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REVIEW ARTICLE

Cholesterol-Dependent Cellular Processes and Peptides Containing Cholesterol-Binding Motifs: Possible Implications for Medicine

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ABSTRACT

The animal cell is a unique system in which the functioning of its constituent molecules is interdependent and coordinated, and the violation of this coordination is fatal for the cell. One example of this coordination and mutual regulation is the functioning of membrane proteins, whose activity depends on their interaction with membrane lipids. This review reminds us of the crucial importance of the lipid component of cell membranes for normal cell function, and in particular looks at the role of the "cholesterol" component. Given a given genome and a corresponding set of proteins, this lipid component provides a wide range of regulation of cellular functions. The review exemplifies cholesterol-dependent membrane proteins and cellular processes and considers their role in microbial infections and some other pathologies. The concept of cholesterol-recognizing/interacting amino acid consensus (CRAC) motifs in proteins as a possible mechanism of these protein–lipid interactions is discussed. Examples of the use of peptides containing such motifs to modulate cholesterol-dependent processes are presented. In summary, consideration of the cholesterol component in disease pathogenesis and understanding the mechanisms of cholesterol–protein interactions represent a significant resource for the development of drugs that affect the protein–lipid interface. Such drugs may include cholesterol-binding peptides that target specific cholesterol-dependent proteins.

Keywords: cholesterol, cholesterol-dependent proteins, cholesterol-recognizing/interaction amino-acid consensus (CRAC), CRAC motifs, peptides

INTRODUCTION

Major progress in genetic engineering and bioinformatics in recent decades have significantly advanced biological science in understanding the molecular basis for the storage, transmission, and evolution of genetic information contained in nucleic acids. However, while this knowledge is undoubtedly and immensely important, it is not sufficient for understanding the mechanisms of existence and functioning of the living cell. The operation of proteins, the main executors of genetic instructions, depends on their interactions with other molecules constituting a live cell. Functioning of all these molecules is interdependent and coordinated, and a violation of this interdependence and coordination is fatal for the cell.

One example of such coordination and mutual regulation is the interactions of proteins with membrane lipids. With a given genome and a corresponding set of proteins, this lipid component offers a wide range of regulation of cellular functions and provides clues to understanding the regulation of protein activity under different physiological and pathological conditions. Furthermore, without consideration of these protein–lipid interactions, the results of genetically engineered interventions may not always meet expectations precisely because the activity of many proteins depends on their interactions with cholesterol and other lipids. The main goal of this work is to emphasize the crucial importance of the lipid component of cell membranes for normal cell function, and in particular to consider the role of the "cholesterol" component for eukaryotic cell.

The activity of many proteins depends on cholesterol, and impairments in cholesterol homeostasis at the cellular level and at the level of the whole organism can be an important factor in pathogenesis of many diseases, including viral and bacterial infections, as well as stroke, heart disorders, type II diabetes, and neurodegenerative and age-related diseases. Many extensive reviews have been devoted to this issue; see, for example, refs.[1–3]. Cholesterol dependence implies that cholesterol-dependent proteins require an optimal concentration of cholesterol to function properly, so not only can excess cholesterol be harmful and destructive, but also its insufficiency. The fight against high "bad" cholesterol is usually given great importance, while the damaging effects of cholesterol deficiency, which can occur in various metabolic and infectious diseases, may be underestimated.

This review is structured as follows. The first part reminds that cholesterol as an important component of membranes of eukaryotic cells and that many processes in cell are cholesterol dependent. Examples of cholesterol-dependent enzymes, receptors, ion channels and cholesterol-dependent processes are given. The second part considers cholesterol-binding motifs in proteins as a possible mechanism of protein–lipid interactions. Examples of cholesterol-dependent proteins with cholesterol-binding motifs are given. The third part gives examples of cholesterol-binding peptides as regulators/modulators of cholesterol-dependent processes. In the fourth part, the involvement of cellular cholesterol in pathogenesis of some infectious and non-infectious diseases is considered. The main conclusion is that understanding the role of membrane lipids, particularly cholesterol, in the pathogenesis of various human diseases, as well as the mechanisms of cholesterol–protein interactions, is essential for successful therapy of these diseases and can be used to develop new drugs targeting the protein–cholesterol interface.

CHOLESTEROL IN CELL MEMBRANES AND CHOLESTEROL-DEPENDENT PROCESSES

Cholesterol in cell membranes. Cell membranes are composed of lipid bilayers, which are formed from a variety of amphipathic lipids[1–6]. The main component of membranes (about 50% on a molar basis) are phospholipids, complex esters of polyatomic alcohols and higher fatty acids. They contain a phosphate group, glycerol (except for sphingomyelin), and two fatty acyl chains esterified to both the *sn*1 and *sn*2 positions of glycerol. The fatty acyl chains can either be saturated or unsaturated. Important phospholipids are phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylglycerol, sphingomyelin, and cardiolipin. Glycolipids (acylglycerols) are another important class of membrane lipids. They feature at least one carbohydrate moiety linked to their hydrophilic head. Examples of glycolipids are cerebrolysides, which are present in the membranes of human muscle and nerve cells. The third class of lipids found in biological membranes are terpene-derived lipoids, sterols in eukaryotic cells. Cholesterol is one of the best known sterols because of its numerous and vital functions in the vertebrate

cell membrane. It makes up about 40 mol% of the cell plasma membrane and is the second most abundant lipid after phospholipids. And by value, cholesterol is "the central lipid of mammalian cells"[6].

Cholesterol (Fig. 1) contains mostly a structural rigid part, a flexible chain, and a hydroxyl group at the 3β position that provides the amphipathicity to the lipophilic molecule. This hydroxyl group is considered as the anchor for its position in the vicinity of the lipid–water interface, while the rest of the hydrophobic core fits between the hydrophobic chains of the lipids[1–5].

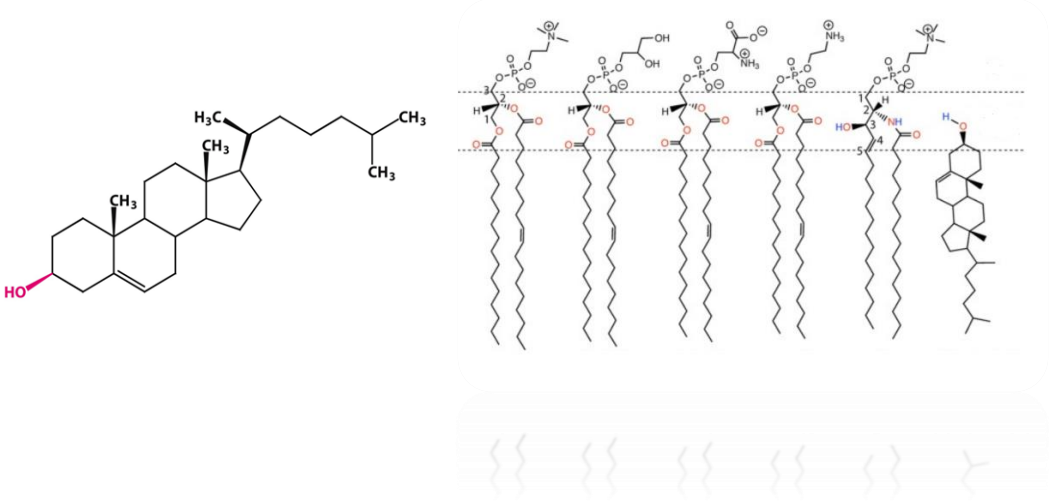

Fig. 1. Cholesterol molecule (a; PubChem CID 5997) and its proposed positioning among other lipids constituting cell membrane[3, 5] (b).

Cells maintain the sterol content of various membranes at very different levels, even though there is extensive vesicular and non-vesicular transport among organelles[6]. The highest content of cholesterol is in plasma membrane (25–50 mol%) and the lowest, in the nuclear membrane and endoplasmic reticulum (1–10 ml%). The endoplasmic reticulum (ER) is the site of much of the regulation of cholesterol levels. It contains ACAT, the enzyme responsible for esterifying excess cholesterol for storage in lipid droplets. It is also the site of cholesterol synthesis, including the key regulated step catalyzed by HMG-CoA-reductase[6]. Deviations from the proper distribution, transport, and metabolism of cholesterol at the cell and whole-body level lead to various pathological conditions[1–4,7].

Cholesterol plays an extremely important role in the organization and functions of membranes, due to its effect on the shape, mobility, and mechanical properties of membranes. Cholesterol affects many physicochemical properties of phospholipid bilayers. Intercalation of hydrophobic cholesterol rings between the acyl chains of phospholipids leads to condensation effects: decreased mobility of acyl chains, decreased fluidity, increased membrane thickness and mechanical stability, and decreased membrane permeability. In addition, cholesterol lowers the temperature at which the membrane transitions to a solid gel state, maintaining membrane fluidity at lower temperatures[1–3,5,6]. Cholesterol also plays an important role in the self-organisation of lipid membranes: due to a particular affinity of cholesterol for sphingomyelin, which has long saturated acyl chains, these two lipids can aggregate and form cholesterol-enriched detergent-resistant membrane (DRM) microdomains, or lipid rafts[8–10]. Certain membrane proteins can accumulate in these lipid microdomains, and the vicinity of these proteins may accelerate and optimize their interactions required for cell metabolism, signalling, or other processes. The importance of rafts in carrying out a variety of biological functions has been demonstrated in many works[8–12].

It has long been observed that various lipids intensely affect the function of membrane proteins[1–3,5,6,8–15]. This lipid regulation ensures the appropriate functioning of the protein depending on the lipid composition of the membrane surrounding the protein. For example, many ion channels are regulated by membrane phosphoinositides[13]; activity of Na^+,K^+-ATPAse is modulated by non-esterified fatty acids and lysophospholipids[14], and the functions of the insulin receptor is modulated by diacylglycerols[15]. As

noted by B. Hille in the review on phosphoinositide-regulated ion channels[13], this lipid regulation ensures proper channel activity and electrical excitability depending on the lipid composition of the membrane surrounding the channel-forming protein. This regulation mechanism certainly applies not just to ion channels but also to other proteins, as well as to other lipids. This review focuses on cholesterol-dependent processes.

Cholesterol-dependent proteins and processes. The activity of many membrane proteins – receptors, ion channels, enzymes, transporters – depends on the presence of cholesterol. One old example is Na^+,K^+-ATPase, a molecular membrane pump that creates a transmembrane gradient of sodium and potassium ion concentrations. The activity of this pump, sensitive to various lipids[14], is largely determined by membrane cholesterol, and cholesterol depletion inhibits Na^+,K^+-ATPase activity[16–19].

Another large group of membrane proteins whose function is regulated by cholesterol are ion channels[20–22], among which are gap junctions that provide intercellular communication[23–26]. Regulation of these channels is of particular significance because of their crucial role in organization of multicellular functional ensembles of excitable and non-excitable cells. The importance of cholesterol dependence of these channels may still be underestimated and insufficiently studied[25].

Membrane receptors involved in hormonal signal transduction and neurotransmission are also regulated by cholesterol, such as, for example, purinergic P2X receptors[27,28], GABAA receptors[29,30], G protein coupled receptors including beta2-adrenergic and serotonin receptors[31–35], as well as acetylcholine receptors[36–38], NMDA receptor[39], glycine receptor[40]. Among cholesterol-dependent receptors there are also phagocytic receptors FcγRIIA[41], scavenger receptor CD36[42–44], and LDL receptor responsible for cholesterol transport and regulation of the cholesterol level in the blood[45]. This is by no means a complete list of cholesterol-dependent molecules; much more can be found in reviews[1,2,5,6,32].

The most important cellular processes are also known to be cholesterol-dependent: adhesion[46], locomotion[47], endocytosis[48,49], and phagocytosis[50–52]. Cholesterol depletion was shown to considerably reduce phagocytic index in macrophages[51]. Synaptic vesicle biogenesis (synaptogenesis) is also cholesterol-dependent[53]: limited cholesterol depletion, which had little effect on total endocytic activity, blocked the biogenesis of synaptic-like microvesicles from the plasma membrane.

As will be discussed further, not only normal physiological processes are cholesterol dependent. Many pathogenic bacteria and viruses use cholesterol and cholesterol-dependent processes to infect cells, and the damage produced by the pathogen to the host organism includes the deterioration in the cholesterol homeostasis. This is well documented both for bacterial[54–57] and viral infections[58,59], including SARS-CoV-2[60–62]. There are also non-infectious diseases in the pathogenesis of which cholesterol plays a prominent role. Understanding the mechanisms of this cholesterol dependence is essential for developing methods to correct the corresponding problems. These mechanisms can be roughly divided into two groups, nonspecific and specific. As was noted above[1–3,5,6], cholesterol affects many physicochemical properties of phospholipid bilayers, such as fluidity, membrane thickness, mechanical stability, membrane permeability, as well as formation of cholesterol-enriched membrane (DRM) microdomains, or lipid rafts[8–12]. Changes of these physicochemical properties of the membrane affect the function of many membrane proteins in a nonspecific way (see a comprehensive review of Song et al, 2014[3] and references therein), which makes it difficult to assess the effect of cholesterol on a particular protein at the living cell level. Another – and much more specific – cause of cholesterol dependence of proteins is the direct interaction of protein with cholesterol, leading to the formation of saturable stoichiometric complexes, in which cholesterol can act as an allosteric modulator of protein function. One of the proposed cholesterol-binding amino acid sequences in proteins is the so called CRAC motif, which will be discussed in the next section.

CHOLESTEROL-BINDING MOTIFS IN PROTEINS AS A POSSIBLE MECHANISM OF PROTEIN–LIPID INTERACTIONS

Cholesterol-binding (CRAC) motifs. A possible mechanism of interaction between proteins and cholesterol is that proteins have special sites, or motifs, that specifically interact with cholesterol molecules. One variant of the structure of such sites, called the cholesterol recognition/interaction amino acid consensus (CRAC), was proposed by Li and Papadopoulos in 1998[63]. The authors investigated the mechanism of cholesterol transport from the

outer mitochondrial membrane to the inner membrane, where cholesterol, a precursor of steroid hormones, undergoes appropriate enzymatic processing. Back in 1990, this group of authors published a paper in JBC[64], which showed that in cells responsible for steroidogenesis (in the adrenal glands), this transfer of cholesterol is performed by the mitochondrial peripheral benzodiazepine receptor protein, PBR, later renamed the translocator protein TSPO[65]. In the paper[63] the authors reported that a cytoplasmic C-terminal domain with the amino acid sequence ATVLNYYVWRDNS in the PBR/TSPO protein is required for cholesterol binding.

The authors also studied the primary sequences of other proteins relevant to cholesterol transport and metabolism (apolipoprotein A1, caveolin, annexin, steroid metabolism enzymes, etc.) and found motifs that were similar to the cholesterol-binding sequence from PBR. The authors proposed a general formula L/V-(X)$_{(1-5)}$-Y-(X)$_{(1-5)}$-R/K and called it "cholesterol-recognition/interaction amino acid consensus sequence" (CRAC), which we will also call here cholesterol-binding motif.

In subsequent discussions, variations of this formula and its name (sterol-sensitive motif (SSM), cholesterol consensus motif (CCM))[1-3,32,66-69] have been proposed, but the general idea has remained: for successful interaction of a protein with cholesterol a certain sequence of amino acids with non-polar (hydrophobic) (isoleucine Ile, valine Val, leucine Leu), aromatic (tyrosine Y, tryptophan W, phenylalanine F) and positively charged (arginine R or lysine K) side chains is required. These motif-forming amino acids are separated by short segments of any 1–5 amino acid residues. So, the general formula for the CRAC motifs, presumably involved in the interaction of protein with cholesterol, presently can be written as follows: V/L/I–X$_{(1–5)}$–W/Y/F–(X)$_{(1–5)}$–R/K, where X stands for any amino acid residue. Although a predictive accuracy of this formula remains the subject of debate, the presence of this motif in many proteins and its involvement in protein-cholesterol interactions has been confirmed by various methods[1,2,3,63,66–69]. Obviously, the formula of the cholesterol-interacting motif can be further developed; what is important is the very concept that a cholesterol-dependent protein has a specific site interacting with cholesterol, so that this interaction leads to changes in the protein function.

Examples of proteins containing CRAC motifs. Further studies revealed the presence of CRAC motifs in many cholesterol-dependent proteins[3,5] and the role of these motifs in cholesterol dependence of the protein was scrutinized. For example, in a study by Singh et al.[70], the structural basis for cholesterol inhibition of large conductance Ca^{2+}- and voltage gated K^+ channels, also known as "big potassium" (BK) channels, was investigated by a combination of MD simulations, site-directed mutagenesis, and single-channel electrophysiology. The study showed that cholesterol action is mediated by the cytosolic C-tail domain of the BK channel-forming protein Cbv1, in which seven CRAC motifs were present and were involved in cholesterol sensing and in the cholesterol–channel interaction. Studies of cholesterol binding sites in another type of K^+ channels, inwardly rectifying potassium channels (Kir2.1)[71], showed that cholesterol modulates channel function by affecting the transmembrane helix limiting the pore, which is responsible for gating the channel. Ionotropic receptors nAChRs directly linked to ion channels have CRAC motifs adjacent to the transmembrane helix adjacent to the cholesterol-binding cavity.[72,73]

Cholesterol-binding motifs are also present in proteins involved in Ca signalling. The influx of Ca^{2+} via store-operated Ca channels (SOCs) is a central component of receptor-induced Ca signals. Orai channels are SOCs that are controlled by STIM1. STIM1 is a Ca^{2+} sensor located in the endoplasmatic reticulum (ER). All Orai channels are activated by the conserved amino acid fragment STIM1 344-442, which is called SOAR (STIM1 Orai activating region). Pacheco et al, 2016[74] showed that the calcium sensor in the ER (STIM1) has a CRAC motif located inside the SOAR region and showed the functional association of STIM1 and SOAR with cholesterol and the change of the SOAR interaction with Orai when cholesterol is depleted. Another group of store-operated Ca channels, Ca-release-activated channels, also have cholesterol-binding motifs[75]. These data suggest a significant role for cholesterol in Ca signalling, which is involved in exocytosis, motility, apoptosis, excitability, transcription, and other vital processes in the cell.

Thus, the cholesterol binding motif (CRAC) has been detected in many cholesterol-dependent proteins, and the involvement of this motif in the function of cholesterol-dependent proteins has been demonstrated by various methods. Yet another way to test and develop the idea of cholesterol-binding

motifs in cellular proteins is to design peptides containing such motifs and study their effect on cholesterol-dependent cellular functions.

EXAMPLES OF PEPTIDES CONTAINING CRAC MOTIFS AND MODULATING CHOLESTEROL-DEPENDENT PROCESSES

CRAC-containing peptides of viral origin. If the concept of cholesterol-binding motifs is correct and such motifs in proteins are involved in protein–cholesterol interactions, then a small peptide containing such motif(s) can compete with this protein for cholesterol, interfere with the protein–cholesterol interactions, and thus modulate the cholesterol-dependent activity of the whole protein. Peptides containing such motifs may prove to be a useful tool not only for research but also for the regulation of cholesterol-dependent processes in cells. The possibility of such regulation in vitro was directly demonstrated by the authors of the CRAC-concept in 2001[76]. It was reported that cell-penetrating peptide TAT-CRAC containing cholesterol-binding motif ATVLNYYVWRDNS inhibited the hCG- and cAMP-stimulated steroid production in Leydig cells MA-10 in a dose-dependent manner. Mutated TAT-CRAC lost its ability to bind steroids and to inhibit the hCG-stimulated steroidogenesis.

Another example of an efficient action of a peptide containing cholesterol-binding motif is a viral protein derived peptide C5A, containing amino acid residues 3–20 of the amphipathic α-helical N-terminal domain of hepatitis A virus protein NS5A[77]. Peptide C5A suppressed the virus replication by more than 5 orders of magnitude. Later, the antiviral activity of this peptide C5A against HIV was also demonstrated[78]. The authors did not mention CRAC motifs; however, the active peptide C5A obviously contains an amino-acid sequence corresponding to the CRAC formula: SWL**R**DIWD**W**ICE**VL**SDFK (motif-forming amino acids are shown in bold, the CRAC motif is underlined). It is possible that peptide C5A inhibits the formation of the viral particle owing to the presence of the cholesterol-binding motif that competes for cholesterol with viral protein.

Matrix protein M1 of influenza virus was also shown to possess cholesterol-binding domains, and their importance for virion structure organization was shown[79,80]. Later it was found[81] that a 26-aa peptide P4 (RT**KLWEML**VELGNMDKA**VKLWRK**LKR) constructed of two short CRAC domains of protein M1 modulates cholesterol-dependent activity of macrophage-like cells IC-21 and is cytotoxic at a concentration of about 50 μM. Substitution of the motif-forming amino acids in P4 abolished the cytotoxic effect of the peptide[82]. Cell cholesterol depletion using methyl-β-cyclodextrin lowered the toxic concentration of P4 by an order of magnitude, which confirms the cholesterol-dependence of the P4 impact and a protective role of cholesterol against the toxic effect of P4. The cytotoxic effect of M1-derived peptide P4 can be explained by sequestration of membrane cholesterol by the peptide and/or dysfunction of cellular cholesterol-dependent proteins. The observed effects of CRAC peptides of viral origin may reflect the influence of viral proteins with CRAC motifs on the infected cells. The formation of a viral envelope with a high cholesterol content occurs with the participation of CRAC-containing viral proteins that sequester cholesterol from cell membranes during viral envelope formation[83–88]. Interestingly, the activity of peptide P4 was significantly higher than its constituent α-helices of the influenza virus M1 protein[89]. This can be explained by the fact that short peptides containing one CRAC motif each could not reach this interface and therefore could not have the expected action on membrane proteins, while in the P4 peptide the CRAC-containing α-helices could be oriented as in the tertiary structure of protein M1 and could interact more efficiently with the cell membrane in the protein–cholesterol interface.

Remarkably, S proteins of coronaviruses SARS-CoV and SARS-CoV-2 also contain cholesterol binding motifs[90–92] (see discussion in[82]). In the case of the S protein of coronavirus SARS-CoV, this motif YI**K**WP**W**Y**V**W is located in the "aromatic" region of the transmembrane domain of the S protein; this highly conserved region of the S protein was shown to be necessary for the infection of cells with coronavirus[90–92]. S protein of SARS-CoV-2 contains overall, 15 CRAC motifs V/L/I__W/Y__R/K, including three "double-CRAC" motifs (Fig. 2): **L**PPA**Y**TNSFT**R**G**V**Y**Y**PD**K** (25L-34R_36V-41K); **V**SNGTH**W**FVTQ**R**NF**Y**EPQ**I** (1096V-1107R_ 1107R-114I), and **L**GK**Y**EQY**IK**WP**W**Y**I**WLGFI (1203L-1211K_1211-1221I). At least some of these motifs can be involved in the formation of cholesterol-rich viral envelope from the host membranes, and the deteriorating effect of SARS-CoV-2 infection and

massive cell death can be related with depletion of cholesterol from ER and other membranes of the host cells during the formation of new viral particles[88]. The presence of CRAC motifs in S protein and their ability to bind cholesterol was also shown in[93, 94]. It can be expected that the sequestration of host cell cholesterol by viral proteins will be suppressed by molecules that prevent the interaction of the viral cholesterol-binding motif with cell cholesterol. Cholesterol-binding peptides could be among such molecules. Design of such peptides on the basis of our knowledge about the cholesterol binding motifs in viral proteins and testing the ability of such peptides to affect the viral replication would be very useful for verification of the CRAC hypothesis and further development of antivirals.

```
ORIGIN
        1 mfvflvllpl vssqcvnltt rtqlppaytn sftrgvyypd kvfrssvlhs tqdlflpffs
       61 nvtwfhaihv sgtngtkrfd npvlpfndgv yfasteksni irgwifgttl dsktqslliv
      121 nnatnvvikv cefqfcndpf lgvyyhknnk swmesefrvy ssannctfey vsqpflmdle
      181 gkqgnfknlr efvfknidgy fkiyskhtpi nlvrdlpqgf saleplvdlp iginitrfqt
      241 llalhrsylt pgdsssgwta gaaayyvgyl qprtfllkyn engtitdavd caldplsetk
      301 ctlksftvek qiyqtsnfrv qptesivrfp nitnlcpfge vfnatrfasv yawnrkrisn
      361 cvadysvlyn sasfstfkcy gvsptklndl cftnvyadsf virgdevrqi apgqtgkiad
      421 ynyklpddft gcviawnsnn ldskvggnyn ylyrlfrksn lkpferdist eiyqagstpc
      481 ngvegfncyf plqsygfqpt ngvgyqpyrv vvlsfellha patvcgpkks tnlvknkcvn
      541 fnfngltgtg vltesnkkfl pfqqfgrdia dttdavrdpq tleilditpc sfggvsvitp
      601 gtntsnqvav lyqdvnctev pvaihadqlt ptwrvystgs nvfqtragcl igaehvnnsy
      661 ecdipigagi casyqtqtns prrarsvasq siiaytmslg aensvaysnn siaiptnfti
      721 svtteilpvs mtktsvdctm yicgdstecs nlllqygsfc tqlnraltgi aveqdkntqe
      781 vfaqvkqiyk tppikdfggf nfsqilpdps kpskrsfied llfnkvtlad agfikqygdc
      841 lgdiaardli caqkfngltv lpplltdemi aqytsallag titsgwtfga gaalqipfam
      901 qmayrfngig vtqnvlyeng klianqfnsa igkiqdslss tasalgklqd vvnqnaqaln
      961 tlvkqlssnf gaissvlndi lsrldkveae vqidrlitgr lqslqtyvtq qliraaeira
     1021 sanlaatkms ecvlgqskrv dfcgkgyhlm sfpqsaphgv vflhvtyvpa qeknfttapa
     1081 ichdgkahfp regvfvsnqt hwfvtqrnfy epqiittdnt fvsgncdvvi givnntvydp
     1141 lqpeldsfke eldkyfknht spdvdlgdis ginasvvniq keidrlneva knlneslidl
     1201 qelgkyeqyi kwpwyiwlqf iagliaivmv timlccmtsc csclkgccsc gscckfdedd
     1261 sepvlkgvkl hyt
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FIG. 2. Surface glycoprotein of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2]. NCBI Reference Sequence: YP_009724390.1. CRAC motifs are underlined.

Antimicrobial peptides with CRAC motifs. A separate group of CRAC-containing peptides with proven biological effects are antimicrobial peptides (AMPs). AMPs are known as amphipathic peptides that usually contain cationic and hydrophobic domains and have an antibacterial activity. AMPs are produced by many bacteria and are also produced in response to microbial invasion into various multicellular organisms (invertebrates and vertebrates, as well as plants) and, therefore, are one of the most important natural components of the humoral immune system[95–102]. Analysis of published data[101–105] shows that many antimicrobial peptides of different origin have cholesterol-binding motifs, which may indicate the participation of sterols in the mechanism of action of these AMPs[105]. This aspect is not always considered in studies devoted to AMPs, although CRAC motifs are present in many AMPs (for example,[103–106]). Such motifs are present in protegrins and their derivatives, which represent a new class of peptide antibiotics based on mammalian antimicrobial peptides[103]. Protegrins contain 16–18 amino acids and well-defined CRAC motifs (for example, protegrin 1, RGGR**L**C**Y**C**R**R**F**C**R**V**CVGR, and protegrin 4, RGGR**L**C**Y**C**R**G**W**ICFC**V**GR). CRAC motifs are also present in AMPs from frog skin, magainins[104] (magainin 1, G**I**GK**F**LHSAG**K**FG**K**A**F**VG**E**IMKS; magainin 2: G**I**GK**F**LHSA**K**KFG**K**A**F**VG**E**IMNS), and in some temporins[105,106]. Among the temporins studied, temporin L containing the CRAC motif (F**V**QW**F**S**K**FLGRIL) has the greatest antibacterial activity against bacterial and fungal strains[106].

In[107] it was shown that peptide RT**K**L**W**EM**L**VELGNMDKA**V**K**L**W**R**K**L**KR (P4)[81,82] containing cholesterol binding motifs from viral protein also exhibits antibacterial activity. The toxic concentration of P4 for Gram-negative *E. coli* was an order of magnitude lower than for macrophages, containing more cholesterol than the bacteria. The "scramble" peptide nScr containing

the same amino acids as P4, but in a random order, and lacking CRAC motifs, was not toxic either for *E. coli* or macrophages[107]. The membranes of many Gram-negative bacteria, including *E. coli,* contain sterols, such as cholesterol or its derivatives and/or hopanoids that exhibit steroid-like properties and can form liquid-ordered lipid domains in the bacterial membranes, just like cholesterol does in animal cells[108–111]. In Gram-negative bacteria, sterols/hopanoids play an important role in the organization and functions of membranes, due to its effect on the shape, mobility, and mechanical properties of membranes. In the case of Gram-negative bacteria, the mechanisms of antibacterial action of AMP containing cholesterol-binding motifs can be similar to the mechanism of cytotoxic effect of peptides containing cholesterol-binding motifs in the case of eukaryotic cells: CRAC-containing peptide can compete with cholesterol-dependent membrane proteins for binding to cholesterol and, by sequestering cholesterol, cause malfunctioning of these proteins and/or cause deleterious permeabilization of the cell membrane. The higher sensitivity of *E. coli* to P4 as compared to macrophages agrees well with the protective role of cholesterol[81,82, 112–114] and, on the one hand, provides a good "therapeutic window" for AMPs, which are not toxic for eukaryotic cells at the concentration that is toxic to the bacteria. Moreover, we have found that at a dose, which was toxic to bacteria, P4 moderately stimulated macrophage activity[81,82,107]; this means that peptide P4 can combine antimicrobial and immunostimulatory effects.

It should be noted that in the case of Gram-positive bacteria the observed toxic effect of CRAC-containing AMP cannot be explained by the presence of CRAC motif and its interaction with sterols, which are absent in the bacterial membrane of Gram-positive bacteria. In[107], we found that the CRAC-containing peptide P4 was also extremely toxic to the Gram-positive bacteria *B. subtilis* (IC50, 0.1 microM). However, the "scrambled" peptide nScr, containing the same amino acids as P4 but in a random order and lacking CRAC motifs, was also toxic to *B. subtilis*, indicating that the CRAC motif is not involved in this effect. Apparently, the toxicity of the two peptides, P4 and nScr, for *B. subtilis* could be due to the presence of cationic amino acids in both amphipathic peptides, which contain 8 cationic amino acids (30% of the total number of amino acids comprising peptide). As was shown in[115], such peptides containing cationic amino acids interact with anionic phospholipids such as phosphatidylglycerol, phosphatidylserine, and cardiolipin present in Gram-positive bacteria and cause the formation of liquid-unordered domains in the bacterial membrane, which leads to a decrease in membrane potential and a fatal increase in membrane permeability of *B. subtilis*. Thus, the presence of a cholesterol-binding motif in an antimicrobial peptide is not yet sufficient to explain the effect of this peptide. On the other hand, the irrelevance of the motif in the antimicrobial effect in some cases cannot exclude its involvement in other conditions. Appropriate control experiments are needed to understand the mechanisms of the effect.

Overall, the accumulated data indicate that peptides containing CRAC motifs exist and can be active in regard of cholesterol-dependent processes, at least in the *in vitro* conditions. Cytotoxicity of peptide P4 derived from virus protein may imply that CRAC-containing viral proteins can have a similar cytotoxic effect on host cells *in vivo*. The antiviral activity of virus-derived CRAC-containing peptide C5A[77,78] may be due to interference of the peptide with interactions of viral proteins and cell membrane cholesterol, which inhibits virus replication. Furthermore, antibacterial effects of CRAC-containing peptides, as well as the presence of CRAC motifs in many naturally occurring AMPs suggests that protein–sterol interface is an essential element in the microbe–host interactions[107]. Therefore, peptides carrying CRAC motifs can be considered as promising antiviral and antibacterial agents. The fact that not all CRAC-containing peptides interact with cholesterol does not invalidate numerous data on the key role of such motifs in protein–cholesterol interactions. Also, the current formula of CRAC motif does not exclude other versions of cholesterol-binding motifs. The design of new peptides requires a combination of molecular dynamics methods and appropriate experimental testing of the peptides.

THE PATHOGENESIS OF SOME INFECTIOUS AND NON-INFECTIOUS DISEASES IS RELATED TO THE IMPAIRMENT OF CELLULAR CHOLESTEROL HOMEOSTASIS

Infectious diseases and cholesterol. As was mentioned above, many bacterial[54–57] and viral[58–62,88] pathogens use cholesterol and cholesterol-dependent processes to infect cells. Interactions of enveloped viruses with the cell during penetration, assembly, budding, and exit from the cell are

known to depend on the presence of cholesterol and lipid rafts in the membranes of the host cells. This has been shown for immunodeficiency viruses (HIV), influenza, herpes, hepatitis C virus (HCV), rotavirus, Yellow fever virus, Zika virus, Dengue virus, West Nile virus, and many other (reviewed in[88]). Moreover, virus–cell interactions lead to significant modulations in the lipid composition of cell membranes. The issues concerning the importance of the host cell membrane cholesterol at different stages of the virus life cycle, as well as the impact produced by viruses on cellular lipids and cholesterol in particular, have been addressed in many works[116–128]. An illustrative example is West Nile virus[116], an enveloped RNA virus, that modulates host cell cholesterol homeostasis by upregulating cholesterol biosynthesis and redistributing cholesterol to viral replication membranes. Virus-induced redistribution of cellular cholesterol downregulated cellular antiviral response to infection, and exogenous addition of cholesterol counteracted this effect[116]. The main conclusion from these works is that in a virus-infected cell, the entire cholesterol homeostasis system is reconfigured to form new viral particles. As a result, cell membrane composition and properties, as well as the functioning of cholesterol-dependent proteins can be violated.

It is noteworthy that, as virus entry requires cholesterol, lowering cholesterol can prevent virus entry, and therefore cholesterol-lowering drugs such as statins can reduce the effectiveness of virus entry[118,119,121,122,124–127]. However, at the virus replication stage, because of the formation of the membrane of new viruses, cholesterol levels in the membrane of the infected cells can drop to dangerously low levels. A decrease in cholesterol in cell membranes owing to the formation of viral envelopes can be one of the most harmful consequences of the virus particle assembly, as the amount of cholesterol removed from the cell membranes by newly formed viruses can exceed the compensatory resources of the cell[88]. If the delivery of cholesterol to the cells is insufficient, deregulation of cholesterol-dependent processes can lead to massive cell death, which manifests itself in the clinical course of the disease and a poor prognosis. In this connection, it should be noted that in patients infected with SARS-CoV-2, a significant decrease (several fold) in total cholesterol and low-density lipoprotein (LDL) cholesterol levels was recorded[129–131], and cholesterol-lowering treatments (such as statins) did not seem advisable for Covid-19 patients with life-threatening infection, at least until they recover from the infection[88,130]. Such a drop of the LDL cholesterol level in Covid-19 patients can reflect an enhanced recruitment of circulating cholesterol by the cells to compensate for its loss associated with virus reproduction. Perhaps the clinical prognosis depends on the timely and successful delivery of cholesterol required for cell membrane repair. Another direction in the development of drugs for the treatment of the disease is the search for agents that interfere with the interactions of viral proteins with cholesterol, and this search should be based on an understanding of the mechanisms of these interactions.

In addition to viral infections, various bacterial infections also affect cholesterol homeostasis, which may be an important component of the disease pathogenesis. One example is *Helicobacter pylori*, a Gram-negative bacterium, that causes chronic gastritis and predisposes to gastric cancers. Gastric colonization by this bacterium is cholesterol dependent[132–134]. *H. pylori* does not synthesize cholesterol on its own but extracts it from the plasma membranes of the gastric epithelial cells, which leads to their disruption[133,134]. Mycotic diseases are also often associated with alterations in cholesterol homeostasis, as pathogenic fungi utilize cholesterol from host cells[51,135,136].

Another classical example of such infection related with host cell cholesterol depletion is tuberculosis. *Mycobacterium tuberculosis* enters the cell by phagocytosis and remains in the phagosome, using the cell's cholesterol as a carbon source and redirecting cholesterol homeostasis to its needs[137,138,139]. During a chronic infection, *M. tuberculosis* further destroys cells, at least partly due to cholesterol depletion. As with viral infections, timely and sufficient delivery of cholesterol is necessary to maintain the integrity of the host cell membrane. It can be recalled that in the pre-antibiotic era, tuberculosis was treated with an enriched diet high in fats and sterols.

One more typical example of cholesterol-dependent infection is malaria[140–143]. Plasmodium is not capable of de novo biosynthesis of fatty acids and cholesterol and gets them from the vertebral host. Metabolic activity of the parasite leads to changes in the amount of fatty acids and cholesterol in the erythrocyte plasma membrane and a

decrease in the cholesterol/phospholipid ratio, and these alterations are responsible for changes in erythrocyte permeability and fragility. Studies of Samuel et al, 2001[143] proved that cholesterol from the erythrocyte plasma membrane rafts is essential for the invasion and growth of *P. falciparum* and demonstrated the involvement of cholesterol from erythrocyte membrane raft in the protozoan infection, which was blocked following raft cholesterol disruption. Interestingly, plasmodium creates a cholesterol gradient in the erythrocyte, so that cholesterol from the erythrocyte membrane, where the cholesterol concentration is higher, could flow to the parasitiform vacuole membrane and then to the parasite membrane, where the cholesterol concentration is lowest[142]. This gradient is created by the removal of cholesterol from the parasite membrane by protein NCR1, the Niemann–Pick type C1-related protein, the human orthologue of which, the Niemann-Pick Type C1 protein (NPC1), binds cholesterol. Inhibition or genetic knock-down of NCR1 is lethal to the parasite. These findings demonstrate that cholesterol plays multiple roles in the Plasmodium growth in erythrocytes.

Thus, even a concise review of the literature shows that at least some viral and bacterial infections, as well as diseases caused by pathogenic fungi and protozoas, have a distinct cholesterol component, which can determine the severity and outcome of disease. Therefore, it is not surprising that some of the drugs used to treat these conditions are similar. One example is chloroquine, a classic antimalarial drug that has proven effective in treating Covid-19[144]. Other examples are polyphenolic substances like quercetin[145] and saponin glycyrrhizin/glycyrrhizinic acid, which has antiviral[146,147], anti-tuberculosis[148], and antimalarial[149] effects. These examples may indicate that in such different diseases these drugs have a similar target, namely the protein–cholesterol interface, and apparently hinder cholesterol binding to the pathogen protein that sequesters cholesterol from the membrane of an infected cell.

However, such agents are not very selective and can affect other cholesterol-dependent proteins and therefore cause side effects. Perhaps specially designed peptides with CRAC motifs corresponding to those in the target protein could specifically interfere with the interactions of the pathogen protein with host membrane cholesterol and thus prevent the loss of cellular cholesterol. It seems appropriate therefore to test for the presence of CRAC motifs in pathogen proteins involved in host cholesterol recruitment. The ability of CRAC-containing peptides to regulate cholesterol-dependent cell functions has been demonstrated in a number of works[76,81,82,107,150,151], and further studies of the activity of these peptides may be useful and promising.

Another important aspect in treatment such diseases is to maintain a safe level of cholesterol in the cell membranes to prevent their permeabilization and destruction due to sequestration and removal of membrane cholesterol by the pathogen. As was mentioned above, significant decrease in total cholesterol and low-density lipoprotein (LDL) cholesterol levels in COVID-19 patients[129,130,131] may be indicative of the critical loss of cholesterol by cells, and an efficient cholesterol delivery to the cholesterol-depleted host cells should be helpful. Cyclodextrins are possible candidates as non-toxic cholesterol transporters[152–154], which can redistribute cholesterol from endogenous and/or exogenous sources and deliver it to cholesterol depleted cells. The use of cyclodextrins increased the lifespan of NPC1−/− experimental mice[155] and improved the condition of patients with Nieman–Pick disease[156]. In diseases caused by cholesterol-consuming pathogens, when cell membranes lose cholesterol to dangerously low levels, it seems necessary (and perhaps critical) to compensate for the loss of cholesterol, including through proper nutrition, for a more successful recovery.

Cholesterol and some non-infectious diseases. The cholesterol component is found not only in the pathogenesis of some viral, bacterial, fungal, or protozoan infections, but also in some cardiovascular and neurodegenerative diseases as well, often age-related, such as atherosclerosis[157,158], Alzheimer disease[1,159–163], Huntington's disease[159,160,164], Parkinson disease[159,160,165], amyotrophic lateral sclerosis[166,167], type 2 diabetes[168], and many others. This subject deserves a separate review and is only mentioned here to illustrate the ubiquitous and versatile role of cholesterol in implementation of normal cell functions. The role of cholesterol in these diseases is quite intricate and not necessarily has to do with increased concentrations of HDL- and LDL-cholesterol in the blood. For example, although cholesterol-lowering drugs have been considered

as potential drug candidates for the prevention of Alzheimer disease[169,170], experimental and computational studies suggested a protective role of cholesterol in terms of Aβ fibril formation[161], as cholesterol was shown to prevent Aβ from leaving the membrane environment and entering solution. Besides, one of the main features of AD is the disfunction of cholinergic system, and acetylcholine receptors are known to be cholesterol and raft dependent[36–38,162,163], so that cholesterol depletion may impair the functioning of cholinergic system.

The significance of cholesterol and rafts has also been shown in Huntington's disease, characterized by neurodegeneration of the striatum and cortex. Studies of postsynaptic membranes showed that the disease marker protein hanttingin (htt) binds to lipid domains[164]. Dramatic alterations in cholesterol metabolism were described in amyotrophic lateral sclerosis[166,167].

Of particular interest in this context are experiments of Fukui et al, 2015[168], who showed that cholesterol depletion in neuron-derived cells, mimicking the reduction of cellular cholesterol in diabetes, results in impaired insulin/IGF-1 and neurotrophin signalling and causes defects in signal transduction and function in neuron-derived cells. The authors concluded that reduced brain cholesterol, similar to that observed in diabetic brain, could contribute to CNS-related complications of diabetes, including increased risk of neurodegenerative diseases, such as Alzheimer disease[161–163,169,170]. In should be mentioned here that persistent cholesterol depletion caused by microbial infections may also cause neurological problems and trigger neurodegeneration processes.

The aforementioned Niemann-Pick disease[156,171] is also a prime example of neurodegeneration associated with cholesterol deficiency caused by impaired cholesterol delivery to target cell membranes. This defect is associated with inactivating mutations in the transmembrane cholesterol transporter NPC1, which is located on the lysosomal membrane and exports cholesterol released from low-density lipoproteins (LDL) to the acceptor compartments (endoplasmic reticulum, Golgi and plasma membrane). In cells devoid of functional NPC1, cholesterol accumulates in lysosomes and is not transported to these compartments, which leads to deterioration of cell structure and function.

Thus, cholesterol is one of the most important lipids involved in vital cell processes, involving modulation of gene expression, membrane compartmentalization, signal transduction, communication between cells, and many others. Because of its structural and physiological role, changes in its metabolism can cause various abnormalities including brain function. This applies to neurodegenerative pathologies such as Niemann-Pick disease, Alzheimer's disease, Huntington's disease, Parkinson's disease and amyotrophic lateral sclerosis, and other diseases where significant disturbances in membrane lipid composition and cholesterol and other lipid homeostasis have been reported. These changes affect the functions of cholesterol-dependent and raft-dependent proteins, which may be an important component of the pathogenesis of neurodegenerative diseases. The development of technologies enabling the study protein–cholesterol interactions and their physiological correction will contribute to the identification of new therapeutic targets for the treatment of these diseases.

CONCLUSION

Cholesterol is an important component of cell membranes, which regulates the activity of many proteins and cell processes. Many cholesterol-dependent proteins contain cholesterol-recognizing/interacting amino acid consensus (CRAC) motifs, which are considered to represent one of the mechanisms of protein–cholesterol interactions. This concept is supported by the ability of CRAC-containing peptides of different origin to interfere with cholesterol-dependent processes in cells. The cholesterol component is present in many infectious and non-infectious diseases, and in some of them, a decrease in cholesterol levels below the optimal physiological level can be detrimental for cells and can be a significant pathogenesis factor worsening the prognosis. Therein lies the difficulty, and it must be carefully studied, so that the struggle to reduce "bad cholesterol" does not turn out to be deteriorating. Among the drugs used to treat diseases exhibiting the cholesterol component there are low-molecular-weight substances that apparently act at the cholesterol—protein interface. Design of peptides containing cholesterol-binding motifs can be another perspective direction for the development of new drugs to treat such diseases. This requires a deeper understanding of

the mechanisms of cholesterol dependence and the interaction of proteins with cholesterol.

CONFLICTS OF INTEREST STATEMENT

The author has no conflicts of interest to declare

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