Virus Research 329 (2023) 199099

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

The proprotein convertase SKI-1/S1P is a critical host factor for Nairobi sheep disease virus infectivity

Caroline Bost [a], Julia Hartlaub [a], Vinícius Pinho dos Reis [b], Thomas Strecker [b], Nabil G. Seidah [c], Martin H. Groschup [a], Sandra Diederich [a], Kerstin Fischer [a,*]

[a] Institute of Novel and Emerging Infectious Diseases, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany
[b] Institute for Virology, Philipps-University Marburg, Germany
[c] Montreal Clinical Research Institute (IRCM), affiliated to the University of Montreal, Laboratory of Biochemical Neuroendocrinology, Montreal, Quebec H2W 1R7, Canada

ARTICLE INFO

Keywords:
Nairobi sheep disease virus
Orthonairovirus
Host cell factor
Infectivity
Glycoprotein proteolytic processing
CCHFV

ABSTRACT

Nairobi sheep disease virus (NSDV) belongs to the *Orthonairovirus* genus in the *Bunyavirales* order and is genetically related to human-pathogenic Crimean-Congo hemorrhagic fever virus (CCHFV). NSDV is a zoonotic pathogen transmitted by ticks and primarily affects naïve small ruminants in which infection leads to severe and often fatal hemorrhagic gastroenteritis. Despite its veterinary importance and the striking similarities in the clinical picture between NSDV-infected ruminants and CCHFV patients, the molecular pathogenesis of NSDV and its interactions with the host cell are largely unknown. Here, we identify the membrane-bound proprotein convertase site-1 protease (S1P), also known as subtilisin/kexin-isozyme-1 (SKI-1), as a host factor affecting NSDV infectivity. Absence of S1P in SRD-12B cells, a clonal CHO-K1 cell variant with a genetic defect in the S1P gene (MBTPS1), results in significantly decreased NSDV infectivity while transient complementation of SKI-1/S1P rescues NSDV infection. SKI-1/S1P is dispensable for virus uptake but critically required for production of infectious virus progeny. Moreover, we provide evidence that SKI-1/S1P is involved in the posttranslational processing of the NSDV glycoprotein precursor. Our results demonstrate the role of SKI-1/S1P in the virus life cycle of NSDV and suggest that this protease is a common host factor for orthonairoviruses and may thus represent a promising broadly-effective, indirect antiviral target.

1. Introduction

Nairobi sheep disease virus (NSDV) is a tick-borne zoonotic bunyavirus of veterinary importance that has been reported to circulate in East Africa and Asia (Montgomery, 1917; Davies, 1997; Marczinke and Nichol, 2002; Krasteva et al., 2020). Infections of naïve small ruminants lead to severe and often fatal hemorrhagic gastroenteritis in these animals (Montgomery, 1917; Bin Tarif et al., 2012; Hartlaub et al., 2021). The virus belongs to the genus *Orthonairovirus* within the family *Nairoviridae* and has a negative-sense RNA genome with a small (S), medium (M) and large (L) segment and is genetically related to human-pathogenic Crimean-Congo hemorrhagic fever virus (CCHFV) (Kuhn et al., 2016). Due to striking similarities in the clinical picture between NSDV-infected ruminants and human CCHFV patients, NSDV has been suggested as a model organism for studying CCHFV pathogenesis (Baron and Holzer, 2015). However, despite the veterinary importance of NSDV, knowledge concerning its pathogenesis and virus-host interactions is limited.

Viral envelope glycoproteins directly interact with the susceptible host cell at different stages of the viral life cycle. They represent key factors in determining host cell tropism through binding to specific host cell receptors and by mediating virus-host membrane fusion (Bossart et al., 2013; Pierson and Kielian, 2013; Albornoz et al., 2016; Hulswit et al., 2021). Overall, most viral glycoproteins are synthesized as inactive precursors and rely on host cell proteases for post-translational processing and activation required for infectious virus (Guardado-Calvo and Rey, 2017; Hulswit et al., 2021). Host proteases within the proprotein convertase (PC) family represent well-known examples with a major impact on viral glycoprotein processing and ultimately on virus assembly, release and infectivity (reviewed in (Seidah and Prat, 2012; Garten, 2018; Seidah et al., 2021). The site-1 protease (S1P), also known as subtilisin/kexin-isozyme-1 (SKI-1), is a membrane-bound serine

* Corresponding author: Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald Insel Riems - Germany.
E-mail address: Kerstin.fischer@fli.de (K. Fischer).

https://doi.org/10.1016/j.virusres.2023.199099
Received 5 January 2023; Received in revised form 14 March 2023; Accepted 18 March 2023
Available online 22 March 2023

endoprotease that belongs to this group of PCs (Sakai et al., 1998; Seidah et al., 1999). SKI-1/S1P is ubiquitously expressed, highly conserved among mammalian hosts and plays a critical role in the proteolytic activation of sterol regulatory element binding proteins (SREBPs), which control the expression of key enzymes involved in cholesterol homeostasis and fatty-acid biosynthesis (Brown and Goldstein, 1997; Seidah et al., 1999). SKI-1/S1P has a unique substrate specificity and cleaves peptides following the hydrophobic consensus sequence R-X-(aliphatic)-Z↓, where X is any residue except Pro or Cys, and Z is any residue except Val, Pro, Cys, or Glu (Seidah et al., 1999; Pasquato et al., 2006; Seidah et al., 2021).

In addition to its function in the cellular lipid and cholesterol metabolism, SKI-1/S1P has been demonstrated to play a central role in the life cycle of several viruses, including arenaviruses, flaviviruses and hantaviruses (Lenz et al., 2001; Blanchet et al., 2012; Kleinfelter et al., 2015; Hyrina et al., 2017). Similarly, SKI-1/S1P has been shown to be involved in proteolytic processing of the CCHFV glycoprotein precursor complex (GPC) encoded by the viral M segment, which undergoes an unusually complex processing to form mature structural glycoproteins Gn and Gc as well as non-structural proteins of rather unknown function (Vincent et al., 2003; Sanchez et al., 2006). Consequently, SKI-1/S1P expression was shown to have a strong impact on CCHFV infectivity (Bergeron et al., 2007). However, whether SKI-1/S1P plays a similarly important role for other orthonairoviruses such as NSDV has not been investigated to date. Therefore, the aim of this work was to study the role of SKI-1/S1P expression in NSDV replication and infectivity.

2. Material and methods

2.1. Cell lines and virus isolate

Human adrenocortical carcinoma (SW13) cells (kindly provided by Ali Mirazimi, National Veterinary Institute, Sweden) were maintained in Leibovitz-15 (L-15) medium supplemented with 5% fetal calf serum (FCS; L-15–5) and incubated at 37 °C without CO_2. Chinese hamster ovary (CHO)-K1 and SRD-12B cells were a generous gift from J. L. Goldstein (Rawson et al., 1998). CHO-K1 cells were grown in DMEM nutrient mixture F12 Ham (GIBCO) supplemented with 10% FCS, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. SRD-12B cells were maintained as CHO-K1 cells with the addition of 5 µg/ml cholesterin (Sigma-Aldrich), 1 mM sodium mevalonate (Sigma-Aldrich), and 20 µM sodium oleate (Sigma-Aldrich). Both cell lines were incubated at 37 °C and 5% CO_2.

The NSDV isolate (Ganjam virus IG619; hereafter referred to as NSDV; GenBank accession number: KU925466, KU925465, KU925464) used in this study was grown on SW13 cells and has been described previously (Hartlaub et al., 2021). All work with live virus were performed under BSL3-conditions at the Friedrich-Loeffler-Institut.

2.2. Plasmids and transfection

The open reading frame (ORF) of the NSDV M segment (GenBank accession no. KU925465) was codon-optimized for expression in human cells and synthesized by Genscript (Netherlands). For detection in subsequent analyses, an HA-epitope tag was added to the C-terminus of the glycoprotein precursor (GPC; GPC-Gc-$_{HA}$). The NSDV GPC-Gc-$_{HA}$ ORF was then subcloned into the pCAGGS expression vector. Human SKI-1/S1P and LASV GP-C_{HA} expression plasmids have been described previously (Lenz et al., 2001; Schlie et al., 2010). All sequences are available upon request. Transfection experiments were performed using Lipofectamine 3000 (Invitrogen) following the manufacturer's protocol. Six hours post transfection (p.t.) the medium was changed to DMEM supplemented with 2% FCS.

2.3. Antibodies

A polyclonal rabbit-derived in-house antiserum was used to stain NSDV nucleoprotein (NP) in western blot (WB; 1:1000) and immunofluorescence analyses (1:200). This hyperimmune serum was collected from a rabbit after three consecutive immunizations with CCHFV NP (Kosova Hoti isolate) and confirmed to cross-react with NSDV NP in the respective assays. Monoclonal anti-ß actin antibodies (MA5–15739, Invitrogen) and rabbit-derived anti-membrane bound transcription factor protease site 1 (MBTPS1) antibodies (PA5–77103, Invitrogen) were diluted in 1:5000 and 1:1000 for WB analysis, respectively. Immunoprecipitation of HA-tagged proteins was performed using HA-specific H6908 antibodies (Sigma-Aldrich; 1:500). Precipitated proteins were detected in WB with monoclonal HA.11 antibodies (#901501, Biolegend; 1:1000). Secondary anti-mouse and anti-rabbit antibodies conjugated either with Horseradish peroxidase (HRP) or Alexa Fluor 488 (AF488; both Invitrogen) were used in WB (1:5000) or IFA (1:500), respectively.

2.4. Virus infection and multi-cycle replication kinetics

Confluent SW13, CHO-K1 and SRD-12B cells were washed once with PBS before inoculation with NSDV at a multiplicity of infection (MOI) of 0.1 for 1 h at 37 °C. After virus adsorption, inoculum was removed and cells washed three times with PBS before DMEM or L-15 supplemented with 2% FCS was added to each well. Supernatants were harvested at 0, 8, 12, 24, 48 and 72 h post infection (p.i.) for multi-cycle replication kinetics. To investigate the effect of SKI-1/S1P expression on NSDV infectivity, SRD-12B cells were transfected with an expression vector encoding human SKI-1/S1P 8 h before inoculation with NSDV as described above. Supernatants were collected at 0, 14, 24, 48 and 72 h p.i.. Virus titers in the supernatants were quantified as described below. Virus titers of three independent experiments performed in duplicates are presented as mean ± standard error of the mean (SEM).

To compare infectivity of virus progeny between SRD-12B and S1P-complemented SRD-12B cells, 100 µl of the supernatants collected at 24 h p.i. were transferred to confluent SW13 cells seeded in a 6-well plate (total volume was filled up to 500 µl with L-15 for inoculation). After an incubation period of 1 h, inoculum was removed and wells were washed three times with PBS before incubation for another 24 h. Then, SW13 cells were fixed and analyzed by immunoblot and immunofluorescence assays.

2.5. Tissue culture infectivity dose 50 ($TCID_{50}$)

Tissue culture infectivity dose 50 ($TCID_{50}$) was used to estimate the endpoint viral titers in supernatants from all infection experiments performed. For this, SW13 cells were seeded in 96-well plates at a concentration of 1.5×10^4 cells per well 24 h prior to virus inoculation. Supernatants were ten-fold serially diluted and added to the wells with eight replicates per dilution. Cells were incubated at 37 °C for 7 days before endpoint read-out via light microscopy. The Spearman-Kärber algorithm was used to determine $TCID_{50}$/ml.

2.6. RNA isolation and RT-qPCR

Viral RNA from cell supernatant was isolated using the QIAmp Viral RNA Mini Kit (Qiagen) following manufacturer's instructions. For the detection of NSDV S segment RNA, an RT-qPCR protocol was used as described previously (Hartlaub et al., 2021). For quantification of viral genome, a standard curve containing serial dilutions of a synthetic RNA harboring the primer/probe binding sites of the primer set used was included in every RT-qPCR run. RT-qPCR reactions were performed using the CFX96 Real-Time PCR System (Bio-Rad).

2.7. WB analysis

To determine the total expression of NSDV NP, β-actin and S1P, cells were lysed in 1% SDS in PBS. For separation in SDS-PAGE, sample loading buffer containing 40% glycerol, 0.1% bromophenol blue, 200 mM Tris (pH 6.8), and 4% β-mercaptoethanol was added. The proteins were separated on a 10% polyacrylamide gel under reducing conditions and transferred onto a nitrocellulose membrane. After blocking with 5% non-fat dry milk solution, the proteins were stained with specific primary antibodies and peroxidase-conjugated secondary antibodies and detected by enhanced chemiluminescence (Clarity Western ECL Substrate, BioRad). Molecular weights of protein bands were determined using the Image Lab software (version 6.0.1, Bio-Rad Laboratories).

2.8. Immunofluorescence analysis (IFA)

For immunostaining of infected cells, cells were grown on glass coverslips and fixed at different times p.i. with ice-cold methanol/acetone (M/A; 1:1). M/A was quenched by 0.1 M glycine in PBS supplemented with $MgCl_2$ and $CaCl_2$ (PBS^{++}). Then, cells were treated with a blocking-buffer containing 0.35% BSA (Sigma-Aldrich). NSDV NP antibodies were added for 1 h (1:200), and followed by incubation with Alexa Fluor 488-conjugated secondary antibodies for 45 min. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Images were acquired with an Eclipse Ti-S inverted microscope system (magnification, 20x) and were processed with the NIS-Elements BR 4.00.07 software (Nikon).

2.9. Immunoprecipitation

Cells transiently expressing LASV GP-C_{HA} or NSDV GPC-Gc-$_{HA}$ proteins were washed with PBS, followed by cell lysis in radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 10 mM EDTA, 50 units/mL aprotinin, and 20 mM Tris–HCl, pH 7.5). Cell lysates were centrifuged for 45 min at 20,000 × g, and GPC proteins were immunoprecipitated overnight using polyclonal anti-HA antibodies (H6908; 1:500). Protein A-Sepharose CL-4B (Sigma-Aldrich) suspension was added for another 45 min followed by three washes of the immune complexes with RIPA buffer. After suspension in sample buffer, the total amount of precipitated proteins per sample was separated on a 10% polyacrylamide gel under reducing conditions. Subsequent western blot analysis was performed as described above.

2.10. Compound

For testing of inhibitory effects on virus replication, infected cells were treated with PF-429242 (Sigma-Aldrich, reconstituted in deionized water) by adding the compound in different concentrations (5 μM, 10 μM, 20 μM) to the cell culture medium 1 h after virus inoculation and three washes. Supernatants were collected at 24 h p.i. and viral titers quantified by $TCID_{50}$. Virus titers of two independent experiments performed in duplicates are presented as mean ± SEM. Data were analyzed by unpaired t-test with Welch's correction.

2.11. Viability assay

Cell viability assay was performed using the Cell Proliferation Kit (MTT Assay; Roche) according to manufacturer's instructions. Briefly, SW13 cells were seeded in a 96-well plate (5×10^4 cells/well) and incubated for 24 h with medium containing PF-429242 in a 10-fold serial dilution (0.01 μM to 1000 μM). Each compound dilution was tested in quadruplicates. Control wells were left untreated. To determine cell viability after 24 h of treatment, 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)−2, 5-diphenyl tetrazolium bromide (MTT) was added to each well and incubated at 37 °C for 4 h. The MTT formazan crystals were extracted by adding the solubilization solution (100 μl/well). Optical density was measured at 590 nm. Cell viability was calculated using the following equation: cell viability (%) = (OD sample)/(OD untreated cell control) x 100.

2.12. Data and statistical analysis

Data was first analyzed using Microsoft Excel. Graphics were designed using GraphPad Prism (version 9.3.1). All statistical analyses were performed using GraphPad Prism (version 9.3.1). Statistical significance is denoted as ** for $P \leq 0.005$.

3. Results

Previous studies have demonstrated that the CCHFV GPC is cleaved by SKI-1/S1P at the consensus motif $RRLL_{519}$ (amino acid position refers to CCHFV-IbAr 10,200), and that this proteolytic cleavage has a strong impact on virus infectivity (Vincent et al., 2003; Bergeron et al., 2007). Interestingly, comparing the amino acid sequences of the CCHFV GPC (IbAr 10,200) with the NSDV GPC (Ganjam virus isolate IG619) revealed at least two potential recognition motifs in the NSDV GPC sequence for proteolytic processing by SKI-1/S1P (Fig. 1). Accordingly, we hypothesized that the proteolytic activity of SKI-1/S1P is required for NSDV GPC processing and thus might play a role for the production of infectious NSDV progeny.

To first determine whether SKI-1/S1P-mediated proteolytic activity is involved in NSDV GPC processing, we used CHO-K1 cells as well as SRD-12B cells, a clonal CHO cell variant with a genetic defect in the S1P gene (Rawson et al., 1998). Both cell lines were transfected with expression plasmids encoding NSDV GPC-Gc-$_{HA}$. At 24 h p.t., cells were lysed and the glycoproteins immunoprecipitated using an anti-HA antibody. In subsequent WB analysis, we observed two HA-tagged bands in SKI-1/S1P-competent CHO-K1 cells (~ 85 kDa and 75 kDa, Fig. 2, lane 1), suggesting the presence of an unprocessed Gc precursor and a fully cleaved Gc, as previously described for CCHFV (Vincent et al., 2003; Zivcec et al., 2016; Freitas et al., 2020). In contrast, we predominantly detected the unprocessed Gc precursor in SRD-12B cells (Fig. 2, lane 2). Interestingly, co-transfection of SKI-1/S1P expression plasmids restored the cleavage of NSDV GPC-Gc in SRD-12B cells,

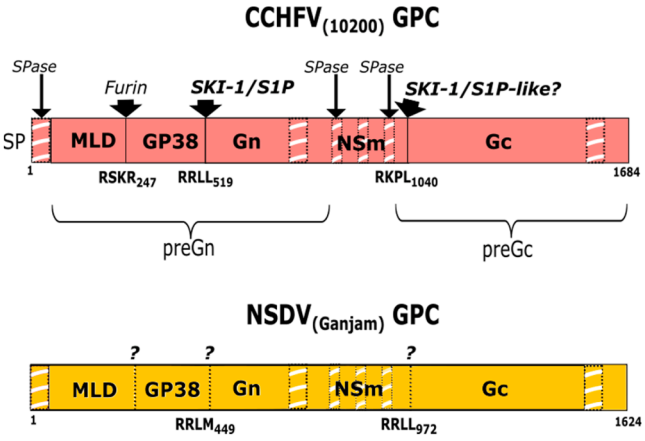

Fig. 1. Schematic of glycoprotein precursors (GPCs) of Crimean-Congo hemorrhagic fever virus (CCHFV; reference isolate CCHFV-IbAr 10,200) and Nairobi sheep disease virus (NSDV; Ganjam virus isolate IG619) with (putative) cleavage products. (Putative) cleavage sites are indicated in one letter code. Numbers represent amino acid positions. Proteases known to be involved in CCHFV processing are displayed. Horizontal white-shaded lines represent (putative) transmembrane domains. kDa: kilodalton; SP: signal peptide; SPase: signal peptidase; MLD: mucin-like domain; GP38: glycoprotein 38; Nsm: non-structural M protein. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

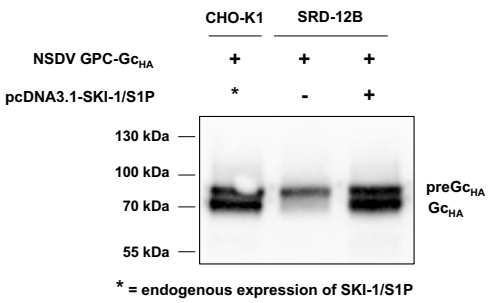

Fig. 2. SRD-12B cells cleave NSDV glycoprotein precursor (GPC) after transfection with recombinant SKI-1/S1P. Non-confluent SRD-12B and CHO-K1 cells were transfected with pcDNA3.1 plasmid encoding human SKI-1/S1P (+) or empty vector (-). After 8 h, cells were additionally transfected with vectors encoding NSDV GPC-Gc-$_{HA}$. At 24 h after transfection, cells were harvested and lysed. Proteins were immunoprecipitated using an antibody against the HA-tag (H6908) and subjected to SDS-PAGE and subsequent western blot analysis. Precipitated GPCs were visualized using a mouse-derived antibody against the HA-tag (HA.11) as well as HRP-labeled secondary anti-mouse antibodies and chemiluminescence. Representative blots are shown from three independent experiments.

resulting in the detection of two protein bands (Fig. 2, lane 3). These data suggest that SKI-1/S1P is critical for proteolytic processing of NSDV preGc to fully cleaved Gc. As a control, we additionally investigated proteolytic processing of LASV GP-C$_{HA}$ in both cell lines (Supplementary Figure 1), which was previously shown to be cleaved by SKI-1/S1P (Lenz et al., 2001). Our data verified SKI-1/S1P activity after transient expression and were consistent with the study by Lenz and colleagues.

In order to further investigate the role of SKI-1/S1P expression for the NSDV life cycle, we next performed multi-cycle replication kinetics in CHO-K1 and SRD-12B cells. SW13 cells were used as reference cell line. All three cell lines were infected with NSDV at a MOI of 0.1. First, cell lysates were collected at indicated time points p.i. to determine intracellular viral NP expression by WB analysis using a rabbit hyperimmune serum. While NSDV NP was detected as early as 12 h p.i. in SW13 control cells, first NP expression in CHO-K1 cells was observed at 24 h p.i., with both cell lines displaying a marked increase in the NP signal by 48 h and 72 h p.i. (Fig. 3A). Initial intracellular NSDV NP was only very weakly detectable in SRD-12B cells at 24 h p.i., and remained consistently low over time (Fig. 3A). In line with these findings, immunolabelling of the viral NP revealed only single infected SRD-12B cells in IFA at 72 h p.i. (Fig. 3B), indicating that NSDV was able to enter SRD-12B cells initially but that subsequent viral spread in this cell line was impaired. Moreover, we detected almost no infectious virus in the SRD-12B cell supernatant at any time point as determined by TCID$_{50}$ (Fig. 3C). In contrast, release of infectious virus in CHO-K1 cells increased steadily over the course of 72 h and only then reached titers comparable to those in the reference cell line SW13 (Fig. 3C), in which infection spread rapidly and viral titer peaked at 48 h (Fig. 3B). In contrast, the level of viral RNA in the supernatant of SRD-12B cells slightly increased over time but at a generally lower level compared to the other cells (Fig. 3D).

We next aimed to investigate whether transient expression of SKI-1/S1P in SRD-12B cells can rescue the production of infectious NSDV progeny. To this end, SRD-12B cells were transfected with a plasmid encoding human SKI-1/S1P 8 h before infection with NSDV (MOI of 0.1). Recombinant SKI-1/S1P overexpression was verified by WB analysis over a period of 72 h after NSDV infection with the strongest signals detectable between 24 h and 48 h p.i. (equaling 32 h and 56 h p.t.;

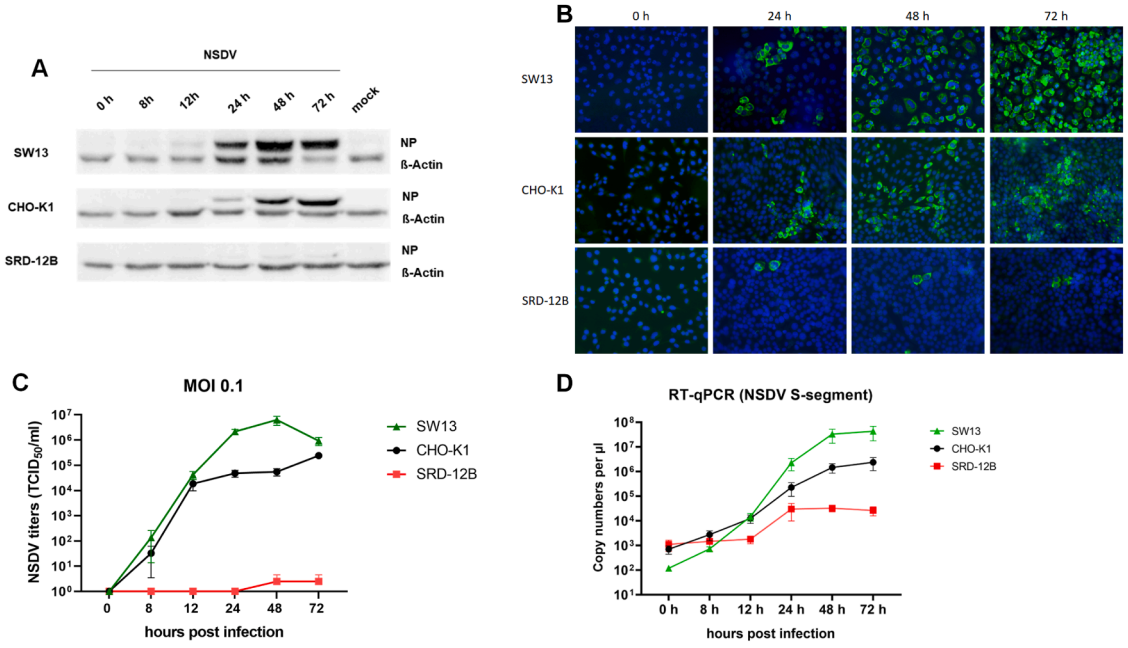

Fig. 3. Multi-cycle replication kinetics of NSDV in SW13, CHO-K1 and SRD-12B cells. Cells were inoculated with NSDV at a multiplicity of infection (MOI) of 0.1 and cultivated for up to 72 h. Samples were collected at the indicated times post infection (p.i.) for subsequent analyses. Three independent experiments were performed in triplicates. (A) Viral nucleoprotein (NP) expression determined by western blot. Cell lysates were collected, proteins separated by SDS-PAGE and viral NP was visualized in subsequent western blot using rabbit-derived anti-NP primary antibodies, HRP-labeled secondary antibodies and chemiluminescence. ß-actin served as loading control. Representative blots from three independent experiments are shown. (B) Virus spread determined by NSDV NP detection in immunofluorescence assay. Infected cells were fixed and incubated with polyclonal anti-NP antibodies. Primary antibodies were stained with anti-rabbit-Alexa Fluor 488 secondary antibodies. Nuclei were counterstained with Hoechst 33,342. Representative images from three independent experiments are shown. Magnification, 20x. (C) Replication of NSDV in SW13, CHO-K1 and SRD-12B cells. Supernatants were harvested and titrated by TCID$_{50}$. The growth curves shown display the mean viral titers ± SEM of three independent experiments. (D) Viral S segment RNA levels in the cell supernatant. The amount of viral S segment RNA in the supernatant was determined by RT-qPCR and expressed as mean copy numbers per microliter. Error bars depict the standard error of the mean (SEM). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

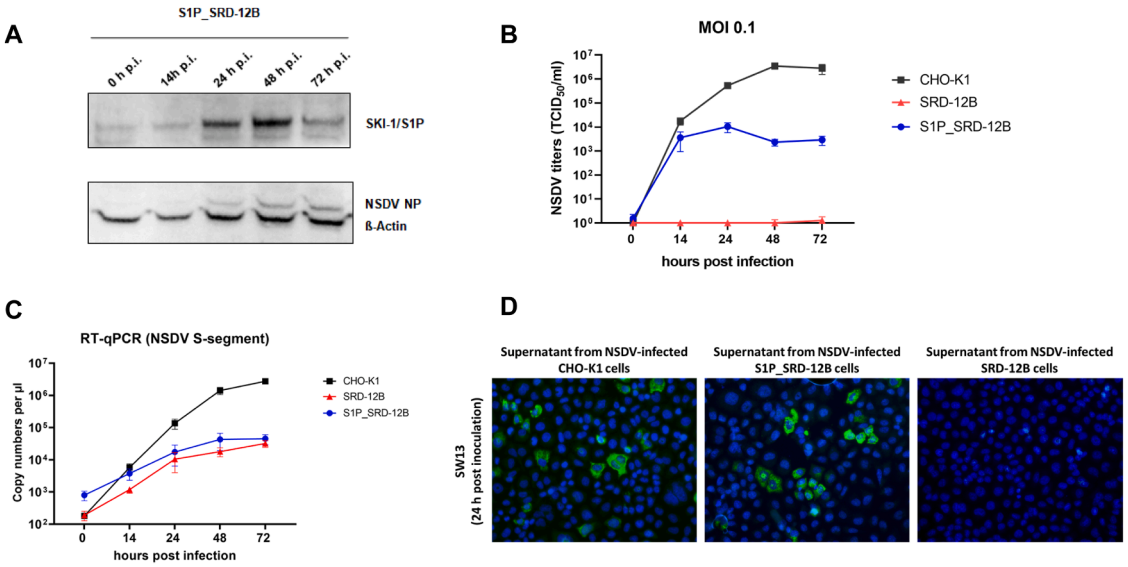

Fig. 4. Production of infectious NSDV progeny in SRD-12B can be restored by SKI-1/S1P complementation. Non-confluent SRD-12B cells were transfected with pcDNA3.1 plasmid encoding human SKI-1/S1P. Transfected cells were infected 8 h post transfection with NSDV at a MOI of 0.1 and cultivated for up to 72 h post infection (p.i.). Samples were collected at the indicated times p.i. for subsequent analyses. Three independent experiments were performed in duplicates. (A) Detection of SKI-1/S1P, viral NP and ß-actin expression in transiently transfected SRD-12B cells by western blot analysis. Cell lysates were collected, proteins separated by SDS-PAGE and target proteins detected using commercial MBTPS1 antibodies (Invitrogen), rabbit-derived anti-NP antibodies and anti-ß-actin antibodies. Labeled proteins were visualized by incubation with HRP-labeled secondary antibodies. One representative blot from three independent experiments is shown. (B) Replication of NSDV in CHO-K1, SRD-12B and S1P-complemented SRD-12B cells (S1P_SRD-12B). Supernatants were harvested and titrated by $TCID_{50}$. The growth curves shown display the mean titers plus SEM of three independent experiments. (C) Viral S segment RNA levels in the cell supernatant. The amount of viral S segment RNA in the supernatant was determined by RT-qPCR and expressed as mean copy numbers per microliter. Error bars depict the standard error of the mean (SEM). (D) Infectivity of virus progeny from cell supernatant of CHO-K1, SRD-12B and S1P_SRD-12B cells was determined by transfer to highly susceptible SW13 cells. Supernatants from NSDV-infected CHO-K1, SRD-12B and S1P_SRD-12B cells were collected at 24 h p.i. and transferred to confluent SW13 cells. Inoculum was removed after 1 h and wells were intensively washed before incubation for 24 h. SW13 cells were then fixed before polyclonal anti-NP antibodies were added. Labeled proteins were visualized using Alexa Fluor 488-conjugated secondary antibodies. Nuclei were counterstained with Hoechst 33,342. Representative images from two independent experiments are shown. Magnification, 20x. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4A, S1P_SRD-12B cells). First NSDV NP expression in SRD-12B cells complemented with SKI-1/S1P was observed at 24 h p.i. and increased only slightly over time (Fig. 4A). Noteworthy, infection of pre-transfected SRD-12B cells with NSDV resulted in the release of infectious virus to the cell supernatant reaching titers of up to 4×10^4 $TCID_{50}$/ml (Fig. 4B). The amount of viral S segment RNA in the cell supernatant also increased, but remained in a range comparable to the level of viral RNA in the supernatant of SRD-12B cells (Fig. 4C). To additionally verify the presence of infectious virus particles, the cell culture supernatant from S1P_SRD-12B cells collected at 24 h p.i. was used to inoculate SW13 cells for further analysis by IFA. Similarly, the supernatant from NSDV-infected SRD-12B cells was transferred to prove the absence of infectious virus progeny, while the supernatant from CHO-K1 cells served as a virus-containing control. Consistent with our previous results, NSDV NP was only detected in SW13 cells after 24 h when inoculated with supernatants from CHO-K1 and S1P_SRD-12B cells (Fig. 4D), indicating successful infection and thus the presence of infectious virus particles in the respective inoculum. In contrast, no NSDV NP was detected in SW13 cells after transfer from SRD-12B cell culture supernatant (Fig. 4D).

Finally, to determine whether the specific inhibition of SKI-1/S1P activity has an impact on NSDV infectivity, SW13 cells were infected with NSDV at an MOI of 0.1, and subsequently treated with different concentrations of PF-429242, a small-molecule inhibitor that has been shown to efficiently block SKI-1/S1P activity (Hawkins et al., 2008). Prior to use, potential cytotoxic effects of the compound were analyzed in the MTT Cell Proliferation Assay (Roche), which revealed that cell viability of SW13 cells was not affected after 24 h incubation with PF-429242 at concentrations up to 100 µM (Supplementary Figure 2). After NSDV inoculation, cells were further incubated for 24 h in the presence of 5 µM, 10 µM or 20 µM of PF-429242, which resulted in a substantial, dose-dependent reduction in NSDV titers compared to the untreated control (Fig. 5A). Interestingly, we detected only a 1 log reduction in RNA levels between treated and untreated SW13 cells (Fig. 5B), suggesting that inhibition of SKI-1/S1P has no substantial impact on RNA replication, whereas production of infectious virus

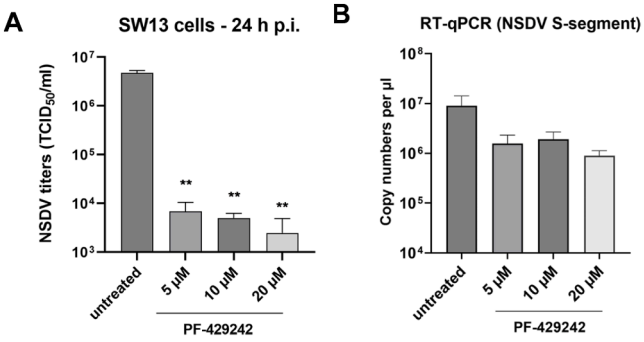

Fig. 5. Inhibition of NSDV by a small-molecule inhibitor. SW13 cells were infected with NSDV at a multiplicity of infection (MOI) of 0.1. After 1 h of incubation, virus inoculum was removed, cells were washed and medium containing the indicated concentrations of PF-429242 (5 µM, 10 µM, 20 µM) was added to the wells. Cells in control wells were left untreated. Supernatants were collected at 24 h post infection (p.i.) and analyzed by (A) $TCID_{50}$ and (B) RT-qPCR targeting the viral S segment. Bars represent mean (A) NSDV titers or (B) mean RNA copy numbers ± standard error of the mean (SEM) of two replicates from two independent experiments. Statistical analysis: Welch's t-test; (**) $p \leq 0.005$.

particles is drastically reduced.

4. Discussion

While SKI-1/S1P, an essential enzyme in the cholesterol regulation pathway of the cell, has been demonstrated to be of importance for the life cycle of human-pathogenic CCHFV (Vincent et al., 2003; Bergeron et al., 2007), its role for other zoonotic orthonairoviruses such as NSDV has been unclear. In this study, we provide first evidence that SKI-1/S1P is critical for NSDV replication and infectivity, and that this may be due to its role in proteolytic processing of the structural glycoprotein Gc.

SKI-1/S1P has been widely discussed as a promising target for indirect-acting antiviral drug development, since in addition to CCHFV, a number of other viruses such as arena- and flaviviruses are known to depend directly or indirectly on the functionality of SKI-1/S1P for their life cycle (Lenz et al., 2001; Beyer et al., 2003; Olmstead et al., 2012; Hyrina et al., 2017). One well-characterized compound in this context is PF-429242, a small-molecule inhibitor of SKI-1/S1P activity (Hay et al., 2007; Hawkins et al., 2008), whose efficacy has been demonstrated for several flaviviruses that rely on the functional host lipid metabolism, and thus indirectly on SKI-1/S1P activity, to complete their replication cycle. For example, PF-429242-mediated inhibition of SKI-1/S1P strongly impaired replication of hepatitis C virus (Blanchet et al., 2012), dengue virus (Uchida et al., 2016) and Zika virus (Raini et al., 2021). Another indirect dependence on SKI-1/S1P activity has been described for hantaviruses, where the inhibition of SKI-1/S1P by PF-429242 prevented hantavirus entry by depleting cellular membrane cholesterol, which was shown to be essential for hantavirus membrane fusion (Kleinfelter et al., 2015). However, even though PF-429242 did not show detrimental effects in a mouse model, the compound also demonstrated no significant protective effects after SFTS infection in mice (Shimada et al., 2015). While PF-429242 has thus exhibited broad antiviral activity against different flavi-, hanta- and also arenaviruses in vitro (Urata et al., 2011; Blanchet et al., 2012; Pasquato et al., 2012; Kleinfelter et al., 2015; Uchida et al., 2016; Raini et al., 2021), its efficacy against orthonairoviruses has not been investigated to date. Here, we demonstrate its efficacy against NSDV as an orthonairovirus representative. Inhibitor treatment resulted in a significant decrease in viral titers, clearly demonstrating the importance of SKI-1/S1P activity for the replication cycle of NSDV. Although the details of the NSDV replication cycle are still to be elucidated, our results point towards the essential role of SKI-1/S1P in glycoprotein processing. While indirect effects of SKI-1/S1P inhibition on virus entry or replication, as discussed earlier for hanta- or flaviviruses, cannot be completely excluded, the slight detection of NSDV NP in SRD-12B cells and the moderate increase of viral S segment RNA indicated the overall susceptibility of the S1P-deficient SRD-12B cells as well as its ability to support viral genome replication. Constantly low viral protein expression levels and only single infected cells during multicyclic replication however pointed towards an impaired viral spread. This assumption was further underlined by the lack of detectable infectious virus progeny in the cell culture supernatant of infected SRD-12B cells. Considering these findings and given the putative SKI-1/S1P recognition motifs found within the NSDV GPC sequence, a direct involvement of SKI-1/S1P in the proteolytic processing of NSDV GPC is more likely, as also observed for the antigenically related CCHFV GPC.

CCHFV GPC is cleaved by SKI-1/S1P at the characteristic consensus motif $RRLL_{519}$ (amino acid positions refer to the GPC of the CCHFV-IbAr 10,200 reference strain), which results in the production of structural glycoprotein Gn (Vincent et al., 2003). Interestingly, a similar SKI-1/S1P recognition motif ($RRLM_{449}$) is also found in the N-terminal region of NSDV GPC suggesting an involvement of the protease in NSDV Gn production. While cleavage of NSDV Gn will be investigated in a future study, the detection of HA-tagged NSDV preGc and Gc after transfection in CHO cells and S1P-complemented SRD-12B cells provided first evidence that SKI-1/S1P is involved in proteolytic processing of the C-terminal portion of NSDV GPC, presumably at the SKI-1/S1P consensus motif $RRLL_{972}$. Noteworthy, it has already been suggested that a SKI-1/S1P-related protease may play a role in the processing of CCHFV Gc, as another putative recognition motif ($RKPL_{1040}$) is found upstream of the N-terminus of CCHFV Gc (Sanchez et al., 2002). However, the corresponding protease involved in cleavage of CCHFV Gc remains to be identified, as cleavage has been shown to occur independently of SKI-1/S1P (Vincent et al., 2003).

SKI-1/S1P has also been described to be directly involved in the maturation of several arenavirus GPCs (Lenz et al., 2000; Lenz et al., 2001; Beyer et al., 2003; Pasquato et al., 2006). Interestingly, proteolytic cleavage of arenavirus GPC by SKI-1/S1P has been shown to be a prerequisite for proper assembly and release of infectious virus particles (Lenz et al., 2001; Beyer et al., 2003; Kunz et al., 2003; Rojek et al., 2008). Accordingly, for a number of different arenaviruses it has been reported that inhibition of SKI-1/S1P activity by PF-429242 resulted not only in impaired GPC processing but also in a lack of infectious virus progeny (Urata et al., 2011; Pasquato et al., 2012). Similarly, in the absence of SKI-1/S1P activity, the production of infectious CCHFV particles was significantly reduced in SRD-12B cells with neither mature nor immature glycoprotein forms detectable in the cell culture supernatant of infected cells, suggesting that GPC maturation plays an essential role in the production of infectious CCHFV progeny (Bergeron et al., 2007). However, in the absence of SKI-1/S1P activity, Bergeron and co-authors observed only nucleoprotein-containing particles in the cell culture supernatant of CCHFV infected cells. Both findings from Bergeron and colleagues are consistent with our data on the detection of NSDV S RNA and a significantly reduced amount of infectious NSDV progeny in the supernatant of S1P-deficient SRD-12B cells, suggesting a similar importance of SKI-1/S1P in the production of infectious virus progeny, possibly due to its role in GPC processing and maturation. In line with these findings, we detected high levels of viral RNA in the supernatant of SW13 cells treated with the SKI-1/S1P inhibitor PF-429242 (Fig. 5B), again indicating that RNA replication appears to occur independently of SKI-1/S1P activity. In contrast, viral titers were significantly reduced, further emphasizing the critical role of SKI-1/S1P in the production of infectious virus progeny. Moreover, in view of our data from the immunoprecipitation experiment, SKI-1/S1P appears to be involved in GPC processing, possibly at an identified consensus motif at the N-terminus of Gc ($RRLL_{972}\downarrow$), which could have significant impact on the incorporation of mature glycoproteins into virus particles and thus, ultimately on infectivity. Alternatively, in absence of SKI-1/S1P cleavage in *cis/medial* Golgi, the SKI-1/S1P processing site $RRLL_{972}\downarrow$ (Seidah et al., 2021) in NSDV GPC may be partially recognized by a Furin-like convertase allowing cleavage at the dibasic recognition motif $RR_{970}\downarrow LL$, likely in the trans-Golgi network (TGN) (Seidah et al., 2021). The significant decrease in infectious NSDV in the supernatant, either after inhibition or in the absence of endogenous SKI-1/S1P expression, as well as the fact that infectious NSDV was produced only in SKI-1/S1P-competent cells are however in line with previous reports on arenaviruses and CCHFV and point towards the importance of SKI-1/S1P in the NSDV replication cycle. Although we did not specifically address virus assembly and release, our results suggest that proteolytic processing and maturation of the NSDV GPC by SKI-1/S1P may play a similarly important role in the production of infectious virus progeny of NSDV. One can speculate that the observed reduction in viral titers obtained from S1P-deficient SRD-12 N cells could result from incorporation of immature glycoproteins into virus particles or even the total lack of glycoproteins in the virus envelope may account for the observed reduction in viral titers obtained from S1P-deficient SRD-12B cells. However, given the unusually complex maturation of the GPC of CCHFV (and thus potentially other orthonairoviruses), it cannot be ruled out that a SKI-1/S1P-mediated processing step other than the NSDV Gc cleavage may account for the observed effects. Thus, further analyses are needed to dissect the detailed mechanisms of proteolytic processing of the NSDV GPC and to elucidate the role of the cleavage products in virus

assembly, release and infectivity.

Overall, our study demonstrates the importance of the host cell protease SKI-1/S1P in NSDV infectivity and replication. Consistent with previous reports on the role of SKI-1/S1P in the viral life cycle of CCHFV, our results suggest that SKI-1/S1P may serve as a critical orthonairovirus host cell factor. Furthermore, this study provides first evidence of the antiviral activity of the small molecule inhibitor PF-429242 against NSDV. This warrants further experimental studies to evaluate its efficacy against other orthonairoviruses such as human pathogenic CCHFV.

Funding

This work was supported by the Friedrich-Loeffler-Institut, intramural funding (K.F.) and by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation)—Projektnummer 197785619/SFB1021 (T.S.). This work was also supported in part by a CIHR Foundation grant (N.G.S.: # 148363) and a Canada Research Chair in Precursor Proteolysis (N.G.S.: # 950–231335)

CRediT authorship contribution statement

Caroline Bost: Investigation, Methodology, Data curation, Writing – original draft. **Julia Hartlaub:** Investigation, Methodology, Writing – review & editing. **Vinícius Pinho dos Reis:** Data curation, Formal analysis, Visualization, Writing – review & editing. **Thomas Strecker:** Conceptualization, Resources, Writing – review & editing. **Nabil G. Seidah:** Resources, Writing – review & editing. **Martin H. Groschup:** Resources, Funding acquisition. **Sandra Diederich:** Conceptualization, Data curation, Writing – review & editing. **Kerstin Fischer:** Conceptualization, Data curation, Formal analysis, Visualization, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors wish to thank Sarah Knapp for excellent technical assistance as well as Bärbel Hammerschmidt, Sven Sander and FLI animal caretakers for their help during rabbit immunizations. We are grateful to J. L. Goldstein for kindly providing SRD-12B cells. The graphical abstract was created with BioRender.com (agreement number VR24LF9XIC).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2023.199099.

References

Albornoz, A., Hoffmann, A.B., Lozach, P.Y., et al., 2016. Early Bunyavirus-Host cell interactions. Viruses 8.
Baron, M.D., Holzer, B., 2015. Nairobi sheep disease virus/Ganjam virus. Rev. Sci. Tech. 34, 411–417.
Bergeron, E., Vincent, M.J., Nichol, S.T., 2007. Crimean-Congo hemorrhagic fever virus glycoprotein processing by the endoprotease SKI-1/S1P is critical for virus infectivity. J. Virol. 81, 13271–13276.
Beyer, W.R., Popplau, D., Garten, W., et al., 2003. Endoproteolytic processing of the lymphocytic choriomeningitis virus glycoprotein by the subtilase SKI-1/S1P. J. Virol. 77, 2866–2872.
Bin Tarif, A., Lasecka, L., Holzer, B., et al., 2012. Ganjam virus/Nairobi sheep disease virus induces a pro-inflammatory response in infected sheep. Vet. Res. 43, 71.
Blanchet, M., Seidah, N.G., Labonte, P., 2012. SKI-1/S1P inhibition: a promising surrogate to statins to block hepatitis C virus replication. Antiviral Res. 95, 159–166.
Bossart, K.N., Fusco, D.L., Broder, C.C., 2013. Paramyxovirus entry. Adv. Exp. Med. Biol. 790, 95–127.
Brown, M.S., Goldstein, J.L., 1997. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell 89, 331–340.
Davies, F.G., 1997. Nairobi sheep disease. Parassitologia 39, 95–98.
Freitas, N., Enguehard, M., Denolly, S., et al., 2020. The interplays between Crimean-Congo hemorrhagic fever virus (CCHFV) M segment-encoded accessory proteins and structural proteins promote virus assembly and infectivity. PLoS Pathog. 16, e1008850.
Garten, W., 2018. Characterization of proprotein convertases and their involvement in virus propagation. In: Böttcher-Friebertshäuser, E, Garten, W., Klenk, H. (Eds.), Activation of Viruses by Host Proteases. Springer, Cham., pp. 205–248 pp.
Guardado-Calvo, P., Rey, F.A., 2017. The envelope proteins of the bunyavirales. Adv. Virus Res. 98, 83–118.
Hartlaub, J., Gutjahr, B., Fast, C., et al., 2021. Diagnosis and pathogenesis of nairobi sheep disease orthonairovirus infections in sheep and cattle. Viruses 13.
Hawkins, J.L., Robbins, M.D., Warren, L.C., et al., 2008. Pharmacologic inhibition of site 1 protease activity inhibits sterol regulatory element-binding protein processing and reduces lipogenic enzyme gene expression and lipid synthesis in cultured cells and experimental animals. J. Pharmacol. Exp. Ther. 326, 801–808.
Hay, B.A., Abrams, B., Zumbrunn, A.Y., et al., 2007. Aminopyrrolidineamide inhibitors of site-1 protease. Bioorg. Med. Chem. Lett. 17, 4411–4414.
Hulswit, R.J.G., Paesen, G.C., Bowden, T.A., et al., 2021. Recent advances in bunyavirus glycoprotein research: precursor processing, receptor binding and structure. Viruses 13.
Hyrina, A., Meng, F., McArthur, S.J., et al., 2017. Human Subtilisin Kexin Isozyme-1 (SKI-1)/Site-1 Protease (S1P) regulates cytoplasmic lipid droplet abundance: a potential target for indirect-acting anti-dengue virus agents. PLoS ONE 12, e0174483.
Kleinfelter, L.M., Jangra, R.K., Jae, L.T., et al., 2015. Haploid genetic screen reveals a profound and direct dependence on cholesterol for hantavirus membrane fusion. MBio 6, e00801.
Krasteva, S., Jara, M., Frias-De-Diego, A., et al., 2020. Nairobi sheep disease virus: a historical and epidemiological perspective. Front. Vet. Sci. 7, 419.
Kuhn, J.H., Wiley, M.R., Rodriguez, S.E., et al., 2016. Genomic characterization of the genus nairovirus (Family Bunyaviridae). Viruses 8, 164.
Kunz, S., Edelmann, K.H., de la Torre, J.C., et al., 2003. Mechanisms for lymphocytic choriomeningitis virus glycoprotein cleavage, transport, and incorporation into virions. Virology 314, 168–178.
Lenz, O., ter Meulen, J., Feldmann, H., et al., 2000. Identification of a novel consensus sequence at the cleavage site of the Lassa virus glycoprotein. J. Virol. 74, 11418–11421.
Lenz, O., ter Meulen, J., Klenk, H.D., et al., 2001. The Lassa virus glycoprotein precursor GP-C is proteolytically processed by subtilase SKI-1/S1P. Proc. Natl. Acad. Sci. U. S. A. 98, 12701–12705.
Marczinke, B.I., Nichol, S.T., 2002. Nairobi sheep disease virus, an important tick-borne pathogen of sheep and goats in Africa, is also present in Asia. Virology 303, 146–151.
Montgomery, E., 1917. On a tick-borne gastroenteritis of sheep and goats occurring in british east africa. J. Comp. Pathol. Ther. 30, 28–57.
Olmstead, A.D., Knecht, W., Lazarov, I., et al., 2012. Human subtilase SKI-1/S1P is a master regulator of the HCV Lifecycle and a potential host cell target for developing indirect-acting antiviral agents. PLoS Pathog. 8, e1002468.
Pasquato, A., Pullikotil, P., Asselin, M.C., et al., 2006. The proprotein convertase SKI-1/S1P. In vitro analysis of Lassa virus glycoprotein-derived substrates and ex vivo validation of irreversible peptide inhibitors. J. Biol. Chem. 281, 23471–23481.
Pasquato, A., Rochat, C., Burri, D.J., et al., 2012. Evaluation of the anti-arenaviral activity of the subtilisin kexin isozyme-1/site-1 protease inhibitor PF-429242. Virology 423, 14–22.
Pierson, T.C., Kielian, M., 2013. Flaviviruses: braking the entering. Curr. Opin. Virol. 3, 3–12.
Raini, S.K., Takamatsu, Y., Dumre, S.P., et al., 2021. The novel therapeutic target and inhibitory effects of PF-429242 against Zika virus infection. Antiviral. Res. 192, 105121.
Rawson, R.B., Cheng, D., Brown, M.S., et al., 1998. Isolation of cholesterol-requiring mutant Chinese hamster ovary cells with defects in cleavage of sterol regulatory element-binding proteins at site 1. J. Biol. Chem. 273, 28261–28269.
Rojek, J.M., Lee, A.M., Nguyen, N., et al., 2008. Site 1 protease is required for proteolytic processing of the glycoproteins of the South American hemorrhagic fever viruses Junin, Machupo, and Guanarito. J. Virol. 82, 6045–6051.
Sakai, J., Rawson, R.B., Espenshade, P.J., et al., 1998. Molecular identification of the sterol-regulated luminal protease that cleaves SREBPs and controls lipid composition of animal cells. Mol. Cell 2, 505–514.
Sanchez, A.J., Vincent, M.J., Erickson, B.R., et al., 2006. Crimean-congo hemorrhagic fever virus glycoprotein precursor is cleaved by Furin-like and SKI-1 proteases to generate a novel 38-kilodalton glycoprotein. J. Virol. 80, 514–525.
Sanchez, A.J., Vincent, M.J., Nichol, S.T., 2002. Characterization of the glycoproteins of Crimean-Congo hemorrhagic fever virus. J. Virol. 76, 7263–7275.

Schlie, K., Maisa, A., Freiberg, F., et al., 2010. Viral protein determinants of Lassa virus entry and release from polarized epithelial cells. J. Virol. 84, 3178–3188.
Seidah, N.G., Mowla, S.J., Hamelin, J., et al., 1999. Mammalian subtilisin/kexin isozyme SKI-1: a widely expressed proprotein convertase with a unique cleavage specificity and cellular localization. Proc. Natl. Acad. Sci. U. S. A. 96, 1321–1326.
Seidah, N.G., Pasquato, A., Andreo, U., 2021. How do enveloped viruses exploit the secretory proprotein convertases to regulate infectivity and spread? Viruses 13.
Seidah, N.G., Prat, A., 2012. The biology and therapeutic targeting of the proprotein convertases. Nat. Rev. Drug Discov. 11, 367–383.
Shimada, S., Posadas-Herrera, G., Aoki, K., et al., 2015. Therapeutic effect of post-exposure treatment with antiserum on severe fever with thrombocytopenia syndrome (SFTS) in a mouse model of SFTS virus infection. Virology 482, 19–27.
Uchida, L., Urata, S., Ulanday, G.E., et al., 2016. Suppressive effects of the site 1 Protease (S1P) Inhibitor, PF-429242, on dengue virus propagation. Viruses 8.
Urata, S., Yun, N., Pasquato, A., et al., 2011. Antiviral activity of a small-molecule inhibitor of arenavirus glycoprotein processing by the cellular site 1 protease. J. Virol. 85, 795–803.
Vincent, M.J., Sanchez, A.J., Erickson, B.R., et al., 2003. Crimean-Congo hemorrhagic fever virus glycoprotein proteolytic processing by subtilase SKI-1. J. Virol. 77, 8640–8649.
Zivcec, M., Scholte, F.E., Spiropoulou, C.F., et al., 2016. Molecular insights into Crimean-Congo hemorrhagic fever virus. Viruses 8, 106.