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The Use of Alphavirus Protease for Inhibition of Virus Replication

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Abstract:

Alphaviruses are positive-strand RNA viruses, 12 kb in length, belonging to *Togaviridae* family. They are transmitted through arthropod vectors, can infect humans, and cause various of diseases. Structurally, they consist of two ORFs, one of which encodes four non-structural proteins that serve as viral replication machinery. Another ORF encodes three structural proteins. Studying the process of correct proteolytic processing of non-structural polyprotein which leads to production of nsPs is crucial for alphavirus replication life cycle. Alphaviral nsP2 protease has diverse functions including regulation of cleavages within the polyprotein and inhibition of cellular activities. The main aim of the thesis was the investigation of the inhibitory effect of six different versions of single-residue substitutions in SINV nsP2 protease to SINV-specific template/replicase RNAs as well as the inhibitory level of SINV nsP2 proteases on replication of different Alphaviruses. According to the results the SINV nsP2 N614D mutant was the strongest inhibitor among other six versions of SINV nspP2 tested, but the inhibition level of other mutants of SINV nsP2 on other Alphaviruses replication was not prominent. Neither vaccine nor antiviral therapy against Alphaviruses is available up to date, together with its frequent incidence of outbreaks have emphasized the necessity to study Alphaviruses. Current study can give an insight into the most important aspects of its replication and give a possibility to use this knowledge in order to elaborate different approaches for antiviral therapy.

Keywords:

Alphavirus, nsP2, inhibition, protease, Sindbis virus

CERCS: B230

Alfaviiruse proteaasi kasutamine viiruse replikatsiooni inhibeerimiseks

Kokkuvõte:

Alfaviirused on 12 kb pikkused positiivse ahelaga RNA viirused, mis kuuluvad *Togaviridae* sugukonda. Need levivad sääsehammustuste teel ja, võivad nakatada inimesi ja põhjustada mitmesuguseid haigusi. Struktuurselt koosnevad need kahest ORF-ist, millest üks kodeerib nelja mittestruktuurset valku, mis toimivad viiruse replikatsioonimehhanismina. Teine ORF kodeerib kolme struktuurvalku. Mittestruktuurse polüproteiini õige proteolüütilise töötlemise protsessi uurimine, mis viib nsP-de tootmiseni, on alfaviiruse replikatsiooni eluea tsükli jaoks ülioluline. Alfaviiruse nsP2 proteaasil on erinevad funktsioonid, sealhulgas polüproteiini lagunemise reguleerimine ja rakulise aktiivsuse pärssimine. Käesoleva lõputöö peamiseks eesmärgiks oli SINV nsP2 proteaasi ühe jäägi asenduste erinevate versioonide inhibeeriva toime SINV-spetsiifilistele matriits/ replikaas RNA-dele uurimine, ning SINV nsP2 proteaaside võime inhibeerida erinevate alfaviiruste perekonda liikmete replikatsiooni. . Eksperimentaalsed tulemused näitasid, et SINV nspP2 kuue versiooni hulgas SINV nsP2 N614D variant oli kõige tugevam inhibiitor, kuid teiste SINV nsP2 mutantide võime inhibeerida teiste alfaviiruste replikatsiooni oli nõrk. Efektiivsete ravimite ega ka toimiva vaktsiini puudus ning sagedased Alfaviirustest põhjustatud haiguste puhangud rõhutavad Alfaviiruste uurimise vajadustKäesolev töö võib anda ülevaate nende viiruste replikatsiooni olulisematest aspektidest ja anda võimaluse neid teadmisi kasutada viirusevastase ravi erinevate lähenemisviiside väljatöötamiseks.

Võtmesõnad:

Alfaviirus, nsP2, inhibeerimine, proteaas, Sindbise viirus

CERCS: B230

TABLE OF CONTENTS

TERMS, ABBREVIATIONS AND NOTATIONS 6

INTRODUCTION 7

1 LITERATURE REVIEW 8

1.1 Molecular structure and genome organization of Alphavirus 8

1.2 Roles and functions of Alphaviral non-structural region........ 9

1.2.1 Non-structural protein 1 (nsP1) 9

1.2.2 Non-structural protein 2 (nsP2) 9

1.2.3 Non-structural protein 3 (nsP3) 10

1.2.4 Non-structural protein 4 (nsP4) 10

1.3 Roles and function of Alphaviral structural region 11

1.3.1 Capsid protein 11

1.3.2 Envelope glycoproteins........ 11

1.3.3 6K protein 11

1.4 The entry of Alphavirus 11

1.5 The replication of Alphavirus in cells........ 13

1.6 Sindbis virus 14

1.7 SINV nsP2 protease and its variants........ 15

2 THE AIMS OF THE THESIS 17

3 EXPERIMENTAL PART 18

3.1 MATERIALS AND METHODS 18

3.2 RESULTS 20

3.2.1 Western blot analysis for protein expression level 20

3.2.2 Inhibition level of different nsP2 protease mutations of SINV in C6/36 cells ... 21

3.2.3 Inhibition level of SINV nsP2 on homologous and heterologous constructs in C6/36 cells 25

3.3 DISCUSSION 29

SUMMARY 31

ACKNOWLEDGMENTS 32

REFERENCES 33

NON-EXCLUSIVE LICENCE TO REPRODUCE THESIS AND MAKE THESIS PUBLIC 41

TERMS, ABBREVIATIONS AND NOTATIONS

EEEV – Eastern Equine Encephalitis Virus

VEEV – Venezuelan Equine Encephalitis Virus

CHIKV – Chikungunya Virus

RRV – Ross River Virus

SFV – Semliki Forest Virus

SINV – Sindbis Virus

MAYV – Mayayo Virus

EILV – Eilat Virus

ORFs – open-reading frames

nsP – non-structural protein

SG RNA – subgenomic RNA

G RNA – genomic RNA

RC – replication complex

Cp – capsid protein

Fluc – Firefly luciferase

Gluc – *Gaussia* luciferase

Cys (C) – Cysteine

His (H) – Histidine

Asn (N) – Asparagine

Asp (D) – Aspartic acid

Pro (P) – Proline

Gln (Q) – Glutamine

NLS – nuclear localization signal

SD – standard deviation

INTRODUCTION

Alphaviruses are widely spread all over the world and can be transmitted to vertebrate hosts by *Aedes albopictus* and *Aedes aegypti* mosquitos (Strauss and Strauss, 1994). Alphavirus infection can persist in insect cells for a long period and does not show deleterious effects, but is characterized by a strong cytopathic phenotype in vertebrate cells.

Depending on antigen analogy, 31 species of alphavirus have been distinguished and separated into eight groups where all of them are similar in both genome organization and virus replication (Rupp *et al*., 2015). Alternatively, they can be differentiated into two groups based on the geographical location where they were initially discovered: New World alphaviruses (for example VEEV, EEEV); Old World alphaviruses (such as SINV, EILV, SFV, CHIKV, MAYV, RRV).

The evidences of reappearance of members of Alphavirus genus such as SINV, CHIKV, RRV, EILV were found in Africa, Europe, and Asia predominantly. In contrast, VEEV and EEEV and MAYV outbreaks were described in America. Such outbreaks emphasize the potential threat that alphaviruses posture to humans.

Since there is no endorsed vaccine or antiviral therapy for alphavirus is available, it is necessary to study the molecular mechanisms behind alphaviruses replication and pathogenesis. In the current study, the possibility of utilizing potential inhibitors for suppression of alphavirus replication was studied where alphaviral nsP2 protease is the focused candidate.

1 LITERATURE REVIEW

1.1 Molecular structure and genome organization of Alphavirus

Alphavirus genome is a single-strand RNA with positive polarity with a 5′ cap and a 3′ poly-adenylated tail (Simmons and Strauss, 1972). The viral genome consists of two open-reading frames (ORFs). The first ORF covers two-thirds of the genome from 5' terminus, which encodes for the non-structural polyproteins; the second ORF covers one-third of the genome from 3' end which contains the genes for structural proteins (Strauss *et al.*, 1984) (Fig. 1A). The four non-structural proteins (nsP1, nsP2, nsP3, nsP4) encoded from the genomic (G) RNA are required for the viral mRNA cytoplasmic activities in the host cells. The subgenomic (SG) RNA is then generated following viral genome synthesis and negative template for RNA transcription, with a capped 5' end and 3′ poly-A tail (Faragher *et al.*, 1988; Strauss and Strauss, 1994). The three structural proteins: Capsid protein (Cp), envelop glycoproteins (E1, E2, E3) and 6K protein, which play essential roles in assembly of virus structure, budding and RNA encapsidation, are the results of the translation of SG RNA. (Foo *et al.*, 2011; Zhang *et al.*, 2002).

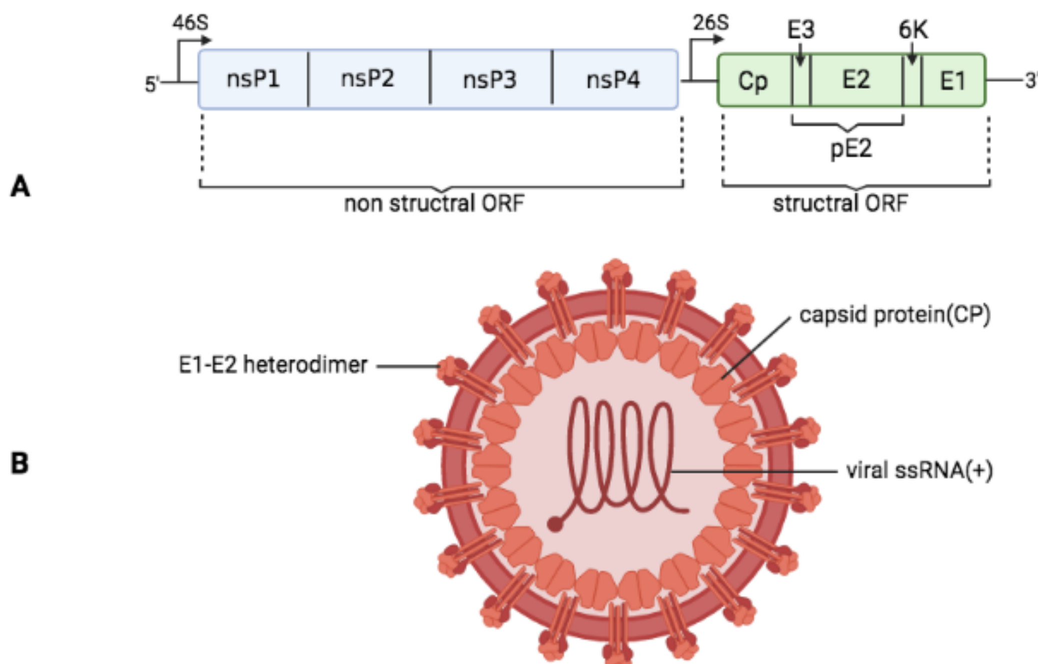

Figure 1. Alphavirus genome and virion structure. (A) The viral RNA is consisted with two open-reading frames (ORFs). The first 2/3 of the genome from 5' terminus encodes for the non-

structural proteins; 1/3 of the genome from 3' end contains the genes encoding for structural proteins. **(B)** The virion has a positive-strand RNA as an inner core encapsulated by capsid proteins and enveloped within a glycoprotein-embedded lipid bilayer.

1.2 Roles and functions of Alphaviral non-structural region

1.2.1 Non-structural protein 1 (nsP1)

nsP1 serves as the methyltransferase (MTase) and guanyltransferase (GTase) during the formation of the genomic and SG RNAs 5' capping (Mi and Stollar, 1991; Scheidel *et al*., 1987). This cap structure keeps the mRNA from degradation by cellular 5' exonucleases thus is important for viral mRNA to be translated. Besides, study in SINV demonstrated that the activity of the nsP2 can be modulated by nsP1 protein since the existence of nsP1 largely diminished the cleavage between nsP2 and nsP3 by nsP2 (de Groot *et al*., 1990).

1.2.2 Non-structural protein 2 (nsP2)

The nsP2 is the biggest non-structural protein and has a variety of enzymatic activities. It contains structural and functional domains: the N-terminal and the C-terminal (Russo *et al*., 2006).

The N terminus has been delineated to regulate ATPase, GTPase (Rikkonen *et al*., 1994) and with nucleoside triphosphate (NTP) binding domains to modulate triphosphatase activities at RNA 5′ terminus (Vasiljeva *et al*., 2000). Likewise, it performs as RNA helicase (Gomez De Cedrón *et al*., 1999) in the process of RNA replication and transcription for loosening the RNA-RNA duplex. Moreover, it is thought that the N-terminal region plays a vital role in the folding of the nsP replicase complex which is obligatory for the SG RNA synthesis (I Dé *et al*., 1996).

The C-terminal domain of the protein can perform proteinase activity and is involved in the regulation of cleavages at different sites within the polyprotein. The engagement of various capacities of the C terminus includes conducting the synthesis of SG RNA (Suopanki *et al*., 1998); downregulation of the synthesis of negative RNA (Sawicki *et al*., 2006); translocation of nsP2 into nucleus (Peränen *et al*., 1990) together with nuclear export pathway regulated by CRM1 protein (Montgomery and Johnston, 2007). It has also

been described that the nsP2 of SINV, CHIKV, SFV can trigger the degradation of RPB1 (a key component of RNA polymerase II) thus inhibit transcription in host cells (Akhrymuk *et al*. 2012).

It has been reported that nsP2 is crucial for suppressing the type I interferon (IFN) response in cells that are infected by Alphavirus (Breakwell *et al*., 2007).

1.2.3 Non-structural protein 3 (nsP3)

The exact role of the nsP3 in the replication of alphavirus remains unclear. The nsP3 protein consists of three domains. The first domain is exceptionally conserved among various viruses such as alphaviruses, hepatitis E virus, rubella virus and coronaviruses (Koonin and Dolja, 1993). The second is preserved in alphaviruses and the third domain varies between viruses (Strauss and Strauss, 1994). It is considered that the phosphorylation of C-terminal domain is associated with minus-stranded RNA synthesis (De *et al*., 2003). Study has suggested that nsP3 and nsP1 acts synergistically to intercede interaction of the RCs with cellular plasma membrane (Peränen, 1991). Additionally, nsP2 protease is presented to be influenced by nsP3 regarding the cleavage specificity (de Groot *et al*., 1990). A few investigations have exhibited that *in vitro* SINV replication (LaStarza *et al*., 1994) and pathogenesis of SFV and virulence in the mouse model may be regulated by the hypervariable region of nsP3 (Vihinen *et al*., 2001).

1.2.4 Non-structural protein 4 (nsP4)

It is thought that nsP4 serves as an alphavirus RNA polymerase since it contains a characteristic of viral RNA polymerases — the GDD motif (Kamer and Argos, 1984). Effective degradation according to the N-terminal rule pathway and translation of the opal stop codon in nsP3 can control the concentration of nsP4 in a strict manner (Strauss and Strauss, 1994). A detailed analysis outlining the essential functions of nsP4 has been performed and showed the fundamen-tal connection between P123 and nsP4. The interaction between RCs and host cell plasma membrane and the potential host factors involved in the process of viral replication, contributing to the development of antiviral targets for alphaviruses (Pietila *et al*., 2017).

1.3 Roles and function of Alphaviral structural region

1.3.1 Capsid protein

Capsid protein acts as an autoprotease and is the first structural protein to be cleaved from a single polyprotein precursor translated from the alphaviral SG RNA. Inside the cytoplasm of infected cells, from the order of Cp-PE2 (E3 + E2)-6K-E1 are the other structural or virion proteins to be cleaved (Melancon and Garoff, 1987; Strauss and Strauss, 1994; Zhang *et al*., 2002).

The alphaviral nucleocapsid is arranged in a $T = 4$ icosahedral lattice, consisting viral RNA as an inner core and a region made up of capsid proteins (Choi *et al*., 1991) (Fig. 1B).

1.3.2 Envelope glycoproteins

The glycoproteins E1 and E2, as heterodimers, are both transmembrane proteins which then combined into trimers that shape the spiky protrusions (Vogel *et al*., 1986; Rice and Strauss, 1982; Wahlberg *et al*., 1989). During virus maturation, E3 plays the crucial role in pE2 folding, pE2–E1 development and regulating the development of functional spikes (Parro *et al*., 2009; Snyder and Mukhopadhyay, 2012).

1.3.3 6K protein

6K is small and cysteine-rich protein (Strauss and Strauss, 1994). It is known for hydrophobicity and capability to interact with the host cell membrane. 6K is mainly participated in virus assembly and budding, translocation of proteins and membrane permeability.

1.4 The entry of Alphavirus

The entry of viruses is achieved by attaching receptors at the cell surface, fusing to plasma membrane or internalization, accompanied by vesicles of endocytosis. The prevalent way of entry, which is reconciled by the development of clathrin-coated pits and following movement to early endosomes, is the receptor-mediated endocytosis and can be activated by the low pH environment (Mayor and Pagano, 2007). The association between the E2 spike component and the protein receptors on the target cells surface promotes the entrance of alphaviruses into the cells (Smith *et al*., 1995; Smith and Tignor, 1980).

Rapid internalizations and fusions with endosomal membranes occur when the interactions between alphaviruses and membrane receptors are established (Fig. 2) (DeTulleo and Kirchhausen, 1998).

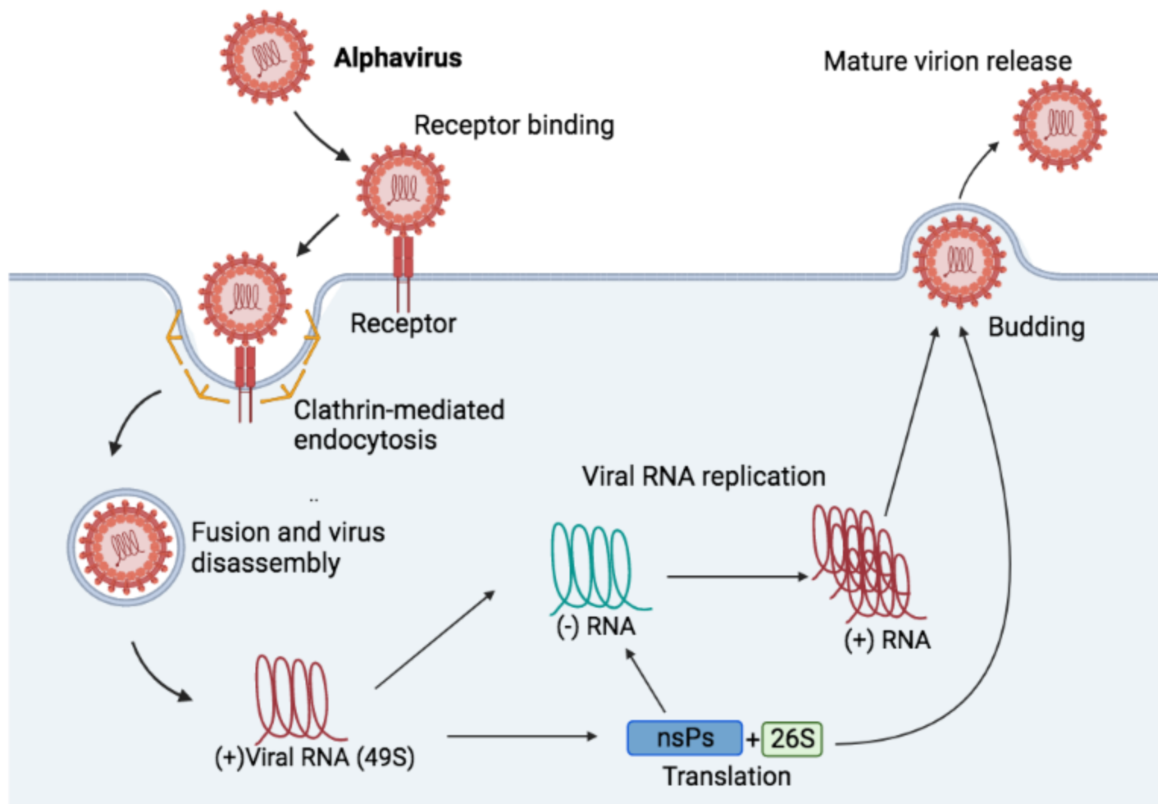

Figure 2. Alphavirus life cycle. Members of Alphavirus genus bind to the receptors on the host cell membrane and enter through clathrin-mediated endocytosis. Following fusion to endosomal membrane the release of G RNA into the cytoplasm occurs. Viral RNA serves as both transcription template for (-) RNA to generate (+) RNA and translation of non-structural and structural proteins. Subsequence budding of the newly synthesized viral particle occurs and virions are released.

Apart from the conventional illustrated model, growing evidence reported implies that there are alternative pathways for alphaviruses to enter the cells. One example to support this hypothesis is an early research showed that SINV can still enter the cytoplasm of mosquito cells and translate viral RNA without being exposed to an acid environment, suggesting that a low pH condition is not obligatory for infection and can be bypassed (Hernandez *et al*., 2001). The same pathway might be applied to SFV (Hase *et al*., 1989) and CHIKV (Bernard *et al*., 2010).

1.5 The replication of Alphavirus in cells

Following entry, genomic RNA is released into the cytoplasm of host cells (Fig. 2). The non-structural and structural polyproteins are then produced as the result of the translation of two ORFs of viral RNA (Glanville *et al*., 1976).

The structural proteins are encoded by SG RNA. The autoproteolytic cleavage of capsid protein (Cp) starts first among structural polyprotein (Garoff *et al*., 1978; Melancon and Garoff, 1987; Aliperti and Schlesinger, 1978). Cp is then released into cytoplasm and integrates with newly synthesized RNA (Kuhn *et al*., 1996; Garo and Li, 1998; Strauss and Strauss, 1994). Cellular proteases cleave the remainder of structural polyprotein (E3-E2-6K-E1) at both ends of 6K, producing PE2 (E3–E2), 6K and E1. PE2 and E1 are combined and form heterodimers that are transferred with 6K in vesicles to the cell surface through ER and Golgi apparatus (Barth *et al*., 1995; Andersson *et al*., 1997; Duffus *et al*., 1995). PE2 precursor is later cleaved at its luminal domain to produce E2 and E3 proteins (Ziemiecki *et al*., 1980; de Curtis and Simons, 1988; Sariola *et al*., 1995). The association between E1 and E2 is thus weakened to enable fusion peptide to be activated at a low pH level (Lobigs and Garoff, 1990). Encapsidation is directed by special packaging signals on the 5' two-thirds of the genomic RNA hence only intact genome can be packed (Owen and Kuhn, 1996; Weiss *et al*., 1989). The envelope around nucleocapsid is generated by the E1 and E2 dimers (Owen and Kuhn, 1997), the following associations between Cp and carboxy domain of E2 protein promote the viral budding action (Vaux *et al*., 1988; Metsikko and Garoff, 1990).

The complicated, highly regulated processing of non-structural polyproteins, which are encoded by G RNA, is crucial for alphavirus replication. The *cis*-cleavage of P1234 by nsP2 at the early stage of infection cleaves nsP4 from P1234 and resulting in the generation of P123 and nsP4 (Fig. 3) (de Groot *et al*., 1990; Takkinen *et al*., 1991). P123 + nsP4 serve as a precursor of replication complex that can synthesize negative-strand RNA using genomic RNA as a template (Shirako and Strauss, 1994; I Dé *et al*., 1996; Lemm *et al*., 1994). Only under the condition of an adequately elevated concentrations of P123 polyprotein can it be cleaved in *trans* and yields nsP1 and P23 (Fig. 3). This cleavage is possessed by a nsP2 protease. Based on previous studies, when this protease was inactivated by mutations, P123 remained uncleaved, causing an increased aggregation of negative-strand RNAs and decreased quantity of genomic and SG RNAs. Subsequently, RCs are formed

by nsP1 and P23 together with nsP4 and are involved in the synthesis of minus-stranded and genomic RNA, but not SG RNA. This initial RC is mostly localized in type I cytopathic vacuoles (CPV I) (I Dé *et al*., 1996; Froshauer *et al*., 1988; Kujala *et al*., 2001; LaStarza *et al*., 1994; Salonen *et al*., 2003). Following complete cleavage of P23, four individual non-structural proteins are fully mature, thus the synthesis of minus-stranded RNA stops and plus-stranded SG and G RNA synthesis initiates (Fig. 3) (Shirako and Strauss, 1994; Lemm *et al*., 1994).

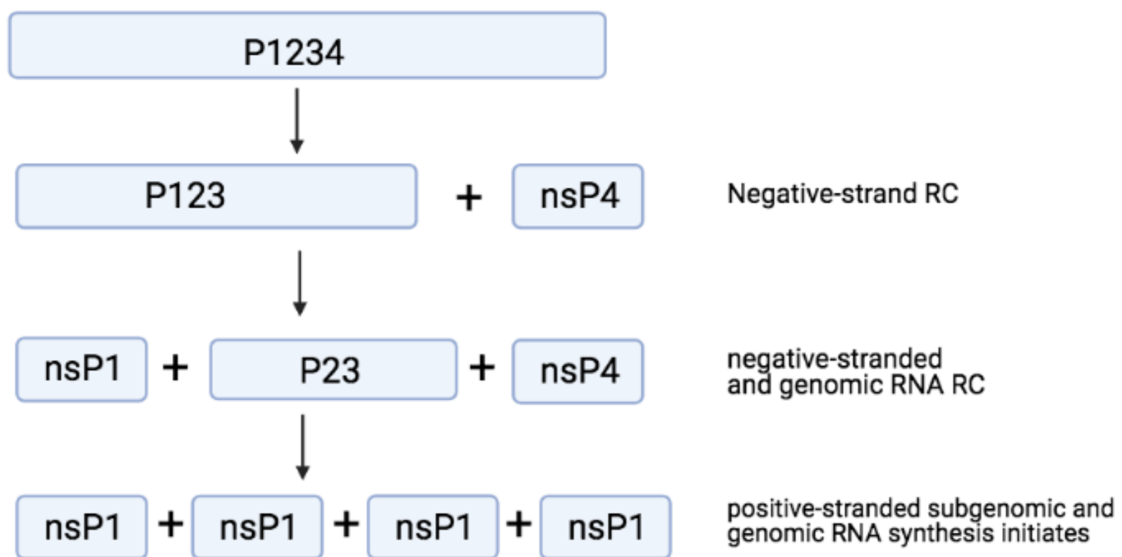

Figure 3. Schematic representation of Alphaviral non-structural polyprotein processing. The cleavage of P1234 at the early stage of infection generates P123 + nsP4. P123 polyprotein is then cleaved and yields nsP1 + P23 together with nsP4 as a RC to synthesize negative-stranded and G RNA. Further processing of P23 generates individual nsPs and positive-strand SG and G RNA synthesis is initiated.

1.6 Sindbis virus

Sindbis virus belongs to the Alphavirus genus and it is a mosquito-borne avian virus. It was first isolated in 1952 from the village of Sindbis in Cairo, Egypt. Infected patients suffered from symptoms such as rash and arthralgia which were characterized as sindbis fever.

SINV is broadly distributed across Eurasia, Oceania, and Africa. However, it is endemic in Northern Europe exclusively where major outbreaks occur occasionally. Cases of

SINV clinical infections are reported in Finland annually. A seven-year SINV infection pattern in Northern Europe was proposed according to a frequent incidence of outbreaks in the 1980s and 1990s (Brummer-Korvenkontio *et al*., 2002).

1.7 SINV nsP2 protease and its variants

It has been demonstrated that nsP2 of SINV is a papain-like cysteine protease (Strauss *et al*., 1992). The catalytic dyad of SINV nsP2 is identified to be composed of Cys-481 and His-558 and are essential for the proteolytic activity of the protein (Strauss *et al*., 1992).

It has been reported that alphaviral nsP2 protease is a vital determinant of alphavirus pathogenesis and cellular inhibition including the development of transcriptional and translational shutoffs in the host cells (Gorchakov *et al*., 2005). To further investigate the inhibitory effect on alphavirus replication due to the present of different mutations in SINV nsP2, the following variants were used.

1.7.1 SINV-WT

Variant SINV-WT refers to original wild-type sequence isolated from Sindbis virus which encodes SINV nsP2. It has variable functions which are important for alphavirus replication and also capable of causing inhibition of cellular activity in host cell.

1.7.2 SINV-N614D

It has been shown that mutation of N614D generates a hyperactivated enzyme and can cause a lethal phenotype of the virus due to the acceleration of SINV non-structural polyprotein processing (Lemm *et al*., 1994; Strauss *et al*., 1992). It is supposed that this mutation can be compensated partially by mutations at the ½ site (Lulla *et al*., 2018).

1.7.3 SINV-noNLS

It is assumed that SINV nsP2 has two SV40 TAg-like nuclear localization signals (NLSs) that are responsible for the migration of nsP2 into the nuclei (Frolov *et al*., 2009). Disruption of this translocation enables the ability of alphaviruses to shut down the transcription in the host cell.

1.7.4 SINV-PQ

The introduced PQ mutation into SINV nsP2 N614D and WT variants of protease preserves cytopathic phenotype and able to decrease the level of viral RNA replication but do not shutoff the transcriptional and translational activity at significant level (Akhrymuk *et al*., 2018).

1.7.5 SINV-CA

Cysteine (C) as one of the important components of catalytic dyad of SINV is essential for the proteolytic activity of the enzyme and the replacement of it with Alanine (A) leads to abolishment of the proteolytic activity of the protease.

2 THE AIMS OF THE THESIS

This study mainly focused on the possibility of utilizing SINV nsP2 protease as an inhibitor for virus replication and transcription of different Alphaviruses because the Sindbis virus is one of the most well-studied Alphaviruses and is a good model for research (Reference!). The activity of SINV nsP2 protease and its variants with different mutations in insect cells and the interactions of SINV nsP2 protease with other Alphaviruses was studied.

Specific aims are:

1. Verification of different versions of SINV nsP2 proteins expression level by Western Blot (with 5 different mutations) and comparison of their expression at different time points;
2. Investigation of inhibition level of different SINV nsP2 mutants (WT, CA, N614D, N614D-PQ, WT-PQ and noNLS);
3. The effect of protease activity on homologous and heterologous template/replicase efficiency.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

Expression Constructs

Expression constructs of corresponding Alphavirus template RNAs were cloned into pUC57Kan plasmid under the control of AlbPolI promoter, containing Fluc and Gluc luciferase markers and was designated as pUC57-AlbPolI-Fluc-Gluc-Alphavirus.

Expression constructs for Alphavirus replicases (pUbi-Alphavirus-WT, pUbi-Alphavirus-GAA) as well as proteases (pMC-Ubi-SINV WT, CA, N614D, N614D-PQ, WT-PQ, noNLS) were cloned under the control of polyubiquitin promoter. The final constructs SINV WT replicase is referred to wild-type sequences encoding for P1234 and GAA referred to mutant lack of polymerase activity (GDD was substituted to GAA at the active site of nsP4).

All constructs were prepared by colleagues at the University of Tartu.

***In vitro* transformation**

E. coli strain XL-10 cells were used to amplify all DNAs construct. After 30 min of incubation on ice and following heat shock at 42 °C, super Optimal Broth (SOB) medium supplemented with 0.4% glucose was added for plasmids recovery for the next 45 min, with following plating on plates containing kanamycin (Km) at a final concentration of 25 μg / ml.

Transfection

C6/36 cells were transfected with 1 μg of each DNA (SINV WT, CA, N614D, N614D-PQ, WT-PQ and noNLS) on 6-well plate using GeneJammer (Agilent) at 28 °C. To determine the optimal time in order to get the maximum expression of the proteases, SINV nsP2 WT protease was used and cells were collected at different time points (6h, 12h, 18h, 24h, 36h, 48h). Another set of transfections was performed to evaluate the level of expression of all protease constructs for SINV. Cell lysates for SINV WT, CA, N614D, N614D-PQ, WT-PQ and noNLS were collected 48 h post transfection.

Cell lines

C6/36 insect cells were maintained in Leibovitz's L-15 medium (PAN Biotech) containing 10% heat-inactivated fetal bovine serum (FBS) and 10% tryptose phosphate broth (TPB) at 28 °C. The media was supplemented with 100 U/m penicillin and 0.1 mg/ml streptomycin.

Fluorescent Western blot

Cells transfected with construct of SINV nsP2 WT protease were lysed after different time points (6h, 12h, 18h, 24h, 36h, 48h) and cells transfected with different constructs of SINV nsP2 proteases (WT, CA, N614D, N614D-PQ, WT-PQ and noNLS) were lysed 48h post-transfection, both with 2xLaemmli: H2O: 1M DTT (5:4:1). Proteins were separated by 10% SDS-PAGE and blotted on to Immobilon-FL membrane (Merck Millipore, ref: IPFL10100) (PVDF membrane). The expression of SINV nsP2 protein was verified with primary polyclonal anti-rabbit nsP2 antibody with following incubation with secondary SINV nsP2 anti-goat antibodies. β-Actin was used as loading control. The signals were visualized with the LI-COR Odyssey Fc Imaging System..

Trans-replicase assay (TRA)

For the first experiment: Cells were grown on 96-well plates (3.5×10^4 cells/well) and co-transfected with the mix of DNAs containing 440 ng of SINV template (pUC57-AlpPolI-Fluc-Gluc-SINV), 440 ng of plasmid encoding SINV replicases (WT and GAA) and 440 ng of different mutants of SINV nsP2 protease (SINV WT, SINV CA, SINV noNLS, SINV N614D, SINV N614D-PQ, SINV WT-PQ) and control transfection with the plasmids expressing a template and a replicase using Lipofectamine LTX (Thermo Fisher Scientific) according to manufacturer's protocol.

For the second experiment: Cells were grown on 96-well plates (3.5×10^4 cells/well) and co-transfected with the mix of DNAs containing 440 ng of virus-specific template (pUC57-AlpPolI-Fluc-Gluc), 440 ng of plasmid encoding virus-specific replicases (WT and GAA) and 880 ng of SINV nsP2 protease (WT and CA) and control transfection (template + replicase) using Lipofectamine LTX (Thermo Fisher Scientific) according to manufacturer's protocol.

All transfected cells were incubated at 28 °C for 48 hours and lysed in 1X Passive lysis buffer (Promega). Fluc and Gluc activities were measured using the dual-luciferase reporter assay system (Promega). All assays were done in triplicate.

Statistical analysis

GraphPad Prism 9 software was used for statistical analysis.

Figures creation

Figure 1, figure 2 and figure 3 were created with BioRender.com

3.2 RESULTS

3.2.1 Western blot analysis for protein expression level

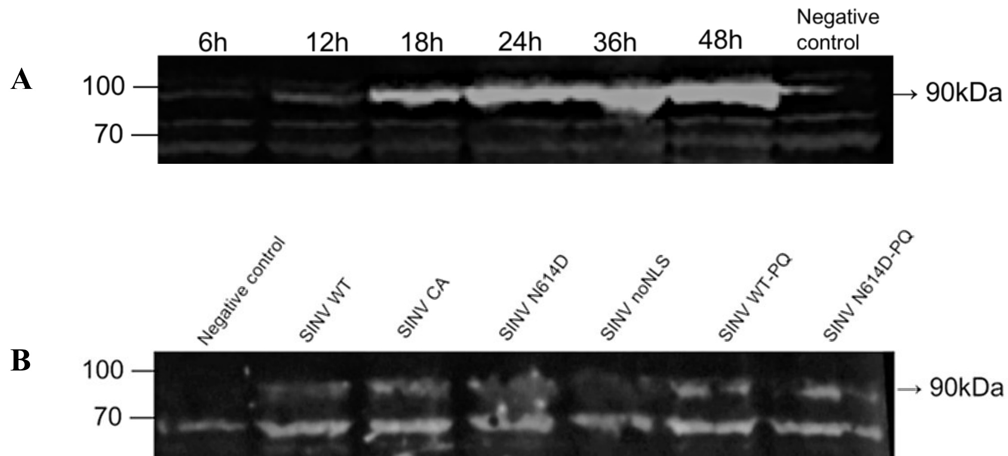

Figure 4. Western blot for verification of SINV nsP2 protein expression level. A. C6/36 cells were transfected with construct of SINV nsP2 WT protease and lysed at different time points (6h, 12h, 18h, 24h, 36h, 48h), corresponding antibodies were used and the size of the target protein is detected (pointed with an arrow). **B.** C6/36 cells were transfected with different constructs of SINV proteases with following lysis after 48 hours. Protein expression was verified with corresponding antibodies. The size of the proteins is indicated with the arrow (~90 kDa).

Preliminary examination of the expression level of SINV nsP2 at different time points was done by Western Blot. The DNA construct encoding SINV nsP2 WT protease was transfected using GeneJammer in C6/36 cells and collected after 6h, 12h, 18h, 24h, 36h and 48h. Based on the results observed (Fig. 4A), transfected cells lysed after 48h has shown the optimal expression level of protein at ~90kDa, this information was used for the following experiments.

After determination of the optimal time point, examination of the expression level of SINV nsP2 carrying different mutations was done. The plasmid constructs encoding different versions of SINV nsP2 proteases were transfected using GeneJammer in C6/36 cells and collected after 48 hours. Western blot analysis confirmed that all versions of SINV nsP2 expressed the protein at a significant level and in correct manner at the expected molecular weight of around 90 kDa (Fig. 4B) at 48 h post transfection.

3.2.2 Inhibition level of different nsP2 protease mutations of SINV in C6/36 cells

It has been published that point mutations in one out of three cleavage sites can lead to more efficient cleavage, however another approach can be used such as mutations in the C-terminal domain of nsP2.

Trans-replicase assay as the main method was chosen to observe the effect of point mutations introduced into SINV nsP2 on replication/transcription excluding the possibility to get adaptive/second-site mutations or any other compensatory changes during viral replication. Using the advantage of this method to uncouple replication from replicase production, we were able to analyse the inhibitory level of different versions of nsP2 proteases on SINV replication and transcription in C6/36 cells successfully.

Based on the experiments which had been done in triplicates, we concluded that the levels of Fluc and Gluc expression in cells indicating the levels of replication and transcription, respectively, under the effects of different nsP2 mutants was different, as the protease activity of different nsP2 variants processed the polyprotein precursor (SINV P1234) in various ways.

Both results of Fluc and Gluc expression have demonstrated that SINV nsP2 protease has the ability of inhibiting viral RNAs replication and transcription. SINV WT nsP2 has reduced the expression level of Fluc and Gluc activities to some extent comparing to the results of control (replicase + template). SINV CA as an inactivated version of SINV nsP2 protease due to the replacement of Cysteine (a vital component of catalytic dyad) to Alanine, has shown little inhibitory effect on replication and transcription comparing to the control but was not significant.

The lowest level of Fluc activity was observed for SINV N614D variant which can be explained with the fact that the effect of enzyme was slightly diminished. The variants of nsP2 harbouring N614D-PQ and WT-PQ performed polyprotein processing similarly to SINV N614D and showed only slight difference from it. These variants demonstrated a similar background, elucidating that the effect of PQ substitution was negligible (Fig. 5A).

The highest fold change of previously calculated impact activity for Fluc was observed for SINV variant of nsP2 lacking NLS which showed around 4 folds reduction of replication activity while the results for WT and N614D variants are 2 and 3 folds, respectively (Fig. 6A).

In contrast to the modest fold change of Fluc which was typically 2 to 4 folds for all mutants, the fold change of impact activity for Gluc expression was significantly higher and varies between different variants. Consistent with the results from Fluc activity, the lowest Gluc expression level (Fig. 5B) as well as the highest fold change of Gluc expression (42 fold reduction of transcription activity) was observed for N614D and showed that it is a stronger inhibitor than WT protease (24 folds reduction of transcription activity) (Fig. 6B). Opposite to the performance of noNLS mutant in the inhibition of replication activity, the lowest fold change of Gluc was observed in noNLS (14 folds reduction) which elucidates that it is not an efficient inhibitor for transcription compared to other mutants (Fig. 6B).

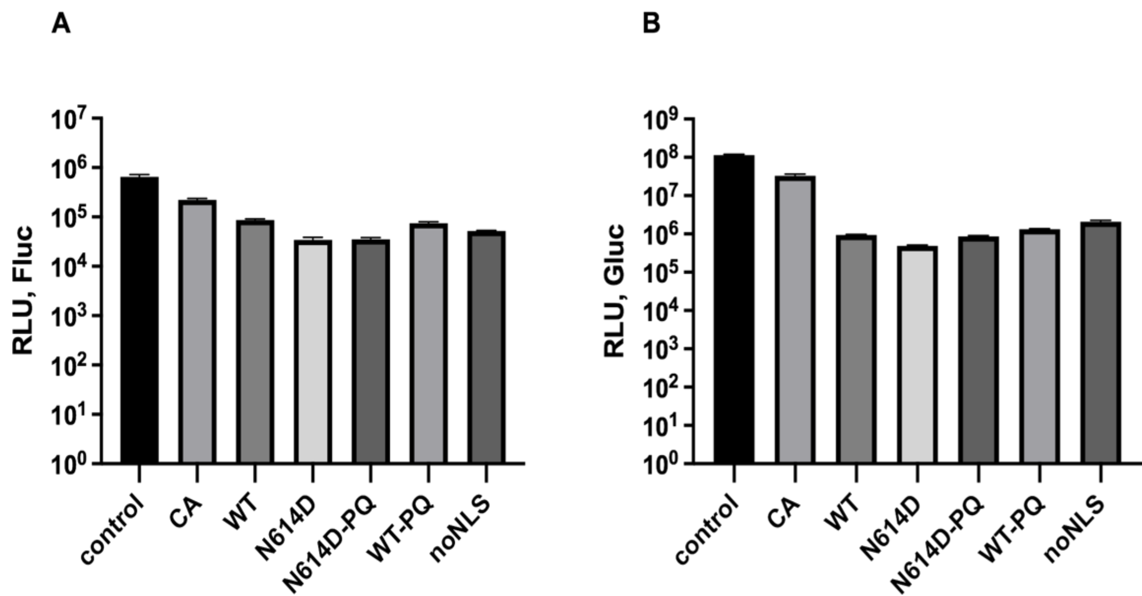

Figure 5. Inhibition level of different nsP2 variants of SINV in C6/36 cells. C6/36 cells in 96-well plates were co-transfected with SINV-specific template + SINV replicase WT (GAA results were not shown) + control (no protease) and different protease mutants shown on graph. Cells were incubated at 28℃ and lysed 48 h post-transfection. Means of relative luminescence units (RLU) of three independent experiments + standard deviation (SD) are shown.

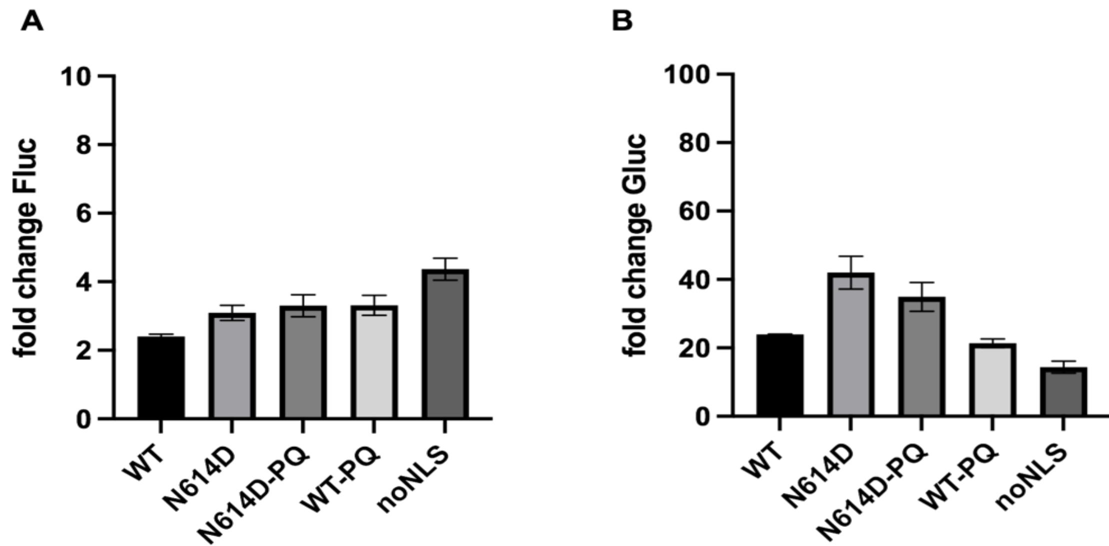

Figure 6. Comparison of impact activity of different SINV nsP2 variants with different substitutions on SINV replication in C6/36 cells. C6/36 cells in 96-well plates were co-transfected with SINV-specific template + SINV replicase WT (GAA results were not shown) + control. Different protease mutants are indicated on the graph. Cells were incubated at 28℃ and lysed 48 h post transfection. Means of impact activity (compared with SINV CA) + SD of three independent experiments are shown.

Overall, mutations in nsP2, as it is shown, resulted in increased or decreased efficiency of alphaviral polyprotein processing at different rates. SINV WT nsP2 protease has shown inhibitory effect on viral RNAs replication and transcription to some extent, while its inactivated version, SINV CA, has also shown little influences but it can be neglected. SINV nsP2 protease without NLS could greatly reduce the level of viral RNAs replication but was ineffective in transcriptional inhibition, and the effect of introduced PQ mutation was small. The result for N614D mutation in nsP2 protease has demonstrated the most competitive effect for inhibition of viral RNAs replication and transcription in C6/36 cells among other nsP2 variants. Such results could be informative for further studies of antiviral targeting in related fields. Nonetheless, the mechanism of hyperactivation of SINV N614D variant is not yet found. One of the proposed explanations is that nsP2 with N614D, affects the cleavage of the polyprotein by atypical increasing of the protease activity of the enzyme.

3.2.3 Inhibition level of SINV nsP2 on homologous and heterologous constructs in C6/36 cells

The efficiency of polyprotein processing by mutations in nsP2 can alter a vital function of the virus or/and effect formation/functional properties of the RC.

Thus, the field of interest was also to observe the possibility of using SINV nsP2 protease as potential inhibitors for the replication of different Alphaviruses. *Trans*-replicase assay was utilized as the main tool for this experiment, since this system was able to separate replicase production from RNA replication which allowed us to study the inhibition level of SINV nsP2 protease on the replication of different Alphaviruses independently. According to the characteristics of DNA constructs being used, the levels of Fluc and Gluc expression in cells indicating the levels of viral RNAs replication and transcription, respectively.

Based on the results of experiments, can be concluded that the effect of SINV nsP2 protease activity on heterologous alphaviral template/replicase RNAs was generally inefficient. The inhibition level on viral replication of this protease to homologous (SINV) constructs was noticed to be similar with the previous experiment (Fig. 7A) at around 3 folds reduction even though the concentration of protein constructs was doubled (880 ng). Interesting-

ly, comparing to the other constructs, the highest fold change of Fluc expression was observed in EILV construct at around 7.6 folds reduction of replication activity (Fig. 8A).

The fold change observed from the Gluc expression presents a striking contrast of inhibitory effect by SINV nsP2 protease to transcription activity between homologous and heterologous template/replicase RNAs. The highest fold change was around 70 folds reduction of viral transcription observed in homologous (SINV) template/replicase, while the inhibitory effect of SINV nsP2 on heterologous alphaviral transcription could be as low as 1.3 folds reduction detected in VEEV constructs. The transcription of EILV was reduced at around 11.7 folds which was 7 times lower than that detected in homologous constructs but was the highest one among all other heterologous template/replicase RNAs (Fig. 8B).

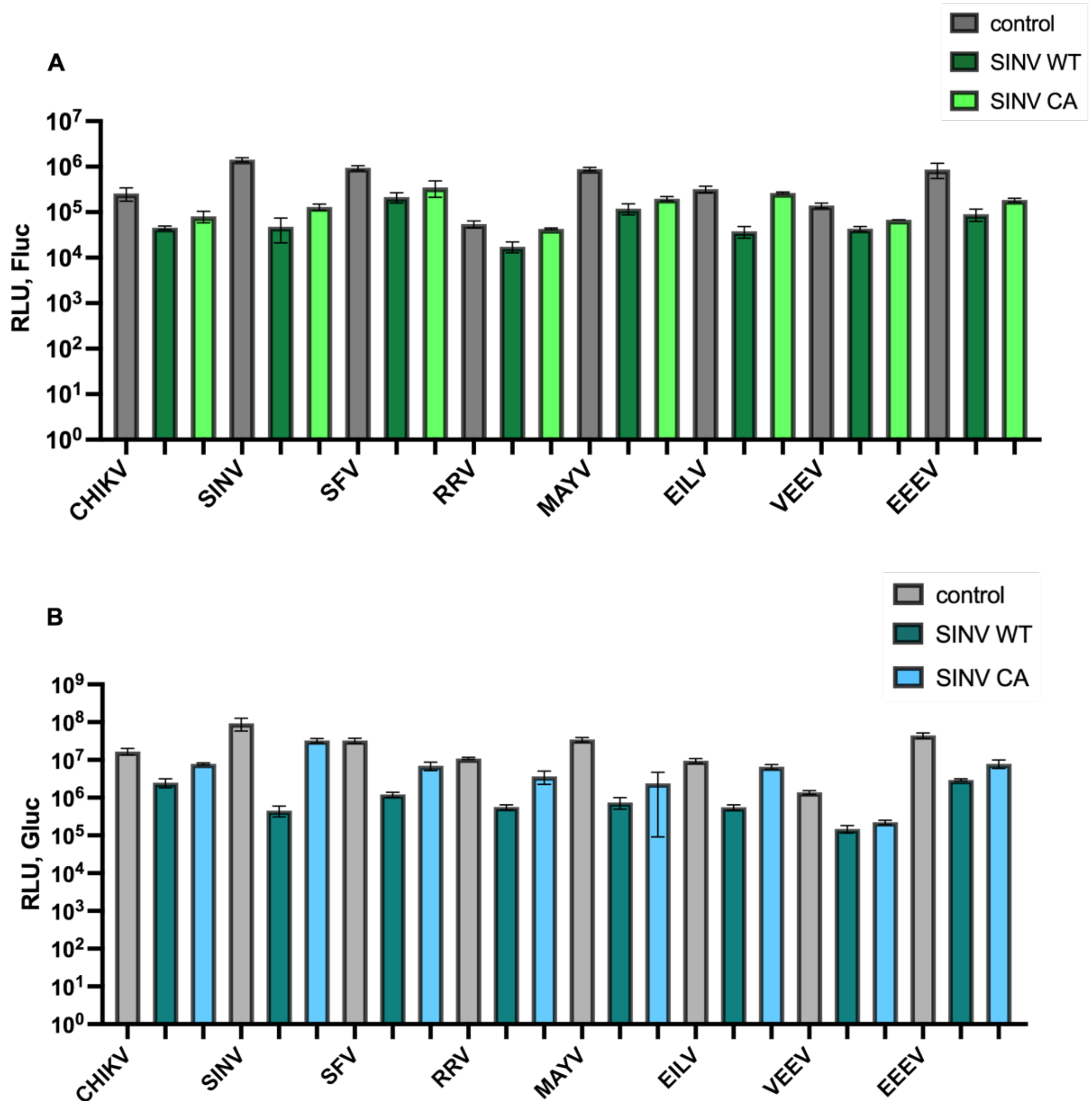

Figure 7. Inhibition level of SINV nsP2 on different Alphavirus constructs in C6/36 cells. C6/36 cells in 96-well plates were co-transfected with virus-specific template + virus-specific replicase WT (GAA results were not shown) + SINV nsP2 protease (WT and CA) and control (no protease). Cells were incubated at 28℃ and lysed 48 h post-transfection. Fluc and Gluc were measured. The average meaning is shown. The SD is presented as error bars.

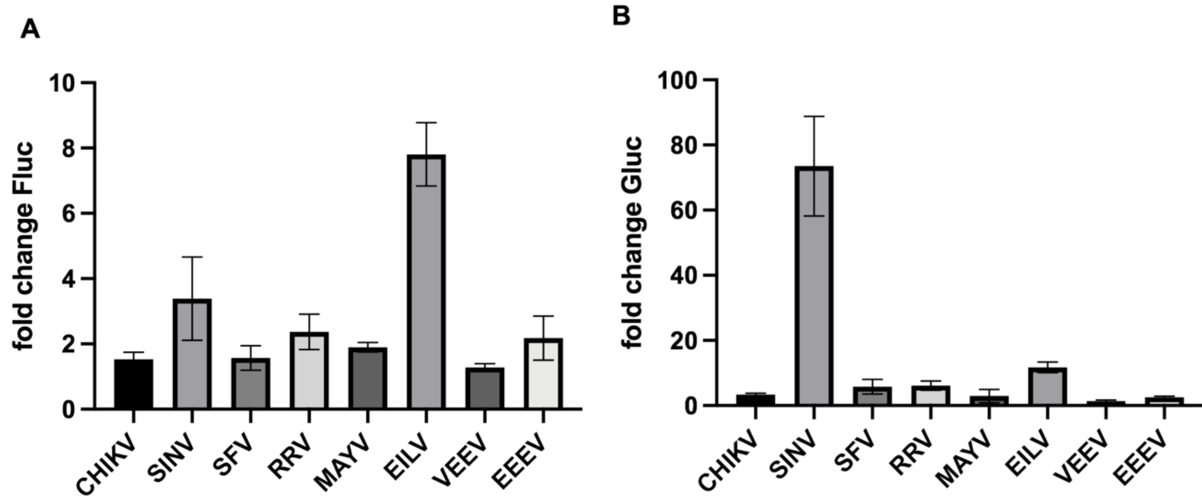

Figure 8. Comparison of impact activity of SINV nsP2 to different alphavirus constructs in C6/36 cells. C6/36 cells in 96-well plates were co-transfected with a mixture of DNAs (template + replicase WT (GAA results were not shown) + SINV nsP2 protease (WT and CA)) and control (no protease). Cells were incubated at 28℃ and lysed 48 h post-transfection. Fluc and Gluc were measured. Means of impact activity (compared with SINV CA) + standard deviation (SD) of three independent experiments are shown.

Based on the experimental results, we can observe that SINV nsP2 protease has inhibitory effect on the replication of heterologous template/replicase to some extent, especially in the unique insect-specific Alphavirus – EILV. The inhibition level of SINV nsP2 on the replication of EILV construct was even 2.5 times more than its effect on the replication of SINV constructs. However, the inhibitory effect of SINV nsP2 on the transcription of different Alphaviruses constructs was extremely weak comparing to the effect on SINV construct, no matter whether the heterologous Alphaviruses belong to the New World (VEEV, EEEV) or Old World Alphaviruses belong to the SFV complex that are closely related (SFV, CHIKV, MAYV, RRV), with the exception of the inhibitory effect on transcription in EILV construct which was slightly stronger. Evolutionary analysis has been performed to construct the phylogenetic tree of replicases of Alphaviruses (Lello *et al.*, 2020), it has been shown that the replicases of SINV and EILV are phylogenetically closer to each other, thus the stronger inhibitory effect of SINV nsP2 on virus replication in EILV construct can be explained by their similar properties of RC. It is possible that there is little or none interaction or cross-reaction between SINV nsP2 protease with other Alphaviruses. Afterall, we summarized that the inhibitory effect of SINV nsP2 to heterologous constructs was insignificant and ambiguous.

3.3 DISCUSSION

The correct processing of Alphavirus polyprotein is crucial during the course of viral replication. It depends on numerous factors, including nsP2, which also perform proteinase activity and involved in the regulation of the formation and processing of RC.

The aim of the study was focused on investigation of properties of SINV carrying different single residue substitutions in the nsP2 and to discover their ability to inhibit the replication of Alphaviruses. *Trans*-replicase assay was chosen to perform experiments due to its efficiency, sensitivity, reliability. It gave a possibility to avoid genetic instability that could be caused by compensations for introduced mutations, since the replicase production and RNA replication are separated, which allows us to analyse the inhibitory effect of nsP2 protease on viral replication independently.

Preliminary experiment showed that optimal protein expression level of SINV nsP2 was observed in *Aedes albopictus* cells lysed 48h post transfection among other five time points tested by Western Blot, thus the time point was determined at 48h for the following

experiments. The Western Blot results also showed that the expression of plasmids encoding nsP2 proteases with mutations were at significant level after transfection in *Aedes albopictus* cells.

Alphavirus nsP2 as a natural inhibitor for cellular activity was utilized to check the inhibition efficiency on virus replication and transcription and nsP2 protease of SINV was chosen. It has been demonstrated that transfection of different versions of SINV nsP2 proteases together with SINV-specific template/replicase reduced the level of viral RNAs replication and transcription at different levels. SINV variant which lacks nuclear localization signals presented stronger effect of inhibiting viral RNAs replication than other variants but not transcription. While the inhibitory efficiency of SINV nsP2 and its mutants on RNA replication was modest, a stronger effect was noticed on transcription activity. The variant of SINV nsP2 protease with N614D mutation has shown the most prominent ability of inhibiting viral RNAs replication and transcription in C6/36 cells.

In contrast, the examination of SINV nsP2 protease interaction with heterologous template/replicase RNAs did not present similar result comparing to homologous constructs. Except for Eilat virus, where SINV nsP2 did not show competent inhibition of viral activity in constructs of other Alphaviruses. An interesting phenomenon was observed when the viral RNA replication of EILV was efficiently inhibited by SINV nsP2 protease and even stronger than that was observed in SINV template/replicase replication (such phenomenon does not apply to the inhibition of transcription activity), which might be correlated with the phylogenetically closer relationship of replicases between SINV and EILV according to the evolutionary analysis being performed. It is also interesting to notice that the template RNA of EILV could be efficiently utilized by other alphaviruses replicases but do not work the other way around (Lello *et al*., 2020), further highlighting the uniqueness of EILV.

Such discovery and information can be useful for the development of antiviral targeting and particular inhibitors, especially for designing drugs as there is no vaccine or antiviral therapy available for currently.

SUMMARY

Alphaviruses are typically transmitted through arthropod vectors and are widely distributed across several continents. It has been proposed that they may follow a seven-year infection pattern while no recognised vaccine and antiviral therapy is available.

The genome of Alphavirus is a positive single-strand RNA with two ORFs, one encodes four non-structural proteins and the other one encodes three structural proteins. One of the crucial determinants of Alphavirus replication is the correct proteolytic processing of non-structural polyprotein that acts as viral replication machinery. It has been demonstrated that alphaviral nsP2 protease is associated with the regulation of RC formation and processing as well as the inhibition of host cell activities. This study is interested in the potential of using SINV nsP2 protease and its variants for inhibition of Alphavirus replication. *Trans*-replicase assay as an efficient and reliable system that has the capacity to separate replicase production from RNA replication and avoid genetic instability, which enables us to evaluate the inhibition level of nsP2 protease and its mutants on virus replication successfully, is chosen as the main approach to perform experiments.

Experimental data also showed that the inhibitory effect of SINV nsP2 and its variants on SINV-specific template/replicase were more significant on viral transcription than RNA replication. N614D variant has shown the most competitive effect of inhibiting viral RNAs activities in C6/36 cells out of six versions of SINV nsP2 tested.

However, the inhibition of heterologous viral activities by using SINV nsP2 in C6/36 cells was ambiguous and insignificant, with the exception of EILV where RNA replication expression was greatly reduced by the addition of SINV nsP2 protease. Nonetheless, further investigations are needed.

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REFERENCES

1. Akhrymuk, I., Kulemzin, S. v., & Frolova, E. I. (2012). Evasion of the Innate Immune Response: the Old World Alphavirus nsP2 Protein Induces Rapid Degradation of Rpb1, a Catalytic Subunit of RNA Polymerase II. *Journal of Virology*, *86*(13), 7180–7191.
2. Akhrymuk, Ivan, Frolov, I., & Frolova, E. I. (2018). Sindbis Virus Infection Causes Cell Death by nsP2-Induced Shutoff. *Journal of Virology*, *92*(23), 1–20.
3. A.lemm, J., Rümenapf, T., Strauss, E. G., Strauss, J. H., & M.rice, C. (1994). Polypeptide requirements for assembly of functional Sindbis virus replication complexes: A model for the temporal regulation of minus- and plus-strand RNA synthesis. *EMBO Journal*, *13*(12), 2925–2934.
4. Aliperti, G., & Schlesinger, M. J. (1978). Evidence for an autoprotease activity of sindbis virus capsid protein. *Virology*, *90*(2), 366–369.
5. Andersson, H., Barth, B. U., Ekström, M., & Garoff, H. (1997). Oligomerization-dependent folding of the membrane fusion protein of Semliki Forest virus. *Journal of Virology*, *71*(12), 9654–9663.
6. Baba, T., Damke, H., Hinshaw, J. E., Ikeda, K., Schmid, S. L., & Warnock, D. E. (1995). Role of dynamin in clathrin-coated vesicle formation. *Cold Spring Harbor Symposia on Quantitative Biology*, *60*, 235–242.
7. Barth, B. U., Wahlberg, J. M., & Garoff, H. (1995). The oligomerization reaction of the Semliki forest virus membrane protein subunits. *Journal of Cell Biology*, *128*(3), 283–291.
8. Bernard, E., Solignat, M., Gay, B., Chazal, N., Higgs, S., Devaux, C., & Briant, L. (2010). Endocytosis of chikungunya virus into mammalian cells: Role of clathrin and early endosomal compartments. *PLoS ONE*, *5*(7).
9. Breakwell, L., Dosenovic, P., Karlsson Hedestam, G. B., D'Amato, M., Liljeström, P., Fazakerley, J., & McInerney, G. M. (2007). Semliki Forest Virus Nonstructural Protein 2 Is Involved in Suppression of the Type I Interferon Response. *Journal of Virology*, *81*(16), 8677–8684.

10. Brummer-Korvenkontio, M., Vapalahti, O., Kuusisto, P., Saikku, P., Manni, T., Koskela, P., Nygren, T., Brummer-Korvenkontio, H., & Vaheri, A. (2002). Epidemiology of Sindbis virus infections in Finland 1981 – 96 : *Epidermiol Infect*, *129*(21), 335–345.
11. Choi, H.K., Tong, L., Minor, W., Dumas, P., Boege, U., Rossmann, M.G., and Wengler, G. (1991). Structure of Sindbis virus core protein reveals a chymotrypsin-like serine proteinase and the organization of the virion. Nature 354, 37–43.
12. Damke, H., Baba, T., Warnock, D. E., & Schmid, S. L. (1994). Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *Journal of Cell Biology*, *127*(4), 915–934.
13. de Curtis, I., & Simons, K. (1988). Dissection of Semliki Forest virus glycoprotein delivery from the trans-Golgi network to the cell surface in permeabilized BHK cells. *Proceedings of the National Academy of Sciences of the United States of America*, *85*(21), 8052–8056.
14. de Groot, R. J., Hardy, W. R., Shrako, Y., & Strauss, J. H. (1990). Cleavage-site preferences of Sindbis virus polyproteins contains the non-structural proteinase. Evidence for temporal regulation of polyprotein processing in vivo. *EMBO Journal*, *9*(8), 2631–2638.
15. Dé, I, Sawicki, S. G., & Sawicki, D. L. (1996). Sindbis virus RNA-negative mutants that fail to convert from minus-strand to plus-strand synthesis: role of the nsP2 protein. *Journal of Virology*, *70*(5), 2706–2719.
16. Dé, Indra, Fata-Hartley, C., Sawicki, S. G., & Sawicki, D. L. (2003). Functional Analysis of nsP3 Phosphoprotein Mutants ofSindbisVirus. *Journal of Virology*, *77*(24), 13106–13116.
17. DeTulleo, L., & Kirchhausen, T. (1998). The clathrin endocytic pathway in viral infection. *EMBO Journal*, *17*(16), 4585–4593.
18. Duffus, W. A., Levy-Mintz, P., Klimjack, M. R., & Kielian, M. (1995). Mutations in the putative fusion peptide of Semliki Forest virus affect spike protein oligomerization and virus assembly. *Journal of Virology*, *69*(4), 2471–2479.
19. Faragher, S. G., Meek, A. D. J., Rice, C. M., & Dalgarno, L. (1988). Genome sequences of a mouse-avirulent and a mouse-virulent strain of ross river virus. *Virology*, *163*(2), 509–526.

20. Foo, S. S., Chen, W., Herrero, L., Bettadapura, J., Narayan, J., Dar, L., Broor, S., & Mahalingam, S. (2011). The genetics of alphaviruses. *Future Virology*, *6*(12), 1407–1422.
21. Frolov, I., Garmashova, N., Atasheva, S., & Frolova, E. I. (2009). Random Insertion Mutagenesis of Sindbis Virus Nonstructural Protein 2 and Selection of Variants Incapable of Downregulating Cellular Transcription. *Journal of Virology*, *83*(18), 9031–9044.
22. Froshauer, S., Kartenbeck, J., & Helenius, A. (1988). Alphavirus RNA replicase is located on the cytoplasmic surface of endosomes and lysosomes. *Journal of Cell Biology*, *107*(6 I), 2075–2086.
23. Garoff, H., & Li, K. J. (1998). Recent advances in gene expression using alphavirus vectors. *Current Opinion in Biotechnology*, *9*(5), 464–469.
24. Garoff, H., Simons, K., & Dobberstein, B. (1978). Assembly of the semliki forest virus membrane glycoproteins in the membrane of the endoplasmic reticulum in vitro. *Journal of Molecular Biology*, *124*(4), 587–600.
25. Glanville, N., Ranki, M., & Morser, J. (1976). Initiation of translocation directed by 42S and 26S RNAs from Semliki Forest virus in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, *73*(9), 3059–3063.
26. Gomez De Cedrón, M., Ehsani, N., Mikkola, M. L., García, J. A., & Kääriäinen, L. (1999). RNA helicase activity of Semliki Forest virus replicase protein NSP2. *FEBS Letters*, *448*(1), 19–22.
27. Hase, T., Summers, P. L., & Cohen, W. H. (1989). A comparative study of entry modes into C6/36 cells by Semliki Forest and Japanese encephalitis viruses. *Archives of Virology*, *108*(1–2), 101–114.
28. Hernandez, R., Luo, T., & Brown, D. T. (2001). Exposure to Low pH Is Not Required for Penetration of Mosquito Cells by Sindbis Virus. *Journal of Virology*, *75*(4), 2010–2013.
29. Kamer, G., & Argos, P. (1984). Primary structural comparison of RNA-dependent polymerases from plant, animal and bacterial viruses. *Nucleic Acids Research*, *12*(18), 7269–7282.

30. Koonin, E. v., Dolja, V. v., & Morris, T. J. (1993). Evolution and taxonomy of positive-Strand RNA viruses: Implications of comparative analysis of amino acid sequences. *Critical Reviews in Biochemistry and Molecular Biology*, *28*(5), 375–430.
31. Kuhn, R. J., Griffin, D. E., Owen, K. E., Niesters, H. G., & Strauss, J. H. (1996). Chimeric Sindbis-Ross River viruses to study interactions between Alphavirus non-structural and structural regions. *Journal of Virology*, *70*(11), 7900–7909.
32. Kujala, P., Ikäheimonen, A., Ehsani, N., Vihinen, H., Auvinen, P., & Kääriäinen, L. (2001). Biogenesis of the Semliki Forest Virus RNA Replication Complex. *Journal of Virology*, *75*(8), 3873–3884.
33. LaStarza, M. W., Lemm, J. A., & Rice, C. M. (1994). Genetic analysis of the nsP3 region of Sindbis virus: evidence for roles in minus-strand and subgenomic RNA synthesis. *Journal of Virology*, *68*(9), 5781–5791.
34. Lello, L. S., Utt, A., Bartholomeeusen, K., Wang, S., Rausalu, K., Kendall, C., Coppens, S., Fragkoudis, R., Tuplin, A., Alphey, L., Ariën, K. K., & Merits, A. (2020). Cross-utilisation of template rnas by alphavirus replicases. In *PLoS Pathogens* (Vol. 16, Issue 9).
35. Lobigs, M., & Garoff, H. (1990). Fusion function of the Semliki Forest virus spike is activated by proteolytic cleavage of the envelope glycoprotein precursor p62. *Journal of Virology*, *64*(3), 1233–1240.
36. Lulla, V., Karo-Astover, L., Rausalu, K., Saul, S., Merits, A., & Lulla, A. (2018). Timeliness of Proteolytic Events Is Prerequisite for Efficient Functioning of the Alphaviral Replicase. *Journal of Virology*, *92*(14), 1–18.
37. Mayor, S., & Pagano, R. E. (2007). Pathways of clathrin-independent endocytosis. *Nature Reviews Molecular Cell Biology*, *8*(8), 603–612.
38. Melancon, P., & Garoff, H. (1987). Processing of the Semliki Forest virus structural polyprotein: role of the capsid protease. *Journal of Virology*, *61*(5), 1301–1309.
39. Merits, A., Vasiljeva, L., Ahola, T., Kääriäinen, L., & Auvinen, P. (2001). Proteolytic processing of Semliki Forest virus-specific non-structural polyprotein by nsP2 protease. *Journal of General Virology*, *82*(4), 765–773.

40. Metsikkö, K., & Garoff, H. (1990). Oligomers of the cytoplasmic domain of the p62/E2 membrane protein of Semliki Forest virus bind to the nucleocapsid in vitro. *Journal of Virology*, *64*(10), 4678–4683.
41. Mi, S., & Stollar, V. (1991). Expression of sindbis virus nsP1 and methyltransferase activity inEscherichia coli. *Virology*, *184*(1), 423–427.
42. Montgomery, S. A., & Johnston, R. E. (2007). Nuclear Import and Export of Venezuelan Equine Encephalitis Virus Nonstructural Protein 2. *Journal of Virology*, *81*(19), 10268–10279.
43. Owen, K E, & Kuhn, R. J. (1996). Identification of a region in the Sindbis virus nucleocapsid protein that is involved in specificity of RNA encapsidation. *Journal of Virology*, *70*(5), 2757–2763.
44. Owen, Katherine E., & Kuhn, R. J. (1997). Alphavirus budding is dependent on the interaction between the nucleocapsid and hydrophobic amino acids on the cytoplasmic domain of the E2 envelope glycoprotein. *Virology*, *230*(2), 187–196.
45. Parrott, M. M., Sitarski, S. A., Arnold, R. J., Picton, L. K., Hill, R. B., & Mukhopadhyay, S. (2009). Role of Conserved Cysteines in the Alphavirus E3 Protein. *Journal of Virology*, *83*(6), 2584–2591.
46. Peränen, J. (1991). Localization and phosphorylation of Semliki Forest virus non-structural protein nsP3 expressed in COS cells from a cloned cDNA. J. Gen. Virol. 72(1), 195–199.
47. Peränen, J., Rikkonen, M., Liljeström, P., & Kääriäinen, L. (1990). Nuclear localization of Semliki Forest virus-specific nonstructural protein nsP2. *Journal of Virology*, *64*(5), 1888–1896.
48. Pietilä, M. K., Hellström, K., & Ahola, T. (2017). Alphavirus polymerase and RNA replication. *Virus Research*, *234*, 44–57.
49. Rice, C. M., & Strauss, J. H. (1982). Association of Sindbis virion glycoproteins and their precursors. *Journal of Molecular Biology*, *154*(2), 325–348.
50. Rikkonen, M., Peränen, J., & Kääriäinen, L. (1994). ATPase and GTPase activities associated with Semliki Forest virus nonstructural protein nsP2. *Journal of Virology*, *68*(9), 5804–5810.
51. Rupp, J. C., Sokoloski, K. J., Gebhart, N. N., & Hardy, R. W. (2015). Alphavirus RNA synthesis and non-structural protein functions. *The Journal of general virology*, *96*(9), 2483–2500.

52. Russo, A. T., White, M. A., & Watowich, S. J. (2006). The Crystal Structure of the Venezuelan Equine Encephalitis Alphavirus nsP2 Protease. *Structure*, *14*(9), 1449–1458.
53. Salonen, A., Vasiljeva, L., Merits, A., Magden, J., Jokitalo, E., & Kääriäinen, L. (2003). Properly Folded Nonstructural Polyprotein Directs the Semliki Forest Virus Replication Complex to the Endosomal Compartment. *Journal of Virology*, *77*(3), 1691–1702.
54. Sariola, M., Saraste, J., & Kuismanen, E. (1995). Communication of post-Golgi elements with early endocytic pathway: Regulation of endoproteolytic cleavage of Semliki Forest virus p62 precursor. *Journal of Cell Science*, *108*(6), 2465–2475.
55. Sawicki, D. L., Perri, S., Polo, J. M., & Sawicki, S. G. (2006). Role for nsP2 Proteins in the Cessation of Alphavirus Minus-Strand Synthesis by Host Cells. *Journal of Virology*, *80*(1), 360–371.
56. Scheidel, L. M., Durbin, R. K., & Stollar, V. (1987). Sindbis virus mutants resistant to mycophenolic acid and ribavirin. *Virology*, *158*(1), 1–7.
57. Shirako, Y, & Strauss, J. H. (1994). Regulation of Sindbis virus RNA replication: uncleaved P123 and nsP4 function in minus-strand RNA synthesis, whereas cleaved products from P123 are required for efficient plus-strand RNA synthesis. *Journal of Virology*, *68*(3), 1874–1885.
58. Simmons, D. T., & Strauss, J. H. (1972). Replication of Sindbis virus. I. Relative size and genetic content of 26 s and 49 s RNA. *Journal of Molecular Biology*, *71*(3), 599–613.
59. Smith, A. L., & Tignor, G. H. (1980). Host cell receptors for two strains of sindbis virus. *Archives of Virology*, *66*(1), 11–26.
60. Smith, T. J., Cheng, R. H., Olson, N. H., Peterson, P., Chase, E., Kuhn, R. J., & Baker, T. S. (1995). Putative receptor binding sites on alphaviruses as visualized by cryoelectron microscopy. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(23), 10648–10652.
61. Snyder, A. J., & Mukhopadhyay, S. (2012). The Alphavirus E3 Glycoprotein Functions in a Clade-Specific Manner. *Journal of Virology*, *86*(24), 13609–13620.

62. Sourisseau, M., Schilte, C., Casartelli, N., Trouillet, C., Guivel-Benhassine, F., Rudnicka, D., Sol-Foulon, N., le Roux, K., Prevost, M. C., Fsihi, H., Frenkiel, M. P., Blanchet, F., Afonso, P. v., Ceccaldi, P. E., Ozden, S., Gessain, A., Schuffenecker, I., Verhasselt, B., Zamborlini, A., … Schwartz, O. (2007). Characterization of reemerging chikungunya virus. *PLoS Pathogens*, *3*(6), 0804–0817.
63. Strauss, E. G., de Groot, R. J., Levinson, R., & Strauss, J. H. (1992). Identification of the active site residues in the nsP2 proteinase of sindbis virus. *Virology*, *191*(2), 932–940.
64. Strauss, E. G., Rice, C. M., & Strauss, J. H. (1984). Complete nucleotide sequence of the genomic RNA of Sindbis virus. *Virology*, *133*(1), 92–110.
65. Strauss, J. H., & Strauss, E. G. (1994). The alphaviruses: Gene expression, replication, and evolution. *Microbiological Reviews*, *58*(3), 491–562.
66. Suopanki, J., Sawicki, D. L., Sawicki, S. G., & Kääriäinen, L. (1998). Regulation of alphavirus 26S mRNA transcription by replicase component nsP2. *Journal of General Virology*, *79*(2), 309–319.
67. van der Bliek, A. M., Redelmeier, T. E., Damke, H., Tisdale, E. J., Meyerowitz, E. M., & Schmid, S. L. (1993). Mutations in human dynamin block an intermediate stage in coated vesicle formation. *Journal of Cell Biology*, *122*(3), 553–563.
68. Vasiljeva, L., Merits, A., Auvinen, P., & Kääriäinen, L. (2000). Identification of a novel function of the Alphavirus capping apparatus. RNA 5’-triphosphatase activity of Nsp2. *Journal of Biological Chemistry*, *275*(23), 17281–17287.
69. Vaux, D. J. T., Helenius, A., & Mellman, I. (1988). Spike - nucleocapsid interaction in Semliki Forest virus reconstructed using network antibodies. *Nature*, *336*(6194), 36–42.
70. Vihinen, H., Ahola, T., Tuittila, M., Merits, A., & Kääriäinen, L. (2001). Elimination of Phosphorylation Sites of Semliki Forest Virus Replicase Protein nsP3. *Journal of Biological Chemistry*, *276*(8), 5745–5752.
71. Vogel, R. H., Provencher, S. W., von Bonsdorff, C. H., Adrian, M., & Dubochet, J. (1986). Envelope structure of semliki forest virus reconstructed from cryo-electron micrographs. *Nature*, *320*(6062), 533–535.

72. Wahlberg, J. M., Boere, W. A., & Garoff, H. (1989). The heterodimeric association between the membrane proteins of Semliki Forest virus changes its sensitivity to low pH during virus maturation. *Journal of Virology*, *63*(12), 4991–4997.
73. Weiss, B., Nitschko, H., Ghattas, I., Wright, R., & Schlesinger, S. (1989). Evidence for specificity in the encapsidation of Sindbis virus RNAs. *Journal of Virology*, *63*(12), 5310–5318.
74. Zhang, W., Mukhopadhyay, S., Pletnev, S. v., Baker, T. S., Kuhn, R. J., & Rossmann, M. G. (2002). Placement of the Structural Proteins in Sindbis Virus. *Journal of Virology*, *76*(22), 11645–11658.
75. Ziemiecki, A., Garoff, H., & Simons, K. (1980). Formation of the Semliki Forest virus membrane glycoprotein complexes in the infected cell. *Journal of General Virology*, *50*(1), 111–123.

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