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Rlip Protein: A Potential Target for COVID-19

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Abstract

On January 30, 2020, the COVID-19 epidemic was declared an international public health emergency by the World Health Organization. Given the growing impact of the pandemic, there is great interest in finding potential targets for treating infected or hospitalized COVID-19 patients. Therapeutic studies have been conducted on pre-existing drugs, which vary by country, including anti-malarial agents, antiviral agents, and convalescent plasma. However, many of these agents are ineffective at reducing mortality or only shorten the severity or duration of COVID-19 illness in hospitalized patients. As such, other alternatives for treating COVID-19 are being investigated. One such target of interest has been clathrin-dependent endocytosis (CDE). Clathrin-dependent endocytosis is the most commonly observed mechanism of viral entry into cells. However, there have been no published studies to date on CDE inhibition strategies against COVID-19. One such target is Rlip or RLIP76 (human gene RALBP1, 18p11.22). Among its many functions, Rlip is a stress-protective, Ral-regulated ATPase of the mercapturic acid pathway that transports glutathione-electrophile conjugates of electrophilic toxins, which are precursors of mercapturic acid that precedes de-glutamylation by gamma-glutamyl transferase. Rlip is also regulated by several G-proteins that coordinate movement of cells, organelles, membranes, cytoskeleton, macromolecules, and other small molecules. Previous studies have link Rlip in the pathogenesis of several viral illness. In this paper, we want to propose that RLIP76 (Rlip or RALBP1) may be a novel target for treating SARS-CoV-2 viral infections.

Keywords: COVID-19, SARS-CoV-2, Rlip, Clathrin-dependent endocytosis (CDE)

1. Introduction

On January 30, 2020, the COVID-19 epidemic was declared an international public health emergency by the World Health Organization. Currently, the SARS-CoV-2 virus has infected over 72 million and killed over 1.5 million people worldwide. Given the growing impact of the pandemic, there is great interest in finding potential cell-surface receptor targets for treating infected or hospitalized COVID-19 patients. Therapeutic studies have been conducted on pre-existing drugs, which vary by country, including antiviral agents and convalescent plasma.[1] However, many of these agents are ineffective and do note reduce the mortality or only shorten the severity or duration of COVID-19 illness in hospitalized patients. As such, other alternatives for treating COVID-19 are being investigated. One such target of interest has been clathrin-dependent endocytosis (CDE).

Clathrin-dependent endocytosis is the most commonly observed mechanism of viral entry into cells.[2] After a viral particle attaches to the plasma membrane surface, the virus is internalized into endosomes and transported to either the plasma membrane/secretory pathway or the late endosomes to be degraded in lysosomes.[3] This pathway has important roles in the infections of several viruses, including coronaviruses, influenza A virus, rhinovirus, and adenovirus 2.[3,4] Specifically, clathrin-dependent endocytosis and cathepsin- mediated S protein cleavage are two important steps for viral entry and pathogenesis of SARS-CoV-2.[3,5] Several coronaviruses have been reported to utilize CDE for viral internalization, including the Middle East respiratory syndrome (MERS) virus,[6] the

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mouse hepatitis coronavirus (MHC),[7] and the original SARS coronavirus which utilizes the ACE2 receptor for entry.[8] The SARS-CoV-2 (COVID-19) coronavirus also utilizes the ACE2 receptor[9] and likely uses CDE for viral internalization. A recent study showed that the top canonical pathways associated with plasma ACE2 included clathrin-mediated endocytosis signaling, actin cytoskeleton signaling, EIF2 (eukaryotic initiation factor 2) signaling, and the protein ubiquitination pathway.[10] However, there have been no published studies to date on CDE inhibition strategies against COVID-19. In this paper, we want to propose that RLIP76 (Rlip or RALBP1) may be a novel target for treating SARS-CoV-2 viral infections.

2. SARS-CoV-2 and endocytosis

The COVID-19 pandemic has left the world in a distressful situation. Treatments are primarily based on supportive care or on drugs with uncertain efficacies, and a lack of strong preclinical evidence makes it difficult to predict the efficacy of the vaccination strategies under development.[11] Therefore, the need for the safe and effective pharmacologic therapies is greater than ever. Clathrin-dependent endocytosis (CDE) is the most commonly observed mechanism of viral entry into cells.[2] SARS-CoV-2 virus binds to the human angiotensin converting enzyme 2 (ACE2) receptor and the neuropillin receptor using viral spike glycoproteins to gain entry into vulnerable cells via CDE.[12,13] Neuropilin is known to initiate endocytosis.[14] Several other studies have shown that the Moesin receptor, an actin anchoring membrane-organizing protein, is also involved in the pathogenesis of the measles, human immunodeficiency virus (HIV), and herpes simplex viruses.[15–22] It is hypothesized that Moesin negatively regulates stable microtubule networks, which determines cellular sensitivity to retroviral infection.[23] However, we argue that another regulator of endocytosis, known as Rlip, may be a potential therapeutic target for COVID-19.

3. Rlip and endocytosis

RLIP76 (Rlip or RALBP1) is a multifunctional membrane ATPase that links the mercapturic acid pathways to clathrin-dependent endocytosis (CDE). RLIP76 (Rlip), the 76KDa product of the RalA Binding Protein 1 (*RALBP1*) gene, is critical for the completion of clathrin-dependent endocytosis.[24–26] Rlip serves as a scaffold for the formation of endocytic complexes during interphase and regulates endocytosis.[25,27–29] Rlip knockout mice have severely deficient CDE resulting in impaired internalization of receptor-ligand complexes. These mice show improved insulin-sensitive, hypoglycemic, hypolipidemic, and concomitantly markedly resistant to obesity and either chemically induced or spontaneous carcinogenesis that accompanies p53 loss.[30] They also have remarkably low levels of numerous inflammatory cytokines.

RNA-Seq and whole-genome bisulfite sequencing studies show that biological processes and signaling pathways that govern viral entry, signaling by TNF, IL-6, and chemokine as well as Ral- and Rho-regulated intracellular trafficking are inhibited.[31–33] Interestingly, there is considerable overlap between Rlip-interacting pathways and moesin, a human spike protein that functions as an actin anchor and membrane-organizing protein for endocytic trafficking of the EGF receptor and the RhoA signaling pathways that have been implicated in SARS virus endocytosis. Using RNA sequencing (RNAseq) data from heterozygous or homozygous depleted mice, we show a highly overlapping interaction network between Rlip and moesin. The network has 229 intersecting nodes of protein-coding and miRNAs genes and Rlip depletion inhibits several nodes important for viral infection, diabetes and cancer with down-regulation **and** multiple key nodes by Rlip depletion.

These findings were supported by proteomic analyses of spleen from $Rlip^{-/-}$ mice showing reduction in the quantity of multiple proteins that are upstream regulators of RNA virus infection per the Ingenuity Pathway Analysis software (Figs. 1–3). Several proteins in this network are directly related to the pathogenesis of other viral infections. The CTSB gene encodes a lysosomal cysteine protease, known as Cathepsin B, which facilitates chikungunya virus infectivity via endocytosis or micropinocytosis.[34] Another study found that Cathepsin B facilitated CD4-independent entry of HIV-1 through endocytosis and endosomal acidification processes.[35] The ANXA2 gene, which encodes annexin II, is a multifunctional calcium- and lipid-binding protein that contributes to the pathogenesis of several viruses (e.g., human papillomavirus, cytomegalovirus, and the influenza A virus) through actin remodeling near the plasma membrane.[36,37] This actin remodeling subsequently influences endocytosis processes, particularly CDE.[36,37] However, many of the targets suggested by the network analysis have not been directly linked to endocytosis and viral pathogenesis.

Therefore, investigating Rlip function with respect to viral infections may describe additional

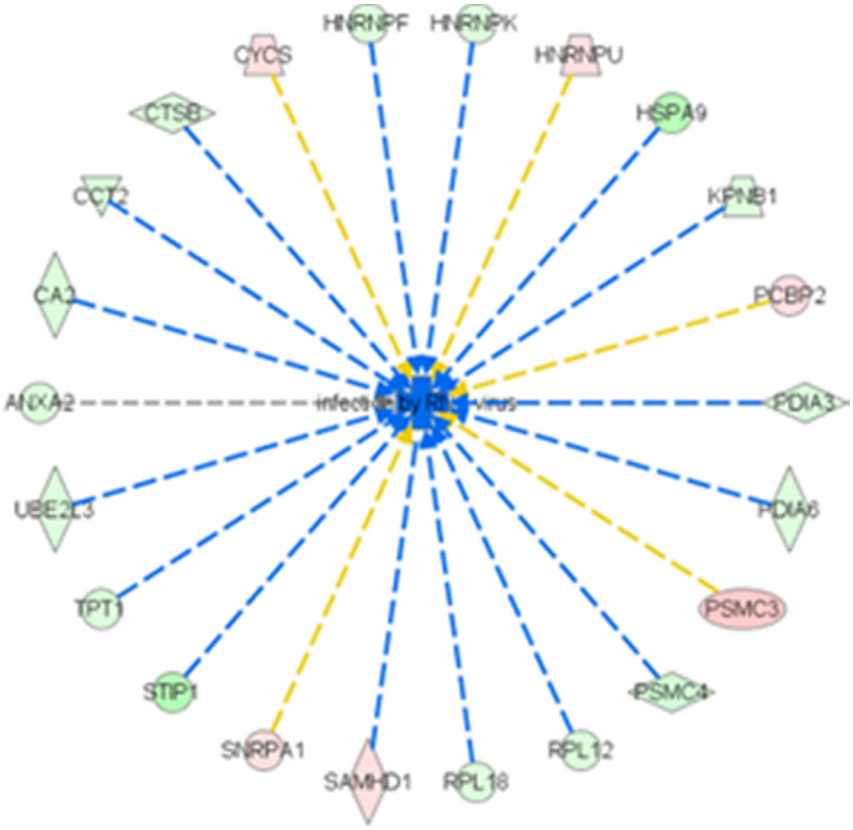

Fig. 1. Upstream regulators of RNA virus infection. Results are from IPA analyses of Rlip$^{-/-}$ C57-Bl/6 mice spleen proteomic analyses normalized to wild-type. Green represents down-regulation and red represents up-regulation. Several proteins (e.g., CTSB) in this network are directly related to the pathogenesis of other viral infections.

mechanisms by which it regulates CDE. Several studies demonstrate the functional importance of heptad repeats of the SARS-CoV spike protein in membrane fusion and viral entry.[38] Using the online tool (http://coiledcoils.chm.bris.ac.uk/Scorer/) we found RalBP1 has long C-terminal heptad repeats. It is yet to be established the functional interactive role of heptad repeats of these two key proteins involved in internalization of coronavirus. Further, hyperglycemia may influence the glycosylation patterns on ACE2 and the viral spikes resulting in increased binding affinity.[39] Research into the mouse hepatitis coronavirus suggests that after internalization, coronaviruses may spread via direct cell-to-cell fusions requiring functional macropinocytosis, an actin-mediated nonspecific uptake mechanism.[40]

Thus, targeting Rlip76 (a protein product of *RALBP1)* may inhibit the successful internalization of coronaviruses into cells by stalling endocytosis and by lowering plasma glucose levels to reduce the viral sugar coating necessary for attachment to the cell surface. Therefore, we predict that therapies targeting this pathway may help reduce viral entry into vulnerable cells. Using the results from this analysis, we propose that there may be benefit in targeting these genes and proteins in the treatment of COVID-19 with regards to Rlip and endocytosis inhibition. However, there are currently no approved inhibitors for Rlip in humans. We have recently shown that the naturally occurring and non-toxic orange peel-derived compound 2'-hydroxyflavanone that inhibits both endocytosis and cancer growth, particularly in lung cancer.[41] Specifically, 2'-hydroxyflavanone effectively inhibits the transport and endocytic functions of Rlip.[42] 2'-hydroxyflavanone (2HF) is highly tolerable and is considered generally regarded as safe by the FDA.[41,42]

Furthermore, 2HF is also capable of inhibiting micropinocytosis.[42] *In vitro* studies have shown that the inhibition of macropinocytosis reduces MHC viral titers.[40] In addition, viral activation of macropinocytosis was found to be dependent on EGF receptor activation.[40] Recently, a clinical trial in Spain

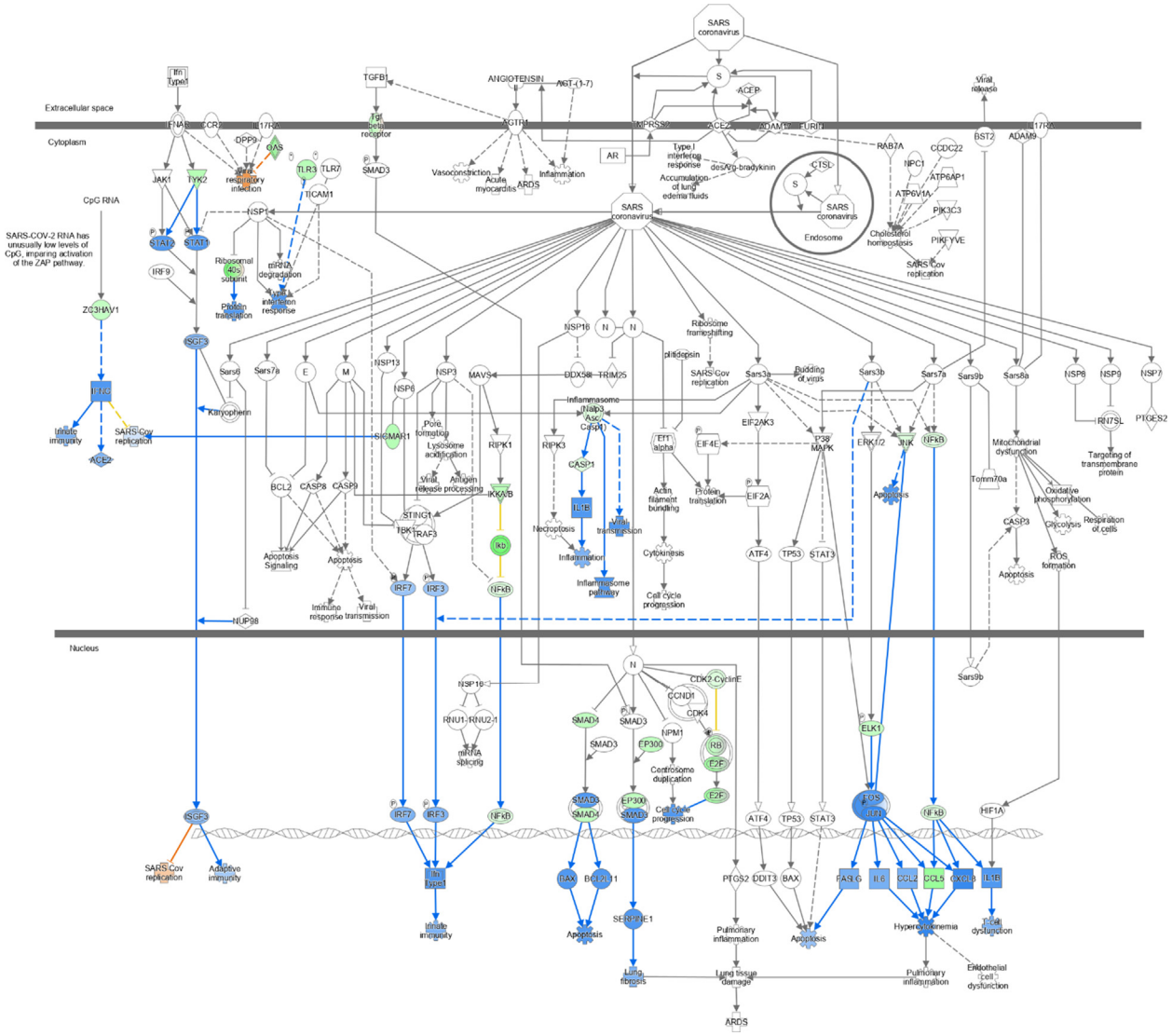

Fig. 2. ***Effect of Rlip loss on COVID-19 pathogenesis pathway****. The red geometric shape indicates an increase in measurement. The green geometric shape indicates a decrease in measurement. The orange geometric shape indicates an increase in prediction of the activity of connected signaling node. The blue geometric node indicates a decrease in prediction of the activity of connected signaling node. The glow around a geometric shape indicates activity when opposite of measurement. The orange line indicates an interaction leading to activation. The blue line indicates an interaction leading to inhibition. The yellow line indicates findings inconsistent with state of downstream molecule. The grey line indicates effect not predicted.*

has begun recruiting subjects to investigate whether combining ruxolitinib with simvastatin may both prevent and treat respiratory failure of in hospitalized COVID-19 patients (ClinicalTrials.gov Identifier: NCT04348695). Similarly, 2'-hydroxyflavanone could be investigated in randomized clinical trials alone or with other treatment regimens to assess whether 2HF would be effective at reducing the morbidity or mortality in hospitalized COVID-19 patients. For these reasons, our primary hypothesis is that inhibition of Rlip will inhibit the successful internalization of coronaviruses into cells, resulting in reduced viral titer and reduced severity of symptoms.

4. Pitfalls, limitations, and alternative strategies

It is possible that the COVID-19 virus does not require the completion of the clathrin-dependent endocytosis process for viral entry into certain types of cells. In this case RLIP inhibition may not be effective against COVID-19 since loss of RLIP stalls endocytosis relatively late in the process. It is also possible that blocking clathrin-dependent viral entry is insufficient to completely abrogate entry, as clathrin-independent viral entry has also been reported for the original SARS virus in HEK293E cells.[43]

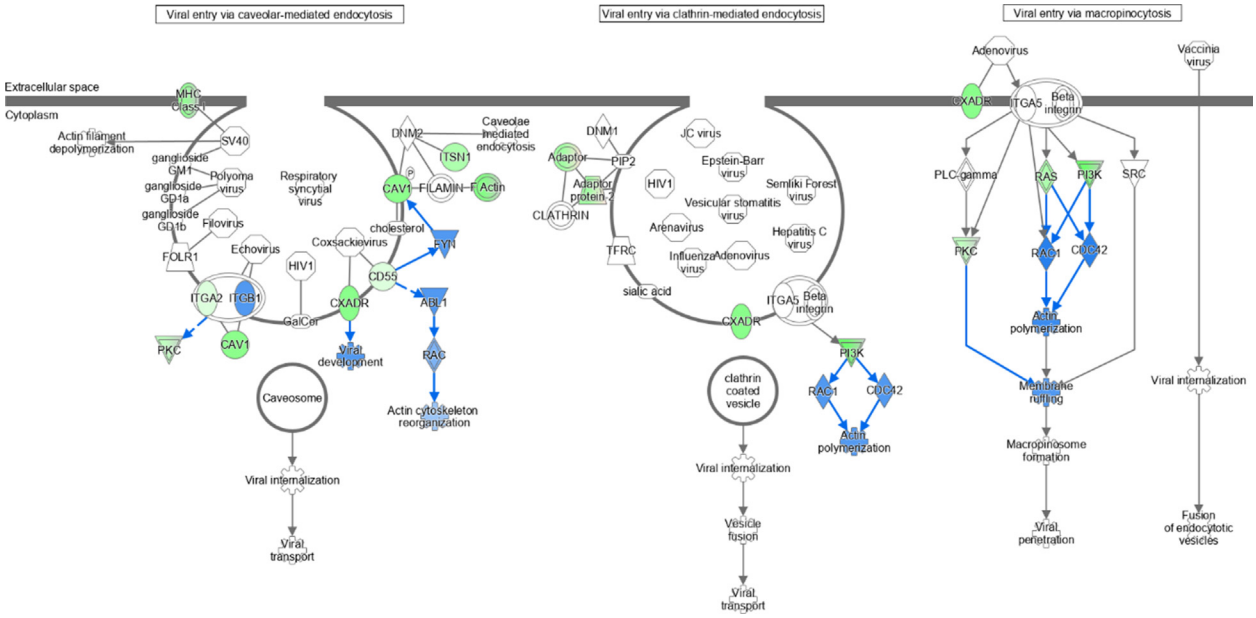

*Fig. 3. **Effect of Rlip heterozygous loss on viral entry pathway.** The red geometric shape indicates an increase in measurement. The green geometric shape indicates a decrease in measurement. The orange geometric shape indicates an increase in prediction of the activity of connected signaling node. The blue geometric node indicates a decrease in prediction of the activity of connected signaling node. The glow around a geometric shape indicates activity when opposite of measurement. The orange line indicates an interaction leading to activation. The blue line indicates an interaction leading to inhibition. The yellow line indicates findings inconsistent with state of downstream molecule. The grey line indicates effect not predicted*

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Ethics statement

Consent statement/Ethical approval: Not required.

Conflict of interest

SA has a conflict of interest because of intellectual property in company (Avesta76 Therapeutics).

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