1 sphingomyelins) that generate bilayer asymmetry by enriching the sphingolipid content of the outer leaflet of the lipid bilayer can favor the partitioning of the nAChR in Lo domains (Perillo, Penalva, Vitale, Barrantes, & Antollini, 2016). This can be correlated with the observation that Lo domains in the outer leaflet of a bilayer can induce liquid order in the inner leaflet by a coupling mechanism involving in-register Lo domains in the two halves of the bilayer (Lin & London, 2015).



6. EVOLUTIONARY CONSERVATION OF STEROL-RECOGNITION MOTIFS

The occurrence of consensus cholesterol-recognition motifs covers a wide evolutionary span, from *H. sapiens* back to the bacterial pentameric channels, structural homologs of the nAChR found in prokaryotes, i.e., the cyanobacterium *Gloeobacter violaceus* and its orthologue from *Erwinia chrysanthemi*. Cyanobacteria possess hopanoids, which are structurally and functionally similar to sterols. The remarkable preservation of the CCMs through millions of years in the evolutionary scale has led us to suggest that this domain has important structural and/or functional roles (Barrantes, 2015; Barrantes & Fantini, 2016). In support of this hypothesis is the extensive experimental work showing that mutations in amino acid residues in the TM regions of the nAChR alter channel gating (see review in Barrantes, 2007). Some of these functionally relevant mutations are very close to or within CARC/CARC-like domains. The CCMs may have had other functional roles in prokaryotic AChR-like and other channel-forming proteins, but upon

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appearance of cholesterol in the course of phylogeny, this lipid probably acquired protagonism in eukaryotes for transducing regulatory signals from the plasma membrane to the protein moiety and, concomitantly, cholesterol-recognizing sequences became integrated into the genes coding for many hormone and neurotransmitter receptors as well as channel proteins.

As discussed in preceding sections, the vectorial topography of the CARC sequence makes this motif ideally suited for interaction with cholesterol in the outer leaflet of biological membranes. This is indeed the case with type I membrane proteins, in which the carbon chain enters the membrane bilayer from the extracellular, outer leaflet in the N- to C-terminus direction, as depicted in Fig. 2. The same vectoriality applies to the TM segments 1, 3, 5, and 7 in the GPCRs (Fig. 4), with the corresponding outer leaflet topography for CARC. Since the cholesterol/sphingolipid-enriched domains ("rafts") stem essentially from the tight contacts between these two lipid molecules in the outer membrane leaflet, it is tempting to speculate that one of the possible evolutionary forces leading to the establishment of the CARC sequence was the need to optimize ordered lipid domains in eukaryotic biomembranes.

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