Serpin-based SKI-1/S1P inhibitors against Old and New World Arenaviruses

by

Mable W.S. Chan, B.Sc. (Hons)

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☐ Thesis

☐ Practicum

Advisor: Dr. T. Blake Ball

Advisory Committee:

Dr. Joerg Stetefeld

Dr. Keith Fowke

Dr. Ute Ströher

Date: _____

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Mable Chan, B.Sc. (Hons), University of Manitoba, 2010

Abstract

The importance of arenavirus glycoprotein processing has only been understood within the past decade, with the majority of work focused on the Old World arenavirus, Lassa virus. Evidence has shown that SKI-1/S1P (subtilisin kexin isozyme-1/site 1 protease) is the cellular protease responsible for glycoprotein cleavage in Old and New World arenaviruses. Furthermore, it has been demonstrated that glycoprotein cleavage is necessary for the production of infectious virus particles in Lassa and Junín viruses. In this thesis, evidence is provided that the recently emerged Chapare virus (New World) is also processed by SKI-1/S1P. Additionally, novel serpin-based SKI-1 inhibitors expressed by recombinant adenoviruses were generated, and were shown to effectively prevent SKI-1 mediated cleavage. Using a wide panel of recombinant vesicular stomatitis viruses expressing New World arenavirus glycoproteins, these inhibitors were capable of significantly reducing viral titres. This provides strong evidence that SKI-1 inhibitors can be used as an effective treatment against the majority of New World Clade B arenaviruses and LASV *in vivo*.

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1.0 Introduction

1.1Taxonomy of Arenaviruses

The family *Arenaviridae* contains the single genus *Arenavirus*, which currently includes 23 species recognized by the International Committee on Taxonomy of Viruses (http://ictvonline.org/virusTaxonomy.asp). Arenaviruses are generally classified into the Old World (Lassa-lymphocytic choriomeningitis virus serocomplex) and New World (Tacaribe serocomplex) groups based on antigenic properties, serological differences, and geographic distribution (Table 1). Although divided into two groups, monoclonal antibody studies have shown the presence of shared epitopes between the Old and New World viruses (Weber and Buchmeier 1988). Phylogenetic analysis based on nucleoprotein and glycoprotein sequences, have led to the finding that New World arenaviruses can be further divided into clades A, B, C and a recombinant A/B clade (Charrel et al. 2002; Charrel et al. 2008). The majority of highly-pathogenic viruses causing hemorrhagic fever in humans are grouped in clade B, which includes Junín (JUNV), Machupo (MACV), Guanarito (GTOV) and Sabia (SABV) viruses (Figure 1) (Bowen et al. 1996). Amapari and Tacaribe are also members of Clade B, however, these viruses are non-pathogenic in humans.

The natural host of almost all arenaviruses are rodents, with the exception of Tacaribe virus, where it has been isolated from the *Artibeus* sp. of fruit bats (Downs et al. 1963). New World arenaviruses are generally associated with rodents within the subfamilies *Sigmodontinae* and *Neotominae*, while Old World arenaviruses are

Table 1. **Comparison of the diseases and symptoms caused by Old and New World arenaviruses.**

Virus		Disease	Distribution	Reservoir	Symptoms	Mortality Rate
Old World arenaviruses						
LASV	Lassa	Lassa fever	West Africa	*Mastomys* sp.	Fever, malaise, hearing loss (~30%)	15-20%
LCMV	Lymphocytic choriomeningitis	Meningitis	Worldwide	*Mus musculus*	Fever, myalgia Aseptic meningitis, encephalitis, CNS disease	< 1%*
LUJV	Lujo	HF	South Africa / Zambia	unknown	Flu-like symptoms, diarrhea, pharyngitis, neurological symptoms, respiratory and circulatory problems	4 / 5 cases
New World arenaviruses						
JUNV	Junín	Argentine HF	Argentina	*Calomys musculinus*	Fever, malaise, myalgia, nausea, vomiting, severe prostration, hemorrhagic and neurological symptoms	15-30%
MACV	Machupo	Bolivian HF	Bolivia	*Calomys callosus*		25-35%
GTOV	Guanarito	Venezuelan HF	Venezuela	*Zygodontomys brevicauda*		25-35%
SABV	Sabia	Brazilian HF	Brazil	unknown		1 / 3 cases
CHPV	Chapare	HF	Bolivia	unknown		1 fatal case

*LCMV generally has <1% mortality rate, with the exception in immunocompromised individuals where there may be a higher risk of death.

HF – hemorrhagic fever

Data compiled from (Buchmeier et al. 2001; Delgado et al. 2008; Briese et al. 2009).

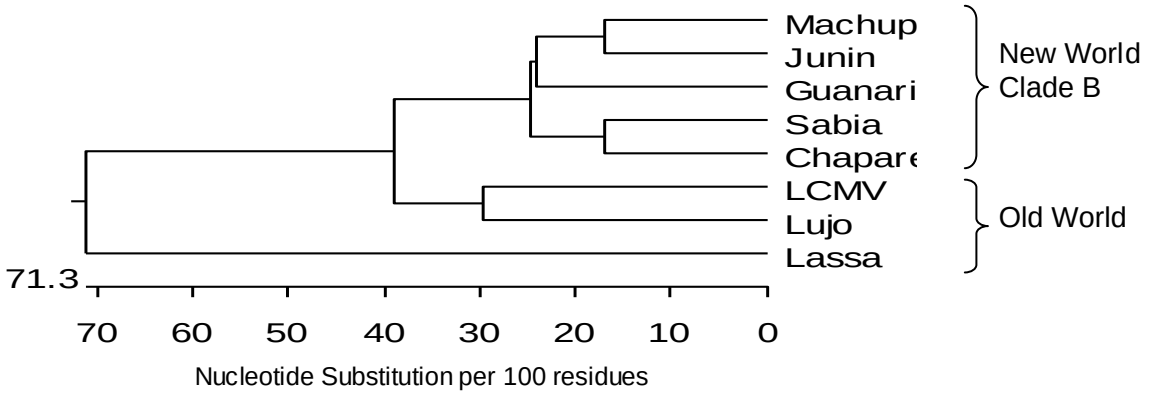


Figure 1. **Arenavirus phylogenetic analysis based on complete S segment.** Phylogenetic relationship was analyzed between members of the Old World and New World Clade B arenaviruses by Clustal W method. Scale bar represents the number of nucleotide substitutions per 100 residues. GenBank accession numbers used for complete S segment analysis include: Machupo (AY129248), Junín (AY619641), Guanarito (AY129247), Sabia (NC_006317), Chapare (EU260463), LCMV (NC_004294), Lujo (NC_012776), and Lassa (NC_004296).

connected with rodents in the subfamily *Murinae* (Emonet et al. 2009). Since the majority of South American viruses that cause hemorrhagic fever are grouped into Clade B, it is suggested that these viruses may have originated from a single common pathogenic ancestor (Buchmeier et al. 2001), however, for Amapari and Tacaribe, these viruses did not acquire the genes necessary for pathogenesis in humans. Evidence supporting the idea of co-evolution in arenaviruses with their rodent hosts is the observation that genetically similar arenaviruses infect closely related rodent species. For example, both JUNV and MACV are found in hosts that belong to the genus *Calomys* while the Clade A viruses Flexal, Parana and Pichinde all share hosts in the genus *Oryzomys* (Buchmeier et al. 2001; Emonet et al. 2009).

1.2Transmission and Clinical Symptoms

Arenaviruses cause chronic infections in rodents and transmission to humans occur through inhalation of aerosolized excretions, secretions or direct contact with an infected host. In addition to horizontal spread, the virus can also spread vertically through infected rodents (Peters 2002). The ability of arenaviruses to form stable, infectious aerosols have led to cases of laboratory infections and deaths (Buchmeier et al. 2001). Due to the possibility of person-to-person transmission, and absence of an effective vaccine or treatment, these viruses are classified as biosafety level 4 (BSL-4) agents, and are also included in the Category A Pathogen List established by the Centers for Disease Control and Prevention for potential bioterrorism use (Charrel and de Lamballerie 2003).

Every year several hundred thousand individuals are infected with Lassa virus (LASV), resulting in thousands of deaths (Salazar-Bravo et al. 2002). Among those

infected with LASV, almost 30% suffer an acute loss of hearing (Cummins et al. 1990). LCMV is the only arenavirus with a worldwide distribution; this is attributed to the broad circulation of its rodent host, *Mus musculus*. LCMV infections are rarely fatal, however, in immunosuppressed or pregnant individuals it can cause neurological diseases such as aseptic meningitis and encephalitis. Congenital LCMV infection may lead to hydrocephalus, chorioretinitis, fetal abnormalities or even death (Buchmeier et al. 2001; Barton et al. 2002; Bonthius et al. 2007). Furthermore, transmission of LCMV to transplant patients is becoming a major public health concern, as the number of deaths caused by LCMV-infected organ transplants is increasing (Fischer et al. 2006). Recently, an LCMV-related arenavirus was identified to be the cause of a group of fatal transplant-associated diseases (Palacios et al. 2008).

In Argentina, JUNV annually infects hundreds to thousands of individuals, and is the causative agent of Argentine hemorrhagic fever (Peters 2002). Human infections caused by JUNV follow a seasonal trend. During the rainy season, rodent hosts tend to invade human habitats more frequently, increasing the chances of infection. In comparison to the harvest season, agricultural workers raise their risk to JUNV exposure from increased contact with rodents out in the field (Buchmeier et al. 2001).

The clinical symptoms of almost all the hemorrhagic fever causing arenaviruses are similar. Generally, the incubation period lasts between 1 - 2 weeks and disease begins often with fever and malaise (Peters 2002). Other minor symptoms include myalgia, back pain, headache, and dizziness. Gastrointestinal distress is also associated with these infections, including abdominal pain, nausea and vomiting (Peters 2002). As the disease progresses, hemorrhagic and neurological signs such as petechiae of the skin,

hemorrhaging from the gums and gastrointestinal tract, tremor of the hands, and difficulty swallowing or speaking may occur. These symptoms may eventually lead to coma, convulsions, and ultimately shock, causing death 7 – 12 days after infection (Charrel and de Lamballerie 2003). The ability to mount an effective immune response is consistent with clinical recovery and the clearance of viremia (Buchmeier et al. 2001). In particular, patients who survive JUNV infections develop protective antibodies against the virus, while for LASV, a strong cell mediated response is more important for protection (de Bracco et al. 1978; Fisher-Hoch et al. 2000).

1.3Treatment of Arenaviruses

There is currently no licensed vaccine available for the treatment of arenaviruses. The most effective antiviral drug used is ribavirin, however, its availability in endemic regions is limited and serious side effects can occur. Rodent control has been shown to decrease arenavirus infections in humans, but the economic circumstances in the affected areas and broad distribution of the rodent host make it impractical. Furthermore, LCMV is also pathogenic for several nonhuman primate species such as marmosets and tamarinds, which have led to outbreaks occurring in zoos and laboratories when naturally infected wild mice enter animal pens (Coggeshall 1939; Peters et al. 1987). Immediate supportive therapy for patients infected with arenaviruses is a key factor in determining disease outcome (Buchmeier et al. 2001). In the early stages of disease, patients are minimally contagious compared to later on, when virus can be detected in target organs and blood (Buchmeier et al. 2001).

Ribavirin is a guanosine analog that is effective against arenavirus infections, as *in vitro* and *in vivo* studies have demonstrated protection against lethal challenge in a number of animal models (Jahrling et al. 1980; Jahrling et al. 1984; Kenyon et al. 1986; McKee et al. 1988; Lucia et al. 1989; Smee et al. 1993). Furthermore, cases of human infection by Machupo and Sabia have been treated with ribavirin, and in all cases the patients recovered with no sequelae (Barry et al. 1995; Kilgore et al. 1997). For treatment with ribavirin to be effective, it must be provided within the first few days of illness. This can be challenging especially when the availability of ribavirin in endemic areas may be limited (Fisher-Hoch and McCormick 2001; Peters 2002). Side effects of using ribavirin may include thrombocytopenia and anaemia, but these will resolve after cessation of drug use (Charrel and de Lamballerie 2003). However, since severe arenavirus disease can be associated with blood loss, the use of ribavirin as the standard treatment for hemorrhagic arenaviruses remains to be approved (Gowen et al. 2008).

Patients who recover from JUNV infection develop antibodies capable of clearing viremia during the second week of disease (de Bracco et al. 1978). Therefore, plasma from recovered patients can be used as a treatment in endemic areas as a source of neutralizing antibodies (Enria and Maiztegui 1994). For JUNV infections, therapy with convalescent plasma in humans and experimental animals have proven effective at reducing mortality to less than 1-2% only when administered early in illness (Maiztegui et al. 1979; Enria et al. 1984). However, about 10% of treated patients developed late neurological signs including fever, headache, cerebellar tremors, and cranial nerve palsies (Maiztegui et al. 1979; Buchmeier et al. 2001). Additionally, with the low number of cases and an absence of a well established program for the collection and storage of

immune plasma, a shortage of plasma will become likely in the future (Charrel and de Lamballerie 2003). Therefore, alternative methods for therapy will be necessary to effectively prevent these infections.

Currently, there is only one successful live attenuated vaccine against JUNV, named Candid 1. The vaccine has low neurovirulence, no evidence of persistence, and no shown phenotypic reversion (Barrera Oro and McKee 1991; Contigiani et al. 1993). Vaccination of guinea pigs and rhesus macaques provided protection from lethal JUNV challenge (McKee et al. 1992, 1993). Large scale Phase I and II human studies have been completed and show that the vaccine has a protective efficacy of 84%, and was also safe and immunogenic in these human volunteers (Maiztegui et al. 1998). Since Candid 1 vaccination began, it has been very successful in reducing the incidence rates of AHF in endemic regions.

Each year, over one hundred thousand people are infected with LASV, causing Lassa fever in endemic regions of Africa. In addition, there is a rising concern of LCMV infections in immunocompromised or transplant patients. Presently, an effective vaccine against these Old World arenaviruses is yet to be produced. Studies using other closely related, non-pathogenic members of the Old World arenaviruses, such as Mopeia virus, to infect Guinea pigs or rhesus monkeys, have shown protection against virulent LASV challenge (Kiley et al. 1979; Walker et al. 1982). Though, the problem with using other seemingly non-pathogenic arenaviruses to provide cross-protection is the difficulty of proving the safety and efficacy of their use in humans.

Other vaccine attempts using a recombinant vaccinia virus to carry LASV genes have also shown protection in animal models. Recombinant vaccinia viruses expressing either

the LASV NP gene or GPC gene were able to provide protection in Guinea pigs challenged with LASV (Clegg and Lloyd 1987; Auperin et al. 1988; Morrison et al. 1989). More extensive studies in non-human primates using different combinations of these recombinant vaccinia viruses demonstrated that the ones expressing full length LASV S (small) RNA segment, which encodes the NP and GPC genes, provided 90% protection against lethal LASV challenge, compared to 88% for GPC alone, or 20% for NP alone (Fisher-Hoch et al. 2000). For LCMV and LASV, there is no correlation between antibody response and protection against disease; instead, a strong cell mediated response likely plays a role in protection (Fisher-Hoch et al. 2000; Fisher-Hoch and McCormick 2001; Botten et al. 2010). Although these results are promising, due to the possible side effects of using recombinant vaccinia viruses, their use will never be approved (Fisher-Hoch et al. 2000). Especially, since high Human Immunodeficiency Virus (HIV) prevalence overlaps with endemic arenavirus regions, the use of live vaccinia viruses as a vaccine platform in immunocompromised HIV patients is a major cause for concern.

More recently, the development of replication-competent recombinant Vesicular stomatitis viruses (rVSVs) as a vaccine platform for LASV and filoviruses such as Ebola virus (EBOV) and Marburg virus (MARV) is under investigation. The establishment of a reverse genetics system for VSV (Lawson et al. 1995), and the ability of these viruses to tolerate expression of foreign genes (Schnell et al. 1996b; Kretzschmar et al. 1997; Haglund et al. 2000) has made this a useful system to study the role of foreign glycoproteins in the context of infectious viral particles (Garbutt et al. 2004). In non-human primates, a VSV-based LASV vaccine is able to elicit a protective immune

response when challenged with a lethal dose of LASV (Geisbert et al. 2005). Other studies using VSV-based EBOV or MARV vaccines have demonstrated the ability to confer complete protection in non-human primates against lethal EBOV or MARV challenge (Jones et al. 2005; Geisbert et al. 2008a). Recently, the VSV-based EBOV vaccine is shown to be safe in immunocompromised non-human primates and additionally, is able to provide protection against EBOV challenge (Geisbert et al. 2008b). Taken together, this data provides evidence that VSV-based vaccines are safe and highly efficacious, showing promise as an attractive vaccine candidate against hemorrhagic fever viruses.

1.4Arenavirus Structure

Purified arenavirus virions often contain electron dense granules that were later discovered to be host ribosomes (Farber and Rawls 1975; Murphy and Whitfield 1975). From the Latin word *arenosus*, meaning "sandy", the term "arena" was, therefore, used to name this family of viruses (Meyer et al. 2002). Arenaviruses are enveloped and contain a bi-segmented, ambisense RNA genome. These viruses are pleomorphic to spherical in shape, and vary in size from 62 to > 200 nm in diameter. The surface of the viral envelope is studded with glycoprotein spikes consisting of GP1 and GP2 subunits (Figure 2A).

1.4.1 Genome organization

The arenavirus genome consists of two single-stranded RNA segments, each encoding for two genes in an ambisense orientation. The small (S) segment (~3.4 kb), encodes for the nucleoprotein (NP) and envelope glycoproteins, GP1 and GP2 (Meyer et al. 2002). The large (L) segment (~7.2 kb), encodes for the RNA-dependent RNA polymerase (RdRP, L) and Z protein (Figure 2B). Ambisense viruses follow a unique transcription strategy in infected cells. The genes found at the 3' end of each segment, NP and L, are read as negative sense RNA that is transcribed into mRNA for expression. The genes at the 5' end of each segment, GPC and Z, are read as positive sense RNA. However, instead of using this genomic RNA as mRNA, transcription of a full length antigenomic negative sense RNA occurs. This antigenomic RNA serves two functions; first to act as a template to generate more full length complementary copies of genomic RNA (for replication), and secondly as a template to transcribe GPC or Z mRNA for protein expression.

At the 5' end of each RNA segment are 19 nucleotides that are semi-complementary to their corresponding 3' end (Perez and de la Torre 2003). The resulting 5' and 3' base pairing between the termini forms a panhandle structure which explains the circular appearance of nucleocapsids by electron microscopy (Buchmeier et al. 2001; Perez and de la Torre 2003). The complementation of these terminal nucleotide sequences is conserved in almost all arenaviruses. The secondary structures formed either

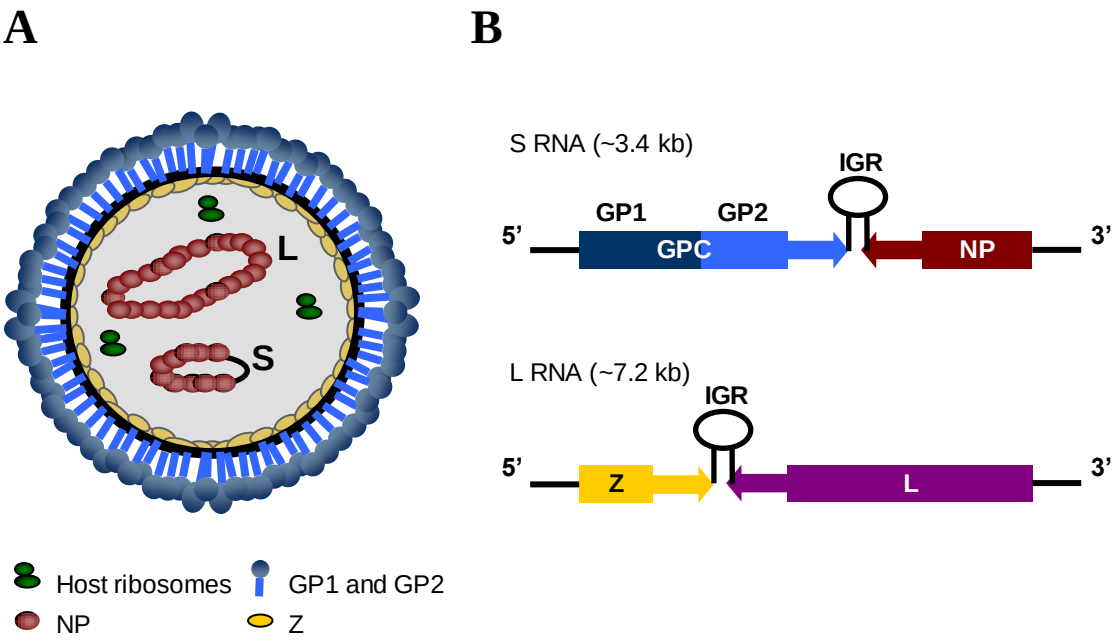


Figure 2. **Arenavirus structure and genome organization. (A)** Schematic drawing of an arenavirus particle. Glycoprotein spikes consisting of GP1 (head) and GP2 (stalk) protrude from the viral envelope. Lining the interior of the viral envelope are Z proteins. The large (L) RNA segment and small (S) RNA segment are found encased by nucleoprotein (NP), which form ribonucleoparticles (RNP) that associate with L (RdRP) to begin transcription. Included are host ribosomes (green) that are often enclosed within the virus particle. **(B)** The ambisense genomic organization of arenaviruses. In the S RNA, the glycoprotein precursor (GPC) is encoded 5' to 3', while the nucleoprotein (NP) is encoded 3' to 5'. A small intergenic region (IGR) separates the two genes, and forms a hairpin structure. The L RNA encodes for the viral RdRP (L) and putative matrix protein (Z).

intramolecularly or intermolecularly, between L and S RNA segments, is suggested to play a role in the binding and or initiation of replication by the viral RdRP (Salvato and Shimomaye 1989; Meyer et al. 2002; Hass et al. 2004).

Separating the two genes on each RNA segment are non-coding, intergenic regions (IGR). These IGR can range from 59 to 217 nucleotides in length and are predicted to form energetically stable G-C rich hairpin structures in both the genomic and antigenomic sense (Auperin et al. 1984; Iapalucci et al. 1989; Wilson and Clegg 1991). The function of these hairpin structures is to regulate transcriptional termination (Meyer et al. 2002). Evidence demonstrating transcription termination at multiple sites in the hairpin structure, supports the theory that arenavirus polymerases dissociate from template RNAs due to RNA structure, instead of recognizing a precise termination site or sequence (Meyer and Southern 1993).

1.4.2 Proteins and their functions

Although arenaviruses are capable of causing a wide variety of diseases ranging from CNS symptoms, to severe hemorrhagic fever, to chronic infections in rodent reservoirs, their structural makeup is quite simple. The two RNA segments encode for four non-overlapping open reading frames, resulting in the expression of five proteins.

The nucleoprotein, or NP (60-68 kDa), is the major structural protein of nucleocapsids. NP is closely associated with virion RNA forming a bead-like structure as seen by electron microscopy (Young and Howard 1983; Buchmeier et al. 2001). NP along with the L protein, are the minimal *trans*-acting factors required for efficient transcription and replication (Lee et al. 2000; Lopez et al. 2001; Hass et al. 2004).

Alignment of NP sequences between several members of arenaviruses, show a high degree of conservation in both sequence and structural motifs (Buchmeier 2002). Furthermore, a stretch of 4 to 5 consecutive basic amino acids near the C-terminal end of NP may function as a RNA-binding domain (Buchmeier 2002). In addition to the involvement of NP in RNA transcription and replication, NP also plays a role in virion assembly. In Tacaribe virus (TCRV), a non-pathogenic New World Clade B arenavirus, two functional domains of NP were identified. The C-terminus of NP is involved in NP-Z interactions, while the N-terminus is involved in NP-NP interactions (Levingston Macleod et al. 2011). Additionally, the interaction between NP and Z is required for the formation of virus-like particles (VLPs) (Casabona et al. 2009).

Arenavirus glycoproteins are synthesized as a precursor protein (pre-GPC, 70-80 kDa) that is processed into three subunits: the stable signal peptide (SSP), GP1 and GP2 (Figure 3). The pre-GPC is co-translationally cleaved by a signal peptidase found within the endoplasmic reticulum (ER), removing a 58 amino acid signal peptide from the N-terminus (Buchmeier et al. 2001). Generally, signal peptides help translocate proteins across the ER membrane destined for the secretory pathway, and also aid in membrane insertion of secretory and membrane resident proteins (Eichler et al. 2003). The SSP contains two transmembrane domains and has both termini in the cytosol, exposing an ectodomain loop on the viral surface (Agnihothram et al. 2007). Evidence has shown that the released SSP serves as a *trans*-acting maturation factor (Eichler et al. 2003). In later studies, it was determined that SSP forms a zinc finger structure with the cytoplasmic domain of GP2, masking endogenous ER

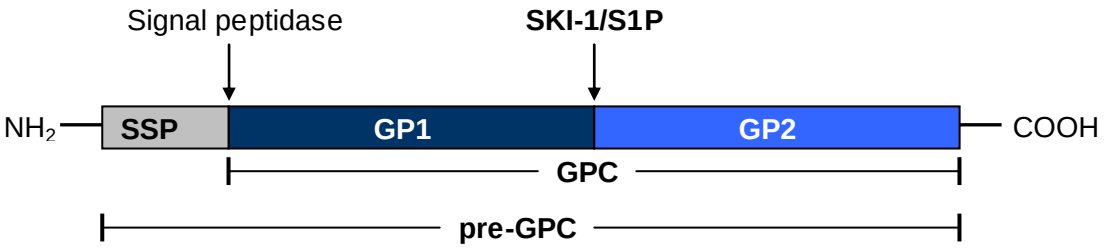


Figure 3. **Schematic diagram of arenavirus glycoprotein precursor structure.** The precursor glycoprotein polypeptide (*pre-GPC*) is first co-translationally cleaved by a signal peptidase at the N-terminal end to release the stable signal peptide (*SSP*). The second cleavage event occurs post-translationally by the cellular protease SKI-1/S1P, generating the two mature subunits GP1 and GP2.

localization signals found on GP2, allowing for further transit of the GPC complex through the Golgi to the plasma membrane where virion assembly occurs (Agnihothram et al. 2006; York and Nunberg 2007). The short ectodomain loop of SSP plays a key role in the ability of the GPC complex to respond to acidic pH, thus influencing pH-dependent fusion of the viral and cellular membranes (York and Nunberg 2006).

As GPC proceeds through the secretory pathway, it is post-translationally processed into two mature subunits, the N-terminal GP1 (40-46 kDa) and C-terminal GP2 (35 kDa). SKI-1/S1P, or subtilisin kexin isozyme-1/site 1 protease, was first identified as the cellular protease responsible for GPC cleavage in the Old World arenaviruses (LASV and LCMV) and later expanded to include the New World arenaviruses (JUNV, MACV and GTOV) (Lenz et al. 2001; Beyer et al. 2003; Rojek et al. 2008c). GP1 is a peripheral membrane protein that mediates viral attachment by interacting with the cellular surface receptors. GP1 assembles into homotetrameric complexes that are held together via disulfide bonds (Buchmeier et al. 2001). The integral membrane glycoprotein, GP2, mediates the fusion of the viral and cellular membranes. The C-terminus of GP2 has a number of highly charged residues that may interact with NP in the cytosol, while at the N-terminus, the presence of two heptad-repeat sequences predict a coiled-coil conformation (Buchmeier 2002). GP2 also assembles into homotetramers that form the stalk of the GP spike, and through ionic interactions, binds with GP1, which forms the 'head' of the spike (Buchmeier et al. 2001).

The L protein (180-250 kDa) is a viral RdRP, and is also a component of the nucleocapsid (Salvato et al. 1989; Lukashevich et al. 1997). RdRP functions in transcribing viral RNA into mRNA, and in the generation of antigenomic RNA to serve

as a template for replication of genomic RNA. The arenavirus L protein contains motifs common to all RdRPs of segmented, negative stranded RNA viruses (Lukashevich et al. 1997). The central domain of L contains the RdRP (Hass et al. 2008), while the N-terminus has RNA endonuclease activity that is essential for the generation of primers to initiate viral mRNA synthesis (Lelke et al. 2010; Morin et al. 2010).

The smallest protein, Z (11 kDa), has been suggested to play both a structural and regulatory role in the arenavirus infectious cycle (Iapalucci et al. 1989; Salvato and Shimomaye 1989; Salvato et al. 1992; Djavani et al. 1997). LASV and LCMV Z protein contains a RING motif that has zinc-binding activity (Salvato et al. 1992). The RING finger domain is essential for Z to bind to L, and may help regulate gene expression by interfering with viral RNA synthesis (Jacamo et al. 2003). Z has also been shown to associate with NP, and together with L they comprise the virion nucleocapsids (Meyer et al. 2002). In LCMV-infected cells, Z protein was localized in the cytoplasm and nucleus (Buchmeier 2002).

1.5 Virus Life Cycle

The arenavirus life cycle occurs within the cytoplasm of infected cells. Within 16 to 24 hours after infection, progeny virions are released from the infected cell by budding from the plasma membrane. Acute infections are generally noncytolytic, and in rodent hosts, there is the possibility of progression into long-term persistent infections (Buchmeier et al. 2001). Not every arenavirus will form plaques, for example LCMV is only moderately cytopathic in cell culture, therefore, other immunological methods are utilized to quantify these viruses (Battegay et al. 1991; Gunther and Lenz 2004).

Arenaviruses are known to have a broad host range and tropism for a variety of cell types. This is in part due to the wide distribution of the two main arenavirus cellular receptors: α-dystroglycan (α-DG) and transferrin receptor 1 (TfR1) (Cao et al. 1998; Radoshitzky et al. 2007). As a cell surface receptor, α-DG links proteins of the extracellular matrix to the intracellular cytoskeleton (Henry and Campbell 1996; Oldstone and Campbell 2011). Dystroglycan is expressed in a wide variety of cells and tissues, including muscle, pancreas, liver, kidney etc. (Durbeej et al. 1998). TfR1 is also expressed ubiquitously, in particular, high levels of expression is found on macrophages, activated lymphocytes and endothelial cells (Jefferies et al. 1984; Soda and Tavassoli 1984; Daniels et al. 2006). Although arenaviruses are able to infect most cells, there are still a number of undefined host-viral interactions that influence the overall outcome of arenavirus infection.

1.5.1 Attachment and Entry

Initial attachment occurs when GP1, the head of the spike protein, binds to the cellular receptor. Studies have shown that antibodies directed against GP1 epitopes can block binding and neutralize the viral infection (Borrow and Oldstone 1992). The receptor of the Old World arenaviruses (LCMV, LASV, Mopeia, and Mobala) and the New World Clade C arenaviruses (Latino and Oliveros) is the cellular glycoprotein α-dystroglycan (α-DG) (Cao et al. 1998; Spiropoulou et al. 2002). Alternatively, the cellular receptor for the New World Clade B arenaviruses (JUNV, MACV, GTOV and SABV) is transferrin receptor 1 (TfR1) (Cao et al. 1998; Radoshitzky et al. 2007). After attachment, virions are internalized into large endocytic vesicles (Buchmeier 2002). For

the New World arenavirus JUNV, virions enter via clathrin-mediated endocytosis (Martinez et al. 2007). In contrast, electron micrographs of LCMV entry demonstrated uptake of virions into non-coated vesicles (Borrow and Oldstone 1994). Subsequent studies involving LASV along with LCMV, revealed that entry of these Old World arenaviruses into host cells occur by a unique clathrin-, caveolin-, dynamin-, and actin-independent endocytic pathway (Rojek et al. 2008a; Rojek et al. 2008b). Acidification of these endosomes will release nucleocapsids into the cell cytoplasm via a pH-dependent fusion of the viral and endosomal membrane (Borrow and Oldstone 1994; Di Simone et al. 1994). At an acidic pH, the GPC complex (GP1, GP2 and SSP) undergoes a conformational change resulting in the exposure of the GP2 fusion peptide, which afterwards inserts itself into the cellular membrane to connect the viral membrane (Di Simone et al. 1994). Once nucleocapsids are released into the cytoplasm, replication begins by transcription of subgenomic and genome-complementary NP and L mRNAs by the viral L protein (Buchmeier 2002).

1.5.2 Transcription and Genome Replication

Arenavirus mRNAs contain a 5' cap structure consisting of 4 to 5 heterogeneous nucleotides, resembling mRNAs from influenza and bunyaviruses (Buchmeier et al. 2001). Therefore, it is suggested that similar to these viruses, arenavirus L proteins may have the ability to 'cap-snatch' from cellular mRNAs in order to prime viral mRNAs for transcription (Buchmeier et al. 2001). Recently, the crystal structure of the N-terminus of LCMV L protein revealed a RNA endonuclease domain that closely resembles the N-terminus of PA protein from Influenza virus, which also has endonuclease activity that

allows it to 'steal' cellular mRNA caps for viral RNA (Dias et al. 2009; Morin et al. 2010). This evidence supports the theory that arenaviruses utilize cap-snatching as a mechanism for transcription; particularly, it is likely the N-terminal domain of L protein that is responsible for this activity.

The first transcriptional products detected are NP mRNAs, and subsequently, NP proteins are the first to appear after arenavirus infection (Buchmeier et al. 2001). The second protein detected is the L protein, about 12 to 24 hours post-infection (Buchmeier et al. 2001). Due to the unique ambisense organization of the genome, the virus can separate the initial expression of NP and L proteins, from GPC and Z expression shortly after replication begins (Buchmeier et al. 2001).

For GPC and Z to be expressed, one round of replication must happen in order to produce full length S and L RNA segment antigenomes. During replication, the viral RdRP does not terminate at the IGR of the template strand, instead it "reads through" to produce the full-length genomic and antigenomic RNAs (Buchmeier et al. 2001). The exact mechanism that regulates the switch from transcription to replication is unknown, however, Z protein may play a role in this regulation (Garcin et al. 1993; Buchmeier et al. 2001). Using the Tacaribe virus (TACV) reverse genetics system, it was demonstrated that TACV NP and L alone are sufficient to drive transcription and RNA replication, and the presence of Z acts as a powerful inhibitor of these processes (Lopez et al. 2001; Jacamo et al. 2003). By co-immunoprecipitation, it was demonstrated that Z did not interact with NP, instead it precipitated with L; the inhibitory activity of Z is dependent on the ability to bind to L (Jacamo et al. 2003). Similar results were also seen in studies using a LCMV minigenome system (Lee et al. 2000; Cornu and de la Torre 2001).

The resulting S and L segment antigenomes serve two functions: they can act as templates for transcription of GPC and Z mRNAs, or as a template to generate more full-length genomic RNAs (Buchmeier et al. 2001). Once GPC is translated, it is glycosylated and transported to the Golgi for proteolytic processing into GP1 and GP2 (Buchmeier et al. 2001).

1.5.3 Virus Assembly and Release

The process of arenavirus assembly is still poorly understood. It is thought that newly synthesized genomic RNAs interact with NP and L to form the nucleocapsid structure. For unknown reasons, host cell ribosomes are packaged into arenavirus particles and the amount packaged varies between virions (Buchmeier et al. 2001). The ability of NP to cross-link to the C-terminus of GP2 suggests that interaction between these proteins may be important in the assembly of viruses (Buchmeier et al. 2001). Newly formed viruses bud out of the plasma membrane by "pinching off" of the lipid bilayer and separation of the virion and plasma membranes (Buchmeier et al. 2001).

The Z protein of several arenavirus species is shown to function as a matrix protein (Strecker et al. 2003). Z is found localized to the inner side of the viral envelope and is able to facilitate budding from the plasma membrane. In the absence of all other viral proteins, expression of Z is sufficient for the release of enveloped Z-containing particles (Strecker et al. 2003). Electron microscopy looking at these virus-like particles compared to Lassa virus particles showed no significant difference in morphology and size (Eichler et al. 2004). It was also demonstrated that Z recruits and interacts with NP at the cell membrane where assembly occurs (Eichler et al. 2004).

A study looking at LASV infection and release from polarized MDCK (Madin-Darby canine kidney) cells found that infection occurs mainly through the basolateral route, this is consistent with the basolateral localization of the cellular receptor α-dystroglycan (Schlie et al. 2010). However, the release of progeny virus was at the apical cell surface (Schlie et al. 2010). Furthermore, using a virus-like particle assay, it was determined that GP mediates the apical release of LASV from epithelial cells by recruiting Z to the assembly site, which in turn interacts with NP for the incorporation of nucleocapsid into virions (Schlie et al. 2010).

Studies on the late-budding domains (L-domain) located at the C-terminus of arenavirus Z protein have demonstrated their important role in virus release. In LASV, the two L-domains PTAP and PPXY are shown to be necessary in the release of VLPs (Strecker et al. 2003). However, in the New World arenavirus Tacaribe (TACV), Z-mediated budding still occurs efficiently despite the absence of these proline-rich L-domains, and additionally, budding is enhanced by NP expression (Urata et al. 2009; Groseth et al. 2010). Data generated by our group also confirms that JUNV and MACV Z proteins are responsible for mediating the budding process, despite the presence of only one L-domain (Chan, Balcewich et al. unpublished data). Furthermore, we were able to produce JUNV and MACV VLPs containing NP and GP (Chan, Balcewich et al. unpublished data). Overall, the presence of L-domains is important in arenavirus budding, although there appears to be different requirements for these domains that are dependent on the virus.

1.6 GPC Processing and Role in Virus Maturation

There are two main processing events that occur during the maturation of arenavirus GPC. The first event occurs when the pre-GPC is co-translationally cleaved by a signal peptidase releasing the signal peptide and GPC. The second, more crucial event occurs later in the secretory pathway where GPC is post-translationally processed into its two mature subunits GP1 and GP2.

In the first studies looking at glycoprotein cleavage in LASV and LCMV, it was discovered that the cellular protease subtilisin kexin isozyme-1 (SKI-1), also known as site 1 protease (S1P), was responsible for proteolytic processing of GP (Lenz et al. 2001; Beyer et al. 2003). Mutational analysis of the LCMV GP cleavage site determined a consensus motif of R-(R/K/H)-L-$(A/L/S/T/F)_{265}\downarrow$ that is important for proteolytic processing by SKI-1 (Beyer et al. 2003). In 2007, the consensus motif of SKI-1 was broadly modified to (R/K)-X-(hydrophobic)-X↓, where X is any amino acid (Pullikotil et al. 2007).

Alignment of the cleavage region of Old and New world arenaviruses show the presence of a SKI-1 consensus motif in all the arenaviruses included, suggesting that SKI-1 may cleave the glycoproteins of all arenaviruses (Figure 4). Supporting this theory

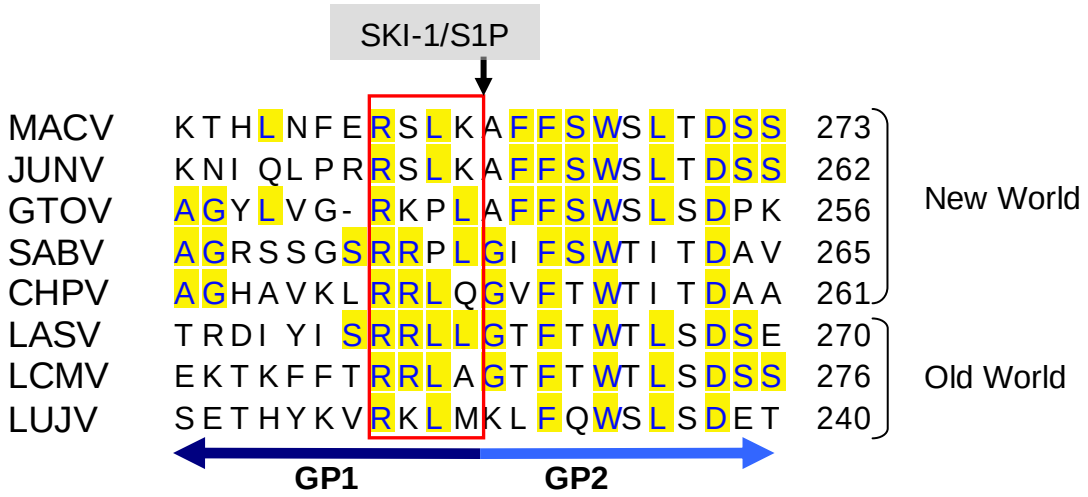


Figure 4. **Alignment of Old and New World arenavirus glycoprotein amino acid sequences at the proposed SKI-1/S1P cleavage site.** All arenaviruses analysed contained the putative SKI-1/S1P consensus motif, enclosed in red. The downward arrow denotes where SKI-1/S1P cleaves the glycoprotein into GP1 and GP2 subunits. Analysis was performed by the Clustal W method of alignment.

was evidence showing that members of the New world arenaviruses such as JUNV, MACV and GTOV were also cleaved by SKI-1 (Rojek et al. 2008c). Interestingly, SKI-1 also processes the GP of Crimean-Congo hemorrhagic fever virus (CCHFV), a member of the *Bunyaviridae* family of viruses. CCHFV is a tick-borne pathogen that causes human hemorrhagic fever in Europe, Asia and Africa (Vincent et al. 2003; Bergeron et al. 2007).

Proteolytic processing of LASV GPC is not required for GPC to be transported to the cell surface (Lenz et al. 2001). Similar to LASV, we demonstrated that the same is true for JUNV and MACV (Chan, Balcewich et al. unpublished data). Although GPC cleavage is not important for transport, the formation of infectious virus particles is absolutely dependent on GPC processing, as only cleaved GP is incorporated into virions (Lenz et al. 2001). The dependence of glycoprotein cleavage for the formation of infectious virions is also true for CCHFV (Bergeron et al. 2007). The recent generation of a reverse genetics system for JUNV also confirms that cleavage is essential for the production of infectious viral particles in New world arenaviruses (Albarino et al. 2009). Therefore, preventing this processing step may be a promising antiviral target that may protect against arenavirus and CCHFV infections.

1.7 Cellular localization and function of SKI-1

SKI-1 belongs to a family of proprotein convertases that consists of nine, subtilisin-like serine endoproteinases (Seidah et al. 2008). These convertases help convert inactive precursor proteins into their mature forms by recognizing general consensus motifs and cleaving the precursors at that site (Seidah et al. 2008). There are seven convertases that

cleave after basic residues, like furin, and two other convertases that prefer cleavage at non-basic amino acids including SKI-1 (Seidah et al. 2006).

SKI-1 is synthesized as an inactive precursor which becomes activated via three sequential autocatalytic events, leading to the formation of intermediate forms located in the ER, and finally an active form found in the cis/medial Golgi (Seidah et al. 1999; Elagoz et al. 2002; Pullikotil et al. 2007). Subcellular localization studies revealed that SKI-1 is located in the cis/medial Golgi as well as endosomal/lysosomal compartments (Pullikotil et al. 2007). In contrast to furin, which cycles from the cell surface to the trans Golgi network, SKI-1 is not trafficked to the cell surface (Thomas 2002; Pullikotil et al. 2007).

In addition to processing viral glycoproteins, SKI-1 naturally plays a role in regulating the synthesis of cholesterol and fatty acids in mammalian cells. As well, SKI-1 processes a number of membrane-bound transcription factors including the sterol regulatory element-binding proteins (Espenshade et al. 1999), pro-brain-derived neurotrophic factor (Seidah et al. 1999), and the activating transcription factor-6 that regulates ER stress response (Haze et al. 1999).

1.8 Serine Protease Inhibitors (Serpins)

Serpins are the largest and most widely distributed superfamily of proteins (Irving et al. 2000). Although the name suggests serine protease inhibitors, cysteine protease inhibitors and other proteins lacking any inhibitory protease activity are also grouped within this superfamily (Gettins 2002). Examples of some well studied serpins are those that inhibit serine proteinases of the chymotrypsin family, including alpha$_1$-antitrypsin

(α_1-AT), an elastase inhibitor, and the closely related antithrombin that inhibits several enzymes in the mammalian coagulation pathway (Law et al. 2006).

Serpins are relatively large molecules that fold into a conserved structure consisting of generally 3 β-sheets and 8-9 α helices (Law et al. 2006). The region of the serpin that interacts with target proteases is the reactive center loop (RCL), which extends out from the main body of the serpin structure and is accessible to binding by target proteases (Law et al. 2006). The target protease recognizes the sequence on the exposed RCL as 'substrate' and binds to it. There are two potential outcomes that occur after binding, the substrate pathway or the inhibitory pathway. In the inhibitory pathway, the target protease cleaves the RCL of the serpin, causing the serpin and the attached target protease to undergo such a significant conformational change that the protease is inactivated and trapped as a complex, eventually leading to proteolytic degradation as a result of the structural disorder (Huntington et al. 2000). In the substrate pathway, the target protease also cleaves the RCL, but instead, escapes the conformational trap and remains as an active protease and a cleaved, inactivated serpin (Law et al. 2006). Due to the irreversibility of the interaction between serpin and protease, serpins are known to function as suicide substrate inhibitors (Gettins 2002).

1.8.1 Serpins against Furin

Furin is a cellular protease that is also a member of the proprotein convertases that cleaves protein substrate after basic residues, R-X-K/R-R↓, where X is any amino acid (Molloy et al. 1992; Thomas 2002). Furin plays a key role in the maturation of a number of viral glycoproteins including Ebola virus and Marburg virus GP (Volchkov et al. 1998;

Volchkov et al. 2000), Human immunodeficiency virus (HIV) gp160 (Hallenberger et al. 1992), Influenza virus HA (Stieneke-Grober et al. 1992), and the Measles virus fusion glycoprotein (Richardson et al. 1986). In addition, furin also processes a number of bacterial toxins, growth factors, serum factors, and receptors (Gettins 2002). Due to the key role furin plays in the activation of a number of pathogens, many furin inhibitors have been developed and studied over the years.

One of the earliest studied serpin-based furin inhibitor was an α_1-antitrypsin Portland variant (α_1-PDX) (Anderson et al. 1993). Based on α_1-AT, the PDX variant was generated by mutating the RCL of α_1-AT to contain the minimum recognition sequence for cleavage by furin, which was an R-X-X-R↓ motif, where X is any amino acid (Molloy et al. 1992). Using this variant, Anderson *et al.* were able to efficiently inhibit furin-mediated cleavage of HIV-1 gp160, and its subsequent membrane fusogenic properties (1993). Further studies looking at the effect of furin inhibition on virus replication revealed that in the presence of α_1-PDX, HIV-1 replication was impaired (Bahbouhi et al. 2001). For human cytomegalovirus, the addition of α_1-PDX also significantly reduced the amount of infectious virus produced (Jean et al. 2000). However, the effect of using α_1-PDX to block filovirus glycoprotein processing had minimal effect on the reduction of viral titres (Stroher et al. 2007). Although, for Ebola virus, it has been demonstrated that proteolytic processing of GP is not required for replication *in vitro*, which is in contrast to many other viruses.

In 2004, Richer *et al.* used a homology-search program to screen for eukaryotic serpin genes that contained an endogenous furin recognition site (R-X-K/R-R↓, where X is any amino acid) in their RCL region. The *Spn4* gene in *Drosophila melanogaster* was

identified to encode a highly effective endogenous serpin (Spn4A) that was able to inhibit furin (Richer et al. 2004). It was also determined that the length of RCL is an important feature of the serpin that influences its inhibitory ability. Therefore, the unusual 18 amino acid length RCL of Spn4A compared to the typical 17 residue RCL common to other inhibitory serpins, makes Spn4A a more potent and effective natural serpin in comparison to α_1-PDX, which was adapted from α_1-AT (Richer et al. 2004). Additionally, as a result of this longer RCL region, Spn4A favours the inhibitory pathway rather than the substrate pathway (refer to section 1.8). Taken together, the ability to mutate the RCL region of Spn4A to encode any general consensus motif, for example, one that is specific for SKI-1, demonstrates the potential and versatility that these serpins have to become powerful antipathogenic agents.

1.9 Research Goals

1.9.1 Significance of research

New arenaviruses are being discovered on an average of one every three years, with the two most recent additions primarily being the New World Chapare virus isolated from Bolivia in late 2003, and Old World Lujo virus, found in South Africa in 2008 (Delgado et al. 2008; Briese et al. 2009; Emonet et al. 2009). Both of these viruses caused cases of severe hemorrhagic fever in humans, resulting in death (Delgado et al. 2008; Briese et al. 2009). Interestingly, Lujo virus is the first Old World hemorrhagic fever-associated arenavirus to be isolated in the past three decades (Briese et al. 2009). Current climate changes have also affected the distribution of the natural reservoirs of these

viruses, increasing the risk of human-rodent contact through agriculture or even invasion of homes by rodents in search of food. In addition, the recent increase in travel to endemic regions such as Africa and South America raises the risk of travel-related imported infections of these viruses to other parts of the world.

High mortality rates, the possibility of person-to-person transmission, the ability of these viruses to be spread through aerosols, and the absence of a vaccine, has led to the classification of several arenaviruses as Category A biological agents that pose a threat due to bioterrorism. The continual emergence of new fatal arenavirus species coupled with the lack of a licensed arenavirus vaccine or effective antiviral treatment, emphasizes the importance to develop new therapies that are able to protect against both Old and New World arenaviruses.

In the past, the majority of research on the significance of arenavirus glycoprotein cleavage was focused on the Old World arenaviruses, like LASV and LCMV (Lenz et al. 2001; Beyer et al. 2003). It was demonstrated for LASV that the formation of infectious virus particles is absolutely dependent on GPC processing, as only cleaved glycoprotein is incorporated into virions (Lenz et al. 2001). Only recently, with the generation of a reverse genetics system for JUNV, the importance of GPC processing by SKI-1 for infectious virus formation in a New World arenavirus was confirmed (Albarino et al. 2009).

The focus of the research in this thesis is on New World arenavirus glycoprotein processing. In particular, how the maturation of this protein through cleavage by SKI-1 plays a key role in the formation of infectious virions. The potential use of GP cleavage inhibitors is currently under investigation as a therapy for many viral infections. It is a

very promising approach because the target is a cellular protein, which makes the development of resistance less likely. Previously, our group has used serpins successfully to selectively inhibit the cellular protease furin (Stroher et al. 2007). These inhibitors can be designed specifically to bind to target proteases, like SKI-1, and inactivate it through a conformational change of its active site.

Since Spn4A is a potent and effective natural inhibitor of furin, it was selected to be modified to become a SKI-1 specific inhibitor. Therefore, by changing the RCL of Spn4A from a consensus furin cleavage site (-RRLR↓), to a consensus SKI-1 cleavage site (-RRLL↓), this serpin will now act as a 'substrate' to SKI-1 and inhibit it. To test the specificity for SKI-1 by these inhibitors, two forms of SKI-1 inhibitor were generated, one that contains an ER retention signal (Spn4-ER) and another with the signal deleted to promote transit through the secretory pathway (Spn4-S). Since active SKI-1 is found mainly in the cis/medial Golgi network, it is expected that Spn4-S will have the major inhibitory affect compared to Spn4-ER. Furthermore, parallel experiments involving the use of a SKI-1 deficient cell line and provision of recombinant SKI-1 to these same cells, will confirm whether results obtained from serpin-based SKI-1 inhibition experiments are indeed due to a lack of SKI-1 specifically. As other controls for the specificity of these serpins, α_1-AT and α_1-PDX are also included in our studies. It is expected that neither inhibition of trypsin or furin will have an effect on arenavirus glycoprotein cleavage or replication. Due to the conserved use of SKI-1 in several arenaviruses and even CCHFV, these serpin-based SKI-1 inhibitors may provide broad protection against members of both the Old and New World arenaviruses, as well as against CCHFV.

1.9.2 Hypothesis

The generation of infectious arenavirus particles is dependent on proper glycoprotein processing by the cellular protease SKI-1, and prevention of GPC processing using SKI-1-specific serpins will effectively inhibit Old and New World arenavirus replication.

1.9.3 Objectives

1) **Determine whether New World Chapare virus GPC is cleaved by SKI-1.** Gene synthesis of Chapare virus GPC will be completed first, followed by site-directed mutagenesis of the proposed SKI-1 cleavage site to determine the effect of mutation on cleavability. Expression of Chapare virus GPC in a SKI-1 deficient cell line and the effect of providing recombinant SKI-1 *in trans* will be studied to confirm that SKI-1 is the protease responsible for GPC cleavage

2) **Characterize the effectiveness of serpin-based SKI-1 inhibitors.** Recombinant adenoviruses expressing SKI-1 specific serpins based on the natural furin inhibitor Spn4A of *Drosophila melanogaster* will be generated, and their potential inhibitory effect will be tested on transiently expressed MACV, JUNV, GTOV, SABV, CHPV, and LASV GPC *in vitro*.

3) **Investigate the role of GPC proteolytic processing on arenavirus replication.** Replication-competent recombinant vesicular stomatitis viruses (VSV) expressing MACV, JUNV, GTOV, SABV, CHPV and LASV GPC will be generated to determine

the effect of SKI-1 inhibition on arenavirus replication. The results from those studies will be confirmed with wild-type viruses in BSL-4.

2.0 Materials and Methods

2.1 Cell culture

Vero E6 (African green monkey kidney) and 293T (human embryonic kidney) cells were grown in Dulbecco's modified Eagle medium (DMEM, Sigma) containing 10% heat inactivated (56ºC for 30 minutes) fetal bovine serum (FBS) (Sigma), 100 U/mL penicillin, 100 ug/mL streptomycin, and 2mM L-glutamine (all from Invitrogen). HEK293 cells used the same medium as Vero E6 and 293T with the addition of 1 mM sodium pyruvate (Sigma).

CHO-K1 (Chinese hamster ovary) cells were maintained in DMEM:Ham's nutrient mixture F-12 (ATCC) supplemented with 10% FBS, penicillin, and streptomycin. SRD-12B (SKI-1 deficient CHO-K1) cells were also grown in DMEM:Ham's nutrient mixture F-12 medium, however, with 5% FBS and the addition of 5 ug/ml cholesterol, 1 mM sodium mevalonate, and 20 uM sodium oleate (all from Sigma) (Rawson et al. 1998).

Cells were passaged every 3-4 days using 0.05% trypsin-EDTA (Invitrogen) and diluted into fresh culture medium. All cells were incubated in a humidified incubator at 37º C with 5% CO_2.

2.2 Virus strains

All recombinant vesicular stomatitis viruses were handled in enhanced biosafety level 2 (BSL-2) laboratory settings. Infectious JUNV strain Rumero (AY619641),

MACV strain Carvallo (AY129248), GTOV (AY129247), SABV (NC006317), LASV strain Josiah (NC004296), and *Lake Victoria marburgvirus* strain Angola (DQ447660) work was performed in BSL-4 conditions. Viral RNA was extracted and used as a template for amplification of the GPC gene. The published sequence of CHPV (EU260463) was used to design primers for gene synthesis of the GPC gene (see section 2.3.3).

2.3 Cloning Arenavirus Glycoproteins

2.3.1 Primer Design

Sequence specific primers were used to amplify MACV, JUNV, SABV, GTOV, and LASV GPC genes. See Appendix A (Table 4) for sequences of primers used. To facilitate cloning of GPC into expression plasmids, specific restriction enzyme sites were included in both forward and reverse primer sets. For cloning into the mammalian expression vector pCAGGS, BsmBI and NheI were used. To clone into the vector which encodes the full length VSV genome, with VSV G deleted and an additional GFP gene (pATX-VSVΔG-XN2-GFP), MluI and AvrII sites were selected.

2.3.2 RT-PCR of arenavirus GPC gene from viral RNA

Arenavirus RNA was extracted in BSL-4 conditions using the QIAamp Viral RNA Mini Kit (Qiagen). Procedure for extraction was as described in manufacturer's manual. RNA was then taken out following standard operating procedures for the removal of samples from BSL-4.

The SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen) was used to generate cDNA that was subsequently used as a template for PCR. Briefly, cDNA synthesis set up included 2 ul each of forward and reverse gene specific primer (1 uM), 1 ul RNA, 1 ul dNTP mix (10 nM), and 7 ul RNase free dH_20. Mixture was heated to 65º C for 5 minutes and then incubated on ice for at least 1 minute. To the cooled mixture, 4 ul of 5X First-Strand Buffer, 1 ul of 0.1 M DTT, 1 ul of RNaseOUT™ Recombinant RNase Inhibitor (40 U/ul) and 1 ul of SuperScript™ III RT enzyme (200 U/ul) was added and mixed gently. Mixture was incubated at 55ºC for 45 minutes and inactivated at 70ºC for 15 minutes. To remove RNA that is complementary to the cDNA, 1 ul of RNase H (Invitrogen) was added and incubated at 37ºC for 20 minutes. The cDNA was then used as a template for PCR amplification (see section 2.3.3).

2.3.3 GPC Amplification by Polymerase Chain Reaction (PCR)

PCR was completed using PfuUltra™ II Fusion Hotstart DNA polymerase (Stratagene). PCR reactions were set up as follows: 30 ng of plasmid DNA template (arenavirus GPC gene in pCR®2.1-TOPO®) or 1 ul of cDNA template (section 2.3.2), 5 µl 10X PfuUltra II reaction buffer, 1.25 µl each of forward and reverse primers (10 µM), 1 µl dNTPs (10 mM), 1 µl PfuUltra II Fusion HS DNA polymerase (Stratagene), and dH_20 up to 50 µl. Primer sets are designated forward and reverse, see Appendix A Table 4 for sequences. Thermocycling parameters: initial denaturation at 95°C for 3 minutes, followed by 18 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute/kb. A final extension of 10 minutes was carried out after cycling. PCR products were stored at -20°C.

2.3.4 CHPV GPC Gene Synthesis

Chapare virus RNA was not available as a template for RT-PCR to amplify GPC. Therefore, the published sequence of the CHPV GPC gene (EU260463) was divided into 80 forward and reverse primers, each approximately 40 nucleotides in length, with 20 overlapping nucleotides using the Gene2Oligo (http://berry.engin.umich.edu/gene2oligo/) software program. All primers were diluted to 150 uM in dH_20 and 5 uL of each primer was combined to generate a master primer mix. Four rounds of PCR using iProof™ High-Fidelity DNA Polymerase (Bio-Rad) were completed to generate the full length CHPV GPC gene.

First round PCR set up was as follows: 5 uL primer mix, 1 uL dNTPs (10 mM), 10 uL 5X iProof GC Buffer, 0.5 uL $MgCl_2$ (50 mM), 1 uL iProof DNA Polymerase (2U/uL), and 32.5 uL dH_20 for a final volume of 50 uL. Annealing temperature for the first 10 cycles was determined using the primer with the lowest Tm +3ºC. The annealing temperature for the next 10 cycles was chosen using the average Tm +3ºC. Thermocycler parameters for all rounds of PCR can be found in Table 2.

Second round PCR set up: 10 uL of round 1 PCR product, 1 uL dNTPs, 10 uL 5X iProof GC Buffer, 0.5 uL $MgCl_2$, 1 uL iProof DNA polymerase, and 27.5 uL dH_20 for a final volume of 50 uL.

Third round PCR set up: 2 uL of round 2 PCR product, 1 uL dNTPs, 1 uL each of CHPV GPC 5’ and 3’ end forward and reverse primers (150 uM), 10 uL 5X iProof GC Buffer, 1 uL iProof DNA polymerase, and 34.5 uL dH_20 for a final volume of 50 uL. The third round annealing temperature used was Tm +2.

Table 2. **Thermocycler parameters used in each round of PCR for the synthesis of Chapare virus GPC.**

PCR Round	# of cycles	Temperature	Time
1	1	98ºC	60 s
	10	98ºC	10 s
		54ºC	30 s
		72ºC	25 s
	10	98ºC	10 s
		60ºC	30 s
		72ºC	30 s
	30	98ºC	10 s
		65ºC	30 s
		72ºC	30 s
	1	72ºC	10 min
2	1	98ºC	60 s
	35	98ºC	10 s
		65ºC	30 s
		72ºC	40 s
	1	72ºC	10 min
3	1	98ºC	60 s
	35	98ºC	10 s
		69ºC	30 s
		72ºC	45 s
	1	72ºC	10 min
4	Same as for Round 3		

Fourth round PCR set up: 3 uL of round 3 PCR product, 1 uL dNTPs, 1 uL of CHPV GPC 5' end forward primer (150 uM), 1 uL of CHPV GPC 3' end reverse primer (150 uM), 10 uL 5X iProof GC Buffer, 0.5 uL iProof DNA polymerase, and 33.5 uL dH_20 for final volume of 50 uL. Fourth round thermocycler parameters were the same as round 3.

2.3.5 Gel extraction

PCR products were run on a 1% agarose gel containing 0.5 g of ultrapure agarose (Invitrogen) dissolved in 50 ml 1X TAE buffer and 5 ul of SYBR®safe DNA stain (Invitrogen). Samples were mixed with 6X DNA loading buffer (Appendix C) and loaded along with a 2-log DNA ladder (NEB). Gels were run in an electrophoresis chamber at 100V for 40 min. Visualization of the DNA gel was done by exposure to UV light and DNA bands of interest were excised using a scalpel. The QIAquick Gel Extraction Kit (Qiagen) was then used to extract the DNA; protocol followed was as described in manual using a microcentrifuge with the exception that 30 uL of dH_20 was used to elute DNA rather than the TE buffer provided.

2.3.6 pCR2.1-TOPO® cloning

To facilitate cloning into the pCR2.1-TOPO® vector, dATP was added to the ends of the PCR product to generate sticky ends. The following reaction was set up: 25 uL of gel extracted DNA (section 2.3.5), 3 uL 10X PCR Buffer with $MgSO_4$ (Roche), 1 uL of Taq polymerase, 1 uL of 100 mM dATPs (Invitrogen) for a final volume of 30 uL. Reaction mix was incubated at 72ºC for 12 minutes.

Using the TOPO TA Cloning® Kit (Invitrogen), the TOPO cloning reaction set-up was as follows: 4 uL of PCR product (Taq incubated), 1 uL Salt Solution, and 1 uL of pCR2.1-TOPO. Reaction mix was incubated at room temperature for 10 minutes and placed on ice until the next transformation step (section 2.3.7).

2.3.7 Transformation of chemically competent bacteria

For each TOPO cloning reaction, a 100 ul vial of chemically competent TOP10 *E.coli* cells (Invitrogen) was thawed on ice. To each vial of cells, 2 ul of TOPO cloning reaction (section 2.3.6) was added and incubated on ice for 30 minutes. Cells were then heat shocked for 30 seconds at 42ºC and transferred back on ice for 2 minutes. Then, 250 ul of SOC medium was added to the cells, and incubated for 1 hour at 37ºC with shaking at 300 rpm. Luria-Bertani (LB) agar plates containing 100mg/ml of ampicillin were pre-warmed at 37ºC for 30 minutes. Then, 40 uL of X-gal Solution (20 mg/ml, Fermentas) was spread onto each plate and allowed to dry at 37ºC until plating. About 150 ul of transformation mixture was plated onto one LB+Amp+X-gal plate and incubated overnight at 37ºC. Next morning, blue/white colonies were screened, and only white colonies were picked for further growth and DNA isolation (section 2.3.9).

For further subcloning into pCAGGS and pATX-VSVΔG-XN2-GFP, chemically competent XLI-Blue *E. coli* cells were transformed. For each ligation reaction, a 100 µl vial of XL1-Blue cells was thawed on ice. Next, 10 µl of a ligation reaction (section 2.3.12) was added and further incubated on ice for 30 minutes. Cells were then heat shocked for 45 seconds at 42ºC and transferred back on ice for 3 minutes. To the cells, 1 ml of LB medium without antibiotics was added and incubated at room temperature with

shaking for 90 minutes. Cells were then centrifuged at 6000 rpm for 2 minutes. The pellet was resuspended in 150 µl of LB medium and spread onto pre-warmed LB plates+amp. Plates were incubated at 37ºC overnight. Next day, isolated colonies were picked and grown up in culture with LB+amp medium for plasmid isolation (see section 2.3.9).

2.3.8 Preparation of Chemically Competent *E. coli.*

All chemically competent *E.coli* XLI-Blue cells were produced in-house using the TSS method. An overnight culture containing 50 ul of a previously thawed vial of *E.coli* XLI-Blue cells in 5 ml LB broth without antibiotics was grown. The next day, 0.5 ml of overnight culture was added to 50 ml of fresh LB broth (1:100 dilution) and grown at 37ºC until an optical density reading at 600 nm (OD_{600}) of 0.4-0.5 was attained. Cells were then incubated on ice for 20 minutes and pelleted by centrifugation at 2500 x g for 10 minutes at 4ºC. The supernatant was discarded and the pellet was gently resuspended in 5 ml of filter sterilized TSS buffer (Appendix C). Cells were then divided as 100 ul aliquots into pre-chilled 1.5 ml eppendorf tubes on dry ice and immediately stored at -80ºC.

2.3.9 Plasmid Isolation

Plasmid isolation from bacterial cultures was done using the QIAprep Spin Miniprep Kit (Qiagen). Procedure used was as described in manual using a microcentrifuge, with the exception of the final elution step where 50 µl of dH_20 was used to elute DNA instead of 50 µl of Buffer TE provided. After plasmids were isolated,

a ~200 ng DNA sample was digested with the same restriction enzymes used to clone the insert into the vector for 1 hour, and subsequently verified on a 1% agarose gel in 1X TAE buffer. If the correct sized plasmid was isolated, samples were sent for DNA sequencing for further confirmation.

2.3.10 Restriction Digests

Once arenavirus GPC genes were cloned into pCR2.1-TOPO®, the GPC gene was cut out using either BsmBI/NheI for subcloning into pCAGGS, or MluI/BlnI for subcloning into pATX-VSVΔG-XN2-GFP. All enzymes and buffers were from New England Biolabs (NEB). BsmBI works by cutting 1 bp downstream of its recognition site. Therefore, the first cloning primers were designed so that digestion by BsmBI would result in an EcoRI compatible end, and allow for ligation into pCAGGS cut with EcoRI and NheI. This was necessary because of an internal EcoRI site within the MACV GPC gene that prevented the use of EcoRI directly for subcloning. Generally, reactions were set up as follows: 5 ug of DNA, 1.5 ul of enzyme, 5 ul of 10X buffer, 5 ul of 10X BSA and dH_20 for a final volume of 50 ul. Reaction mixtures were digested for 4 hours at the specified temperature and heat inactivated. If separate digest reactions were required due to temperature incompatibilities, a PCR purification step was performed to remove used enzyme and buffer before the second digest reaction (see section 2.3.11). After double digest, product was run on a 1% agarose gel and the target band was excised for gel extraction (section 2.3.5).

2.3.11 PCR Purification

For the purification of DNA from restriction enzymes and buffers that may interfere with subsequent cloning procedures, the QIAquick PCR Purification Kit (Qiagen) was used. The protocol followed was as described in the manual using a microcentrifuge with the exception of the final elution step, where 30 µl of dH_20 was used to elute DNA from the column instead of using TE buffer provided.

2.3.12 Ligation

Prior to ligation, dephosphorylation of the vectors pCAGGS and pATX-VSVΔG-XN2-GFP was done to enhance efficient subcloning. To the entire digested pCAGGS sample, 1 µl of Shrimp Alkaline Phosphatase (Fermentas) was added and incubated for 4 hours at 37ºC. Phosphatase catalyzes the release of 3' and 5' phosphate groups. Following dephosphorylation, plasmid was purified using the QIAquick PCR Purification Kit (Qiagen) (see section 2.3.11).

Gel extracted glycoprotein DNA was then ligated into dephosphorylated pCAGGS using T4 DNA ligase (Invitrogen). The following reaction mixture was set up: 1 µl of vector, 6.5 µl of insert, 2 µl of 5X Buffer and 0.5 µl of T4 DNA Ligase for a final reaction volume of 10 µl. Reaction mix was incubated at 14ºC overnight for 16 hours. Two control reactions were also set up, first one lacking T4 DNA Ligase to determine amount of undigested plasmid, and a second reaction lacking insert to determine amount of re-ligated plasmid. Ligation reactions were then used directly for transformation into XL1-Blue *E. coli* cells (section 2.3.7).

2.3.13 Site directed mutagenesis

To mutate amino acids in the putative SKI-1 cleavage site in Chapare virus GPC to alanine residues, point mutations were introduced into the gene by site directed mutagenesis. Generally, the entire plasmid was amplified by PCR using two complementary primers that contained the desired mutation. Primer designs were based on suggestions made by Stratagene's QuikChange® Site-Directed Mutagenesis kit instructor's manual. Briefly, the primers were designed such that the mutation site was situated in the middle, with at least 15 bp to the left and right to attain a $T_m \geq 78^oC$. See Appendix B Table 5 for list of mutagenesis primers used. The PCR reaction set up and thermocycling parameter was as described in section 2.3.3. After PCR amplification, removal of the template DNA was achieved by adding 1 ul of DpnI to the sample and incubating 37ºC for 8 hours. DpnI digests the methylated bacterial plasmid DNA of the template, while leaving the non methylated PCR product intact. The resulting nicked, circular PCR product was directly transformed into bacteria (see section 2.3.7), colonies were picked, plasmid DNA isolated (section 2.3.9) and sent for sequencing to confirm that the desired mutation occurred (see section 2.3.13).

2.3.13 DNA Sequencing

The DNA Core Facility at the Public Health Agency of Canada sequenced all DNA samples using the Sanger method of sequencing. Sequenced data of all cloned or subcloned vectors confirmed whether the arenavirus glycoprotein gene was correctly inserted within the vector and if any mutations were present.

2.4. Protein Expression

Expression of arenavirus glycoproteins were tested in mammalian 293T, Vero E6, SRD-12B and CHO-K1 cells using Lipofectamine™ 2000 (Invitrogen) transfection reagent. For 293T cells, the 6-well plates were coated with 0.1 mg/ml poly-D-lysine (Sigma) in PBS and incubated for 20 minutes at 37ºC. Plates were then washed twice with PBS before use. All cells were seeded one day prior to transfection so that cells were ~60% confluent the next day.

A recombinant plasmid encoding human SKI-1 (pIR-hSKI) was kindly donated by Dr. Nabil Seidah (Laboratory of biochemical neuroendocrinology). This plasmid was transfected into SKI-1 deficient SRD-12B cells to recover the function of SKI-1. Generally, 4 ug of pIR-hSKI was transfected into SRD-12B cells using Lipofectamine™ 2000 transfection reagent.

2.4.1 Transfection using Lipofectamine™ 2000

Transfection of eukaryotic cells using Lipofectamine™ 2000 involved the set up of two mixtures. Mixture A consisted of a tube containing 250 µl of Opti-MEM®1 reduced Serum Media (Opti-MEM, Gibco), where 1.5 ug of plasmid DNA was added. For mixture B, 10 ul of Lipofectamine™ 2000 was added to 250 ul of Opti-MEM in a separate tube. Both mixtures were incubated at room temperature for 5 minutes, then combined together, mixed gently, and incubated for a further 30 minutes at room temperature. The final volume was adjusted to 1 ml by adding 500 ul of Opti-MEM to each tube. Old media from wells were removed, cells were rinsed with plain DMEM, and finally transfection mixture was added drop wise into the well. Cells were incubated

overnight in a humidified chamber at 37ºC with 5% CO_2. The next morning, 1 ml of DMEM supplemented with 2% FBS was added to the wells without removal of the transfection mixture. Cells were incubated for another 24 hours before harvesting.

At 48 hours post-transfection, supernatant was removed and cells were rinsed once with PBS. Cells were lysed using 500 µl of Triton-X 100 lysis buffer (Appendix C) per well for 20 minutes on ice with gentle rocking. Cell lysate was scraped off each well, transferred to a 1.5 ml tube, and centrifuged at 10,000 rpm for 20 minutes at 4°C. In a new tube, 300 ul of the cell lysate supernatant was combined with 100 ul of 4X SDS-PAGE sample buffer (Appendix C). Samples were then analyzed by SDS-PAGE and Western blot (see sections 2.4.2 and 2.4.3).

2.4.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were separated based on molecular weight by SDS-PAGE. All gels used consisted of a 10% resolving gel and 4% stacking gel (Appendix C). As a standard, one lane of each gel was loaded with 3 ul of Odyssey® Two-Color Protein Molecular Weight Marker (LI-COR) to confirm protein sizes. The gels were then run using the mini-PROTEAN 3 electrophoresis system (Bio-Rad). Before loading, all protein samples were heated for 5 minutes at 95ºC and then transferred immediately to ice. Generally, 20 ul of each sample was loaded and run at 130V for 1-1.5 hours.

2.4.3 Western Blot

After electrophoresis, protein gels were transferred to a polyvinylidene fluoride (PVDF) (Millipore) membrane using a mini Trans-Blot Cell (Bio-Rad) for detection by specific antibodies. Each PVDF membrane was soaked in methanol for 5 minutes and then placed in fresh Transfer Buffer (Appendix C). All filter papers and fibre pads were also pre-soaked in Transfer Buffer for 5 minutes. The gel sandwich was set up as follows: Cathode (black end), grey side of cassette, fibre pad, filter paper, acrylamide gel, PVDF membrane, filter paper, fibre pad, white side of cassette, Anode (red end). After second filter paper was placed on the gel and membrane, a roller was used to remove any bubbles. Finally, the transfer apparatus was assembled and run at 100 V and 250 mA for 80 minutes or 30 V and 40 mA overnight. After transfer, the membrane was blocked for 1 hour in Odyssey® Blocking Buffer (OBB, LI-COR) at room temperature with rocking, or overnight at 4ºC.

The primary antibody was diluted using 5 ml of OBB (0.1%Tween20) per membrane, and was incubated with the membrane for 1-2 hours at room temperature (see section 2.4.4 for antibodies and dilutions used). The membrane was then washed four times for 5 minutes each in PBS (0.1%Tween20). The secondary antibody was diluted in 7 ml of OBB (0.1%Tween20) and incubated for 1 hour at room temperature while protected from light. The membrane was washed four times for 5 minutes each using PBS (0.1%Tween20), and one final wash in PBS for 5 minutes. Membranes were then visualized using a LI-COR® Odyssey Infrared Imaging System.

To strip membranes of antibodies for further detection using other primary antibodies, a Stripping Buffer (Appendix C) was used. Each membrane was immersed in

20 ml of Stripping Buffer containing 140 ul of β-mercaptoethanol, and incubated at 50˚C for 30 minutes. Membranes were then washed twice with PBS (0.1% Tween20) for 10 minutes each, and blocked again with OBB either for 1 hour at room temperature or overnight at 4˚C. Using the same procedure described, subsequent immunoblotting with a different antibody was done.

2.4.4 Antibodies

To detect JUNV, GTOV, SABV and CHPV glycoprotein, a monoclonal mouse anti-MACV GP2 (F100G4) antibody known to cross-react with other new world arenaviruses was used at a dilution of 1:100. Alternatively, for MACV, the monoclonal mouse anti-MACV GP2 (F106G3) antibody was used at a dilution of 1:100 because of the higher affinity towards MACV glycoproteins. These antibodies were generated at the Public Health Agency of Canada and have been described recently by York *et al*. 2010. For the detection of LASV GP, a polyclonal rabbit anti-LASV G2 serum (L4) was kindly provided by Thomas Strecker and Wolfgang Garten (University of Marburg). The L4 antibody was used at a dilution of 1:2000.

To detect the Flag-tagged serpins (Spn4-ER, Spn4-S, α_1-antitrypsin, and anti-furin), a polyclonal rabbit anti-Flag antibody (Sigma F7425) diluted 1:500 was used. As a loading control, a monoclonal mouse anti-β-actin antibody (Sigma A3854) was used at a dilution of 1:20000.

Secondary IRDye® infrared fluorescent antibodies (LI-COR) were used to visualize proteins using the LI-COR® Odyssey Infrared Imaging System. Detection of

proteins using near-infrared fluorescence has demonstrated lowered background signals, high signal-to-noise ratio, and equal or increased sensitivity in comparison to chemiluminescence methods (http://biosupport.licor.com/docs/odysseybrochure.pdf). IRDye 800 Goat Anti-Mouse IgG (LI-COR) and IRDye 680 Goat Anti-rabbit IgG (LI-COR) were diluted 1:15000.

2.5 Generation of Recombinant Adenoviruses encoding Serpins

Spn4A was originally isolated from *Drosophila melanogaster* as described by Han *et al.* (2000). More detailed analysis of the inhibitory function of Spn4A to inactivate furin was discussed later by Richer *et al.* (2004). The RCL of Spn4A was changed from a consensus furin cleavage site -RRLR↓ to a consensus SKI-1 cleavage site -RRLL↓. Furthermore, two forms of SKI-1 specific Spn4A were generated, one with the ER retention signal –HDEL located at the C-terminus (Spn4-ER), and one with the signal deleted to allow further transport through the secretory pathway (Spn4-S). As controls for the specificity of these serpins, a α_1-antitrypsin serpin (α_1-AT) and anti-furin serpin (α_1-PDX) were also included in our studies (Anderson et al. 1993; Jean et al. 2000). The α_1-PDX is a variant of α_1-AT, where the RCL was mutated from -AIPM↓ to encode -RIPR↓, a consensus furin cleavage site (Anderson et al. 1993). Using the AdEasy™ Adenoviral Vector System (Stratagene), recombinant adenoviruses expressing the various forms of Spn4-based SKI-1 inhibitors were generated. Procedure as described in manufacturer's manual.

2.5.1 Generation of recombinant adenovirus stocks

The recombinant adenoviruses used in this study were replication deficient due to the deletion of the E1 and E3 genes. Therefore, to grow viral stocks, HEK293 cells that express the adenovirus E1A gene in *trans* were used for viral amplification. Cells were grown in T150 cm^2 flasks to 90% confluency, and then infected at a MOI of 0.1 for 3 hours. The virus was removed and 30 ml of DMEM supplemented with 10% FBS, Pen/Strep and L-Glutamine was added. Virus was harvested from the cells 2-3 days post-infection when the majority of cells were rounded and lifted from the flasks.

To harvest the adenovirus, cells were resuspended in the medium in the flask and transferred to a tube to be pelleted by centrifugation at 1000 x rpm for 5 minutes at 4ºC. The supernatant was removed, and an aliquot was stored at -80ºC until the viral titre could be measured. For every T150 cm^2 flask, 0.5 ml of previously removed supernatant was used to resuspend the pellet. The cell pellet was rapidly frozen in an ethanol/dry ice mixture, and then thawed in a 37ºC water bath. Four rounds of freeze/thawing were performed to lyse the cells. After the final round, cell debris was pelleted by centrifugation at 12,000 x rpm for 10 minutes at 4ºC. Supernatant containing the viral stock was kept frozen in aliquots at -80ºC.

Titration of viral stocks was done using the TrueBlue Peroxidase Substrate (KPL). Briefly, a 96-well plate of Vero E6 cells at 100% confluency was prepared. Viral stocks were diluted and used to infect Vero E6 cells for 3 hours at 37ºC. Virus was removed and replaced with DMEM containing 5% FBS. Cells were infected for 48 hours, fixed with 10% formalin for 30 minutes at room temperature, and washed three times in PBS. Next, cells were permeabilized using 0.1% Triton X-100 in PBS for 15 minutes at room

temperature, and washed three times in PBS. To block any endogenous peroxidase activity in the cells, 50 ul of Blocking Solution Concentrate (KPL) diluted 1:10 in dH_20, was added to each well for 45 seconds at room temperature. Cells were rinsed in dH_20 for 5 minutes and soaked in PBS for 10 minutes. To detect the Flag-tagged serpin expressed by the recombinant adenoviruses, a monoclonal anti-Flag peroxidase antibody (Sigma A8592) diluted 1:3000 in PBS was incubated with the cells for 30 minutes at room temperature. Cells were washed in PBS for 10 minutes, 100 ul of TrueBlue Peroxidase Substrate was added per well and incubated for 10 minutes at room temperature. Finally, cells were washed for 5 minutes in dH_20 and the cells stained blue were counted to determine adenovirus titres.

2.6 Recombinant Vesicular Stomatitis Virus (rVSV) System

We thank John Rose (Yale University) for providing us with the vesicular stomatitis virus reverse genetics system. Since the first establishment of the replication competent rVSV system by JK Rose's group (Lawson et al. 1995), the rVSV system has been modified and enhanced for the expression of foreign genes, in particular the glycoproteins of filoviruses and arenaviruses (Schnell et al. 1996a; Garbutt et al. 2004). Cloning of the full length VSV genome to contain arenavirus GPC in exchange for VSV G was described in section 2.3. In Figure 5A, a schematic representation of the VSV genomes used are shown, and the resulting recombinant VSV particle expressing arenavirus glycoproteins on its surface is depicted in Figure 5B. The full length VSV genome was also modified to include a green fluorescent protein (GFP) gene, which was inserted after the GPC gene and before the

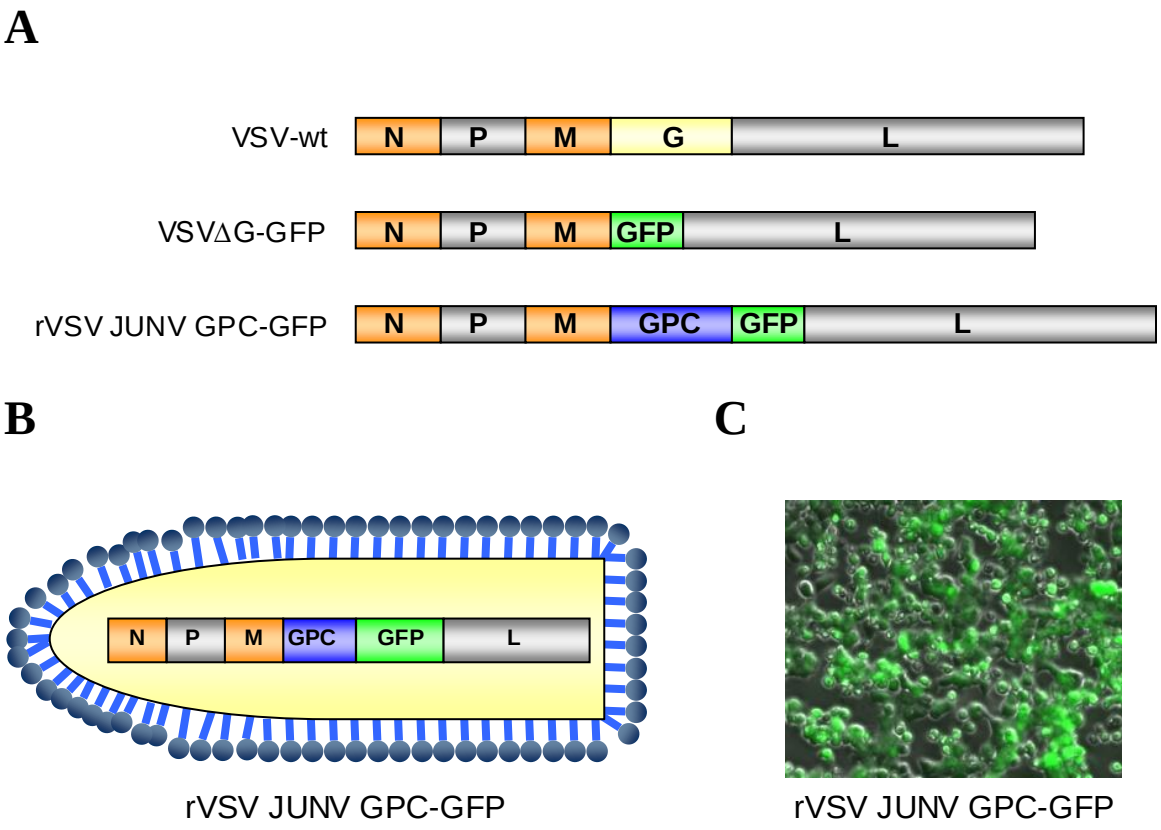


Figure 5. **Recombinant vesicular stomatitis viruses encoding arenavirus GPC and GFP genes. (A)** Schematic representation of the various recombinant VSV genomes used. VSV wild-type (*VSV-wt*) genome organization and the genes that encode the nucleoprotein (*N*), phosphoprotein (*P*), matrix protein (*M*), glycoprotein (*G*) and RNA-dependent RNA polymerase (*L*). VSVΔG-GFP contains the VSV-wt genome except the VSV G gene has been deleted, and the GFP gene has been inserted between the M and L. The rVSV JUNV GPC-GFP genome is where JUNV GPC has been inserted between the M and GFP genes. **(B)** A recombinant VSV virus particle expressing arenavirus glycoproteins on the outer surface. **(C)** GFP expression in Vero E6 cells infected with rVSV JUNV.

VSV L gene (Figure 5A). Expression of GFP was included to enable easy visualization of infected cells by fluorescent microscopy and for quantification by flow cytometry (Figure 5C).

2.6.1 Transfection and rescue of rVSV

Recombinant VSV were rescued in a mixture of 293T (60%) and Vero E6 (40%) cells. Cells were seeded one day prior in medium lacking antibiotics and transfected when cells were ~90% confluent. Cells were transfected using Lipofectamine™ 2000 (section 2.4.1) with four helper plasmids (2 ug of pBS-T7, 0.5 ug of pBS-VSV N, 0.25 ug of pBS-VSV L, and 1.25 ug of pBS-VSV P) and 2 ug of full length VSV genome encoding arenavirus GPC (pATX-VSVXN2ΔG-GFP containing either MACV, JUNV, GTOV, SABV, CHPV, LASV GPC). Figure 6 illustrates the procedure of transfecting 293T/Vero E6 cells for the generation of recombinant VSV expressing arenavirus GPC and GFP.

Briefly, one tube containing 90 ul of Opti-MEM was combined with DNA plasmids. In a separate tube, 14 ul of Lipofectamine™ 2000 was added to 90 ul of Opti-MEM. Both tubes were incubated for 5 minutes at room temperature, mixed together and further incubated for another 15 minutes. The final volume of each tube was adjusted to 1 ml by adding 720 ul of Opti-MEM. Spent media in the wells were removed, and cells were washed with 1 ml of plain DMEM before transfection mix was added to the cells drop wise. Plate was transferred immediately into an enhanced BSL-2 laboratory where it was incubated at 37ºC with 5% CO_2 in a humidified chamber overnight. The next morning, 1 ml of DMEM supplemented with 10% FBS was added to each well and the

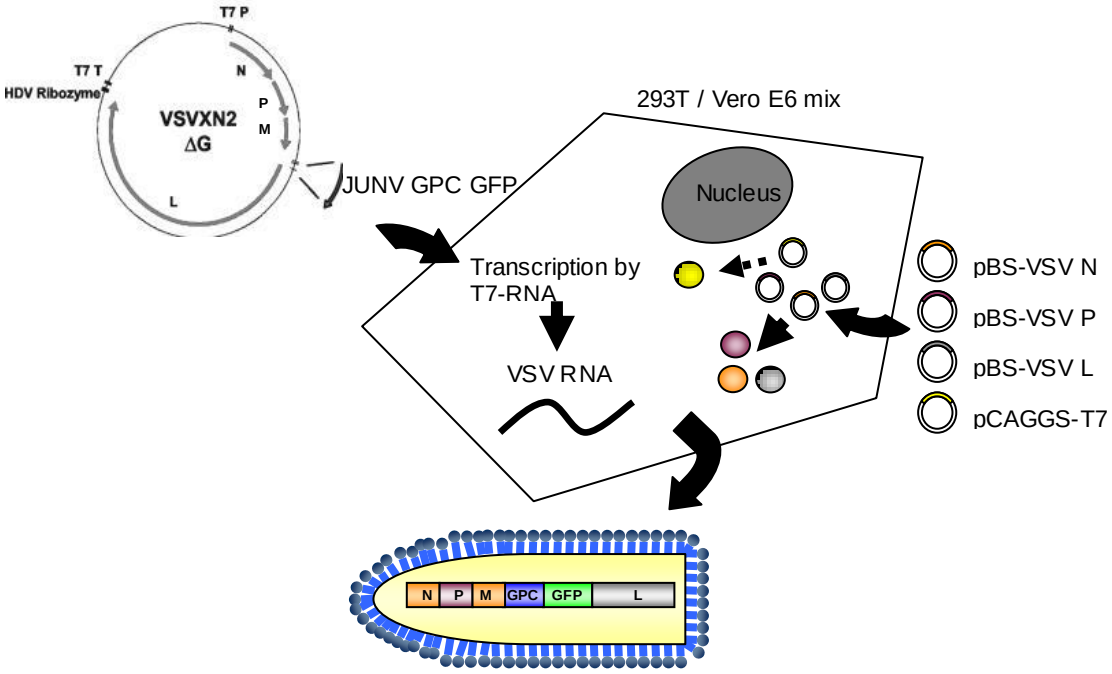


Figure 6. **Schematic drawing of the generation of infectious recombinant VSV expressing arenavirus GPC and GFP from plasmids.** A mixture of 293T and Vero E6 cells were transfected with helper plasmids encoding VSV nucleoprotein (*pBS-VSV N*), phosphoprotein (*pBS-VSV P*), RNA-dependent RNA polymerase (*pBS-VSV L*), and T7 polymerase (*pCAGGS-T7*). Additionally, a plasmid encoding the full length VSV genome with VSV G replaced by JUNV GPC and GFP (*VSVXN2-JUNVGPC-GFP*) was transfected into the same cells.

plate was further incubated for 48 hours or until signs of cytopathic effect (CPE) were seen. After 72 hours post-transfection, or the first signs of CPE, supernatants were blind passaged onto fresh Vero E6 cells (100% confluent). Rescue of infectious rVSV was confirmed by scanning the new Vero E6 monolayer for the presence of CPE.

2.6.2 Growing of rVSV stocks and plaque titration

Rescued rVSV from transfection was passaged onto Vero E6 cells in T75 cm^2 flasks to amplify the virus to generate virus stocks. Generally, a dilution of 1:1000 of the original rescued virus would cause significant CPE (~80-90%) in Vero E6 cells within three days post-infection. For larger scale amplification, T150 cm^2 flasks of Vero E6 cells were infected and the supernatant was harvested for viral stocks.

Titration of rVSV's was done by plaque assay. Vero E6 cells in 12-well plates were infected with 1 ml of virus for 1 hour at 37ºC, then virus inoculum was removed and replaced with 1 ml of 1.5% Carboxymethylcellulose (CMC, Sigma) overlay in 1X Minimum essential medium (MEM, Gibco) supplemented with 2% FBS (Appendix C). Infected cells were then incubated at 37ºC for 2-3 days and plaques were visualized by adding 1 ml of crystal violet stain (Appendix C). The next day, stain was rinsed off with water and viral titres were determined by the number of plaque forming units per ml (PFU/ml).

2.7 Virus Infections

All rAdV infections were either done at a MOI of 1, for subsequent transient arenavirus GPC expression, or an MOI of 5 for subsequent infection by rVSV. Generally, cells were infected in 6-well plates at a confluency of 90% for 4 hours at 37ºC. Subsequent rVSV infections were done at a MOI of 1 (for Western blot samples) or 1 x 10^{-4} (for replication studies) for 1 hour at 37ºC. All rVSV infections were performed in an enhanced BSL-2 laboratory setting.

All wild-type arenavirus infections were performed in BSL-4 laboratory facilities. VeroE6 cells were first infected with rAdV expressing Spn4-ER or Spn4-S for 4 hours at 37ºC at a MOI of 5. Cells were then transferred into BSL-4, and infected for 1 hour at 37ºC with wild-type JUNV and LASV diluted 1:100 in DMEM containing penicillin, streptomycin and L-glutamine. After 1 hour, virus inoculum was removed, cells were washed three times with plain DMEM, and 3 ml of SRD-12B medium was added to the cells (see section 2.1)

2.8 Immunofluorescence assays

To study the cellular localization of the two different forms of serpins (Spn4-ER, Spn4-S), immunofluorescence assay (IFA) was performed by using specific fluorescent antibodies to stain for cellular markers and the flag-tagged serpins. Glass cover slips were placed into the wells of a 24-well plate, and Vero E6 cells were seeded onto them so that a confluency of 90% was reached the next morning. Cells were infected with 1 ml of rAdV at a MOI of 5 for 4 hours at 37ºC, virus was then removed and replaced with 1 ml

DMEM supplemented with 5% FBS. After 48 hours, cells were fixed onto the cover slip using 1 ml/well of 4% paraformaldehyde (PFA) in PBS for 15 minutes at room temperature or overnight at 4ºC. Cells were then washed three times in PBS for 3 minutes each. Cell membranes were permeabilized by incubation with 0.1% Triton X-100 in PBS for 15 minutes at room temperature, and washed three times in PBS for 3 minutes. Next, 25 µl of primary antibody per cover slip was prepared and incubated on the cells for 1 hour at room temperature in a humid chamber. Primary antibodies used were rabbit Anti-Protein Disulfide Isomerase (ER marker, Sigma) and rabbit Anti-Giantin (Golgi marker, Abcam); both diluted 1:300 in PBS+1%BSA. Cells were then washed three times in PBS for 5 minutes each. Next, a secondary anti-rabbit Alexa Fluor 568 (Invitrogen) diluted 1:2000 was combined with a mouse anti-Flag M2 Monoclonal Antibody-FITC conjugate (Sigma) diluted 1:300 in PBS. Both secondary antibodies were applied to the cells and incubated for 1 hour at room temperature in a dark, humid chamber. Cells were washed three times in PBS for 5 minutes. Cover slips were rinsed in dH_20 to remove any excess PBS, and finally mounted onto glass slides using a small droplet of ProLong Gold antifade reagent with DAPI (Invitrogen) to stain the cell nuclei. Cover slips were allowed to dry for 10-15 minutes and edges were sealed with nail polish to prevent cells from drying out. Slides were stored in the dark until analysis using a Zeiss Axiovert 200 M inverted microscope.

2.9 Flow cytometry

To determine the efficiency of infection by rVSV containing GFP in cells expressing SKI-1 inhibitors (Spn4-ER, Spn4-S), flow cytometry was utilized to count the

number of GFP positive cells. First, Vero E6 cells were infected with rAdV (Spn4-ER, Spn4-S) at a MOI of 5 for 4 hours at 37ºC, then super-infected with rVSV (MACV, JUNV, GTOV, SABV, CHPV, LASV) at a MOI of 1 x 10^{-4} for 1 hour at 37ºC. The virus inoculum was replaced by DMEM supplemented with 2% FBS. Cells were incubated for 48 hours at 37ºC and then stained for flow cytometry.

To differentiate between live and dead cell populations, a LIVE/DEAD Fixable Red Dead Cell Stain Kit (Invitrogen) was used. Live cells have a more intact cellular membrane, therefore, are less permeable to the red dye and stain lighter. On the other hand, the membranes of dead cells are much more permeable to the red dye and thus, stain darker. In brief, supernatant was removed and 800 ul of PBS-0.02%EDTA was added to each well for 10 minutes at 37ºC to detach cells from the well. The removed supernatant was stored at -80ºC for analysis by plaque assay to determine viral titres (section 2.6.2). The resuspended cells from each well were centrifuged at 2500 x rpm for 8 minutes at 4ºC. The pellet was washed once in 1 ml PBS, centrifuged again, and resuspended in 1 ml PBS. Cells were diluted to 1 x 10^{6} cells/ml in PBS, 1 ul of dye was added to 1 ml of cell suspension, mixed and incubated at 4ºC protected from light for 30 minutes. After staining, cells were pelleted, washed once with PBS, pelleted again, and finally resuspended in 900 ul of PBS. The entire cell suspension was added into 5 mls of 4% PFA in PBS to fix overnight at 4ºC. The next day, cells were pelleted and resuspended in 500 uL of fresh 4% PFA.

All samples were run on a BD FACSCalibur™ flow cytometer. GFP positive cells were detected in the FL1 channel, while red stained cells were counted in the FL4 channel. All data was analyzed using the FlowJo Version 7 software program. Live and

dead cell populations were determined based on uninfected, red stained Vero E6 cells. Within the live and dead cell populations, the number of GFP positive cells was also determined.

2.10 Statistics

All graphs were generated using GraphPad Prism 5 software program. Unpaired t test comparing the standard error of mean between samples were determined, where sample data was obtained from three to five independent experiments completed in duplicate. Data was considered statistically significant when p value < 0.05.

3.0 Results

3.1 Role of SKI-1/S1P in Chapare virus GPC cleavage

3.1.1 Chapare virus GPC gene synthesis

In 2003, a new arenavirus named Chapare (CHPV) was identified in Bolivia as the cause of a fatal case of severe hemorrhagic fever (Delgado et al. 2008). Phylogenetic analysis of full length S and L RNA segments classified Chapare with other Clade B, New World arenaviruses (Delgado et al. 2008). As our group did not have CHPV RNA available, gene synthesis of CHPV GPC was necessary (refer to section 2.3.4). After four rounds of PCR, CHPV GPC was successfully generated using 80 overlapping forward and reverse primers (Figure 7). The amplified CHPV GPC was confirmed by sequencing and then cloned into pCAGGS for expression in mammalian cells.

3.1.2 Residues P_2 and P_4 in the SKI-1 consensus motif are important for cleavage

To determine whether SKI-1/S1P plays a role in CHPV GPC cleavage, mutational analysis of the proposed SKI-1 consensus motif was completed. Each amino acid of the putative SKI-1 cleavage site in CHPV GPC was mutated to an alanine residue by site directed mutagenesis (see section 2.3.13). Furthermore, three amino acids prior to, and three amino acids following the cleavage site were also mutated to alanine residues (Table 3). The first residue closest to the N-terminus of the cleavage site is termed P_1; the

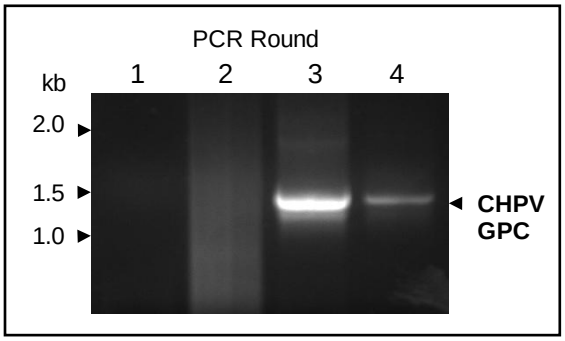


Figure 7. **Chapare virus GPC gene synthesis.** GenBank accession number EU260463 was the CHPV GPC gene sequence used and divided into 80 overlapping forward and reverse primers. GPC gene was generated through four rounds of PCR and visualized on a 1% agarose gel.

Table 3. **Alanine scanning of the putative SKI-1/S1P cleavage site of CHPV GPC.**

Position	CHPV GPC	Sequence at cleavage site										Cleavage
		P_7	P_6	P_5	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	
	Wild-type	V	K	L	R	R	L	Q↓	G	V	F	+
P_7	V244A	A	.	.	.	.	.	.	.	.	.	+
P_6	K245A	.	A	.	.	.	.	.	.	.	.	+
P_5	L246A	.	.	A	.	.	.	.	.	.	.	+
P_4	R247A	.	.	.	A	.	.	.	.	.	.	-
P_3	R248A	.	.	.	.	A	.	.	.	.	.	+
P_2	L249A	.	.	.	.	.	A	.	.	.	.	-/+
P_1	Q250A	.	.	.	.	.	.	A	.	.	.	+
P_1'	G251A	.	.	.	.	.	.	.	A	.	.	+
P_2'	V252A	.	.	.	.	.	.	.	.	A	.	+
P_3'	F253A	.	.	.	.	.	.	.	.	.	A	+

* Each amino acid was sequentially mutated to an alanine residue by site-directed mutagenesis and the putative SKI-1/S1P cleavage site is underlined

** (+) denotes cleavage, (-/+) reduced cleavage, and (-) no cleavage

residue N-terminal to P_1 is called P_2, etc. The amino acids at the C-terminal end of the cleavage site are thus named P_1', P_2', etc.

Each mutant generated was then transfected into VeroE6 cells (see section 2.4.1), and analysis of the cell lysates by Western blot (see section 2.4.3) revealed the effect of mutation on GPC cleavage (Figure 8). The antibody used detects both GP2 and GPC (see section 2.4.4), therefore, it is able to show whether cleavage of GPC into the smaller GP2 subunit occurs or not. The larger band at 65 kDa corresponds to the expected size of CHPV GPC, while the smaller band at 35 kDa matches the predicted size of CHPV GP2. Of all amino acids mutated, only residues P_2 and P_4 had an effect on GPC proteolytic processing into GP1 and GP2. Mutant L249A (P_2) had reduced GP2 expression in comparison to the expression of GP2 in the wild-type lane (Figure 8, *lanes P_2 and wt*). Mutant R247A (P_4) had an even greater effect on GPC cleavage, as it completely lacked any GP2 expression when compared to the wild-type lane (Figure 8, *lanes P_4 and wt*). All other alanine mutations in the SKI-1 consensus region had no effect on cleavage, as GP2 was detected in all those lanes. To ensure equal sample loading per lane, β-actin was used as a loading control. To confirm that SKI-1 is specifically the protease responsible for CHPV GPC cleavage, later experiments involving a SKI-1 deficient cell line were completed (see section 3.3.4).

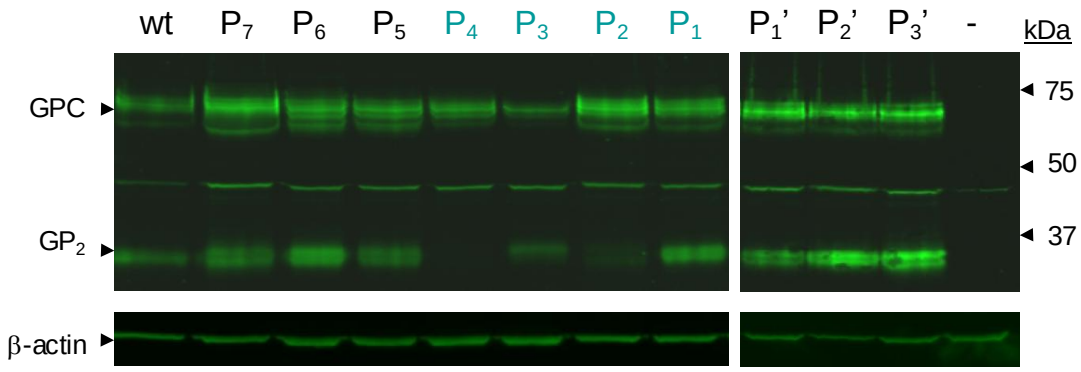


Figure 8. **Proteolytic processing of CHPV GPC mutants generated by alanine scanning of the putative SKI-1/S1P cleavage site.** Amino acids within the SKI-1/S1P consensus region were all mutated to alanine residues by site-directed mutagenesis. VeroE6 cells were transfected using Lipofectamine 2000 transfection reagent, and cell lysates were collected 48h post-transfection and analyzed by Western blot. CHPV GPC was detected using monoclonal Ab F100G4, and β-actin was used as a loading control. Lanes: wild-type CHPV GPC (wt), V244A (P_7), K245A (P_6), L246A (P_5), R247A (P_4), R248A (P_3), L249A (P_2), Q250A (P_1), G251A (P_1'), V252A (P_2'), F253A (P_3'), mock infected (-).

3.2 Effect of SKI-1 inhibitors on arenavirus GPC processing

3.2.1 Expression of recombinant adenoviruses encoding for SKI-1 specific serpins

As a robust method of foreign gene delivery into mammalian cells, recombinant adenoviruses (rAdVs) carrying SKI-1 specific serpins were used to infect cells to facilitate inhibitor expression. These rAdVs have a higher efficiency of gene expression in contrast to transient expression; additionally, expression of inhibitor in nearly every cell is possible. The deletion of E1 and E3 genes in the adenovirus genome renders the virus replication defective; therefore, these viruses are safe to handle under BSL-2 conditions.

Due to the localization of active SKI-1 to the cis/medial Golgi, two forms of the Spn4-based SKI-1 inhibitor was generated to test their ability to inhibit SKI-1. The first form (Spn4-ER) contained the ER retention signal –HDEL, while the second form (Spn4-S) had the retention signal removed to enable transit to the Golgi and secretory pathway (Figure 9A). For antibody detection, each construct included a Flag-tag at the N-terminus of the serpin. Since active SKI-1 is found mainly in the cis/medial Golgi network, it was expected that Spn4-S would have the major inhibitory affect compared to Spn4-ER.

To confirm the different cellular localizations of the two serpin forms, immunofluorescence assays were performed (see section 2.8). VeroE6 cells were infected with rAdVs encoding either Spn4-ER or Spn4-S, and cellular expression of these inhibitors were detected by flag specific antibodies conjugated to FITC. Cellular markers for the Golgi and ER were detected using antibodies specific for Giantin and Protein disulfide isomerase, respectively. Images were taken with a Zeiss Axiovert 200 M

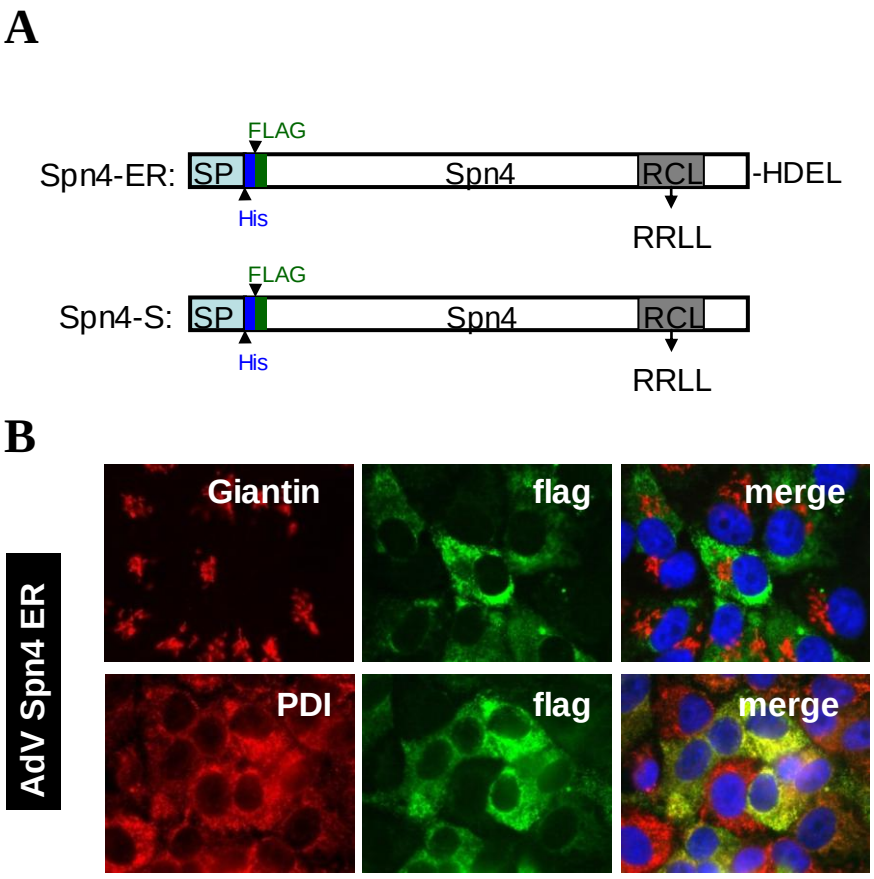


Figure 9. **Expression of Flag-tagged SKI-1/S1P inhibitors Spn4-ER and Spn4-S. (A)** Schematic of SKI-1/S1P inhibitors based on Spn4 gene of *Drosophila melanogaster*. The RCL of Spn4 was mutated to contain a -RRLL SKI-1 consensus motif. At the N-terminus is the signal peptide (SP), followed by both a His- and Flag- tag. At the C-terminus, the HDEL- retention signal is only found in the ER-form of inhibitor, Spn4-ER, while the sequence is deleted in the secretory form of inhibitor Spn4-S. **(B)** Immunofluorescence assay of VeroE6 cells infected with recombinant adenovirus encoding Spn4-ER. Localization of flag-tagged Spn4-ER (*middle panels*) with the cellular markers Giantin (Golgi, *top left panel*), and Protein disulfide isomerase (PDI, ER lumen, *bottom left panel*) was imaged. Colocalization of Spn4-ER with cellular compartments was visualized as a merged image (*right panels*).

inverted microscope. Spn4-ER co-localized completely with the ER marker and did not co-localize with the Golgi marker (Figure 9B). On the other hand, Spn4-S appeared to be localized to the late secretory pathway, however, it did not co-localize with the ER marker or the Golgi marker chosen (data not shown). Overall, the ER retention signal (HDEL) was sufficient to restrict the SKI-1 inhibitor Spn4-ER to only the ER, while the Spn4-S form was free to transit through the ER and Golgi, where active SKI-1 resides.

3.2.2 Spn4-S efficiently inhibits proteolytic processing of arenavirus GPC

The ability of serpin-based SKI-1 inhibitors to prevent arenavirus GPC cleavage was studied using rAdVs expressing the serpins Spn4-ER, Spn4-S, α_1-antitrypsin (α_1-AT), and anti-furin (α_1-PDX). VeroE6 cells were first infected with rAdVs so the cells would begin expressing the inhibitors (see section 2.7). After 4 hours of infection, the same cells were transfected with arenavirus GPC (see section 2.4.1). For negative controls, the α_1-AT and α_1-PDX serpins were used. After 48 hours of transient arenavirus GPC expression in the presence of the various serpins, the effect of inhibition on GPC processing was verified through SDS-PAGE and Western blot (see sections 2.4.2 and 2.4.3). New World arenavirus GPC cleavage was detected by using a monoclonal antibody against MACV GP2 (F1103G), which also detects GPC, and is known to cross react with the GP2 regions of JUNV, SABV, CHPV, and GTOV. For the Old World arenavirus LASV, a polyclonal LASV GP2 (L4) antibody was used to detect GPC cleavage. To determine if Spn4-ER, Spn4-S, α_1-AT and α_1-PDX were expressed, membranes were also stained with a Flag specific antibody.

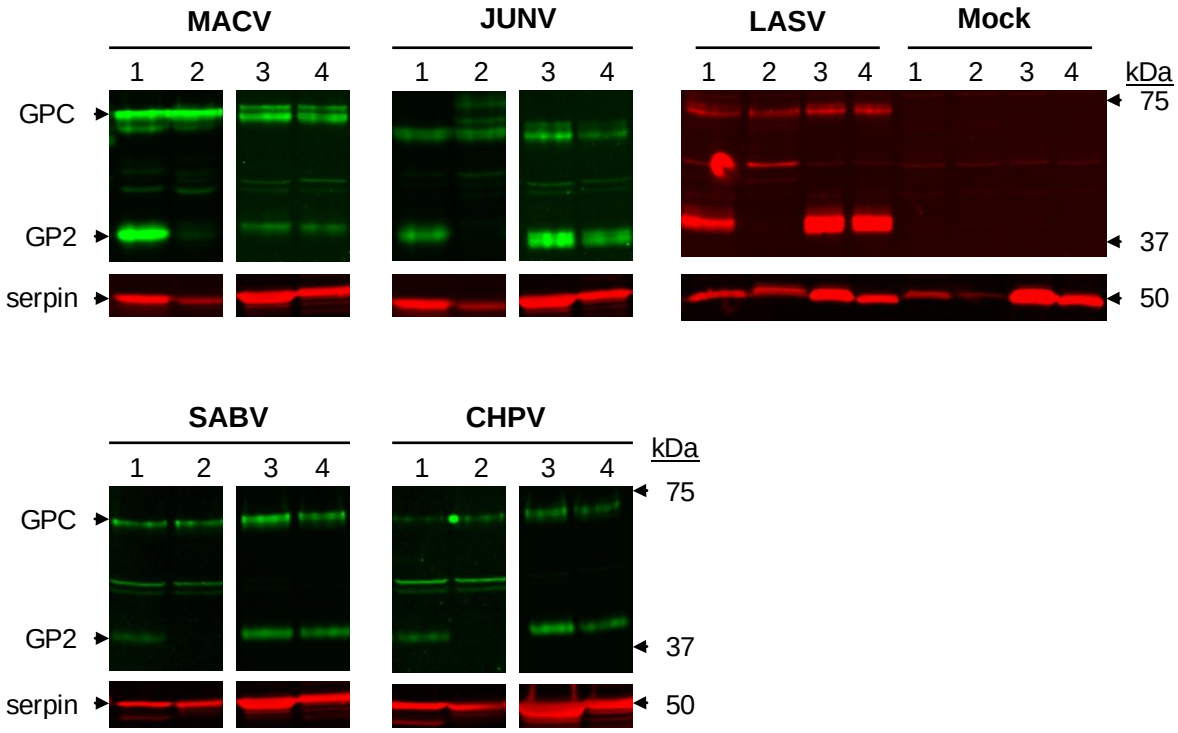


Figure 10. **Effect of various serpins on the proteolytic processing of arenavirus GPC.** VeroE6 cells were infected with rAdV expressing SKI-1 inhibitors Spn4-ER (*lanes 1*) or Spn4-S (*lanes 2*), a α_1-antitrypsin serpin (*lanes 3*), or an anti-furin serpin (*lanes 4*) at a MOI of 1 for 4 hrs. Cells were then transiently transfected with arenavirus GPC and cell lysates were analysed by Western blot 48h after. Arenavirus GP (MACV, JUNV, SABV, and CHPV) was detected using a monoclonal Anti-MACV GP2 (F1103G) Ab. To detect LASV GP, a polyclonal Anti-LASV GP2 (L4) Ab was used. Serpins were visualized using an anti-Flag antibody.

Spn4-ER had no influence on GPC processing; as a GPC band (~65 kDa) along with an equally intense GP2 band (35 kDa) could be seen in every lane for all the arenaviruses studied (Figure 10, *lanes 1*). Both the controls, α_1-AT and α_1-PDX, also had no effect on arenavirus GPC processing (Figure 10, *lanes 3 and 4*). Only Spn4-S effectively blocked GPC processing for all arenaviruses investigated, whether Old or New World, as GPC could be detected but not GP2 (Figure 10, *lanes 2*). Finally, the presence of Spn4-ER, Spn4-S, α_1-AT and α_1-PDX could be seen in every lane by the bands detected using a Flag specific antibody at the expected size of ~48 kDa (Figure 10). Taken together, these data demonstrates that only Spn4-S is able to efficiently inhibit GPC cleavage for JUNV, MACV, SABV, CHPV and LASV. Furthermore, to address whether this inhibition of cleavage is a result of Spn4-S specifically inhibiting SKI-1, experiments using SKI-1 deficient cells were performed later and are discussed in sections 3.3.3 and 3.3.4.

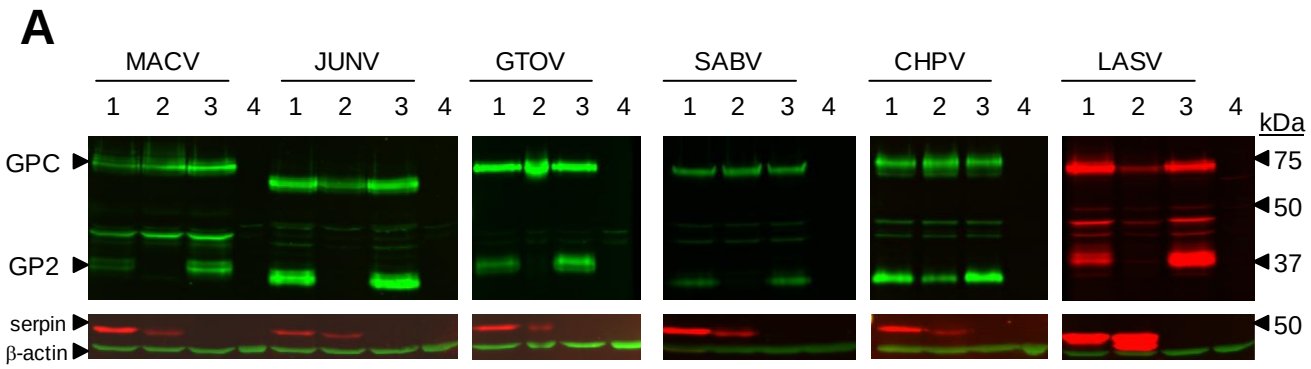
3.3 The role of GPC processing on arenavirus replication

3.3.1 Inhibition of GPC processing reduces the number of GFP$^+$ cells

To investigate the role of GPC processing on New World arenavirus replication, a number of recombinant Vesicular Stomatitis viruses (rVSVs) expressing arenavirus GPC instead of VSV G were generated. These rVSVs are replication competent and dependent on the arenavirus glycoprotein for cell binding and entry to initiate the replication cycle. To easily identify infected cells a GFP gene was included in the rVSV genome, so any cell infected by these viruses fluoresced green.

First, VeroE6 cells were infected with rAdVs expressing Spn4-ER or Spn4-S, or no rAdV at all, to produce these serpins. To study the effect of SKI-1 inhibition on viral replication, these same cells were superinfected with rVSVΔG-MACV/JUNV/GTOV/SABV/CHPV or LASV. Cell lysates were analyzed 2 days post-infection for the presence of GPC cleavage by Western blot as described previously (sections 2.4.3 and 2.4.4). For rVSVΔG-MACV/JUNV/GTOV/SABV and LASV, the presence of Spn4-S was sufficient to inhibit GPC proteolytic processing, as demonstrated by the detection of only the GPC band at ~65 kDa (Figure 11A, *lanes 2*). Interestingly, in the case of rVSV CHPV, Spn4-S did not prevent GPC cleavage, as a second band representing cleaved GP2 was seen. This result is in contrast to cells expressing Spn4-S and transfected with CHPV GPC, where Spn4-S inhibited GPC cleavage (Figure 10, *CHPV lane 2*). The inconsistent effect of Spn4-S on CHPV GPC cleavage is discussed later (see section 4.4). Spn4-ER had no effect on GPC cleavage for all arenaviruses studied (Figure 11A, *lanes 1*), as a strong GP2 band can be seen in every lane that is comparable to the control GP2 bands, where no inhibitor was added (Figure 11A, *lanes 4*). Serpins were detected with a Flag-specific antibody, and was seen in both the Spn4-S and Spn4-ER expressing lanes (Figure 11A, *lanes 1 and 2*). As a loading control, β-actin could be observed in every lane.

To measure the effect of SKI-1 inhibition on recombinant arenavirus VSV replication, flow cytometry was utilized to count the number of GFP positive cells in a population of live or dead rVSV-infected VeroE6 cells (Figure 11B). Spn4-ER and Spn4-S were expressed in VeroE6 cells by rAdV infection, and subsequently superinfected with rVSVΔG-MACV/JUNV/GTOV/SABV/CHPV or LASV at a MOI of 1×10^{-4} (see


A
MACV
JUNV
GTOV
SABV
CHPV
LASV
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
kDa
GPC
GP2
serpin
β-actin
75
50
37
50

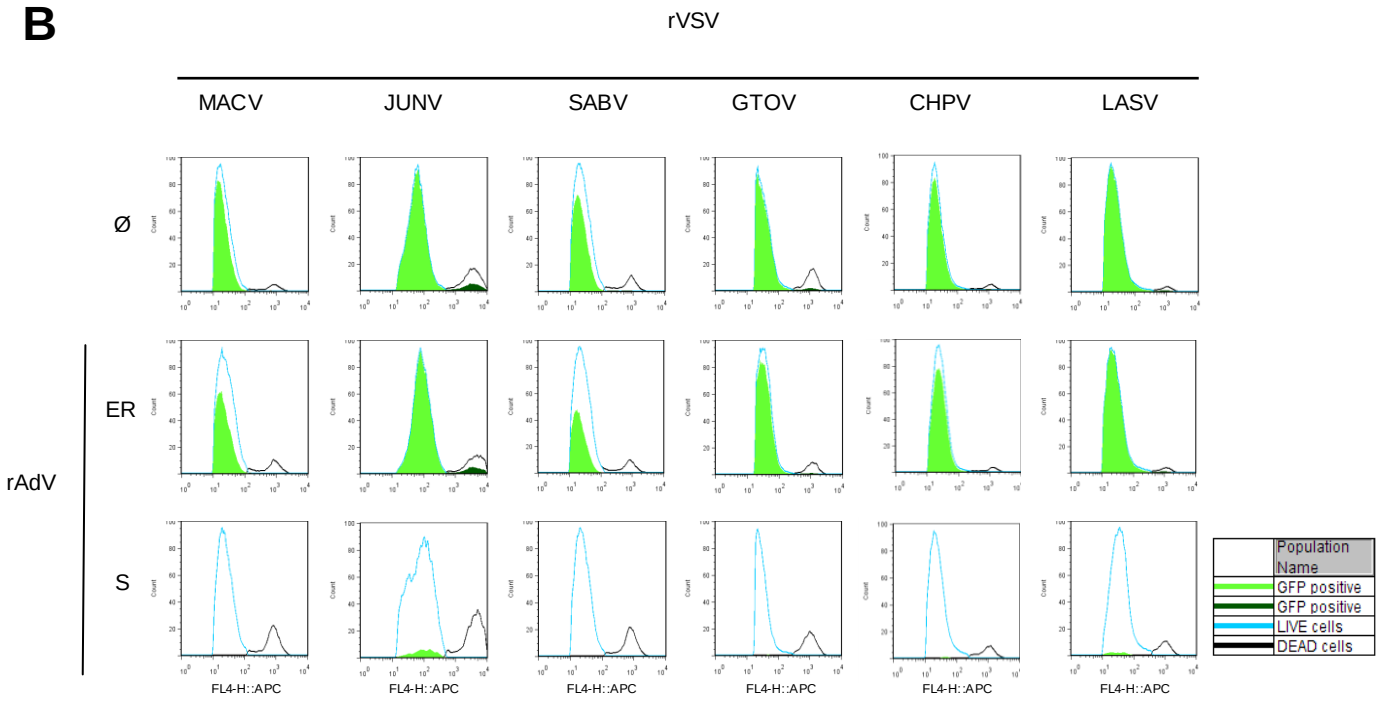

B
rVSV
MACV
JUNV
SABV
GTOV
CHPV
LASV
rAdV
Ø
ER
S
FL4-H::APC
Population Name
GFP positive
GFP positive
LIVE cells
DEAD cells

Figure 11. **Effect of SKI-1 serpins on the infectivity of recombinant VSV encoding arenavirus GPC. (A)** Western blot analysis of VeroE6 cells infected with rAdV expressing serpins Spn4-ER (*lanes 1*), Spn4-S (*lanes 2*) or no inhibitor (*lanes 3*) at a MOI of 5, followed by infection with rVSV encoding arenavirus GPC at a MOI of 1 x 10^{-4}. Cells that were neither infected with rAdV or rVSV are shown in *lanes 4*.Cell lysates were harvested 48h post-infection, and antibodies used to detect arenavirus glycoproteins and serpins as described previously. **(B)** VeroE6 cells infected as described for part A were stained using Invitrogen's LIVE/DEAD Fixable Red Dead cell stain kit. By flow cytometry, live (blue) and dead (black) cell populations were separated, with the number of GFP positive cells shown as green within each population. Cells without any inhibitor (*row Ø*), cells expressing Spn4-ER (*row ER*), and cells expressing Spn4-S (*row S*).

section 2.7). As these rVSVs are replication competent, infection with a low MOI allowed multiple rounds of replication to occur. This allowed the effect of SKI-1 specific serpins on viral replication to be studied 48 hours later. The number of GFP positive cells, representative of cells infected with rVSV, was counted within the live and dead cell populations (Figure 11B). In the positive control, where cells did not express any inhibitor (Figure 11B, *row Ø*), almost 100% of the live cell population were GFP positive. Cells expressing Spn4-ER (Figure 11B, *row ER*) showed a minimal reduction in the number of GFP positive cells, however, cells with Spn4-S (Figure 11B, *row S*) had completely reduced the number of GFP positive cells to almost 0% in some cases. Consequently, Spn4-S was able to negatively effect viral replication.

In summary, Spn4-S was able to inhibit GPC cleavage of rVSVΔG-MACV/JUNV/GTOV/SABV and LASV in infected cells (Figure 11A, *lanes 2*). As a result of blocking GPC cleavage, Spn4-S also reduced the number of cells infected by rVSVΔG-MACV/JUNV/GTOV/SABV and LASV (Figure 11B, *row S*). In the case of rVSVΔG-CHPV, GPC cleavage was not inhibited by Spn4-S (Figure 11A, *CHPV lane 2*). However, rVSVΔG-CHPV replication continued to be inhibited by Spn4-S despite the presence of a cleaved GP2 product (Figure 11B, *CHPV row S*). As mentioned earlier, cells that transiently expressed CHPV GPC in the presence of Spn4-S showed that cleavage of GPC was prevented (Figure 10, *CHPV lane 2*), which is in contrast to the Western blot data generated here by rVSVΔG-CHPV infection (Figure 11A, *CHPV lane 2*). These inconsistencies that are unique to CHPV are addressed later (see section 4.4). Overall, despite cleavage of rVSVΔG-CHPV GPC, Spn4-S was able to effectively prevent viral replication in all arenaviruses studied.

3.3.2 SKI-1 inhibition reduces recombinant arenavirus VSV titres by up to 5-logs

As an alternative method to more accurately quantify the effect of SKI-1 inhibition on recombinant arenavirus VSV replication, viral titres were determined by plaque titration (Figure 12). VeroE6 cells were mock infected, or infected with either rAdV expressing Spn4-ER or Spn4-S, followed by superinfection with rVSVΔG (MACV/JUNV/GTOV/SABV/CHPV or LASV GPC) at a MOI of 1 x 10^{-4} as described (see section 2.7). As a control, rVSV MARV was also used to infect the cells, since filovirus glycoproteins are not proteolytically processed by SKI-1, but are cleaved by furin instead (Volchkov et al. 2000). Supernatants were collected 48 hours after infection, and viral titres were determined. Each recombinant virus titration was done in duplicate, and each experiment was repeated independently at least three times.

For rVSVΔG MACV/GTOV/ and MARV, control cells that did not express any SKI-1 inhibitor produced viral titres of 5 x 10^6 PFU/ml, while rVSVΔG SABV grew to a titre of 1 x 10^6 PFU/ml, and rVSVΔG CHPV was at 5 x 10^5 PFU/ml (Figure 12, *lane No AdV*). This was followed by both rVSVΔG JUNV and rVSVΔG LASV that grew to titres of 1 x 10^5 PFU/ml (Figure 12, *lane No AdV*). Cells expressing Spn4-ER and infected with rVSVΔG MACV or SABV showed 1-log reduction in viral titre in comparison to their respective control titres. For rVSVΔG JUNV/GTOV/CHPV/LASV and MARV, < 1-log reduction in viral titre was seen when compared to control titres (Figure 12, *lane AdV ER*).

For all the recombinant arenavirus VSVs, the most dramatic results were seen in the cells that expressed Spn4-S (Figure 12, *lane AdV S*). The greatest effect was seen in rVSVΔG MACV and rVSVΔG SABV, where viral titres were reduced by ~5-logs

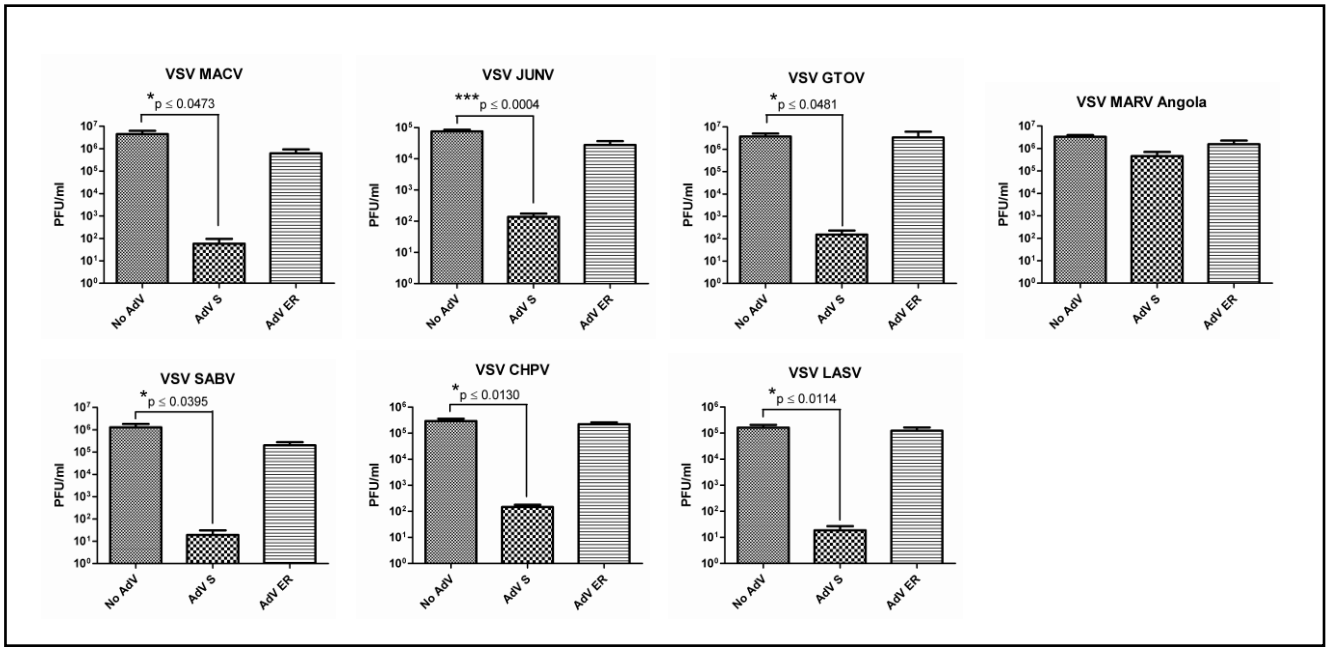


Figure 12. **Effect of SKI-1 inhibitors (Spn4-S or Spn4-ER) on recombinant arenavirus VSV replication.** Cells were infected with rAdV expressing SKI-1 inhibitor Spn4-S, Spn4-ER or no inhibitor at a MOI of 5 for 4 hrs. Cells were then infected with rVSV containing arenavirus GPC or MARV Angola GP (control) at a MOI of 1 x 10^{-4} for 1 hr. Viral titres were determined by plaque assays 48h post-infection. Unpaired t test comparing the standard error of mean between samples were determined, where n = 3 to 5.

compared to cells without any inhibitor (Figure 12, *lane No AdV*). This was followed by rVSVΔG GTOV and rVSVΔG LASV with a reduction of ~4-logs. Next was rVSVΔG CHPV with a reduction of 3.5-logs, and finally rVSVΔG JUNV with a 3-log difference (Figure 12, *lane AdV S*). Since SKI-1 plays no role in MARV GP processing, Spn4-S had the least effect on rVSVΔG MARV, where only a 1-log difference in viral titre was noted compared to the control titre (Figure 12, *VSV MARV*). Taken together, inhibition by Spn4-S was able to significantly reduce recombinant VSVΔG -MACV, -JUNV, -GTOV, -SABV, -CHPV and -LASV replication.

3.3.3 Arenavirus replication is inhibited in SKI-1/S1P deficient cells

To prove that inhibitory effects of Spn4-S are SKI-1 specific, a SKI-1/S1P deficient cell line named SRD-12B was infected with the same recombinant VSVs (rVSVΔG MACV/JUNV/GTOV/SABV/CHPV/LASV or MARV). Since the original cell line that SRD-12B cells were derived from was CHO-K1 cells, these were chosen to be the control cell line used in this experiment. Both CHO-K1 and SRD-12B cells were infected with recombinant arenavirus VSVs and rVSV MARV as a control, at a MOI of 1 x 10^{-4}. Supernatants were collected and viral titres were measured by plaque assay 48 hours after infection. Each virus titration was done in duplicate, and at least three independent experiments were performed for each virus.

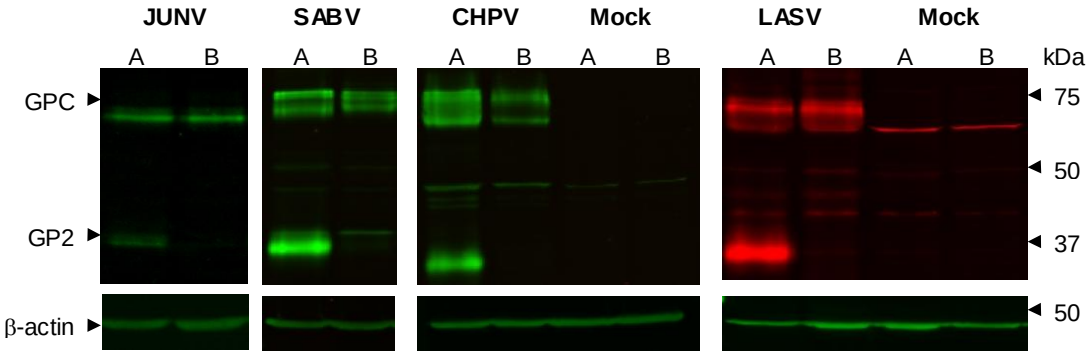
To confirm that SKI-1 mediated processing of arenavirus GPC is inhibited in SRD-12B cells, Western blot analysis of the cell lysates examining GPC cleavage were done (Figure 13A). In CHO-K1 infected cells, JUNV, SABV, CHPV and LASV GPC all showed GPC cleavage, as two bands representative of GPC (~65 kDa) and GP2 (35 kDa)

were detected (Figure 13A, *lane A*). In comparison to SRD-12B infected cells, JUNV, SABV, CHPV and LASV only expressed the GPC band, providing evidence that cleavage into GP2 was inhibited (Figure 13A, *lane B*). This lack of GPC cleavage was similar to when cells were expressing Spn4-S in VeroE6 cells infected with rVSVΔG-JUNV, -SABV, or –LASV (Figure 11A, *lane S*). Once again, contradictory results involving rVSVΔG-CHPV was observed. When rVSVΔG-CHPV was used to infect VeroE6 cells expressing Spn4-S (Figure 11, *CHPV lane 2*), CHPV GPC was still cleaved. However, when rVSVΔG-CHPV was used to infect SKI-1 deficient SRD-12B cells, CHPV GPC was not cleaved (Figure 13A, *CHPV lane B*). Although, this lack of GPC cleavage is consistent with earlier results in cells transiently expressing CHPV GPC in the presence of Spn4-S (Figure 10, *CHPV lane 2*). The unique differences in CHPV GPC cleavage will be discussed later (see section 4.4).

To determine whether SKI-1 deficient cells were able to propagate recombinant arenavirus VSVs, viral titres were measured 48 hours after infection. In CHO-K1 cells, rVSVΔG-MACV, -JUNV, -GTOV, -SABV, -CHPV, and –LASV grew to titres of at least 10^4 PFU/ml. For rVSVΔG-MARV, the viral titre was 10^6 PFU/ml, which was comparable to the 5 x 10^6 PFU/ml titre observed in VeroE6 cells (Figure 13B and Figure 12, *VSV MARV lane No AdV*). In general, these viral titres are ~1 to 2 logs lower when compared to the titres grown in control VeroE6 cells infected with the same viruses (Figure 12, *lanes No AdV*).

Not surprisingly, in SRD-12B cells, titres varied from 0 – 10^2 PFU/ml (Figure 13B), supporting the fact that SKI-1 mediated cleavage is necessary for the formation of infectious arenavirus particles. For rVSVΔG-JUNV/SABV and CHPV, replication in

A


JUNV
SABV
CHPV
Mock
LASV
Mock
A
B
kDa
GPC
GP2
β-actin
75
50
37
50

B

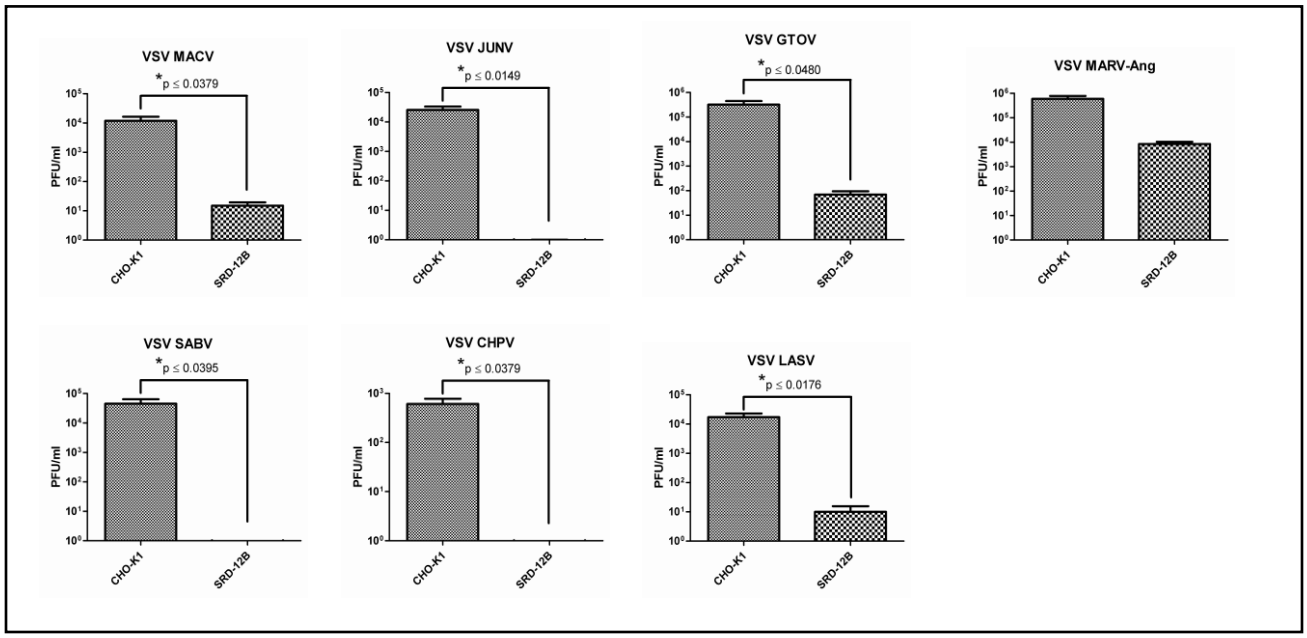

VSV MACV
*p ≤ 0.0379
VSV JUNV
*p ≤ 0.0149
VSV GTOV
*p ≤ 0.0480
VSV MARV-Ang
VSV SABV
*p ≤ 0.0395
VSV CHPV
*p ≤ 0.0379
VSV LASV
*p ≤ 0.0176
PFU/ml
CHO-K1
SRD-12B

Figure 13. **Replication of recombinant vesicular stomatitis viruses in SKI-1 deficient SRD-12B and CHO-KI cells. (A)** Western blot analysis of cell lysates collected 24h post-infection. CHO-K1 cells (*lane A*), and SRD-12B cells (*lane B*), were infected with rVSV containing arenavirus GPC at a MOI of 1. Antibodies used for GP detection as described previously. **(B)** CHO-K1 and SRD-12B cells were infected with rVSV containing arenavirus GPC, or MARV Angola GP (control) at a MOI of 1×10^{-4}, with the exception of rVSV LASV where a MOI of 1×10^{-2} was used. Viral titres were determined by plaque assays 48h post-infection. Unpaired t test comparing the standard error of mean between samples were determined, where n = 3 to 5.

SRD-12B cells was completely inhibited, while replication was mostly inhibited for rVSVΔG-MACV/GTOV and LASV (Figure 13B). More specifically, viral titres were reduced by 4.5-logs for rVSVΔG-JUNV and SABV, 3.5-logs for rVSVΔG-GTOV, and 3-logs for rVSVΔG-MACV and CHPV (Figure 13B). In SRD-12B cells infected with VSVΔG-MARV, the viral titre reached 10^4 PFU/ml, which was 2-logs lower than the titre observed in CHO-K1 cells. This 2-log difference was greater than the 1-log reduction seen in VSVΔG-MARV infected VeroE6 cells expressing Spn4-S (Figure 13B and figure 12, *lane AdV S*). Since VSVΔG-MARV replication was not expected to be affected by a lack of SKI-1, the greater reduction in titre observed here may be due to non-specific effects of growing in SRD-12B cells. In summary, the absence of SKI-1 resulted in a lack of GPC cleavage for JUNV, SABV, CHPV and LASV (Figure 13A, *lanes B*). Furthermore, uncleaved GPC led to a 3 to 4.5-log reduction in viral titres of recombinant arenavirus VSV, which correlated with the 3 to 5 log reduction seen in cells expressing Spn4-S infected with the same viruses (Figure 13B and Figure 12).

3.3.4 GPC processing is restored when SKI-1/S1P is provided *in trans*

To validate that inhibition of GPC processing in SRD-12B cells is due to the absence of functional SKI-1, a plasmid encoding human SKI-1 (pIR-hSKI) (Seidah et al. 1999) was transfected into the cells to see whether GPC cleavage could be restored. SRD-12B cells were transfected with pIR-hSKI for 24 hours, and subsequently infected with rVSVΔG-JUNV and rVSVΔG-CHPV at a MOI of 1. Cell lysates were analyzed by Western blot 24 hours after infection, and the presence of GPC cleavage was detected using antibodies as described previously (see section 2.4.4).

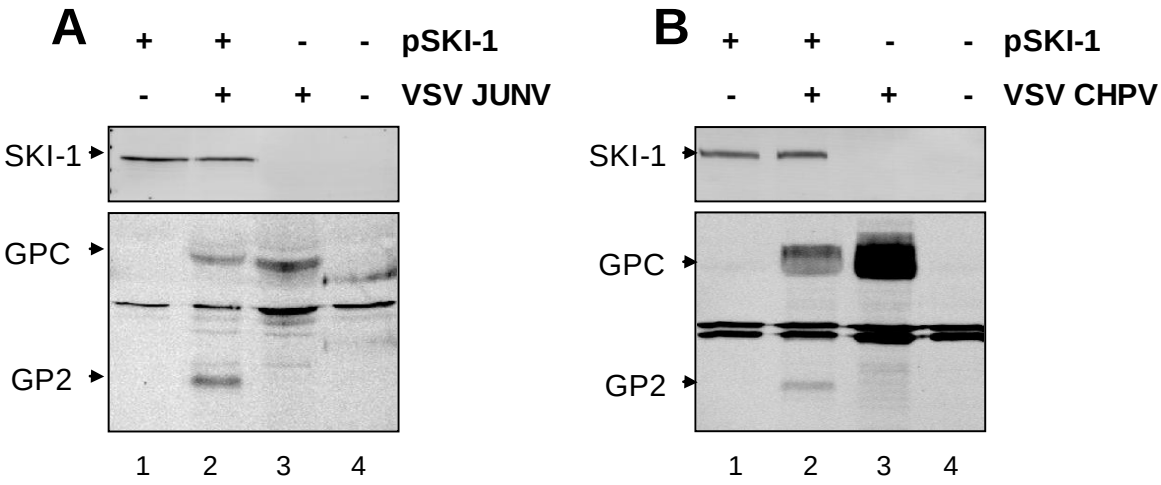


Figure 14. **Effect of providing SKI-1/S1P *in trans* to SKI-1 deficient cells infected with recombinant arenavirus VSVs.** SRD-12B cells were transfected with 4 ug of pIR-hSKI using Lipofectamine 2000 transfection reagent for 24 hours, then subsequently infected with rVSV JUNV **(A)**, and rVSV CHPV **(B)** at a MOI of 1. After 24 hours of infection, cell lysates were analyzed by Western blot using antibodies to detect for arenavirus GP2 as described previously. Detection of SKI-1/S1P was by using a rabbit polyclonal to human SKI-1.

Recombinant SKI-1 was added to lanes 1 and 2, and was detected by a polyclonal antibody specific for SKI-1 (Figure 14) (see section 2.4). Cells infected with rVSVΔG-JUNV or rVSVΔG-CHPV is shown in lanes 2 and 3, and detected for the presence of GPC and GP2 (Figure 14A and B). Lane 4 was the mock, and did not contain recombinant SKI-1 or any glycoprotein. Normally, in SRD-12B cells, arenavirus GPC cleavage does not occur (Figure 13A, *lanes B*, and Figure 14A and B). However, when SKI-1 was provided *in trans* to SRD-12B cells, JUNV and CHPV GPC cleavage was restored, as seen by the presence of both the GPC and GP2 band (Figure 14A and B). SKI-1 was detected in cells transfected with pIR-hSKI by a rabbit polyclonal specific to human SKI-1 (Figure 14A and B). Taken together, this data confirms that the absence of arenavirus GPC cleavage in SRD-12B cells is due to a lack of functional SKI-1.

3.3.5 Effect of SKI-1 inhibition on wild type Junín and Lassa virus *in vitro*

Before testing these SKI-1 inhibitors *in vivo*, it is necessary to verify these results using the wild type arenaviruses in BSL-4. Preliminary *in vitro* experiments involving VeroE6 cells expressing Spn4-ER or Spn4-S were infected with wild type LASV and JUNV. Plaque titration showed a 3-log reduction in LASV infected cells expressing Spn4-S, while a 2-log reduction was observed for JUNV. Although the titre reductions are not as significant as the 5-log difference seen with rVSV, it could be attributed to the slower growth kinetics of wild-type arenaviruses. Although these experiments are preliminary, the 2 to 3-log reduction seen in wild type viruses show the potential of Spn4-S as an effective inhibitor of arenaviruses.

In summary, CHPV GPC was shown to be proteolytically processed by the cellular protease SKI-1 for the first time. This was supported by the lack of CHPV GPC cleavage after mutagenesis of the putative SKI-1 consensus motif (Figure 8), the absence of cleavage in SKI-1 deficient SRD-12B cells (Figure 14B), and the restoration of GPC cleavage in the same cells by the addition of recombinant SKI-1 *in trans* (Figure 14B). SKI-1 inhibitor Spn4-S was demonstrated to efficiently inhibit MACV, JUNV, GTOV, SABV, CHPV, and LASV GPC cleavage, while Spn4-ER had no effect on cleavage (Figure 10). To study the effect of Spn4-S on arenavirus replication, the replication-competent recombinant VSV system was used to generate rVSVΔG-MACV/JUNV /GTOV/SABV/CHPV/ and LASV. It was shown for MACV, JUNV, GTOV, SABV, and LASV, that inhibition of GPC cleavage by Spn4-S resulted in a 3 to 5-log reduction of viral replication (Figure 12). Interestingly, for CHPV, a 3-log reduction in viral titre was observed despite detection of a cleaved GP2 product by Western blot (Figure 12, *CHPV lane AdV S* and Figure 11A, *CHPV lane 2*). Studies using SRD-12B cells confirmed for MACV, JUNV, GTOV, SABV, LASV, and CHPV that an absence of SKI-1 resulted in no GPC cleavage and a similar 3 to 4.5-log reduction in viral titres (Figure 13A and B). It was also demonstrated that SKI-1 function in SRD-12B cells can be restored by the addition of recombinant SKI-1 *in trans* (Figure 14A and B). Taken together, we have shown that Spn4-S specifically inhibits SKI-1 and functions as an efficient inhibitor against Old and New World arenaviruses.

4.0 Discussion

4.1 Role of SKI-1/S1P in Chapare virus GPC maturation

For more than a decade, it has been known that the glycoprotein of Old World arenaviruses LASV and LCMV are cleaved by SKI-1/S1P, however, no data was available for any of the New World arenaviruses. In early 2008, it was still unclear whether Clade B New World arenaviruses (MACV, JUNV, GTOV, and SABV) were proteolytically processed by SKI-1. Therefore, part of this project was intended to prove whether SKI-1 is also responsible for New World arenavirus GPC cleavage. However, shortly after initial experiments were underway, Rojek *et al.* published their work demonstrating that SKI-1/S1P is indeed, the protease responsible for cleavage of JUNV, MACV and GTOV GPC (Rojek et al. 2008c). To study the role of SKI-1 in proteolytic processing of GPC, they used recombinant retroviruses pseudotyped with arenavirus GP (Rojek et al. 2008c). These viruses are not replication competent, and represent a more artificial system compared to the recombinant VSV system used by our group. They did, however, demonstrate that the vaccine strain of JUNV, Candid 1, replicated to lower titres in SRD-12B cells in comparison to CHO-K1 (Rojek et al. 2008c). Since we already started to investigate the role of SKI-1 in the same New World arenaviruses with the addition of SABV, we felt that the replication-competent recombinant VSV was a better system than the pseudotyped retroviruses to study the effect of inhibiting SKI-1 mediated cleavage. Given that the recombinant VSV system allows the measurement of virus replication as opposed to only reporter gene expression by the pseudotyped retrovirus system, we therefore decided to continue with these viruses and confirm that SKI-1 is

required for GPC cleavage. Additionaly, we decided to include and focus more closely on Chapare virus in our study.

Since the emergence of Chapare virus in late 2003, little research has been done to learn more about this New World arenavirus. Although CHPV is phylogenetically most closely related to SABV, it was isolated from Bolivia, the same region where MACV is found (Cajimat et al. 2009). To verify that CHPV is also proteolytically processed by SKI-1, like many of the other arenaviruses, gene synthesis of CHPV GPC was completed first, using a set of 80 overlapping primers (Figure 7). Once the gene was successfully generated, alanine scanning of the putative SKI-1 consensus motif region was performed, and the effect of each mutation on GPC processing was studied by expression in VeroE6 cells (Figure 8).

From the Western blot data, it was clear that only residues P_2 and P_4 were critical in facilitating GPC cleavage (Figure 8). The reduction or absence of GP2 in lanes P_2 and P_4, as well as the similar expression level of GPC in these lanes compared to all other mutants and wild-type GPC, supported the fact that GPC was prevented from being processed into GP2 and GP1. Previous mutagenesis studies on the SKI-1 consensus motif of Old World arenaviruses LASV and LCMV also corroborate the importance of the leucine residue at P_2, and the arginine residue at P_4 (Figure 4) (Lenz et al. 2001; Beyer et al. 2003). However, in LCMV the arginine residue at P_3 and phenylalanine at P_7 also have an influence on GPC processing, which differs from LASV and CHPV (Beyer et al. 2003). On the other hand, in LASV, mutation of the leucine in P_1 to alanine also prevented GPC processing (Lenz et al. 2001). Depending on the virus, there seems to be variance in the residues that influence GPC processing, although the significance of

residues P_2 and P_4 are conserved amongst CHPV, LASV and LCMV. When considering the SKI-1 consensus motif (R/K)-X-(hydrophobic)-X↓, where X is any amino acid (Pullikotil et al. 2007), P_2 and especially P_4 have limited amino acid restrictions, thus all the data including ours would suggest that these residues play a more central role in recognition by SKI-1.

4.1.1 Unique proteolytic processing of CHPV GPC

To further investigate the role of SKI-1 in CHPV GPC maturation, replication of recombinant arenavirus VSV in the presence of SKI-1 inhibitors was examined. For MACV, JUNV, GTOV, SABV and LASV, it was confirmed that GPC cleavage by SKI-1 is crucial for the generation of infectious virions, with the exception of CHPV. In the presence of Spn4-S, transiently expressed CHPV GPC was not proteolytically processed (Figure 10, *CHPV lane 2*), suggesting that GPC cleavage is dependent on the presence of SKI-1. However, when rVSVΔG-CHPV was used to infect cells expressing Spn4-S, GPC cleavage was not inhibited and GP2 could be detected (Figure 11A, *CHPV lane 2*). Nevertheless, viral titres (Figure 12, *CHPV lane AdV S*) and the amount of GFP positive cells (Figure 11B, *CHPV rAdV S*) in rVSVΔG-CHPV were comparable to what has been seen for the other recombinant arenavirus VSVs, where cleavage is completely inhibited by Spn4-S (Figure 11A, *JUNV lane 2*).

It is difficult to explain these conflicting results, although a possibility may involve the appearance of rVSVΔG-CHPV escape mutants capable of cleavage by an alternative cellular protease, such as furin. When considering the SKI-1 consensus motif in CHPV GPC, -RRLQ↓, the current glutamine residue at P_1 is encoded by the

nucleotides –CAA (Figure 4). Importantly, a single mutation from –CAA to –CGA, results in an amino acid substitution from a glutamine to an arginine residue. This minor mutation changes the SKI-1 consensus motif to -RRLR↓, which can now be recognized and cleaved by furin. To prove whether mutations did arise in rVSVΔG-CHPV, viral RNA was extracted from cells infected with rVSVΔG-CHPV expressing Spn4-S, Spn4-ER or no inhibitor. The extracted RNA was then used as a template to amplify CHPV GPC by RT-PCR and sent for sequencing. However, analysis of the sequencing results did not reveal any mutational changes, particularly around the SKI-1 consensus motif region.

Another plausible explanation continues with the thought that furin may be cleaving CHPV GPC in the absence of SKI-1. Although the minimal furin recognition motif is -RXXR↓, a comprehensive study involving a series of furin substrate mutants led to the elucidation of a more defined sequence specificity of furin (Molloy et al. 1992; Nakayama 1997). The arginine residue at P_1 is essential, additionally, a minimum of two out of the three residues at P_2, P_4, and P_6 must be basic in order for efficient cleavage to occur (Nakayama 1997). With these requirements in mind, the residue at P_4 is not restricted to only arginine; instead a lysine residue may be a suitable substitution. Upon further analysis of the SKI-1 consensus motif region in CHPV GPC, a lysine residue was found in the P_6 position (Figure 4). Thus, it is possible that SKI-1 inhibition by Spn4-S may lead to recognition by furin two residues upstream of the regular cleavage site, at -K_{245}LRR↓ (Figure 4). Furin-mediated cleavage at this point would result in the generation of a GP2 fragment that is two amino acids longer at the N-terminus. This may influence the function of the fusion peptide to cause improper folding or inactivation,

thus affecting virus entry (Klewitz et al. 2007). Therefore, despite CHPV GPC cleavage, infectious viral particles are still not produced, supporting the reduction in viral titres observed (Figure 12, *VSV CHPV*).

Additional evidence supporting this theory is shown in Figure 11A, the GP2 band detected in lanes 1 to 3 for CHPV are all the same size, however, the intensity of GP2 in lane 2 where Spn4-S is present indicates a reduced efficiency of GPC cleavage. This experiment was repeated several times, each time producing the same results as seen in Figure 11A. Processing of GPC two amino acids upstream of the normal cleavage site would result in such a minimal size shift that would not be detectable by Western blot. Furthermore, the reduced intensity could correspond to furin-mediated cleavage at the lysine in the P_4 position; this is expected to have a lowered cleavage efficiency compared to an arginine at P_4 (Nakayama 1997).

Although our data suggests alternative cleavage by furin when SKI-1 is inhibited by Spn4-S, it is unknown why infection of SKI-1 deficient SRD-12B cells with rVSVΔG-CHPV did not result in cleavage (Figure 13A, *CHPV lane B*). An attempt to explain these discrepancies may come down to the possibility that certain co-factors necessary for cleavage can vary between cell lines, or perhaps selective pressure for glycoprotein cleavage for virus survival may play a role. These viruses are continually co-evolving with their rodent hosts, thus, it is possible that adaption to cleavage by multiple cellular proteases to promote persistence may occur over time. The successful generation of infectious JUNV by furin-mediated GPC processing supports this idea (Albarino et al. 2009). Interestingly, Lujo virus also contains a cluster of basic amino acids, -K_{224}VRK↓, that is two residues upstream of the SKI-1 consensus motif (Figure 4). Although this

motif does not contain an arginine at P_1, because it is found in the same location upstream of the cleavage site and contains a sequence similar to what is found in Chapare virus, -K_{245}LRR↓, it is possible that Lujo virus may also be cleaved by an alternative cellular protease (Fig. 4). Taken together, our data demonstrates, for the first time, that CHPV is also cleaved by the cellular protease SKI-1, and like other New and Old World arenaviruses, proper GPC cleavage is necessary for the generation of infectious virions. However, unlike the other arenaviruses studied, CHPV and possibly LUJV may be cleaved by other cellular proteases, which remain to be identified.

4.2 Effect of Serpin-based SKI-1 inhibition on viral replication

4.2.1 Cellular localization of SKI-1 specific serpins

Immunofluorescence assays looking at the localization of the two SKI-1 specific serpins, Spn4-ER and Spn4-S, confirmed the ability of the –HDEL retention signal to retain the Spn4-ER inhibitor in the ER (Figure 9B). Identical co-localization studies were performed using Spn4-S, although this inhibitor did not co-localize with the ER marker or the Golgi marker. Numerous attempts with other Golgi or endosome markers were also tested, however, none showed complete co-localization (data not shown). Despite the inability to find a cellular marker that co-localized with Spn4-S, when comparing the localization pattern between Spn4-ER and Spn4-S, it is clear that the two inhibitors are different. Spn4-ER appeared punctate throughout the cell with stronger expression closer to the nucleus, whereas Spn4-S appeared as larger vesicular formations scattered throughout the cell. The exact reason why there was difficulty co-localizing Spn4-S with

Golgi/endosome markers remains unclear; however, the time at which the experiment was performed may play a possible role. At 48 hours post-infection, when the immunofluoroscence assay was performed, it is possible that a significant amount of Spn4-S has been produced and has already passed through the Golgi and entered the late secretory pathway. Therefore, if the inhibitor has been sorted from the Golgi to other compartments including lysosomal or endosomal vesicles, then a defined localization to only the Golgi may not be possible. SKI-1 is synthesized as an inactive precursor which becomes activated via three sequential autocatalytic events, where inactive intermediate forms are found in the ER, and a final active form is located mainly in the cis/medial Golgi (Seidah et al. 1999; Elagoz et al. 2002; Pullikotil et al. 2007). Therefore, it is expected that Spn4-ER can only interact with the inactive intermediate forms of SKI-1 in the ER, whereas Spn4-S is free in the Golgi and secretory pathway to inhibit the active form of SKI-1.

4.2.2 Spn4-S efficiently inhibits GPC processing, not Spn4-ER

Only Spn4-S was able to effectively block SKI-1 mediated cleavage in cells transiently expressing arenavirus GPC (Figure 10, *lanes 2*). The inability of Spn4-ER to have an effect on GPC processing can be attributed to its cellular localization. The majority of active SKI-1 is found mainly in the cis/medial Golgi network and has also been found within endosomal compartments (Pullikotil et al. 2007). Therefore, it is hypothesized that the inactive form of SKI-1 in the ER does not have the catalytic activity to cleave the RCL region of Spn4-ER, which is required by the serpin to inhibit SKI-1 by conformational inactivation. A study looking at the need for transport of

SREBP (sterol regulatory element-binding protein) to the Golgi where it is cleaved by SKI-1 showed that when active SKI-1 was relocated from the Golgi to the ER, by fusion of an ER retention signal to SKI-1, it resulted in cleavage of SREBP in the ER (DeBose-Boyd et al. 1999). SREBP is a transcription factor that regulates lipid homeostasis (Eberle et al. 2004). This data supports the theory that enzymatically active SKI-1 is found in the Golgi and not the ER. Thus, the ability of Spn4-S to transit to the Golgi and secretory pathway allows it to interact with and inhibit SKI-1 mediated GPC cleavage. In figure 10, the distinct absence of a GP2 band in every lane expressing Spn4-S clearly shows the highly efficient inhibitory function of this SKI-1 specific serpin. The control serpins, α_1-AT and α_1-PDX, had no effect on arenavirus GPC processing as expected. The ineffectiveness of the furin inhibitor α_1-PDX to block GPC cleavage demonstrates that furin is not important in arenavirus GPC processing.

4.2.3 Spn4-S effectively reduced the number of rVSV-infected GFP positive cells

Since the establishment of a reverse genetics system for VSV, recombinant VSVs became a preferred tool for molecular biologists due to the simplicity of its viral structure and the ability to grow rapidly to high titres (Lawson et al. 1995; Garbutt et al. 2004). These replication competent viruses are a useful system to study the role of foreign glycoproteins in the generation of infectious viral particles (Garbutt et al. 2004). Furthermore, the classification of recombinant arenavirus VSVs as enhanced BSL-2 agents allow for more expedient studies to be performed, instead of the restrictive BSL-4 requirements. The rVSV encoding arenavirus GPC generated in our study also included a GFP gene, this allowed for easy visualization of infected cells by fluorescent microscopy.

In addition, the number of infected cells could be quantified by flow cytometry by counting the number of GFP positive cells. Furthermore, these recombinant viruses grow faster and to higher viral titres, have strong CPE, and are easy to plaque titre.

To support previous data that only Spn4-S blocked GPC cleavage in cells expressing arenavirus GPC transiently (Figure 10, *lanes 2*), this effect was also confirmed in recombinant arenavirus VSV infected cells, with the exception of rVSVΔG-CHPV. In figure 11A, only cells that were expressing Spn4-S were able to prevent SKI-1 mediated GPC processing for rVSVΔG-MACV/JUNV/GTOV/SABV/ and LASV, as no GP2 band was detected (*lanes 2*). All cells containing Spn4-ER had no effect on GPC processing as expected (Figure 11A, *lanes 1*), since active SKI-1 is not located in the ER and therefore, cannot be inhibited by Spn4-ER.

To examine the effect of SKI-1 specific serpins on the production of infectious viral particles, VeroE6 cells expressing Spn4-S or Spn4-ER were infected with rVSVΔG-MACV/JUNV/GTOV/SABV/CHPV or LASV. At 48 hours after infection, all the rVSVs showed a dramatic reduction in the number of GFP positive cells when Spn4-S was present in comparison to cells without any inhibitor (Figure 11B, *rows rAdV S and rAdV Ø*). Although a slight reduction in the number of GFP positive cells was seen when Spn4-ER was present, the difference compared to cells without any inhibitor is minimal (Figure 11B, *row rAdV ER*). Studies looking at the effectiveness of the HDEL signal for ER retention found that when an HDEL-tagged protein was overexpressed, it resulted in the slow release of this fusion protein out of the ER (Dean and Pelham 1990). Further analysis revealed that saturation of the HDEL receptor in the ER occurs when high levels of fusion protein are expressed, resulting in 'leakage' of HDEL-tagged proteins to the

Golgi (Dean and Pelham 1990). Therefore, it is possible that some Spn4-ER is escaping to the Golgi, where it can interact with active SKI-1 and inactivate it. Overall, by Western blot and the detection of cells infected with rVSV, we show that Spn4-S is able to effectively inhibit arenavirus GPC cleavage, and as a result, reduce the number of cells infected by these viruses.

4.2.4 Inhibition of GPC processing reduces rVSV titres

To more accurately determine the effect of inhibiting arenavirus GPC processing on rVSV replication, viral titres were measured by plaque titration. When inhibited by Spn4-S, rVSV viral titres were significantly reduced compared to when no inhibitor was present (Figure 12, *lanes AdV S and No AdV*). The presence of Spn4-ER had a negligible effect on viral reduction, as titres were similar to the positive control data (Figure 12, *lane AdV ER and No AdV*). As a negative control, rVSVΔG-MARV Angola was used to infect cells expressing either Spn4-ER or Spn4-S (Figure 12, *VSV MARV Angola*). Since Marburg viruses are known to be processed by furin, a member of the same family of proprotein convertases as SKI-1, it was expected that the presence of SKI-1 specific serpins would have no effect on rVSVΔG-MARV Angola replication (Volchkov et al. 2000). In the presence of Spn4-ER, VSVΔG-MARV grew to 10^6 PFU/ml compared to 10^5 PFU/ml when Spn4-S was added. Proteolytic processing of MARV or EBOV is not crucial in virus replication, as α_1-PDX was shown to reduce recombinant VSVΔG-MARV and VSVΔG-EBOV titres by only 1-log *in vitro* (Stroher et al. 2007). This was also confirmed *in vivo*, where an EBOV mutant lacking a furin cleavage site was successfully produced and proven lethal in non-human primates (Neumann et al. 2007).

The 1-log titre reduction of VSVΔG-MARV caused by Spn4-S is similar to the 1-log reduction caused by α_1-PDX (Stroher et al. 2007); therefore, it is possible that the expression of these serpins may have a cytotoxic effect on the cells, which may affect the production of infectious virus. For example, SKI-1 naturally plays a role in the biosynthesis of cholesterol and fatty acids, therefore, inhibiting this protease did cause some cytotoxicity to the cells that was observed. However, by supplementing the culture medium with cholesterol and other fatty acids, the cytotoxicity caused by SKI-1 inhibition diminishes.

For rVSVΔG-MACV/SABV/ and GTOV, the almost 5-log reduction in viral titres caused by Spn4-S demonstrates the efficient nature of this serpin to inhibit their target protease. Furthermore, preliminary *in vitro* experiments with wild type JUNV and LASV showed a 2 to 3-log reduction in viral titre in the presence of Spn4-S. This data additionally supports the fact that GPC cleavage is necessary for the generation of infectious arenaviral particles. Thus, our findings support our original hypothesis and show that proper arenavirus GPC processing by SKI-1 is required for the generation of infectious virions, and prevention of GPC cleavage with the SKI-1 specific serpin Spn4-S effectively inhibits Old and New World arenavirus replication.

In terms of disease progression, reducing viral titres can be especially important early in infection. The provision of immediate supportive therapy for infected patients is known to be a key factor that influences disease outcome (Buchmeier et al. 2001). If viral titres are reduced to a manageable amount, the ability of the patient to clear infection on their own increases, leading to a higher chance of survival. Therefore, the 3 to 5-log

reduction in viral titres caused by Spn4-S supports the use of SKI-1 inhibitors as a promising antiviral agent against arenaviruses.

4.2.5 SKI-1 deficient SRD-12B cells do not support arenavirus replication

Infection of SKI-1 deficient SRD-12B cells with the same recombinant arenavirus VSVs demonstrated that in the control CHO-K1 cells, the higher viral titres are directly attributed to the ability of arenavirus GPC to be proteolytically processed (Figure 13A *lanes A* and Figure 13B *lanes CHO-K1*). In contrast to SRD-12B cells, the absence of GPC processing explains the ~5-log reduction in viral titres for some of the rVSVs, such as rVSVΔG-JUNV and rVSVΔG-SABV (Figure 13B).

An interesting observation of a 2-log reduction in SRD-12B cells infected with rVSVΔG-MARV was seen compared to only a 1-log reduction in rVSVΔG-MARV infected VeroE6 cells expressing Spn4-S (Figure 13B and Figure 12, *lane AdV S*). Since glycoprotein processing is dispensable for MARV and EBOV, the 2-log reduction in rVSVΔG-MARV was surprising (Neumann et al. 2007; Stroher et al. 2007). The exact reason why a greater decrease in viral titre was observed in SRD-12B cells is unknown; however, the inherent cytotoxicity caused by the absence of SKI-1 may play a part. It was noted that all rVSVs grew to titres that were 1-log lower in CHO-K1 cells compared to VeroE6 cells infected at the same MOI (Figure 12 and 13). Recent studies have identified that EBOV GP processing by the endosomal protease cathepsin L plays a critical role in viral entry, in particular by exposing the receptor binding domain (Chandran et al. 2005; Hood et al. 2010). Although furin cleavage is not necessary for processing by cathepsin L, it is unknown whether proteolytic processing by other endosomal enzymes prior to

cathepsin L exist (Neumann et al. 2007). Therefore, it can be speculated that SKI-1 may play a minor role in the maturation of EBOV GP, although there is no evidence supporting this.

To validate that SKI-1 is the limiting protease in SRD-12B cells, an experiment was conducted where recombinant SKI-1 was transfected into the deficient SRD-12B cells to restore SKI-1 function. In figure 14, the observation of the cleaved GP2 band in the lane where SKI-1 was added confirms that SKI-1 is the cellular protease responsible for GPC cleavage in all the arenaviruses studied.

4.3 Summary

New arenaviruses will continue to emerge in the future, whether as sporadic outbreaks in endemic regions or imported cases in more developed countries. The classification of these viruses as Category A biological agents that must be handled in BSL-4 facilities has limited research involving these viruses. Although a significant amount of research has been done to understand the importance of glycoprotein processing in the Old World arenaviruses, only recently has the focus been switched to the pathogenic New World arenaviruses. In this study, we have confirmed the hypothesis that glycoprotein processing by the cellular protease SKI-1/S1P plays a crucial role in the generation of infectious arenaviral particles, particularly in several members of the New World arenaviruses. We also show that SKI-1 specific serpins are highly efficient inhibitors of this critical processing step.

Here, we have generated a wide panel of replication competent, recombinant arenavirus VSV expressing GFP, including the New World rVSVΔG-

JUNV/MACV/GTOV/SABV/CHPV and the Old World rVSVΔG-LASV. The recombinant VSV system is an excellent alternative model for analyzing potential antivirals that target the glycoprotein, and to examine their effects on virus replication. These rVSVs are safe to be studied under enhanced BSL-2 conditions, making them very practical tools to study glycoprotein functions of BSL-4 agents.

To inhibit glycoprotein cleavage, an endogenous furin-specific serpin isolated from *D. melanogaster* was genetically modified to contain a SKI-1 consensus motif within its reactive site. These SKI-1 specific serpins, Spn4-ER and Spn4-S, were then cloned into an adenovirus vector system to generate recombinant adenoviruses that expressed these two inhibitors. It was demonstrated that Spn4-S could successfully inhibit Old and New World arenavirus glycoprotein processing, and that the use of recombinant adenoviruses as a method to express these inhibitors proved highly effective. The inhibitory ability of Spn4-S, not Spn4-ER, was directly attributed to the location of active SKI-1 to the cis/medial Golgi network and secretory pathway.

The capability of SKI-1 specific serpins to reduce viral titres by up to 5-logs emphasizes the key role glycoprotein processing plays in arenavirus infectivity. When GPC processing is prevented, infectious virus particles are not produced as only cleaved glycoproteins are incorporated into mature virions (Lenz et al. 2001; Rojek et al. 2008c; Albarino et al. 2009). In this research, we confirm for several members of the New World arenaviruses, in particular the more recently isolated Chapare virus, that inhibition of GPC processing by SKI-1 specific serpins also reduces the amount of infectious virions produced. In the unique case of Chapare virus, it appears that cleavage by an alternative

protease is occurring, particularly when selective pressure for virus replication is present; however, cleavage by this protease still results in non infectious virus particles.

The persistent nature of arenaviruses in their reservoirs and the broad distribution of these rodent hosts ensure that human arenavirus infections will continue to occur. The absence of a licensed vaccine or effective antiviral treatment emphasizes the need to develop novel therapies capable of protecting against all arenaviruses. The conserved use of SKI-1 by arenaviruses together with the data showing significant viral titre reduction by SKI-1 specific serpins strongly supports the use of SKI-1 inhibitors as an effective therapy against Old and New World arenavirus infections *in vivo*.

4.4 Future Work

The recombinant arenavirus VSV system was able to demonstrate effective reduction in viral titres caused by inhibition of SKI-1 mediated GPC processing. Before testing these SKI-1 inhibitors *in vivo*, it is necessary to repeat the *in vitro* studies in BSL-4 as well as include other New World arenaviruses, such as MACV, SABV and GTOV. In the case of Chapare virus, it would be interesting to see if the wild type virus can also be cleaved by an alternative cellular protease when SKI-1 is inhibited by Spn4-S, and whether these processed glycoproteins can form infectious particles. To test whether the alternative protease is furin, experiments involving dual expression of both Spn4-S and α_1-PDX will show if GPC cleavage can be prevented completely, or whether the possibility of a third cellular protease exists. Based on sequence analysis of the cleavage region for Lujo virus, similar experiments with this newly emerged virus would also provide evidence that evolution of these viruses towards GPC cleavage by other cellular

proteases, in addition to SKI-1, may be occurring. Although these alternatively cleaved glycoproteins remain inactive, the significance of using other cellular proteases for maturation remains to be determined.

The final step would be to test the SKI-1 inhibitors in an animal model. Currently, the most relevant model for Lassa fever is to use non-human primates, particularly rhesus and cynomolgus monkeys (Moraz and Kunz 2011). However, work with non-human primates can be costly and restricted to larger BSL-4 facilities, favouring the use of smaller animal models. Many laboratory mouse strains are resistant to LASV, therefore, a suitable mouse model to study LASV is yet to be found. Recently, mice expressing humanized MHC-1 succumbed to LASV infection and developed severe Lassa fever (Flatz et al. 2010). Other animal models, including two strains of guinea pigs, were shown to be susceptible to lethal JUNV infection (Yun et al. 2008). Therefore, using one of these animal models, it is possible to test the protective efficacy of these SKI-1 specific serpins against lethal arenavirus challenge. Due to the natural role of SKI-1 in the biosynthesis and metabolism of cholesterol and fatty acids, it is expected that some side effects will occur, and supplementation of these key components may be required to reduce cytotoxic effects. Not surprisingly, pharmaceutical companies have shown great interest in developing SKI-1 inhibitors to reduce liver and plasma cholesterol, and triglyceride levels in humans (Hawkins et al. 2008). By discovering the important role of SKI-1 in the arenavirus life cycle, future collaboration with the pharmaceutical industry to develop a SKI-1 inhibitor capable of treating high cholesterol levels, and additionally to prevent arenavirus infections would be most advantageous.

5.0 Appendix

Appendix A – Primer sequences for GPC cloning

Table 4. Primer sequences used to amplify Junín (JUNV), Machupo (MACV), Guanarito (GTOV), Sabia (SABV), Chapare (CHPV) and Lassa (LASV) virus glycoproteins by PCR.

Primer name*	Sequence
VSV JUNVGPC NheI R	GCGCGCGCTAGCTTAGTGTCTTCTACGCCAAACTGTTG
JUNVGPC BsmBI **EcoRI** F**	GCGCGCCGTCTCA**AATTC**ACCATGGGGCAGTTCATTAGC
JUNVGPC MluI F	GCGACGCGTATGGGGCAGTTCATTAGCTTC
JUNVGPC BlnI R	GCGCCTAGGTTAGTGTCTTCTACGCCAAACTGTTG
VSV MACVGPC NheI R	GCGCGCGCTAGCTTAATGTCTTTTGTGCCAGATGGTG
MACVGPC BsmBI **EcoRI** F	GCGCGCCGTCTCA**AATTC**ACCATGGGGCAGCTTATCAGC
MACVGPC MluI F	GCGACGCGTATGGGGCAGCTTATCAGCTTC
MACVGPC BlnI R	GCGCCTAGGTTAATGTCTTTTGTGCCAGATGGTG
LASVGPC MluI F	GCGACGCGTATGGGACAAATAGTGACATTCTTC
LASVGPC BlnI R	GCGCCTAGGTTATCTCTTCCATTTCACAG
GTOVGPC BsmBI **EcoRI**	GCGCGCCGTCTCA**AATTC**ACCATGGGACAGTTAATCAGTTTC
GTOVGPC NheI	GCGCGCGCTAGCTCAATGCTTTCTCTTCCAGGTTAC
GTOVGPC MluI	GCGCGCACGCGTACCATGGGACAGTTAATCAGTTTC
GTOVGPC BlnI	GCGCGCCCTAGGTCAATGCTTTCTCTTCCAGGTTAC
SABVGPC BsmBI **EcoRI**	GCGCGCCGTCTCA**AATTC**ACCATGGGTCAATTGTTCAGC
SABVGPC NheI	GCGCGCGCTAGCCTAGTGGTTCTTGTGCCAGGTG
SABVGPC MluI	GCGCGCACGCGTACCATGGGTCAATTGTTCAGC
SABVGPC BlnI	GCGCGCCCTAGGCTAGTGGTTCTTGTGCCAGGTG
CHPVGPC BsmBI **EcoRI**	GCCGCCCGTCTCA**AATTC**ACCATGGGTCAACTTGTGAGTTTC
CHPVGPC NheI	GCCGCCGCTAGCTCAGTGTTTCCTGTGCCAGGTTGTTGG

*The restriction enzyme site encoded in each primer sequence is identified in the primer name and their respective recognition site is underlined within the sequence.

**BsmBI cuts 1 bp downstream of its recognition sequence; therefore, primers containing this enzyme site were designed to generate an overhang that is compatible with an EcoRI site, shown in bold.

Appendix B - Primer sequences for Chapare virus GPC mutagenesis

Table 5. Primer sequences used to mutate amino acids in the putative SKI-1 cleavage site of Chapare virus GPC into alanine residues.

Primer name	Sequence
SKI-1_V244-A_F	AATGCTGGGCATGCTGCTAAATTAAGAAGATTGCAAGG
SKI-1_V244-A_R	CCTTGCAATCTTCTTAATTTAGCAGCATGCCCAGCATT
SKI-1_K245-A_F	TGCTGGGCATGCTGTTGCGTTAAGAAGATTGCAAGGGGTG
SKI-1_K245-A_R	CACCCCTTGCAATCTTCTTAACGCAACAGCATGCCCAGCA
SKI-1_L246-A_F	GCTGGGCATGCTGTTAAAGCGAGAAGATTGCAAGGGGTG
SKI-1_L246-A_R	CACCCCTTGCAATCTTCTCGCTTTAACAGCATGCCCAGC
SKI-1_R247-A_F	CTGGGCATGCTGTTAAATTAGCGAGATTGCAAGGGGTGTTCAC
SKI-1_R247-A_R	GTGAACACCCCTTGCAATCTCGCTAATTTAACAGCATGCCCAG
SKI-1_R248-A_F	GGCATGCTGTTAAATTAAGAGCGTTGCAAGGGGTGTTCACTTG
SKI-1_R248-A_R	CAAGTGAACACCCCTTGCAACGCTCTTAATTTAACAGCATGCC
SKI-1_L249-A_F	GCTGTTAAATTAAGAAGAGCGCAAGGGGTGTTCACTTGG
SKI-1_L249-A_R	CCAAGTGAACACCCCTTGCGCTCTTCTTAATTTAACAGC
SKI-1_Q250-A_F	CTGTTAAATTAAGAAGATTGGCAGGGGTGTTCACTTGGACG
SKI-1_Q250-A_R	CGTCCAAGTGAACACCCCTGCCAATCTTCTTAATTTAACAG
SKI-1_G251-A_F	ATTAAGAAGATTGCAAGCGGTGTTCACTTGGACGATC
SKI-1_G251-A_R	GATCGTCCAAGTGAACACCGCTTGCAATCTTCTTAAT
SKI-1_V252-A_F	GAAGATTGCAAGGGGCGTTCACTTGGACGATC
SKI-1_V252-A_R	GATCGTCCAAGTGAACGCCCCTTGCAATCTTC
SKI-1_F253-A_F	AGATTGCAAGGGGTGGCCACTTGGACGATCACAG
SKI-1_F253-A_R	CTGTGATCGTCCAAGTGGCCACCCCTTGCAATCT

Appendix C – Recipes

TSS Buffer (filter sterilized)

- 85 ml LB broth
- 10 g polyethylene glycol-8000
- 5 ml dimethyl sulfoxide
- 1 g $MgCl_2$-hexahydrate
  dH_20 up to 100 ml

10% Resolving polyacrylamide gel

- 4.9 ml sterile dH_20
- 2.5 ml 40% acrylamide/bis (BioRad)
- 2.5 ml 1.5M Tris-HCl (pH 8.8)
- 100 ul 10% SDS
- 50 ul 10% ammonium persulfate
- 10 ul tetramethylethylenediamine (TEMED)

4% Stacking polyacrylamide gel

- 3.2 ml sterile dH_20
- 0.5 ml 40% acrylamide/bis
- 1.25 ml 0.5M Tris-HCl (pH 6.8)
- 50 µl 10% SDS
- 25 µl 10% ammonium persulfate
- 5 µl TEMED

Western blot lysis buffer

- 1 ml 1M Tris-HCl (pH 8.4)
- 1.5 ml 5M NaCl
- 0.1 ml 1M $CaCl_2$
  dH_20 up to 50 ml

*1% Triton X-100 (added only prior to use)
*1% Complete Protease Inhibitor Cocktail (Roche) (added only prior to use)

6X DNA loading buffer

- 0.025g bromophenol blue
- 4 g sucrose
- 10 ml dH_20

4X SDS-PAGE sample buffer

- 20 ml 1 M Tris-HCl (pH 7.5)
- 20 ml 20% SDS
- 35 ml glycerol
- 5 mg bromophenol blue
  dH_20 up to 80 ml

*20% β-mercaptoethanol (added only prior to use)

Western blot Transfer Buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3)

- 6.06 g Tris base
- 28.8 g glycine
- 400 ml methanol
  dH_20 up to 2 litres

Stock Western Blot stripping buffer (62.5 mM Tris-HCl pH6.7, 2% SDS)

- 100 ml 0.625M Tris-HCl (pH 6.7)
- 20 g SDS
  dH_20 up to 1000 ml

Working solution of Western Blot stripping buffer (per membrane)

- 20 ml stock stripping buffer
- 140ul β-mercaptoethanol

Stock solution of Crystal Violet Stain

- 20 g Crystal violet
- 200 ml 100% Ethanol
- 100 ml 37% formalin in PBS
  dH_20 up to 1000 ml

Working solution of Crystal Violet Stain

- 90 ml 10% formalin
- 10 ml stock crystal violet stain

Carboxymethylcellulose (CMC) Overlay

- 25 ml 3% CMC dissolved in dH_20
- 25 ml 2X Minimal essential medium (MEM, Gibco) supplemented with 4% FBS

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