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Current Opinion in Virology

Genetic deficiency and polymorphisms of cyclophilin A reveal its essential role for Human Coronavirus 229E replication

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Replication of coronaviruses is inhibited *in vitro* by cyclosporin A, a well-known immunosuppressive drug which binds to cellular cyclophilins thus inactivating their enzymatic cis-trans peptidyl-prolyl isomerase function. Latter is required for proper folding of cellular proteins and of proteins of several viruses. Here, we summarize present knowledge on the role of cyclophilin A during coronavirus replication. We present data on the effect of cyclophilin A single nucleotide polymorphism mutants on the replication of human CoV-229E demonstrating the requirement of proper cyclophilin A function for virus propagation. Results define cellular cyclophilin A as a host target for inhibition of coronaviruses ranging from relatively mild common cold to highly pathogenic SARS-CoV and MERS-CoV viruses with the perspective of disclosing non-immunosuppressive cyclosporin A analogs to broadly inactivate the coronavirus family.

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Introduction

Coronaviruses (CoVs) infect a variety of mammalian species including bats, mice, cats, birds and humans causing infection of respiratory and gastrointestinal tracts and the central nervous system [1]. CoVs are enveloped viruses containing the largest known single-stranded RNA genomes (25–32 kb) with positive-sense orientation. They are divided into four genera: *Alpha-* (HCoV-229E), *Beta-* (SARS-CoV: lineage B; MERS-CoV: lineage C), *Gamma-* and *Deltacoronavirus* [2]. The six human CoVs, namely HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, SARS-CoV and MERS-CoV mainly target the respiratory tract. 15–30% of common colds are caused by HCoVs (229E, OC43, NL63, HKU1) with mostly seasonal occurrence. Whereas 229E and OC43 are known since the mid-1960s, SARS-CoV appeared first in China causing a worldwide outbreak with 8098 cases and 774 deaths in 2002/03 and with enormous socio-economic impact [3]. Arising interest in CoVs led to the discovery of NL63 in 2004 [4] and HKU1 in 2005 [5]. MERS was identified in 2012 in Saudi Arabia. By 31 May 2015 MERS-CoV infections rose to 1154 cases with 431 deaths [6]. In May 2015 a new outbreak occurred in South Korea with over 120 reported cases, 10 deaths as of 11 June [7], and over 2300 individuals placed under quarantine, making it the largest outbreak outside Saudi Arabia.

Until now no effective drug treatment is available neither against the common cold nor the highly pathogenic CoVs. Development of antivirals has concentrated on the development of protease [8,9] and helicase inhibitors [10,11]. Great efforts have been made to discover anti-MERS agents by screening defined drug libraries [12–14]. Although, CoVs display some proofreading activities during replication viral targets are usually prone to develop resistance mutations rather quickly. Therefore, defining cellular co-factors required for viral replication as targets is rather intriguing. Screening 16,671 diverse compounds for anti-229E activity Lundin *et al.* have identified an inhibitor (K22) specifically targeting membrane-bound coronaviral RNA synthesis at an early step of viral replication [15]. Using unbiased high-throughput protein–protein interaction screening methods we identified cyclophilins as binding factors for CoV proteins and its inhibitor cyclosporin A (CsA) as broad-range anti-coronaviral agent [16••]. CsA binding and inactivation of CypA as cellular co-factor for virus propagation is summarized in the accompanying article in the October issue of Current Opinion in Virology by von Hahn and Ciesek [17]. Here we summarize the inhibitory effect of CsA and non-immunosuppressive derivatives thereof on CypA function during CoV replication. We further describe the effect of the genetic deficiency and of individual CypA SNP mutants on the replication of HCoV-229E indicating the requirement of correctly folded enzymatic groove of CypA.

Cyclophilins and inhibitors

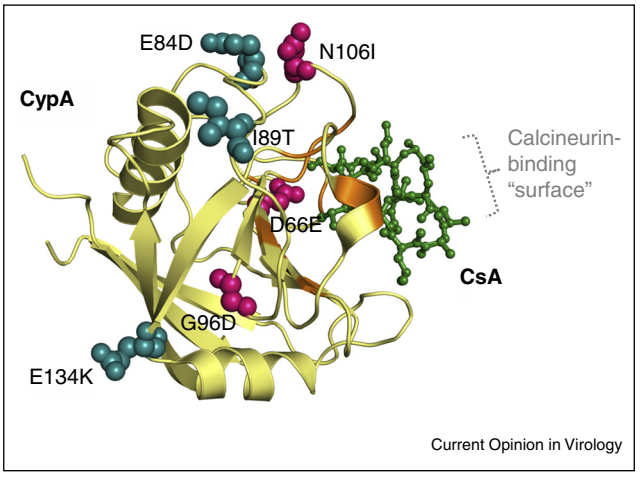
Cyclophilins and FKBPs are members of two ubiquitously distributed PPIase families, collectively called immunophilins [18••]. They are important for a number of cellular processes, for example, protein folding, maturation, trafficking, signal transduction, cell differentiation, apoptosis and infections. CypA and CypB were recognized in 1993 by Y2H techniques to specifically bind to the HIV-1 Gag polyproteins Pr55gag and to capsid p24, but only CypA was demonstrated to be specifically incorporated into HIV-1 virions [reviewed by 19••]. Binding of the amino-terminal domain of HIV-1 capsid to the active groove of CypA was demonstrated by crystal structure [20] and by mutational analysis [21,22]. Proline-containing peptide substrates bind to this hydrophobic pocket conferring them enzymatic peptidyl-prolyl *cis/trans* isomerase (PPIases, EC number 5.2.1.8) activity. The isomerase function of Cyp was identified already in 1989 [23•]. It was first shown for HIV-1 that CsA also binds to the groove thus interfering with proper capsid formation and virus replication. In the case of Hepatitis C Virus (HCV) the involvement of Cyps was shown by several groups. Initially conflicting results on which CypA or B was supporting HCV replication could be clarified in favor of CypA [24–26].

A very important, but from PPIase activity completely independent feature of CsA binding to CypA is the formation of a tri-molecular complex with the cellular phosphatase Calcineurin (Cn). This is a natural coincidence with far-reaching consequences on the immune system: Cn normally dephosphorylates the important immunologic transcription factor NFAT (Nuclear Factor of Activated T-cells), which can then translocate to the nucleus and act as a key regulator of T-cell development and Interleukin-2 production [27•]. CypA/CsA/Cn complexes prevent NFAT dephosphorylation and translocation to the nucleus thus leading to the suppression of the immune system. CsA as a 11mer cyclic peptide displays a 'surface' for binding to the PPIase groove of CypA [28] and one for complexing with Cn (Figure 1). Intensive efforts were made to separate the PPIase blocking from the immunosuppressive functions of CsA. Modifying side chains of the CsA molecule allowed the development of non-immunosuppressive analogs NIM811 [29,30], Alisporivir [ALV, Debio-025] [31], SCY-635 [32], sangliferins [33] and a series of newly synthesized CsA position 1-modified compounds [34–36]. Alisporivir has experienced substantial clinical testing and safety database development with more than 2000 patients treated for up to 48 weeks. NIM811 and SCY-635 have been administered in a very small number (<50 patients) only in short proof-of-concept trials.

Immunophilins and CoV replication

A first hint on the possible involvement of a cyclophilin, namely CypA, in SARS-CoV replication came from an educated guess finding, which demonstrated interaction of the SARS-CoV nucleocapsid (N) protein with CypA by surface plasmon resonance biosensor technology paralleling the binding of HIV-1 gag to CypA [37]. This finding was supported by a proteomics study which identified CypA as one of a number of cellular proteins incorporated into purified SARS-CoV particles by spectrometric profiling [38]. Inhibitory effects of CsA on CoV replication was reported by several laboratories: (1) using unbiased high throughput Y2H protein–protein interaction screening methods we have noticed the binding of several cyclophilins to SARS-CoV nsp1, and CsA as pan-CoV inhibitor including SARS-CoV, HCoV-229E/-NL63, Feline CoV (FCoV) serotypes I and II [strains Black and 791146], Transmissible Gastroenteritis Virus (TGEV PUR46) and Infectious Bronchitis Virus (IBV Beaudette) [16••]. In a follow-up study it could be demonstrated that, at least for replication of HCoV-NL63 CypA, not CypB is the cyclophilin required for virus replication [36]. As also the immunophilins FKBP1A and FKBP1B showed up as nsp1 interaction partners in the Y2H virus–host protein interaction screens mentioned above, SARS-CoV, HCoV-NL63 and HCoV-229E-GFP/-LUC were tested for sensitivity to FK506. The drug could effectively inhibit replication of these viruses, and HCoV-NL63 did not replicate in FKBP1A/B knockdown CaCo2 cell lines [39]. Thus, PPIase activities of CypA and FKBPs seem to be

Figure 1



Crystal structure of human CypA complexed with CsA (1CWA, pdb database, modified) and with coding non-synonymous PPIA gene SNPs. SNPs with accompanying amino acid exchanges introduced in Huh-7.5 PPIA manipulated cell lines [46] are Rs61747111 (D66E), rs17850033 (I89T), rs1059983 (E84D), rs11547706 (G96D), rs17850166 (N106I), rs9769523 (E134K). CypA is shown as a β-sheet structure with the SNP amino acids strongly, or only slightly affecting CoV replication in ball format, colored red and blue, respectively. Active site residues are depicted in orange. CsA binding to the PPIase active pocket of CypA is shown in green. The calcineurin-binding surface of CypA/CsA complex is indicated schematically.

required for CoV replication but they do not substitute for each other. The current interpretation is that the two classes of PPIases act on different viral proteins. (2) Replication inhibition by CsA up to 4 logs was also shown for GFP-expressing SARS-CoV, 229E and MHV [Mouse Hepatitis Virus] [40] as well as for MERS-CoV [41]. In these studies a specific cyclophilin could not be attributed to SARS-CoV replication. (3) Replication of the highly pathogenic cat CoV Feline Infectious Peritonitis Virus (FIPV) was shown to be sensitive to CsA, but not to FK506 [42]. Rather interestingly, when performing the CsA inhibition experiments with animal CoVs [16••] we also did not find FK506 inhibition of the two FCoV serotypes (Black/791146; unpublished and Heinz-Jürgen Thiel, personal communication), and of TGEV PUR46 and IBV Beaudette strains (unpublished; Christel Schwegmann-Weßels/Georg Herrler, personal communication). (4) Cyclophilin D was shown to play a central role in HCoV-OC43-induced neuronal programmed cell death by CypD knockdown and CsA inhibition. Here, CsA in combination with CypD acted as an inhibitor of the mitochondrial permeabilization transition pore [43].

PPIA gene knockout and coding non-synonymous SNP variants limit HCoV-229E replication

For HCoV-NL63 we have recently shown that replication in CaCo2 cells depends on cyclophilin A (CypA) but not CypB [36]. Human hepatocellular carcinoma cells (Huh-7) and derivatives support the replication of a number of viruses including HCV [44] and HCoV-229E [45]. We utilized Huh-7.5 CypA variant cell lines originally constructed for the analysis of HCV infection [46]. The variants used were Huh-7.5KD-PPIA (CypA knockdowns), Huh-7.5sh-Ctr (non-target controls), Huh-7.5-CypA-KD + wtCypA (CypA knockdowns, reconstituted with CypA) and Huh-7.5-CypA mutants encoding individual, non-synonymous PPIA gene SNPs with amino acid exchanges (D66E, N106I, G96D, E134K, E84D, or I89T; see also Figure 1).

For HCV viral growth behavior was shown to depend on protein stability of the individual SNP variants. Although mRNA levels determined by qPCR analysis were comparable in Huh-7.5wt and CypA variants protein levels of D66E were reduced and G96D and N106I appeared nearly absent [44]. For the infection experiments with HCoV-229E relative CypA mRNA expression levels were analyzed in the various cell lines by qPCR in relation to the house keeping gene hTOP1 with the level in Huh-7.5 set to 1.0 (Figure 2a). CypA mRNA levels in the non-target control (sh-Ctr) and in the D66E, G96D, E134K and I89T variants were slightly decreased. Levels in E84D and in the wtCypA-reconstituted Huh-7.5PPIA-KD (sh-CypA-KD) cells were increased. In Huh-7.5KD-PPIA (sh-CypA-KD) cells PPIA mRNA was close to background confirming that the knockdown was very efficient although not complete.

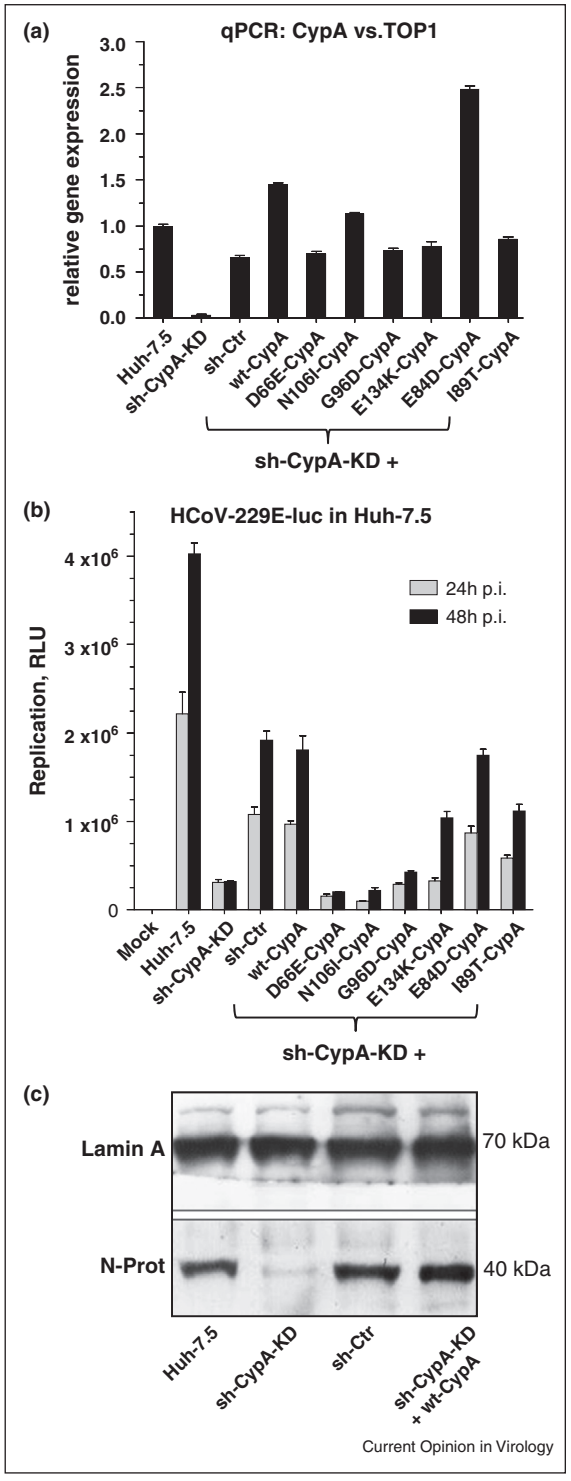
In order to assess growth properties of HCoV-229E-LUC the various cell lines were infected with 0.1 MOI of the virus and grown for 24 and 48 h (Figure 2b). As judged by Luciferase protein expression levels (RLU) virus grew best in Huh-7.5 cells with an about two-fold increase over the sh-CypA-KD + wt-CypA and the non-target sh-Ctr cells. In sh-CypA-KD cells virus replication was decreased by factors of 10 and 20 at 24 h and 48 h time-points, respectively. In case of the E134K, E84D and I89T mutant virus growth was slightly decreased as compared to the sh-CypA-KD + wt-CypA and the sh-Ctr cells. Very interestingly, in D66E, N106I and G96D, 229E replication was almost completely abolished as it was in the case of sh-CypA-KD cells. To assess viral replication at the level of essential N protein production in HCoV-229E-LUC virus-infected cells (MOI 0.01) Western blot analysis of viral N protein two days p.I. was performed (Figure 2c). N protein levels were similar in Huh-7.5, sh-Ctr and sh-CypA-KD + wt-CypA reconstituted cells. In sh-CypA-KD cells N was almost absent confirming the requirement of CypA for HCoV-229E replication.

Discussion

Genetic variation of host genes involved in virus infection and also in other human diseases [47] is of highest clinical interest as such proteins represent potential molecules for host-targeting (therapeutic) agents (HTAs) with broad-range antiviral activity. Even in the case of rare genetic variants their analysis might give important clues to disease mechanism. In the case of HCV understanding the functional architecture of type III IFN genomic regions and SNPs have improved the knowledge on the pathogenetic mechanism of HCV infection [48]. However, studying the effect of SNP mutations on infection has to be interpreted carefully considering composition of cohorts, differences in disease progression, or duration times of follow-up studies.

For CoVs few host SNP data are available which might give clues on resistance to or promotion of viral infection. For example, SNPs of genes involved in innate immunity were correlated with SARS-CoV load during the initial phase of infection [49]. A 336A > G promoter polymorphism in the gene encoding CD209 (DC-SIGN) was correlated with clinical-pathologic outcomes in 824 serologically confirmed SARS patients [50]. The -336AG/GG genotype was associated with lower standardized lactate-dehydrogenase (LDH) levels compared with the '-336AA' genotype carrying patients with a 60% chance of having a poorer prognosis because of higher LDH levels. In cats, several SNPs have been described in CD209, TNF-α [51] and IFN-γ [52] genes. They were found to be associated with the outcome of FCoV infection, that is, with the

Figure 2



Replication analysis of HCoV-229E-LUC in Huh-7.5-KD and single SNP variant mutants. **(a)** qPCR analysis of CypA expression in sh-CypA-KD, non-target control (sh-Ctr) and with wtCypA reconstituted sh-CypA-KD cells, as well as sh-CypA-KD cells reconstituted with CypA SNP variants carrying amino acid exchanges at D66E, N106I, G96D, E134K, E84D and I89T. Huh-7.5 cells and subclones were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (both Gibco LifeTechnologies), L-glutamine, non-essential amino acids, penicillin, and streptomycin. Cells harboring small hairpin RNA (shRNA) constructs were kept in the presence of blasticidin (5 μg/mL). G418 (750 μg/mL) was additionally added to cells carrying pWPI-encoded CypA variants [46]. hTOP1 was used in qPCR to standardize cyclophilin expression. **(b)** Replication was measured by determining Renilla Luciferase activity in cell extracts after 24 and 48 h p.I. Values are given as relative light units (RLU). **(c)** HCoV-229E-LUC N-protein expression as replication measure in Huh-7.5, sh-CypA-KD, non-target control (sh-Ctr) and with wtCypA reconstituted Huh-7.5 cells. qPCR/Western blot methods and materials used are the same as described in [36].

susceptibility for or the resistance to Feline Infectious Peritonitis (FIP), an immune-mediated, highly lethal disease without effective therapy and prevention. A genome-wide association study identified 20 SNPs with significant effect for the antibody level against IBV in chicken [53].

By demonstrating the inhibitory potential of CsA and non-immunosuppressive derivatives as effective inhibitors of CoV replication we and others have introduced cyclophilins, the targets of these compounds, as possible host targets for preventing CoV infection. Using CypA knockdown or knockout mutant cell lines we have shown the requirement of the protein for CoV replication.

Most intriguingly, the individual CypA mutations [D66E]/[G96D]/[N106I] did not support 229E-LUC replication as opposed to the [E84D]/[I89T]/[E134K] variants, which is in close agreement with the HCV replication studies in these cell lines [46]. As discussed by these authors the first three functional (with respect to suppression of virus replication) amino acid exchanges are located near the isomerase active site whereas the non-functional second mutation set is located remote (see Figure 1). Destabilization of CypA was identified as the underlying mechanism, resulting in near-complete intracellular CypA depletion. Before infection of the mutant cell lines with HCoV-229E, CypA mRNA levels detected by qPCR analysis were tested and found to be (with the exception of mutant E84D) quite comparable. It is reasonable to assume the similar mechanisms for the reduced and differential replicative behavior of the coronaviruses.

Even though it could be expected that depletion or destabilization of the highly prominent CypA as a house-keeping gene would be detrimental to cell growth it is clear that the mutated Huh-7.5 cell lines proliferate quite normal. Reasons could be either that, as opposed to a knockout the knockdown of CypA is not complete and the activity of the residual molecules suffices for cell growth, or the PPIase functions could be overtaken by other isomerases as was already shown for prolyl isomerases of the Pin1 type [54,55]. In any case, CypA is a prolyl isomerase required for propagation of HCV and CoVs. From both, the HCV and the HCoV studies it is clear that expression of correctly folded, stable CypA is essential and that blockade of the active groove by CsA or

its derivatives blocks enzymatic functions essential for replication of both viruses. For coronaviruses it has to be clarified whether other viral proteins, apart from the CypA binders nsp1 and nucleocapsid, require the proline-directed binding and PPIase activity of CypA and what the underlying mechanism for their involvement in virus replication is.

Due to the fact that CypA SNPs are very rare in the human population and CypA can be effectively inhibited by CsA or non-immunosuppressive derivatives thereof, it is even more intriguing to put efforts into the development of those compounds into broad-spectrum anti-coronaviral drugs.

Conclusions

Several viruses, including coronaviruses and HCV require functional CypA for replication. SNP variants causing amino acid exchanges around the PPIAse active site destabilize the protein and contribute to lower virus replication. Its role during CoV replication needs to be clarified. CypA represents an important host factor whose activity can efficiently be blocked by HTAs like CsA and non-immunosuppressive derivatives thereof.

Conflicts of interest

The authors disclose no conflicts of interest.

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- •• of outstanding interest

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