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Cellular peptidyl-prolyl *cis/trans* isomerase Pin1 facilitates replication of feline coronavirus

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ABSTRACT

Although feline coronavirus (FCoV) causes feline infectious peritonitis (FIP), which is a fatal infectious disease, there are no effective therapeutic medicines or vaccines. Previously, *in vitro* studies have shown that cyclosporin (CsA) and FK506 inhibit virus replication in diverse coronaviruses. CsA and FK506 are targets of clinically relevant immunosuppressive drugs and bind to cellular cyclophilins (Cyps) or FK506 binding proteins (FKBPs), respectively. Both Cyp and FKBP have peptidyl-prolyl *cis-trans* isomerase (PPIase) activity. However, protein interacting with NIMA (Pin1), a member of the parvulin subfamily of PPIases that differs from Cyps and FKBPs, is essential for various signaling pathways. Here we demonstrated that genetic silencing or knockout of Pin1 resulted in decreased FCoV replication *in vitro*. Dipentamethylene thiuram monosulfide, a specific inhibitor of Pin1, inhibited FCoV replication. These data indicate that Pin1 modulates FCoV propagation.

1. Introduction

Coronaviruses (CoVs) cause severe diseases of the respiratory system, gastrointestinal tract, and the central nervous system in animals (Perlman and Netland, 2009). Feline CoVs (FCoVs) have been classified into two biotypes comprising the ubiquitous feline enteric CoV (FECV) and feline infectious peritonitis virus (FIPV) (Pedersen, 2009). Feline infectious peritonitis (FIP) is one of the most frequent causes of death in young cats, and classical symptoms of effusive/wet FIP, non-effusive/dry form of FIP, or a combination of the two can develop (Berg et al., 2005). Mortality is extremely high once clinical signs appear, although some cats can live with the disease for weeks, months, or even years (Pedersen, 2014). Nevertheless, FIP is currently incurable by drug treatment, and there are no effective prophylactic vaccines. Virus replication depends on a variety of host factors (de Haan and Rottier, 2006; Vogels et al., 2011; Zhang et al., 2010), which consequently represent potential antiviral targets. We have reported that cyclosporin A (CsA), a cellular cyclophilin (Cyp) inhibitor, can inhibit FCoV replication in cell culture (Tanaka et al., 2012). Although FK506 suppresses calcineurin and the nuclear factor pathway of activated T-cells (NFAT) at the same stage as CsA, FK506 did not affect FCoV replication (Tanaka et al., 2012). These data indicate that CsA does not exert inhibitory effects via the NFAT pathway. We have reported that CsA treatment caused a sustained reduction in pleural fluid volume and viral copy number in a cat diagnosed with effusive FIP (Tanaka et al., 2015). CsA is well known as a potent replication inhibitor of various human and animal CoVs (de Wilde et al., 2013; Pfefferle et al., 2011). Regarding requirements of Cyps in CoV replication, using small interfering RNA (siRNA) experiments, de Wilde et al. (2011) reported that both CypA and CypB did not affect severe acute respiratory syndrome CoV (SARS-CoV) replication (de Wilde et al., 2011). In contrast, human CoV NL63 replication depends on CypA but not CypB (Carbajo-Lozoya et al., 2014). FK506 inhibits human CoVs, SARS-CoV, NL63, and 229E (Carbajo-Lozoya et al., 2012), but each CoV requires different immunophilins as described above.

Cyps and FKBPs, two major families of peptidyl-prolyl *cis-trans* isomerase (PPIase) that catalyze the *cis-trans* isomerization of the prolyl peptide bond preceding proline residues, are targets of clinically relevant immunosuppressive drugs, CsA and FK506, respectively (Siekierka et al., 1989a, 1989b). The immunosuppressive activity of these drugs is unrelated to inhibition of PPIase

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activity, and neither Cyp nor FKBP genes are essential (Yaffe et al., 1997). In contrast, Protein Interacting with NIMA (Pin1), a member of the parvulin subfamily of PPIases that differs from Cyps and FKBPs, is essential for cell growth and requires a catalytically competent PPIase domain (Lu et al., 1996; Lu and Hunter, 1995). Its catalytic site is unique among other PPIase enzymes because it recognizes an unusual phosphorylated Ser/Thr–Pro motif in its substrates (Lu and Zhou, 2007; Lu et al., 2002; Wulf et al., 2005). Pin1 plays important roles in many cellular events, including cell cycle progression, cell proliferation, transcriptional regulation, and neoplasmic transformation. The protein has been linked to several diseases, such as cancer, Alzheimer's disease, and asthma (Lu and Zhou, 2007). Regarding infectious diseases, Pin1 directly interacts with the hepatitis C virus (HCV) NS5A and NS5B proteins and plays unique roles in HCV replication (Lim et al., 2011). Pin1 modulates human immunodeficiency virus type1 (HIV-1) infection by interaction with the capsid protein by uncoating and regulating APOBEC3G (Misumi et al., 2010; Watashi et al., 2008). However, there are no reports exploring the role of Pin1 in CoV replication.

In the present study, we examined the roles of Pin1 in FCoV replication. In conclusion, we report that Pin1 facilitates replication of FCoV, and a specific inhibitor of Pin1 inhibits both virus replication and protein expression *in vitro*. Therefore, Pin1 may be a potential target for FIP treatment as well as Cyps.

2. Materials and methods

2.1. Cell culture of virus

Felis catus whole fetus-4 (fcwf-4; American Type Culture Collection, VA, USA) cells were maintained in Dulbecco's modified Eagle's medium (D-MEM, Sigma–Aldrich, Tokyo, Japan) supplemented with 10% fetal bovine serum (Life Technologies, Tokyo, Japan). We propagated FIP virus FCoV (79-1146 strain was a gift from Dr. Tsutomu Hodatsu, Kitasato University, Japan) in fcwf-4 cells and then purified the virions by linear sucrose gradient ultracentrifugation.

2.2. Plasmid constructs

We isolated the feline peptidyl-prolyl *cis/trans* isomerase Pin1 gene from fcwf-4 cells, using the polymerase chain reaction (PCR) with primers 5′-TACCGAGCTCGGATCCACCATGGCGGACGAAG AGAAGCTG-3′ and 5′-GATATCTGCAGAATTCTCACTCCGTGCGCAG GATGATG-3′ for amplification. Total RNA of fcwf-4 cells was isolated using Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's protocol. The RNA was reverse transcribed using a PrimeScript reverse transcriptase (RT)-PCR kit (Takara-bio, Shiga, Japan) before complementary DNA (cDNA) of Pin 1 was amplified with PrimeSTAR Max DNA Polymerase (Takara-bio). Pin1 specific primers are described above. The Pin1 gene was cloned into pEF6/Myc-His A vector (Invitrogen, Tokyo, Japan) which was digested with EcoRI and BamHI restriction enzymes using In-fusion HD Cloning Kit (Takara-bio) according to the manufacturer's protocol. The Pin1 sequence cloned into the vector was confirmed by Big-Dye sequencing analysis (Applied Biosystems Japan, Tokyo, Japan). For genetic knockout (KO) experiments with fcwf-4 cells, we constructed the plasmids using CRISPR/Cas9 systems. Briefly, we synthesized oligonucleotides to guide RNA to target Pin1 DNA (Table 1), and these were sub-cloned into the vector, pSpCas9 (BB)-2A-Puro (pX459: Addgene, Cambridge, MA, USA). The sequences of all constructed plasmids were confirmed using a Big-Dye Terminator v1.1 Cycle Sequencing Kit (Life Technologies, CA, USA).

Table 1
Oligonucleotide sequences for single guide RNA targetting Pin1 gene.

Oligonucleotide name	Oligonucleotide sequence
Feline Pin1 Cas9-5 FW	5′-caccgaagagaagctgccgcccggc-3′
Feline Pin1 Cas9-5 RV	5′-aaacgccgggcggcagcttctcttc-3′

2.3. Transient expression of the Pin1 gene and infection with FCoV

After the cells were seeded 1 day prior to transfection at 2.5×10^5/well in 12-well plates, the plasmid vector containing the c-Myc-tagged Pin1 gene was transfected into fcwf-4 cells using Xtreme HD transfection reagent (Roche diagnosis, Tokyo, Japan) according to the manufacturer's instructions. The empty vector pEF6/Myc-His A was transfected into the cells to normalize the total amount of DNA per transfection. The cells transfected with the plasmids were infected with FCoV at a multiplicity of infection (MOI) of 1 plaque-forming unit (pfu) per cell, in order to study their effects on FCoV infection 24 h after transfection. The infected cells were collected after 20-h incubation and used for analysis.

2.4. Cell viability assay

We assayed WST-8 to evaluate the cytotoxicity of dipentamethylene thiuram monosulfide [DTM, (MP Biomedicals, LLC; Solon, OH, USA)] for fcwf-4 cells using the Cell Counting Kit-8 (Dojin Chemical Inc., Wako, Japan) according to the manufacturer's instructions.

2.5. Cells treated with dipentamethylene thiuram monosulfide or interferon-ω

fcwf-4 cells were incubated with or without various concentrations of DTM or interferon (IFN)-ω (Intercat, TORAY, Tokyo, Japan) for 30 min at 37 °C before we inoculated fcwf-4 cells with FCoV at a MOI of 1 pfu per cell to study their effects on FCoV infection. After adsorption for 1 h at 37 °C, medium containing the virus was removed, and the cells were rinsed three times with phosphate-buffered saline (PBS) and incubated with or without various concentrations of DTM or IFN-ω for 20 h before analysis by Western blotting and a quantitative (qRT-PCR) assay.

2.6. Real-time, quantitative reverse transcriptase-polymerase chain reaction

The fcwf-4 cells were infected at a MOI of 1 pfu per cell and then incubated with or without DTM. The medium was removed 20 h post-infection, and RNAiso-plus (Takara Bio) was added to the cells for RNA preparation according to the manufacturer's protocol. Total RNA was quantified using the One Step PrimeScript RT-PCR kit (Perfect Real Time; Takara-Bio). Viral cDNAs were quantified by real-time PCR using the forward and reverse primers for the FCoV-N gene (5′-TGGCCACACAGGGACAAC-3′) and (5′-AGAACGAC-CACGTCTTTTGGAA-3′) and the TaqMan probe (FAM-TTCATCTCCC-CAGTTGACG-BHQ-1). Reaction mixtures were prepared according to the manufacturer's protocol, and sequences were amplified using a 7500 Sequence Detection System (Applied Biosystems, Tokyo, Japan). cDNA to the FCoV-N gene was cloned into the pcDNA3.1 vector (Invitrogen), which was then serially diluted to provide standards for FCoV gene quantification. The viral RNA copy number was normalized using the feline β-2-microglobulin (β2M) gene (GenBank accession no. **NM_001009876**). The β2M gene derived from fcwf-4 cells was cloned by PCR amplification using the following primers: fβ2M-F 5′-GGCGCGTTTTGTGGTCTTGGTC-3′ and