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Cholesterol Recognition Motifs (CRAC) in the S Protein of Coronavirus: A Possible Target for Antiviral Therapy?

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Abstract

Some interactions of enveloped viruses with the host cell membrane have a cholesterol-dependent component, which may account for clinical manifestations of the infectious disease and can be used for the development of antiviral drugs. These cholesterol-dependent interactions can be mediated by cholesterol-recognition amino-acid consensus (CRAC) motifs present in viral proteins. The S protein of the SARS-CoV and SARS-CoV2 coronaviruses contains CRAC motifs that can be involved in the process of virus entry into the cell. Besides, during viral envelope formation, CRAC motifs can be responsible for binding of cell membrane cholesterol, leading to depletion of cell membrane cholesterol and subsequent malfunctioning of cellular cholesterol-dependent proteins, destabilization and permeabilization of cell membranes and, ultimately, to the death of infected cells. Understanding the mechanisms of cholesterol-dependent virus–cell interactions and the role of CRAC-containing viral proteins in the pathogenesis of the disease can serve as the basis for the development of new drugs that prevent both coronavirus entry into the cell and the damage of the infected cell during the viral morphogenesis. The target for such drugs can be the S-protein/cholesterol interface. CRAC-containing peptides derived from viral proteins may be among these agents. These peptides can also be used as experimental tools to study cholesterol-dependent virus–cell interactions.

Keywords: peptides, cholesterol-recognition motif (CRAC), CRAC-containing peptides, coronavirus, S-protein, SARS-CoV2, COVID-19

1. Introduction

The COVID-19 pandemic caused by the SARS-CoV2 coronavirus has resulted in almost hundred of millions of infections and about two millions of deaths in 2020 [1, 2]. According to the WHO data as of January 4, 2021, there have been 85 929 428 confirmed cases of COVID-19 reported to WHO, including 1 876 100 deaths [2]. It has been shown that in patients with laboratory-confirmed COVID-19, clinical results correlate with the presence of concomitant diseases, among which hypertension and diabetes mellitus, as well as old age, atherosclerosis, cardiovascular and cerebrovascular diseases worsening prognosis [3–9]. Notably, these factors are commonly associated with the impairments in the lipid/cholesterol metabolism and transport or are their direct consequence [3–5].

At present, a lot is known about the coronavirus SARS-CoV2. It is an enveloped single-stranded RNA virus belonging to the Betacoronavirus genus of the Coronaviridae family. The virus contains a 30 kb genome encoding four major viral structural proteins: spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins [9, 10]. According to modern concepts, the cellular receptor for S protein is angiotensin converting enzyme 2 (ACE2); binding of the S protein to this receptor allows the internalization of the virus and triggers the disease. Significant progress has been made in the development of vaccines against SARS-CoV2 and mass vaccination of people in different countries begins [11, 12]; this gives hope that the pandemic will stop. However, the issues of treating patients with various forms of COVID-19 and the development of drugs based on knowledge of the mechanisms underlying this pathology still need to be addressed. This chapter is mainly focused on the lipid aspects of the interactions of coronaviruses with host cells and, in particular, draws attention to the fact that interactions with host cells of many enveloped viruses, including coronaviruses SARS-CoV and SARS-CoV2, are cholesterol dependent. Moreover, these interactions lead to significant and potentially deleterious alterations in the cholesterol status of the infected cells. These cholesterol-dependent processes play a significant role both at the stage of the virus entry and during the development of severe respiratory syndrome (SARS) and other health problems caused by coronaviruses SARS-CoV and SARS-CoV2. Therefore, understanding this component is necessary for the development of additional approaches both to prevention and treatment of these diseases, and the attempts in this direction are being made (e.g., [13–15]). This review focuses on the fact that the coronavirus S protein, which is involved in cholesterol-dependent virus–cell interactions during entry and replication stages, contains the so-called cholesterol recognition amino-acid consensus (CRAC) motifs [16, 17] that can actually mediate these interactions. A hypothesis is put forward suggesting that binding of cell membrane cholesterol by CRAC-containing S protein (and possibly by other viral proteins) and subsequent removal of cholesterol from intracellular membranes by newly formed viral particles can affect normal functioning of cellular cholesterol-dependent proteins (receptors, ion channels, enzymes, etc.) and can eventually cause cell death due to destabilization and permeabilization of cell membranes. This deteriorating effect of CRAC-containing viral proteins can be counteracted by agents that prevent binding of membrane cholesterol to viral proteins and/or compensate for the membrane cholesterol depletion produced by the forming viral particles. It is possible that specially designed CRAC-containing peptides that specifically block interactions of S protein with cholesterol can expand the range of antiviral agents.

2. Some interactions of enveloped viruses with host cells are cholesterol-dependent

The viral life cycle includes four steps: entry, replication, assembly, and egress [18–21] (**Figure 1**). At the entry step, an enveloped virus binds to a target receptor, the viral envelope fuses with the host cell membrane, and the viral nucleic acids are released into the cytoplasm. At the replication step, the nucleic acid is replicated in cytoplasmic replication organelles and viral proteins are synthesized. At the assembly step, viral proteins and nucleic acids are packed into a viral particle and the viral envelope is formed. At the egress step, mature viral particles leave the cell through the cellular membrane [18–21].

Some interactions of enveloped viruses with the cell in the course of penetration and during assembly, budding, and exit of the virus from the cell are known to

Figure 1.

Life cycle of an enveloped virus as exemplified by coronavirus (based on [18–21]). To enter a cell, virus binds via S protein to the receptors on the host cell and the viral envelope fuses with plasma membrane or membrane of endolysosome (EL) and releases the genomic nucleic acid into the cell cytoplasm. Viral membrane is shaded. Note that viral envelope remains in cell plasma membrane both after direct fusion and after endosomal membrane recycling (MR). At the replication stage, replication–transcription complex is formed and viral proteins and copies of RNA are produced. At the assembly stage, viral particles are formed from nucleocapsid (genomic RNA and N protein) and intracellular cell membranes (endoplasmic reticulum (ER) and/or ER-to-Golgi intermediate compartment (ERGIC)), containing viral membrane-associated proteins (S, M, and E). During the assembly process, double-membrane vesicles (DMV) are formed. At the exit stage, viral particles are released by secretory pathway.

depend on the presence of cholesterol and lipid rafts in the membranes of the host cells [21–26]. This has been shown for immunodeficiency viruses (HIV) [27–31], influenza [32–37], herpes [38], Newcastle disease virus [39], and rotavirus [40], as well as hepatitis C virus (HCV) [41–43] and some other viruses of the Flaviviridae family (Yellow fever virus, Zika virus, Dengue virus, West Nile virus [44, 45]). For example, the vital need of cholesterol for replication of hepatitis C virus (HCV) was shown by different methods in [41, 43]. Lipid withdrawal from the medium considerably suppressed the virus replication, which was restored to normal levels upon addition of exogenous LDL. Moreover, virus replication was suppressed by knockdown or pharmacological inhibition of Niemann–Pick type C1 protein (NPC1) – cell protein mediating the endosomal cholesterol transport [41, 43].

The cholesterol dependence of virus–cell interactions has also been demonstrated for various coronaviruses [14, 15, 46–52], including SARS-CoV and SARS-CoV2 [50–52]. Li et al. 2007 [50] reported that the production of SARS-CoV particles released from the infected Vero E6 is notably suppressed following cholesterol depletion by cell pretreatment with methyl- β -cyclodextrin (m β CD), and the addition of cholesterol to the culture medium reversed this effect. Later, Glende et al. 2008 [51] showed that the removal of cholesterol from cell membranes using m β CD reduces the efficiency of infection of cells not only with the SARS-CoV but also with a non-replicating pseudotype of vesicular stomatitis virus containing the surface glycoprotein S of the SARS-CoV virus (VSV- Δ G-S), which confirms the key

role of the S protein in the virus entry. The authors also reported that the cellular receptor of the SARS-CoV virus, angiotensin-converting enzyme (ACE2), is co-localized with Flotilin2 and LAMP2, the protein markers of the detergent-resistant membrane domains (rafts) [51].

The issues concerning the importance of the host cell membrane lipids, rafts, and cholesterol at different stages of the virus life cycle have been addressed in numerous comprehensive reviews, and the dependence of the viral life cycle on cellular cholesterol, as well as the impact produced by viruses on cellular lipids and cholesterol in particular is regarded as a basis for antiviral therapy [15, 30, 31, 45, 53]. For example, cholesterol-lowering treatments are considered as a possible prophylactic or preventive measures [45, 54]. However, alterations in the cell lipid status produced by viruses that have entered a cell impose more complex requirements on potential medicines.

3. Virus-induced modulations of the lipid composition of cell membranes. Formation of viral envelope can result in a hazardous decrease in the cholesterol content in cell membranes

Viruses not only depend on cholesterol but also significantly modulate the lipid composition of cell membranes [53, 55–63]. This occurs both at the stage of virus internalization and during the synthesis of viral proteins and intracellular assembly of new viral particles. The consequences of these changes can determine the clinical course and severity of the disease. The release of the gene material of many enveloped viruses into the cytoplasm of the cell occurs by fusion of the viral envelope with plasma membrane or with membranes of late endosomes (endolysosomes) formed during receptor-mediated endocytosis [18–21, 64–70]. The inclusion of viral envelopes into the host cell membrane (either after direct fusion or after endosomal membrane recycling) should change both the lipid and protein composition of the cell membrane and cause rearrangements in the lipidic milieu and antigenic profile of the host cell membrane (**Figure 1**). Further, at the stage of the assembly of new viral particles, their envelopes are formed from cellular membranes [14, 15, 45, 55, 57–65, 67, 68]. Some viruses bud from the plasma membrane (e.g., togaviruses, rhabdoviruses, paramyxoviruses, orthomyxoviruses, and retroviruses, including HIV), others use the endoplasmic reticulum (ER) (coronaviruses and flaviviruses) or/and a Golgi complex (bunyaviruses), some (e.g., herpes virus) have more complicated budding scenario [59–62]. The formation of the viral envelopes can involve lipid sorting and, in particular, accumulation in the viral envelope of cholesterol and sphingolipids that are acquired from the host cell membranes [33, 61–65, 69, 70]. For example, HIV-1 selectively buds from membrane domains enriched in cholesterol and sphingolipids (rafts); as a result, host cell rafts become a viral coat and the level of cholesterol and sphingolipids and the cholesterol/phospholipids ratio in the viral envelope is higher than in the plasma membrane where they originate, and also notably higher than in the intracellular membranes [53, 61, 62, 65]. Another example – bovine viral diarrhea virus (BVDV) of the Flaviviridae family budding from the endoplasmic reticulum (ER): the content of cholesterol, sphingomyelin, and hexosyl-ceramide in the BVDV particles was shown to be more than twofold higher than in the infected cells [69]. As the cholesterol concentration in the ER is significantly (several times) lower than in the plasma membrane [71–74], the loss of cholesterol due to the formation of viral envelopes can be destructive for the ER membranes.

For lipid sorting necessary for the virus envelope formation, various mechanisms are used to manipulate synthesis, metabolism, and transport of host lipids,

cholesterol in particular, and lead to significant changes in the lipid status of the host cell. HIV-1 infection is known to induce various alteration of cellular lipids, including increased cholesterol synthesis and uptake [75], suppressed cholesterol efflux [76], as well as a shift in phospholipid synthesis to neutral lipids and peroxidation of polyunsaturated fatty acid [53, 65, 75, 76]. Hepatitis C virus (HCV) also causes massive rearrangements of intracellular membranes leading to the formation of double-membrane vesicles (DMVs) enriched with cholesterol. As was shown in [41], HCV ‘usurps’ cholesterol transporter proteins, such as NPC1, in order to deliver cholesterol to the viral replication organelle where cholesterol is needed, and blockage of this transporter suppresses the virus replication. Coronaviruses, like Flaviviruses, are assembled and bud from the membranes of Golgi complex and ER [41, 60] and also form double-membrane vesicles [77]. At the same number of newly formed viral particles, the consequences of removing cholesterol from ER membranes by double-membrane vesicles can be more severe than in the case of single membrane vesicles; a quantitative assessment of this process is necessary.

A possible mechanism stimulating the delivery of cholesterol to ER from plasma membrane during coronavirus replication was demonstrated by Wang et al. 2020 [78]. The authors reported that SARS-CoV2 activates the host cell gene encoding cholesterol 25-hydroxylase and induces the formation of 25-hydroxycholesterol, which increases cholesterol availability [79] and triggers its delivery from the plasma membrane to the endoplasmic reticulum, where cholesterol is required for the viral envelope formation. Although, as is known [14, 23–29, 32–41, 46–55], the depletion of cholesterol in the plasma membrane suppresses the virus entry into the cell, the observed trafficking of cholesterol into the ER (normally the flow goes in the opposite direction [71, 72]) can reflect an increased uptake of cholesterol for the formation of envelopes of new viruses. After the release of newly formed viruses, this depleted cell will not be susceptible to new infection.

Thus, the formation and release of viral particles from the cell cannot but affect the composition of the host cell membranes. It can be expected that after a full replication cycle of viruses with high envelope cholesterol, the level of cholesterol in the membranes of the host cell will be reduced, and this depletion of cholesterol can lead to a significant deregulation of cholesterol-dependent processes, including intracellular signaling and metabolic pathways. At a high rate of assembly of a large number of viruses, infected cells may not be able to compensate for the loss of cholesterol in their membranes, and this can lead to cell death due to destabilization and permeabilization of cell membranes. Indeed, ample evidence indicates that lowered membrane cholesterol is associated with altered mechanical properties and increased permeability of the membrane [80–83]. Some viral and bacterial proteins trigger apoptosis through lysosomal membrane permeabilization leading to release of cathepsins [81]. The human immunodeficiency virus type 1 (HIV-1) protein Nef is one of such proteins: when entering mammalian cells, it causes permeabilization of the lysosomal membrane [81]. It seems appropriate at this point to recall that permeabilization of intracellular membranes due to cholesterol depletion underlies the cytotoxic effect of some anticancer drugs [81, 84–87]. As was shown by Appelqvist et al. 2011 [84], the mechanism of action of cisplatin and some other lysosomotropic drugs at least partially is based on the permeabilization of lysosomal membranes leading to cell death; cholesterol accumulation in lysosomal membranes caused by inhibition of cholesterol transporting protein NPC1 prevented the lysosome-dependent cell death [84]. Note that in the case of virus infection, inhibition of the NPC1-dependent cholesterol transport suppressed the virus replication [41] and rescued the infected cells. When NPC1 functions normally and cholesterol is delivered from lysosomal compartment to ER for the formation of viral envelopes, lysosomal membranes lose cholesterol and become leaky; in a way,

virus acts like a lysosomotropic drug. Cholesterol supplementation was also shown to reverse a strong cytotoxic effect on colon cancer cells caused by a low molecular weight compound TASIN-1 producing cholesterol-dependent ER stress triggering oxidative stress and JNK-dependent apoptosis [85].

Thus, virus–cell interactions lead to significant modulations in the lipid composition of cell membranes. A decrease in cholesterol in cell membranes owing to the formation of viral envelopes can be one of the most dangerous 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. 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 COVID-19, a significant decrease (several fold) in total cholesterol and low-density lipoprotein (LDL) cholesterol levels was recorded [13, 88], and cholesterol-lowering treatments (such as statins) may not be advisable for patients with life-threatening COVID-19 infection, at least until they recover from the infection [88]. Such a drop of the LDL cholesterol level in COVID-19 patients may 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. So far, the only drugs for which clinically significant results have been demonstrated against COVID-19, are dexamethasone and some other corticosteroids [89–91]; secosteroids (vitamin D) are shown to help, too [92]. The use of dexamethasone led to a reduction in mortality to one third of hospitalized patients with severe respiratory complications from COVID-19. It seems possible that the action of steroids may be associated with the repair of a cell membrane damaged by virus-induced depletion of cholesterol.

How does the virus manage to bind and remove cholesterol from cell membranes?

4. Cholesterol recognition/interaction amino acid consensus (CRAC) motifs in viral proteins. Possible uses of CRAC-containing peptides

As was shown earlier [16, 17, 93], some proteins involved in cholesterol-dependent cell functions possess the so-called cholesterol recognition/interaction amino acid consensus (CRAC) motifs – small regions with a specific set of amino acid residues involving a branched apolar amino acid residue (Val (V), Leu (L), or Ile (I)), aromatic residue (Tyr (Y)), and cationic amino acid residue (Arg (R) or Lys (K)); these motif-forming amino acids are separated by short segments of any 1–5 amino acid residues. In subsequent discussions, more candidates of aromatic amino acid residues were proposed, and the general formula for the CRAC motifs presumably involved in the interaction of protein with cholesterol presently appears as follows: V/L/I–X_(1–5)–W/Y/F–(X)_(1–5)–R/K, where X stands for any amino acid residue [94–100]. Although the predictive value of this formula has been questioned [95–97], the presence of this motif in many proteins and its participation in the protein–cholesterol interactions has been confirmed by different methods [16, 17, 93–95]. The formula of the CRAC motif can be further developed [94]; important is the very concept of a motif mediating the interactions of cholesterol-dependent proteins with cholesterol.

CRAC motifs are found in many viral proteins, and their role in cholesterol-dependent virus–cell interactions have been demonstrated. For example, CRAC motifs are present in the HIV matrix protein p17, which was shown to participate in virus entry through the raft domains of the cell membranes [27, 101]. The α -helical domain of the hepatitis C virus nonstructural protein NS5A, which is anchored at the cytoplasmic leaflet of the endoplasmic reticulum and is involved in replication hepatitis C virus, also contains CRAC motif [102]. Importantly, peptides derived from this domain were shown to exhibit a broad-spectrum anti-viral activity. Cheng et al. 2008 [103] reported that peptide C5A containing amino acid residues 3–20 of the amphipathic α -helical N-terminal domain of hepatitis A virus protein NS5A suppressed the virus replication by more than 5 orders of magnitude. The authors did not mention the CRAC concept; however, the active peptide C5A (SWLRDIWDWICEVLSDFK) clearly contains the CRAC motif: RDIWDWICEV. Later, the antiviral activity of this peptide C5A against HIV was also demonstrated [104]. It is possible that peptide C5A, owing to the presence of the CRAC motif, binds cholesterol and competes for cholesterol binding with viral protein and thus inhibits the formation of the viral particle.

CRAC motifs are found in alpha-helices of matrix protein M1 of influenza A virus [105–107]. An important role of these CRAC motifs in the organization of the raft structure of the virion membrane was substantiated by using the method of directed point mutations in the CRAC-containing α -helices in the M1 protein [106, 107]. Further studies revealed that M1-derived peptides containing CRAC motifs LEVLMEWLKTR, NNMDKAVKLYRKLK, GLKNDLLENLQAYQKR, corresponding to α -helices 3 (aa 39–49), 6 (aa 91–105) and 13 (aa 228–243), respectively, to a different extent modulate cholesterol-dependent interactions of cultured macrophages with 2- μ m particles that mimic bacteria (phagocytic index). Of the three peptides, NNMDKAVKLYRKLK was most potent and stimulated the cell activity by 50–60% at 35 μ M [108]. Peptide RTKLWEMLVELGNMDKAVKLWRKLKR obtained by combining two of these short peptides and containing two CRAC motifs produced much stronger and more complex effect: in a narrow range of low concentrations (1–5 μ M) the peptide exerted a stimulatory effect and at 50 μ M the peptide was cytotoxic [109]. Reducing the cholesterol content in the cells with methyl- β -cyclodextrin (m β CD) abolished the stimulatory component and lowered the peptide concentration required for the toxic effect. Substitution of the motif-forming amino acids abolished these effects [110]. The cytotoxic effect of the M1-derived peptide RTKLWEMLVELGNMDKAVKLWRKLKR can be explained by the binding (sequestration) of membrane cholesterol by the peptide; this can imitate removal of cholesterol from cell membranes, which occurs during the formation of the viral envelope.

S proteins of coronaviruses SARS-CoV and SARS-CoV2 also contain CRAC motifs [51, 109, 110]. For example, in the case of the S protein of coronavirus SARS-CoV, the CRAC motif YIKWPWYVW 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 [111, 112].

If the assumption about the essential role of sequestration and removal of membrane cholesterol by viral CRAC-containing proteins in the COVID-19 parthenogenesis is correct, then in order to prevent this destructive action for the cell, it is necessary to maintain a safe level of cholesterol in the plasma membrane. A significant decrease in total cholesterol and low-density lipoprotein (LDL) cholesterol levels in COVID-19 patients [13, 88] may be indicative of the critical loss of cholesterol by cells, and an efficient cholesterol delivery to the cholesterol-depleted cells may be helpful. Cyclodextrins are possible candidates as non-toxic

cholesterol transporters [113–116], which can redistribute cholesterol from endogenous and/or exogenous sources. The use of cyclodextrins increased the lifespan of NPC1^{-/-} experimental mice [117] and improved the condition of patients with Nieman–Pick disease [118]. Another alternative is to prevent viral proteins from interacting with membrane cholesterol. At least some of the drugs that are currently tested – for example, polyphenolic substances like quercetin and saponin glycyrrhizin [119, 120] – can act at the protein/cholesterol interface and hinder cholesterol binding by the CRAC motif of the viral S protein and thus inhibit the assembly of new viral particles. Glycyrrhizin, an active component of liquorice roots, was shown to inhibit SARS-CoV replication in Vero cells and replication of SARS-associated coronavirus [121, 122]. However, such agents are not very selective and can affect other cholesterol-dependent proteins and therefore cause side effects. Perhaps specially designed CRAC-peptides specifically blocking the interactions of S-protein with cholesterol will prevent the cellular cholesterol loss leading to permeabilization of membranes, oxidative stress, and cell death. The ability of CRAC-containing peptides to regulate cholesterol-dependent cell functions has been demonstrated in a number of works [17, 109, 122, 123], and studies of the antiviral activity of these peptides may be useful and promising.

5. Conclusions

The SARS-CoV2 pandemic has sparked a brainstorming session over the underlying mechanisms of viral diseases. Many assumptions have been made. This chapter considers possible consequences of cholesterol depletion in the membranes of infected cells due to the formation of cholesterol-rich viral envelopes. At a high viral load and high replication rate the reduction in the cholesterol level in the cell membranes can lead to their permeabilization and subsequent cell death, and this can be one of the factors in pathogenesis of diseases induced by SARS-CoV2. Cholesterol-recognition/interaction (CRAC) motifs in viral proteins may represent a mechanism for the binding of the viral protein with cholesterol. Substances preventing these interactions of viral proteins with cholesterol can suppress the formation of the viral envelope and therefore can be studied as possible antiviral drugs. Peptides containing CRAC motifs from viral proteins may be among these substances.

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Conflict of interest

The author declares that there is no conflict of interest.

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