Title
Stable Signal Peptide Requirement for Lymphocytic Choriomeningitis Virus Glycoprotein Synthesis and Maturation

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Stable Signal Peptide Requirement for Lymphocytic Choriomeningitis Virus Glycoprotein Synthesis and Maturation

DISSEPTION

submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Lydia Helena Bederka

Dissertation Committee:
Professor Michael J. Buchmeier, Chair
Professor Hung Y. Fan
Associate Professor Christine Sütterlin
Assistant Professor Melissa B. Lodoen

2014
DEDICATION

To

My father

For your support, mentorship, and friendship

Thank you for everything
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<td>alpha-Dystroglycan</td>
</tr>
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<td>BHK-21 cells</td>
<td>Baby Hamster Kidney-21 cell line</td>
</tr>
<tr>
<td>CAT</td>
<td>chloramphenicol acetyltransferase</td>
</tr>
<tr>
<td>CHC</td>
<td>clathrin heavy chain</td>
</tr>
<tr>
<td>CHO cells</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COP</td>
<td>coatamer proteins</td>
</tr>
<tr>
<td>CPE</td>
<td>cytopathic effect</td>
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<tr>
<td>CTL</td>
<td>cytotoxic T Lymphocyte</td>
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<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole, Dihydrochloride</td>
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<td>DBT cells</td>
<td>Delayed Brain Tumor cells – mouse astrocytoma</td>
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<td>DMEM</td>
<td>Dulbecco’s modification of Eagle’s medium</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>ERAD</td>
<td>ER-associated degradation</td>
</tr>
<tr>
<td>ERGIC</td>
<td>ER-Golgi intermediate compartment</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FITC</td>
<td>fluorescein Isothiocyanate</td>
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<tr>
<td>Acronym</td>
<td>Full Name</td>
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<td>---------</td>
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</tr>
<tr>
<td>GP1</td>
<td>spike glycoprotein subunit 1</td>
</tr>
<tr>
<td>GP2</td>
<td>transmembrane glycoprotein subunit 2</td>
</tr>
<tr>
<td>GPC</td>
<td>glycoprotein precursor</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin – influenza protein or protein epitope</td>
</tr>
<tr>
<td>HEK 293T cells</td>
<td>Human Embryonic Kidney</td>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<td>HBV</td>
<td>Hepatitis B virus</td>
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<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
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<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>hTfR1</td>
<td>human transferrin receptor 1</td>
</tr>
<tr>
<td>IBD</td>
<td>inclusion body disease</td>
</tr>
<tr>
<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
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<tr>
<td>MannII</td>
<td>mannosidase II</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>ML</td>
<td>milliliter</td>
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<td>MOI</td>
<td>multiplicity of infection</td>
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<td>MTT</td>
<td>3,4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide</td>
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<td>MVB pathway</td>
<td>multivesicular body pathway</td>
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<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<td>NP</td>
<td>nucleoprotein</td>
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<td>NW</td>
<td>new world arenaviruses</td>
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<td>ORF</td>
<td>open reading frame</td>
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old world arenaviruses
pathogen-associated molecular patterns
phosphate-buffered saline solution
polymerase chain reaction
paraformaldehyde
plaque-forming units
peptide N-glycosidase F
peptide-conjugated phosphorodiamidate morpholino oligomers
ribonucleic acid
ribonucleoprotein complex
really interesting new gene
recombinant Lassa virus
recombinant LCMV
ribonucleic acid
sodium dodecyl sulfate – polyacrylamide gel electrophoresis
small interfering RNA
single strand RNA
Subtilisin kexin isozyme-1/Site-1-Protease
CHO-K1 cell line variant that is SKI-1/S1P deficient
sterol regulatory element binding protein
stable signal peptide - glycoprotein subunit
single-strand ribonucleic acid
trans Golgi network
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<tr>
<td>VLP</td>
<td>virus-like particle</td>
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<tr>
<td>VP-SFM</td>
<td>virus production serum free media</td>
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I give many thanks to my thesis advisor, Michael Buchmeier, for initially allowing me to rotate through his laboratory. Being a member the Buchmeier laboratory for these last six years has been an amazing experience in terms of my professional and scientific growth. With every project I undertook, Dr Buchmeier fostered my ideas and allowed me to make the projects my own. Thanks, Mike.

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To the administrators of the GK-12 program, thank you. I had an incredible experience sharing my research interests with energetic and eager to learn students at Costa Mesa Middle School and at Middle College High School. I had the great honor of working with two fantastic teachers, Mrs Kelly and Ms Groff, to create innovative and hands-on projects in order to foster an appreciation for the sciences to students ranging from grades 7-10. Watching, first-hand, students change their attitudes towards, and develop an interest in, biology – this was awesome.

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ABSTRACT OF THE DISSERTATION

Stable Signal Peptide Requirement for Lymphocytic Choriomeningitis Virus Glycoprotein Synthesis and Maturation

By

Lydia Helena Bederka

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2014

Professor Michael J. Buchmeier, Chair

Arenaviruses persist in distinct rodent hosts in regions defined by the host rodent’s geographic distribution. The prototype arenavirus, the lymphocytic choriomeningitis virus (LCMV) uses the house mouse, Mus musculus, as its reservoir host, and as a result, LCMV has a near-global geographic distribution. In general, LCMV causes non-cytolytic infections and the resulting tissue pathology is caused by immune cell-mediated mechanisms rather than direct viral cytopathology. LCMV has also been implicated in the deaths of organ recipients upon transplantation of infected donor tissue. Several arenaviruses, namely the Lassa Virus and the Junín Virus, are causative agents of viral hemorrhagic fever.

Arenavirus infections are initiated by the binding of the viral surface glycoprotein complex to receptors on the target host cell. A series of protein interactions ensues with the ultimate release of the viral core into the cytoplasm of the newly infected cell. The work presented here targets two aspects of the arenavirus life cycle that, due to their conservation across the Arenaviridae family, are attractive targets for therapeutic developments: the genome promoter element and the
glycoprotein stable signal peptide (SSP).

The 5’ and 3’ termini of each genome segment share sequence complementarity, as each drive expression of the viral open reading frames in an ambisense manner. This highly conserved genome element was the target for antisense peptide-conjugated phosphorodiamidate morpholino oligonucleotide inhibitor development (P-PMO). Cells treated with P-PMO compounds, followed by infection with LCMV resulted in a decrease in viral protein and RNA production. Total viral protein and titer inhibition was observed with 2 uM P-PMO treatment.

In addition to my contribution to the development of antisense oligonucleotide compounds with antiviral activity, I created a panel of glycoprotein tools that directly target SSP with the goal of elucidating this glycoprotein subunit’s role in the synthesis and maturation of the entire glycoprotein complex. SSP is a fascinating component of arenaviruses. This highly conserved 58 amino acid long polypeptide is retained as a critical factor for several steps in the production of a mature glycoprotein complex, which studs the surface of nascent virions originating at the infected cell plasma membrane. Further, I created a glycoprotein plasmid containing an epitope-tagged SSP for experimental manipulation and detection of SSP. Using a series of biochemical assays, confocal microscopy, and flow cytometry, I present compelling evidence that SSP is the driving force for the synthesis of a properly organized and functional glycoprotein complex. The tools I have developed will aide future studies to further elucidate the mechanism and cell biology behind how this viral protein borrows cellular machinery for virus propagation. Since SSP is a highly conserved arenavirus component, a better understanding of how this subunit functions has the potential to become a target for antiviral therapeutic development.
Chapter 1

The *Arenaviridae*: A virus family with multiple ‘Swiss Army Knife’ proteins
Abstract

A common theme centered on the arenavirus family has been that of neglect. The first arenavirus to be identified, lymphocytic choriomeningitis virus (LCMV) is an underestimated causative agent of human congenital central nervous system abnormalities. LCMV is a noted teratogen though is often overlooked as a member of the vertically transmitted TORCH group of pathogens. Lassa Virus is the causative agent of Lassa hemorrhagic fever in western Africa, a neglected viral hemorrhagic fever pathogen, and causes hundreds of thousands of human infections annually, though the prevalence of illnesses caused by malaria or the human immunodeficiency virus gains more attention. Within the arenavirus particle, the overlooked viral glycoprotein stable signal peptide is frequently disregarded in both the structural organization of virion particles we well as in the biosynthesis of the viral glycoprotein though is required for much more than its canonical function. The recent emergence of several human pathogenic arenaviruses, as well as the discovery of distantly related arenaviruses within boid snakes, has allowed for a renaissance in arenavirus research. Since the Arenaviridae as a family contain several conserved features, understanding the mechanism by which these viruses operate will allow for the development of novel, pan-arenavirus therapeutics.
Introduction

The viruses within the Arenaviridae family are simply elegant. This family of chiefly rodent-borne viruses is capable of causing a myriad of human illnesses including non-descript flu-like symptoms, renal failure, to aseptic meningitis or hemorrhagic fever by means of four viral genes. These four open reading frames encode a total of six proteins that orchestrate a non-overtly lytic infection. In cell culture, infected monolayers do not display significant cytopathic effect, indicating a stealth method of infection that does not significantly disrupt normal cellular processes. In infected tissue, pathology occurs due to the immune response mediated by activated CD8\(^+\) T lymphocytes. In nature, these viruses are capable of establishing persistent and asymptomatic infections in rodents if the said rodents are infected before the establishment of a functional immune system (1).

The Arenaviridae family consists of a single Arenavirus genus comprised of greater than 20 virus members with several awaiting official recognition by the International Committee for the Taxonomy of Viruses. These viruses are divided into two serocomplexes: the Old World Lassa-LCMV and the New World Tacaribe arenaviruses (Figure 1.1) (2). With the exception of the Tacaribe virus, which was isolated from a fruit bat, the Arenaviridae has historically been comprised of rodent-borne viruses. Old World viruses predominate in Africa, where Lassa virus causes an estimated hundreds of thousands of hemorrhagic fever cases annually and research settings require biosafety level-4 (BSL-4) containment. Lassa Virus is, however, a neglected pathogen due to the higher magnitude of illness caused by HIV, Yellow Fever, or malaria pathogens within the same geographic region. Several New World viruses including the Junín Virus, Machupo Virus, Guanarito Virus, and Sabia Virus, also cause hemorrhagic fever in South
America and are also considered BSL-4 pathogens (3). The Junin Virus is the only arenavirus to which there exists a vaccine, the attenuated Junin Candid-1 strain; this vaccine is administered to agricultural communities to protect against Argentine hemorrhagic fever though it is not approved for use outside Argentina (4). Ribavirin, the nucleoside analog and antiviral of choice for the treatment of several viral infections, has been shown to be effective in the treatment of Lassa fever when administered early post-infection (5).

The archetype arenavirus, lymphocytic choriomeningitis virus (LCMV), was isolated from an outbreak of St. Louis encephalitis in 1933, and has proven an invaluable tool to the field of viral immunology (1, 6, 7). LCMV uses the common rodents Mus musculus or Mus domesticus as its reservoir, which affords this virus global distribution (8). LCMV infections range in severity, from non-descript malaise to aseptic meningitis. LCMV is a noted teratogen, capable of crossing the placenta and causing irreversible central nervous system damage to the developing fetus. It maintains an underrepresented diagnosis outside the established list of TORCH pathogens (Toxoplasmosis, Other infections, Rubella, Cytomegalovirus, Herpes simplex virus – 2) (9-11).

While arenaviruses are defined by serocomplex, by the ability to infect humans versus restricted to rodent populations, or by the degree of sequence homology that separates virus family members, there is a great deal of conservation in virus organization and the mechanism of action these viruses employ which enables them to endure. Arenaviruses ultimately received their name from the modified Latin (arenosus) translation for the ‘sandy’ appearance of purified virions from electron micrographs. This distinguishes this family from the double strand DNA Adenoviridae family. Arenavirus particles are pleomorphic and range from 90-300 nm in
diameter (12). The helical nucleocapsid contains the single strand RNA tightly wrapped around and protected by the nucleoprotein. The complementary 3’ and 5’ termini contain the promoter elements and are presumed to allow intra or inter-genome segment Watson-Crick base pairing for virion encapsidation (13-15). Virions contain a lipid envelope, derived from infected cell membranes, coated by the trimeric and non-covalently interacting glycoprotein complex (12, 16).

There have been several instances of LCMV transmission from hamsters to humans. The research setting has shown transmission from hamsters contaminated with LCMV-tainted tumor cell lines (17). LCMV-contaminated tumor cell lines were the culprits for a hamster-to-human outbreak at the University of Rochester Medical Center vivarium whereby human infections occurred both by aerosol and direct contact (18, 19). Hamsters are also guilty of causing human deaths due to LCMV-contaminated organ transplantations. Within the past decade, several clusters of solid-organ transplantation recipients have included LCMV-induced fatalities stemming from infection in the organ donor via pet hamsters, where the hamster likely contracted the virus due to contact with infected mice, or from close proximity to rodent excreta (20-22).

**Gene expression is both elegantly designed and complex**

The bi-segmented genome, ambisense expression strategy, intergenic region, and complementary termini allow for the characterization and identification of novel arenaviruses (Figure 1.2). Within the last decade, the ability to clone viral genome segments and to perform genetic manipulations has allowed for the production of recombinant arenavirus from standard plasmid transfections (23). This genetically defined in vivo setting has proven invaluable for
characterizing distinct viral protein functions within the context of a viral infection and holds potential for the arenavirus antivirals and vaccine development (24-26).

Though distantly related based on phylogeny, receptor use, and host tropism, all arenaviruses retain conserved, ambisense genome architecture. Both single strand RNA segments encode two open reading frames separated by a highly structured intergenic region, which serves as a transcription termination signal in lieu of polyadenylation (27). Additionally, the 3’ and 5’ termini share reverse complementarity and this panhandle formation is presumed to base pair for genome packaging as well as to drive gene expression (15, 28).

The large (L) and small (S) genome segments encodes the zinc-binding (Z protein) matrix protein and the glycoprotein complex (GPC) in a pseudo-positive sense, respectively. The RNA-dependent RNA polymerase (L protein) and the nucleoprotein (NP) are expressed in a negative sense from the L and S genome segments. Recognition of the 3’ terminus of each genome strand by the L polymerase drives discontinuous viral gene expression where both viral mRNA transcription and genome replication require an intricate and coordinated balance of viral protein function to complete one infection cycle.

Both viral replication and transcription require coordination between the NP and the L polymerase for distinction between gene regulatory functions. Using the S segment gene products as an example, NP transcription occurs directly from the genome segment as the L protein recognizes the 3’ terminus promoter element (Figure 1.3). Transcription termination occurs within the intergenic hairpin structure as arenavirus messages lack polyadenylation signals (29, 30). As with other negative sense virus polymerases, and despite the lack of a fully
understood mechanism, the L protein utilizes its endonuclease activity only during transcription and not in the course of antigenome replication to pirate cellular mRNA cap structures (31-33).

Prior to GPC transcription, the L protein must morph into a replicative form and synthesize a full-length antigenome complementary S segment. To do this, the polymerase incorporates a non-template pppG primer residue to the 3’ terminus to prime the RNA segment and to realign the polymerase for replication (34-36). Further, the polymerase and the RNP complex cooperate to collapse the intergenic hairpin structures for polymerase read through for full-length complementary antigenome RNA segment replication. The newly replicated, full-length antigenome segment now contains the 3’ element required for glycoprotein complex (and L segment encoded Z) transcription (Figure 1.2).

**Arenavirus entry requires the acid endosome for membrane fusion**

All arenaviruses contain their replication cycle within the cytoplasm, indirectly involving nuclear functions. Arenavirus binding to cellular receptors, mainly α-Dystroglycan (α-DG) for the OW arenaviruses and the human transferrin receptor 1 (hTfR1) for the pathogenic clade B NW viruses, triggers an entry pathway not commonly shared throughout this virus family (37, 38). Initial receptor binding and entry routes aside, arenaviruses ultimately reach the acidified late endosomes for pH-dependent fusion between virion and host endosomal membranes, a process that is mediated solely by the glycoprotein complex (39, 40). NW arenaviruses utilize clathrin-coated endosomal vesicles and rely on the cytoskeletal network for entry and virus proliferation whereas OW arenaviruses deploy a mechanism to arrive at late endosomes independent of clathrin and several players of the endocytic pathway (41-44). Advances in the technologies
available to the research community have allowed for the identification of host factors involved in virus entry. A recent siRNA screen identified voltage-gated calcium channels as a co-factor for Junin virus entry whereas a haploid genetic screen implicated the lysosome protein Lamp-1 as a secondary, intracellular receptor for Lassa virus entry (45, 46).

Viral proteins and their roles within the arenavirus life cycle

Nucleoprotein
The main function of this most abundant viral protein is to encapsidate viral RNA. Within both infected and transfected cells, the NP induces discrete cytoplasmic vesicles, which function as sites for viral replication and transcription (47). Structural and biochemical data support a two-domain protein where the N-terminal domain is essential for maintaining NP-NP interactions, binding to RNA, as well as maintaining a trimeric structure for viral replication and transcription while the C-terminal domain mediates NP mechanisms for innate immune suppression (48-52). Insights from NP structural data not only allow for targeted therapeutic developments but also allow for details on the mechanisms used by this multi-functional protein.

The large (L) protein
The arenavirus polymerase (L protein) is a ~ 2200 amino acid multi-domain protein factory that functions to transcribe viral mRNA independently from replicating genome and/or antigenome RNA segments (Figure 1.3). Considering the size (circa 240 kDa) of this multi-domain and multifunctional protein, the overall structure and comprehensive role of the L protein remains nebulous. Model genome assays indicate L protein oligomerization and multiple domain coordination are required for functional enzyme activity (53, 54). Moreover, residues within the
central portion of the L protein are important for RNA-dependent RNA polymerase activity. Specific point mutations within this core domain suggest structural rearrangements within the L protein for the regulation of mRNA transcription versus genome (or antigenome) replication (55). Additionally, the amino terminus contributes to mRNA transcription, likely by possessing endonuclease functions for cleaving the cap structures from cellular mRNA (32, 33).

Z protein
The 11kDa zinc-binding (Z) matrix protein is quite extraordinary due to its interaction with each viral protein – the glycoprotein complex, the L polymerase, and the nucleoprotein – in addition to cellular factors so as to orchestrate its diverse and regulated functions within the arenavirus life cycle (Figure 1.4). Z plays an inhibitory role in the regulation of arenavirus transcription/replication while orchestrating viral budding from the plasma membrane. Within intact virions, the Z protein provides structural reinforcement via its myristoylation modification and interacts with both the glycoprotein and the nucleoprotein (12, 56-58). In infected cells, Z is involved with distinct processes within separate cellular compartments. Within transcription/replication centers, the Z protein is a key factor in both the regulation and inhibition of viral RNA synthesis (59, 60). Additionally, Z encodes late domains that enable interaction with the cellular multivesicular body pathway (MVB) proteins such as Tsg101 for virus budding from the plasma membrane (61, 62)(56, 63).

Glycoprotein complex
The glycoprotein complex is expressed as a polyprotein, which undergoes two proteolytic cleavage events. The glycoprotein complex encodes a stable signal peptide (SSP) that is cleaved
within the endoplasmic reticulum by cellular signal peptidase (64) (65). The remaining precursor glycoprotein (GPC) is post-translationally modified with asparagine-linked glycans within the ER but must traffic to specific regions of the Golgi stacks for proper glycan trimming prior to precursor cleavage and transport to the plasma membrane (66). GPC cleavage is mediated within various regions of the Golgi apparatus by the cellular SKI-1/S1P enzyme (67), (68). This GP complex consisting of the stable signal peptide, GP1, and GP2 subunits non-covalently assembles at the plasma membrane to stud the surface of newly synthesized virus particles (Figure 1.5).

The soluble spike GP1 subunit directly engages the cellular receptor to initiate contact. Upon internalization, the ensuing acidification disrupts the non-covalent interactions between the GP1 and the transmembrane GP2 subunit. Lack of GP1 binding induces a structural rearrangement within the GP2 subunit to expose hydrophobic heptad repeat fusion peptides (69-71). These heptad regions form a core α–helical bundle that, along with SSP, brings the viral and host membrane to proximity for the creation of a fusion pore to allow the deposition of the viral ribonucleoprotein complex within the newly infected host cytoplasm (72, 73). Upon release of the virion core into the cytoplasm for virus replication, each arenavirus protein executes its army of functions before ultimately reconvening near the plasma membrane for virion packaging and egress.

**Arenaviruses encode a unique signal peptide**

The glycoprotein signal peptide is termed ‘stable’ due its retention as an essential glycoprotein subunit within infectious virions upon signal peptidase cleavage (74, 75). The importance of the
stable signal peptide (SSP), in terms of glycoprotein biology, was overlooked for several years simply due to its size, 6 kDa. Interestingly, SSP had been a useful tool to immunology for almost a decade prior to its characterization as a bona fide glycoprotein subunit due to the presence of an immunodominant T cell epitope (gp33) widely used to study immune memory and virus persistence (64, 76).

This 58-amino acid protein plays a critical role not only in the synthesis and maturation of progeny glycoprotein but also in virion structure and infectivity (77). SSP is critical for trafficking of the GP complex through the secretory pathway and is packaged into infectious virions along with the GP1 and GP2 subunits (78) (79). In terms of virus entry and infectivity, the signal peptide mediates pH-dependent membrane fusion in concert with the GP2 subunit (73, 78).

**Useful tools for understanding the arenavirus life cycle**

**Recombinant arenaviruses to dissect molecular mechanisms of action**

The ability to rescue recombinant lymphocytic choriomeningitis virus (rLCMV) from plasmid-driven expression of each genome segment provided a straightforward genetics system to expand our understanding of several aspects of arenavirus biology and pathogenesis (80-82). Using this plasmid-based system, amino acid differences within both the glycoprotein complex and the L polymerase have been implicated in influencing viral clearance or pathogenicity (83).

The *in vivo* molecular mechanism of attenuation used by the vaccine strain of Argentine Hemorrhagic Fever, Junin Candid-1, was mapped to the glycoprotein transmembrane domain
using recombinant Junin virus particles (84). rLCMV containing point mutations that either destroyed or introduced specific glycosylation sites was demonstrated to affect the behavior and cell tropism while recombinant Lassa virus (rLASV) expressing point mutations within the NP determined amino acid residues involved in innate immune suppression and chemokine activation (23, 85). Additionally, the use of rLCMV to characterize viral elements that contribute to T cell activation, the mechanisms by which these viruses evade the host immune system and establish persistent infections, and the use of rLCMV as vectors to induce both cellular and humoral immunity shows promise for the development of attenuated arenaviral vaccines using FDA-approved cell lines (26, 86, 87). Chimeric rLCMV expressing the Lassa virus glycoprotein provided a means for both \textit{in vivo} analysis of Lassa virus cell tropism and T cell responses for viral clearance without the use of high containment laboratory settings (88).

**Cellular proprotein convertase SKI-1/S1P for cleavage of arenavirus glycoproteins**

Trafficking of the glycoprotein complex through the secretory pathway for synthesis and maturation occurs independently of other viral proteins. The cellular enzyme responsible for cleaving arenavirus glycoproteins is the proprotein convertase subtilisin/kexin-isozyme-1/Site-1 Protease (SKI-1/S1P). The activity of this cellular enzyme differs from other, more-well studied enzymes such as furin in that SKI-1/S1P operates by a regulated mechanism as opposed to a constitutive cleavage function (89). This enzyme plays a key role in the maintenance of cellular cholesterol levels by regulating the activational cleavage of the transcription factor sterol regulatory element binding protein (SREBP) in response to low cellular cholesterol levels (90). Additional substrates for this enzyme are the ER-resident unfolded protein response transcription
factor ATF6, the pro-form of the brain-derived neurotrophic factor (proBDNF), and the Crimean-Congo Hemorrhagic Fever Virus glycoprotein (91-93).

One tool that has benefited the arenavirus community is the creation of the hamster SRD-12B cell line, a Chinese hamster ovary (CHO) cell line defective in the SKI-1/S1P (94). This cell line was initially created for research pertaining to cholesterol regulation and biosynthesis and has become an essential tool for probing glycoprotein processing as an antivirals target and the cellular protease (67, 94). Though the relationship between cholesterol and glycoprotein maturation remains unclear, membrane cholesterol is key for the infectivity of arenavirus particles (43, 95). The interaction between arenavirus glycoproteins and the SKI-1/S1P serves as a viable drug target for anti-arenavirus therapies since infected SKI-1/S1P-defective cells do not produce escape variants, indicating arenaviruses are not capable of altering their glycoprotein maturation pathway (96, 97).

The impact of LCMV to the fields of viral immunology and pathogenesis

Several advances in modern immunology have come from using LCMV in the context of its natural reservoir, the mouse - its own tool for modeling human diseases. The course of infection with LCMV depends on the virus strain and the mouse strain, as well as the age of the mouse. The ability to isolate both viral and cellular factors that contribute to disease outcome has proven valuable to our understanding of the immune response to a viral infection. Arenavirus infections do not result in cell lysis, rather it is the over-reaction of the immune response driven by cytotoxic T lymphocytes (CTLs), that causes tissue injury (98). The mouse model for LCMV infection demonstrated that CTLs require the recognition of the ‘self’ major histocompatibility
complex (MHC) as well as the presented ‘foreign’ LCMV epitope in order to execute killing activities (99, 100). Additionally, the ability of LCMV to establish non-lytic, persistent, chronic within its mouse reservoir serves as a natural model used to study chronic human infections such as HIV, HBV, and HCV (1).

The most frequently used LCMV strains, the neurotropic Armstrong (Arm) and the viscerotropic Clone-13 (Cl-13), differ by single point mutations each within the glycoprotein and the polymerase, though the mechanism by which these amino acid variations drive the two distinct clinical phenotypes remains unclear (101-103). The glycoprotein substitution F\textsubscript{260}L allows LCMV Cl-13 binding to the a-Dystroglycan receptor with higher affinity than LCMV Arm while the Cl-13 polymerase variant has an increased replicative ability for amplified viral load (83, 104, 105). Cl-13 is viscerotropic and fails to mount a CTL-mediated immune response sufficient for virus clearance, thus T cell exhaustion leads to a persistently infected state (104, 106). Conversely, Arm is neurotropic and induces a robust, acute CTL response that results in viral clearance by 10 days post-infection (107, 108).

In nature, mice infected up to 24h post birth fail to mount an arenavirus-specific immune response. The established systemic and chronic infection allows for the production of infectious mouse excreta, whereby allowing continued viral dissemination. The suppressed response to Cl-13 infection, however, leads to a persistent viral infection and lack of viral clearance up to 90 days post infection. The exhausted, anergic T cell state resulting from Cl-13 infection is mediated by the up-regulation of inhibitory factors IL-10 and PD-1, factors that utilize different signaling pathways to control the extent of positive immune response stimulators (109, 110).
Individuals suffering from various chronic viral infections have elevated IL-10 or PD-1 levels. In the Cl-13 model, the use of antibodies to block the IL-10 receptor or to neutralize PD-1 prevented the establishment of persistence by restoring exhausted CTL function to promote viral clearance (111, 112). Further, blocking the activation of the innate interferon receptor (IFNAR1) in an established Cl-13 infection prevented pro-inflammatory cytokine production while allowing for IFN gamma activation, which resulted in decreased tissue and serum viral titers (113, 114).

**Snake arenavirus and evolution from a common ancestor with filoviruses**

The recent discovery of arenaviruses within boid snakes sick with inclusion body disease (IBD) marked the first identification of this virus family in a non-rodent host (115-117). The snake arenaviruses contained features conserved with their distant rodent relatives, namely an ambisense, bi-segmented ssRNA genome, genome termini conservation, and intergenic hairpin structures. A noted difference between rodent and snake arenaviruses is the lack of a myristoylation motif within the Z protein, as well as the lack of Z protein late domains. The Z myristoylation motif is replaced with a transmembrane domain, though the late domains reside within the snake nucleoprotein. The most significant difference between rodent and snake arenaviruses lies with the glycoprotein. Phylogenetic analysis of the snake arenaviruses show a glycoprotein more closely related to filoviruses. The notion of a common ancestor between arenaviruses and filoviruses is a concept previously proposed based on molecular modeling (118).
Since the initial discovery of arenaviruses as a causal agent of disease in reptiles, several reports have emerged to second this observation, which have allowed for snake arenavirus phylogenetic analysis. As with their rodent counterparts, there exists high diversity within the snake arenavirus population accompanied by the observation of intrasegment recombination events within the genome S segment, a phenomenon also observed within the rodent NW clade A/B cluster (119-121). Given that these two viral families are thought to have arisen from a common ancestor, the discovery of snake arenaviruses bearing a filovirus-like glycoprotein is an added tool for understanding virus evolution from a common ancestor.

**Finishing thoughts**

Arenaviruses are composed of simple genomes easily amendable to genetic manipulations. Combine this feature with their natural rodent reservoirs and there exists an elegant system to dissect and understand several aspects of molecular medicine and host-pathogen interactions. The application of next generation technologies has aided in increasing the tool kit for arenavirus research and discoveries. The first report indicating the presence of arenaviruses in reptiles used deep sequencing analysis of RNA extracted from several reptile tissues (115). Further, the human pathogenic Old World Lujo virus was identified and its genome characterized using high throughput pyrosequencing and phylogenetic analysis using RNA collected from two virally infected donor livers (122). Recent studies using high throughput screens have contributed to our understanding of several aspects of arenavirus biology. These screens, though varied in their experimental design, have shed light on co-receptor candidate molecules as well as conserved cellular mechanisms shared by several viral families (45, 123-125). Additionally, structure-
based antiviral development aided by *in silico* drug synthesis allowed a high throughput screen for competitive inhibitors against Tsg101 and the inhibition of VLP egress (126).

Arenaviruses infections start by using a stealth method of entry (127). Upon release of the virion core into the cytoplasm, each viral protein executes myriad functions until convening near the plasma membrane for packaging and viral egress. Understanding how these viruses borrow cellular machinery will not only enable the development of novel anti-arenaviral therapeutics but will provide insight into cellular function and biology.
The Old World (OW) arenaviruses reside within Africa while the New World (NW) arenaviruses are distributed throughout the Americas. Each virus in red type represents an arenavirus that causes disease in humans. The lymphocytic choriomeningitis virus, whose rodent reservoir is the common house mouse *Mus musculus*, is distributed globally.
Figure 1.2 Ambisense arenavirus genome organization
All arenaviruses encode two non-overlapping reading frames on each of two segments of single strand RNA. The 5’ and 3’ terminal nuclei are complementary, and are predicted to Watson-Crick base pair for polymerase recognition. The large RNA segment encodes the large (L protein) RNA-dependent RNA polymerase and the zinc-binding, matrix-like protein (Z protein). The small RNA segment encodes the nucleoprotein (NP) and the glycoprotein (GPC). Each gene is separated by at least one stable intergenic hairpin structure, which serves as a steric block to transcription in lieu of polyadenylation signals. Each RNA strand comprises an ambisense expression strategy. The L and NP proteins are expressed in a negative sense while the Z and GP proteins are expressed in a pseudo-positive manner.
The viral L polymerase serves two functions: to replicate the viral genome/anti-genome and to transcribe viral messenger RNA (mRNA). In order to replicate the genome, the polymerase modifies the 3’ terminal promoter region with a non-template encoded guanine residue (red). The L protein reads through the entire genome strand, collapsing the intergenic region in the process, to produce an anti-genome replicate strand. This anti-genome segment serves as the template RNA strand for the two open reading frames that are expressed in a pseudo-positive sense, the Z and GP genes. The anti-genome now encodes the appropriate 3’ terminus for the polymerase, which must switch from its replication to transcription function. Arenaviruses pirate 5’ cap structures from the cellular RNA pool to modify viral mRNA. The 3’ ends of viral mRNA are of variable length and are not polyadenylated. Transcription terminates within varied regions of the intergenic hairpin structure.
Figure 1.4 Multiple roles for the Zinc-binding matrix protein

The arenavirus Z protein is the viral factor that orchestrates virion budding by interacting with the cellular multi-vesicular body (MVB) pathway, specifically the cellular factor Tsg101. Z is myristoylated, thus anchored to membranes affording a structural function within virion particles. Further, The Z protein plays a role in regulating arenavirus gene expression by interfering with polymerase activity as well as sequestering the translation factor eIF4e, a feature in hand with the delayed expression of the Z protein during the infection cycle.
The glycoprotein complex is the major virus structural component and tropism determinant. This transmembrane protein undergoes two cleavage events for maturation via the secretory pathway. The signal peptide is cleaved from the precursor glycoprotein by cellular signal peptidases. The signal peptide and the remaining precursor glycoprotein traffic to the Golgi apparatus where the cellular SKI-1/S1P enzyme cleaves the precursor into mature subunits. The three glycoprotein subunits: the signal peptide, GP 1, and GP 2 traffic to the plasma membrane where the mature complex is assembled via non-covalent inter-subunit interactions. The GP 1 subunit is the soluble portion and interacts with the cellular receptor. The transmembrane GP 2 subunit encodes meta-stable fusion motifs and, along with the signal peptide, brings about the fusion between the viral and cellular membranes during virus infection and entry. Depicted are proposed orientations the signal peptide may use for membrane orientation. Highlighted in red is the signal peptide myristoylation modification.

Figure 1.5 Secretory pathway maturation for the arenavirus glycoprotein complex
Chapter 2

Controlling Elements for Arenavirus Replication

Experiments and data presented within this chapter were performed and collected by Lydia H Bederka and analyzed by Lydia H. Bederka, Benjamin W Neuman, and Michael J. Buchmeier. Results were analyzed and presented within the manuscript by Neuman BW et al., Antimicrobial Agents and Chemotherapy, 2011 Oct;55(10): 4631-8.
Abstract

Peptide-conjugated phosphorodiamidate morpholino oligomer (P-PMO) compounds are chemically synthesized nucleic acid analogues. P-PMOs are conjugated to a cell-penetrating peptide to deliver nucleotide analogs to the intended target RNA sequence. This nucleotide specificity allows for Watson-Crick base pairing and a steric block to gene expression at the RNA level. The P-PMOs used in this work were synthesized to target the ultimate 3’ nucleotide sequence to block viral protein expression. Arenaviruses are small, single-strand, bi-segmented RNA viruses that infect and propagate within the cytoplasm of infected cells. The ultimate 19 nucleotides of both genome segments are complementary and highly conserved across the entire Arenaviridae family. This complementarity allows for the termini to form a panhandle structural element for viral polymerase recognition and serves to drive both genome replication and transcription. Cell culture toxicity was observed at greater than 5 µM P-PMO concentrations. Treatment with 2 µM P-PMOs resulted in a three log-fold reduction of viral titers, as determined by plaque assay. Additionally, western blot analysis of cells treated with the same concentration of P-PMO treatment resulted in complete inhibition of viral protein expression. These data reveal potential target areas for the design of novel anti-arenaviral therapeutics.
Introduction

Human infections with arenaviruses occur most frequently via close contact with the rodent reservoir hosts, which define the distribution of arenaviruses (3). Zoonotic infection and human transmission requires close contact with either infected animals or aerosolized excreta and no arthropod vector. Due to the aerosol transmission and the ability to cause hemorrhagic fever, several arenavirus members are classified by the Centers for Disease Control and Prevention (CDC) as Category A bioterrorism pathogens that pose a risk to national security.

Arenaviruses persist within rodent reservoirs, which define the geographic distribution of each arenavirus (3). In these rodents, arenaviruses possess a unique life cycle characterized by benign and persistent infection yet infection may cause serious illness in humans. The prototypic arenavirus, lymphocytic choriomeningitis virus (LCMV), uses the common mouse *Mus musculus* as its reservoir host; thus, obtaining a near global geographical distribution. The endemic region for the Argentine hemorrhagic fever causing Junín virus is also this country’s agricultural heartland, with agricultural workers being the most at risk target population for infections. The attenuated Junín Candid-1 vaccine strain is only licensed for use within Argentina and does not have FDA approval for use within the United States (4).

Human infections may result from inhalation of rodent excreta or, rarely, from direct human-to-human transmission. Illness may range from mild, flu-like symptoms to aseptic meningitis (3). LCMV infections rarely result in death, although recent incidences of fatal arenaviral infections involving organ transplant recipients have been recognized (21, 128). The Old World Lassa Virus, as well as several New World arenaviruses such as Junín Virus, Machupo Virus,
Guanarito Virus, and Sabia Virus, cause severe illness, vascular permeability and ultimately hemorrhagic fever (3). Treatment of human infections is mostly limited to supportive therapies, however intravenous administration of the nucleoside analog Ribavirin has been shown to decrease mortality if administered early after onset of symptoms, since late neurological symptoms have been shown to present with NW hemorrhagic fever patients regardless of treatment (5, 129).

All arenaviruses contain negative single strand, bi-segmented RNA genomes encoding a total of four genes using an ambisense expression strategy. The large (L) segment encodes for a RING domain protein (Z protein, 11 kDa) and an RNA-dependent RNA polymerase (L protein, 200 kDa). The Z protein provides matrix-like structural functions and is essential for virus budding from infected cells (57, 62, 130). The small (S) segment encodes for a glycoprotein precursor complex (GPC, 75 kDa) and the viral nucleoprotein (NP, 63 kDa). The glycoprotein precursor retains a 58-amino acid stable signal peptide (SSP) while cleavage by cellular SK1/S1P processes the precursor glycoprotein into the GP1 (41 kDa) and GP2 (35 kDa) subunits (68, 75, 77). GP1 functions as the globular protein involved in receptor recognition (38, 131) while the SSP and the GP2 subunit, a type 1 transmembrane protein, mediate pH-dependent membrane fusion (40, 70).

The virally encoded polymerase and nucleoprotein are the minimal required trans-acting factors required for viral replication and transcription whereas the mechanism for cap-dependent translation is poorly understood (132). Studies originally based on the LCMV genome have revealed complementary 3’ and 5’ terminal un-translated sequences within each genome
segment, which have been hypothesized to form panhandle structures in addition to serving as 
*cis*-acting elements indicative of viral promoters (28). The arenaviruses as a family contain 
extensive sequence conservation at the termini of each segment (13). Also, both the L and S 
genome segments contain intergenic regions predicted to form at least one hairpin structure, 
providing a steric block for transcription termination in lieu of polyadenylation signals (133). 
Due to extensive sequence conservation across the *Arenaviridae*, antiviral therapies designed for 
use with one family member may afford broad-spectrum antiviral activities.

Phosphorodiamidate Morpholino Oligomers (PMOs) are single-strand deoxyribonucleic acid 
analog compounds where the native phosphodiester bond is replaced by a phosphorodiamidate 
linkage and a six-membered morpholine ring substitutes for the deoxyribose sugar to which 
nitrogenous bases are attached. PMOs are uncharged, nuclease resistant compounds that, upon 
cellular uptake, bind their target mRNA sequence via Watson-Crick base pairing and inhibit gene 
expression without induction of mRNA degradation (134, 135). Cellular uptake of antisense 
PMOs is augmented due to a 5’ arginine-rich peptide conjugate (P-PMO) that mimics the 
membrane transport function of the HIV Tat protein (136). Binding of antisense PMOs to 
specific mRNA sequences creates a steric barrier to protein translation resulting in the repression 
of gene expression.

PMOs have been designed to target gene expression for numerous viral families including, but 
not limited to, the *Flaviviridae*, the *Coronaviridae* and the *Arteriviridae*. In addition to the 
genomic termini, genomic sequences such as the AUG start codon and the first 20 nucleotides of 
a coding sequence are critical for the proper regulation of gene expression, thus serving as targets
for antisense PMOs. Previous studies have demonstrated the reduction of viral protein expression when PMOs target the 5’ and 3’ un-translated regions of viral genomes (137-142). Previous work from our group, using both mouse hepatitis virus and severe acute respiratory syndrome coronavirus models, has shown in vitro PMO antiviral activity demonstrating sustained virus inhibition with continued virus passage, a decrease in viral titers and cytopathic effects (CPE), as well as in vivo reductions in viral titer and pathology (143-146). In vivo antiviral activity was more profound when antisense PMOs were administered before infection; however, antiviral PMO activity showed more robust activity in in vitro applications (144). Additionally, PMO technologies have been used in the treatment of Duchenne muscular dystrophy, which resulted in the amelioration of aberrantly spliced dystophin expression, both in vitro and in vivo (147-149).

Based on the sequence conservation across Old World and New World arenaviruses, we have designed antisense PMOs targeting portions of each genome segment critical for gene expression. Antisense PMOs targeting replication differed from PMO targeting both replication and transcription activity by accounting for a non virally-encoded guanine residue found at the 3’ end of each genome segment, which enables replication via an uncharacterized ‘prime and realign’ mechanism (34, 35). For all PMOs, antisense activity was tested against both human and non-human pathogenic arenaviruses to determine broad-spectrum, anti-arenaviral activity (150). PMOs targeting the 5’ un-translated region, affecting both replication and transcription, were the most effective at reducing viral titers and inhibiting viral protein expression.
Results

Design of arenavirus P-PMOs

P-PMOs were designed against the Junín Candid-1 vaccine strain and were complementary to several conserved elements on viral RNA genome segments (Figure 2.1). These P-PMOs that showed greatest antiviral activity were directed against the 5’ terminal promoter element (Figure 2.1). The P-PMO compounds targeting the genomic termini (TERM-L-REP and TERM-S), TERM-L REP compared to the TERM-S, was extended by one nucleotide in order to hybridize more efficiently with the template strand for RNA replication, which is not packaged in viral particles.

Cytotoxicity testing

To determine the concentration of the peptide-conjugated PMO (P-PMO) that could be safely used in Vero-E6 cell culture, we performed MTT cytotoxicity assays 24h after P-PMO incubation. The TERM-L-REP, TERM-L and TERM-S P-PMOs showed toxicity starting at concentrations of 20 μM and mild toxicity at 50 μM (Figure 2.2). Based on the toxicity results, subsequent assays received P-PMO treatment with a maximum concentration of 20 μM P-PMO.

PPMOs inhibit viral protein synthesis

To investigate the mechanism of P-PMO antiviral activity, we assessed the expression of the LCMV nucleoprotein (NP) and glycoprotein (GPC) in infected cells. Treatment with 2 μM P-PMO reduced expression of NP and GPC below the threshold of detection at 24h and 48h post inoculation (Figure 2.3A) in addition to the decrease of viral titers to below the detection threshold (Figure 2.3B). These results suggest that genome termini-binding P-PMOs are
effective anti-arenaviral agents though over-dosing resulted in cellular toxicity, based on the reduction in actin expression levels from the higher concentration P-PMO treated samples.

**P-PMOs interfere with viral RNA synthesis**

We used qRT-PCR to measure the extent of RNA suppression resulting from increasing P-PMO concentrations. Due to the nature of arenavirus gene expression, the probes used for this assay detected both the viral antigenome and nucleoprotein mRNA species. Pre-treated Vero E6 cells were infected with LCMV and RNA was collected 24h post infection. Analysis of viral RNA relative to GAPDH RNA resulted in a dose-dependent inhibition of viral NP RNA at 1uM, a lower inhibitory concentration when compared to NP protein expression levels via western blot (Figure 2.3C).

**Discussion**

To date there does not exist an antiviral therapeutic to effectively treat human arenavirus infections. The only vaccine available, the attenuated Junín virus Candid #1 strain, is solely available in Argentina to protect against Argentine hemorrhagic fever and does not afford cross-protection to arenaviruses other than to its closest relative the Bolivian hemorrhagic fever Machupo virus (4, 151). An attenuated chimeric, laboratory-produced Lassa virus/Mopeia virus, the ML29 strain; containing the Mopeia virus L genome segment and the Lassa virus S genome segment, is currently in use with animal trials for its protective capacity (152). This attenuated virus has been shown to elicit both humoral and cell-mediated immunity against Lassa virus in rhesus macaques though this work remains preliminary (153, 154).
The nucleoside analog Ribavirin is not approved by the FDA to treat these viral infections though may be obtained for compassionate use, but not without nephrotoxic side effects (155). Mirroring efforts focusing on the lung and vascular damage induced by influenza virus and dengue virus, respectively, additional therapeutics to combat the breakdown of vascular permeability produced during viral hemorrhagic fever focus on the maintenance of host cell integrity (156, 157). As discussed by Armstrong et al., re-enforcing VE-Cadherin and cell-cell junction integrity may decrease vascular leakage by mitigating inflammation, thus providing reinforcement for the host defenses against infection (158, 159). Additionally, no diagnostic assays are currently available for rapid and effective identification of arenavirus culprits.

Given that arenaviruses utilize several conserved elements and cellular processes, several of these are currently targets for antiviral therapeutic development. Here, we targeted the conserved genome promoter element with antisense oligonucleotide compounds. The P-PMOs were not cytotoxic at the levels that displayed anti-viral activity. Since antisense oligonucleotides bind to their target sequence by Watson-Crick base pairing, the most likely mechanisms by which these P-PMOs inhibited viral RNA synthesis is by interfering with translation initiation or by inhibiting the binding of the viral polymerase to the genome segment promoter (Figure 2.4) (150). The P-PMO compounds resulted in complete inhibition of viral protein expression and the reduction of viral titers to the limit of detection while siRNA activity targeting the same elements demonstrated the partial reduction in gene expression (160). Given the conserved sequence complementarity at both the 5’ and 3’ termini, targeting these promoter elements serves as a viable target for pan-arenavirus therapeutic development.
Arenaviruses enter cells by interactions between the glycoprotein GP1 subunit binding to cellular receptors and the ensuing acidified endosomes. The decrease in pH within the acidified, late endosome disrupts the non-covalently interacting glycoprotein subunits, which allows the transmembrane subunit GP2 to expose heptad fusion domains. Together with the glycoprotein stable signal peptide, the GP2 subunit fusion domains interact with the endosome membrane to unite the viral and cellular membrane and deliver the viral ribonuclear particle (RNP) core into the newly infected cell cytoplasm. Small molecule inhibitors targeting viral particles to inhibit virus attachment similar to the effects of neutralizing antibodies have been screened for several NW arenaviruses. Drug resistant viruses resulted in amino acid variations within the GP2 transmembrane domain, indicating the compounds interfere with pH-dependent fusion (161). Several additional fusion inhibitors have been in development with similar transmembrane domain or GP1 subunit shedding targets in order to interrupt the conformational changes the glycoprotein employs to interact with the host lipid bilayer (162, 163).

Compounds targeting viral replication, similar to the mechanism used by the nucleoside analog Ribavirin, have also been shown to incorporate modified nucleic acid analogs and inhibit RNA synthesis while not interfering with cellular de novo guanosine precursor synthesis (164, 165). The influenza drug Favipiravir, a nucleoside analog that directly inhibits viral polymerase activity, is currently in Phase II clinical trials for use as an arenavirus therapy after having demonstrated protection using the Pichinde virus and guinea pig in vivo model for acute arenavirus disease (166-168). An additional target for blocking arenavirus infections is against the budding activity of the Z protein and its interaction with the host protein Tsg101, a member of the cellular ESCRT vesicle trafficking complex. Recent in silico screens identified a reversible
inhibitor against the Z late domain-Tsg101 interaction, though animal proof of concept studies has yet to be performed (126).

The arenavirus RNA-dependent RNA polymerase, like that of many ssRNA viruses, is error-prone and lacks proofreading functions. This feature aids in the adaptability and the survival of viruses due to selective pressures. The development of successful anti-arenaviral therapies therefore must target several viral functions or pathways to prevent escape or resistant viral variants. Also, the optimization of in vivo delivery systems to target organs is something that remains to be developed. Here, we focused on one conserved arenavirus element for the efficacy of antiviral compound activity.
Figure 2.1 Conserved arenavirus genome features as target sites by antisense P-PMO compounds
(A) The representative chemical structure of phosphorodiamidate morpholino oligonucleotide compounds. (B) The arenaviruses RNA genome including target sites for antisense compounds. The red bars indicate P-PMO target regions: terminal promoter elements, ORF start codons, and intergenic hairpin structure. The P-PMOs we used to target the genome terminal promoter elements are labeled with a yellow star.
Figure 2.2. P-PMO treatment and cellular viability
Vero E6 cells were incubated with various concentrations of each indicated P-PMO for 24h. Cells were incubated with the MTT reagent and allowed to incubate for 40 minutes. Cells were solubilized and 560nm absorbance/photometry readings were measured using the Victor plate reader. The TERM-S and -L P-PMO targeted viral transcription while the TERM-L-REP targeted viral genome replication.
Figure 2.3 P-PMO treatments inhibit viral protein expression
(A) Western blot analysis of viral nucleoprotein and glycoprotein complex expression using Vero E6 cells treated with the TERM-L P-PMO. Both viral NP and the glycoprotein GP2 subunit expression levels were detected at 24h post-infection. (B) Viral titers were determined via plaque assay using fresh Vero E6 monolayers. Significance levels were determined using the ANOVA method. (C) qRT-PCR was performed to determine viral RNA levels at 24h post-infection with the incubation of each noted TERM-L concentration.
Antiviral compounds targeting the terminal nucleotides potently inhibited RNA synthesis, thus suppressing viral protein translation. The exact mechanism for the overall reduction in viral protein expression, interference of polymerase binding or host factors for viral mRNA translation has not been fully elucidated.
Chapter 3

Production and Validation of a Modified Glycoprotein for Stable Signal Peptide (SSP) Identification and Manipulation

The experimental data presented in this chapter were produced, collected, and analyzed by Lydia H. Bederka. Ms Emily Ling assisted in the development of several plasmid DNA constructs. Dr. Nori Ueno assisted with the flow cytometry experimental setup and data analysis. Data within this chapter is presented in the manuscript by Bederka et al., mBio 2014 Oct 28;5(6).
Abstract

Arenaviruses encode a non-canonical signal peptide that drives the expression and maturation of the entire glycoprotein complex. There exists a shortage of tools and reagents for directly assessing SSP functions. We created and verified the expression of plasmid-derived glycoprotein constructs encoding either a modified SSP both in the context of the entire GPC gene (HA SSP GPC) or with an individual SSP plasmid (SSP-HA). HA SSP GPC expression levels mirrored those of the wild type GPC when assessed by confocal microscopy and flow cytometry assays. SSP-HA, when expressed solely or with the GP1/2 plasmid construct lacking its cognate signal peptide, showed intracellular glycoprotein accumulation and SSP-HA was not able to direct GP1/2 maturation and processing by the cellular SKI-1/S1P enzyme. The HA SSP GPC plasmid we created will allow us to directly study SSP functions from the same translated polyprotein while eliminating the requirement for multi-plasmid transfections.
Introduction

Arenaviruses are elegant in their simple design while being capable of causing severe disease in humans. In nature, these viruses required geographically defined rodent reservoirs (3). Human infections most frequently occur due to close contact with rodent excreta. Several arenaviruses, such as the Old World (OW) Lassa Virus or Lujo Virus, as well as the New World (NW) arenaviruses such as Junín Virus, Machupo Virus, Guanarito Virus, or Sabia Virus, are causative agents of viral hemorrhagic fever (3). Lymphocytic choriomeningitis virus (LCMV), the prototypical and most researched arenavirus, is a neglected human pathogen capable of causing illness spanning non-descript flu-like symptoms, to aseptic meningitis, to multi-organ failure and death in organ transplant recipients from undiagnosed, infected organs (20, 21, 169, 170). Given that the principal rodent host for LCMV is the common house mouse *Mus musculus*, this rodent species allows for the near global distribution of LCMV (171, 172).

Arenavirus infections are initiated by attachment of the viral glycoprotein GP1 subunit to cellular receptor molecules expressed on the plasma membrane (37). The entry mechanism that follows includes a fusion event between the glycoprotein subunits GP2, the stable signal peptide and the cellular membrane within the ensuing acidified endocytic vesicle (173, 174). Fusion of the cellular and viral membranes allows the release of the viral core into the cytoplasm, the site for virus replication (3).

The arenavirus glycoprotein complex is expressed as a polyprotein precursor, which undergoes two cleavage events within the cellular secretory pathway. First, its signal peptide is cleaved from the remaining glycoprotein precursor (GPC) by cellular signal peptidases within the
endoplasmic reticulum (ER). Second, GPC is processed within the Golgi stacks by the enzyme subtilisin kexin isoenzyme 1/Site – 1 protease (SKI-1/S1P) into the GP1 and GP2 subunits (67, 68). The glycoprotein subunits, consisting of SSP, GP1, and GP2 ultimately traffic to the plasma membrane for virion assembly and egress (130, 175, 176). The hydrophobic SSP and the transmembrane domain of GP2 anchor the glycoprotein complex in the membrane and virion bilayer while the GP1 subunit produces the globular spike subunit (16).

The glycoprotein, as its name suggests, is a heavily post-translationally modified protein. Addition of oligosaccharide modifications occurs within the endoplasmic reticulum (ER) during co-translational translocation into the ER lumen and the addition of N-linked sugars is thought to direct and maintain protein folds, as well as antigenicity (177). Studies using VSV-G indicate that oligosaccharide modifications occur almost immediately upon translocation into the ER lumen and are crucial for proteins to attain their proper conformation (178). Many viral glycoproteins, including LCMV, Lassa Virus, Newcastle Virus, Influenza C, and HIV gp120 require N-linked glycoprotein modifications for proper protein structure as well as neutralizing epitope formation (179–184). The LCMV Armstrong-4 strain (Arm-4) GP1 subunit is asparagine-link (N-link) glycosylated at each of its five predicted sites whereas the GP2 subunit harbors N-linked glycans at two of its three predicted sites (16, 179, 185). These post-translational modifications are added to the glycoprotein precursor (GPC) within the endoplasmic reticulum and are necessary for proper protein folding prior to GPC cleavage into GP1 and GP2 (68, 186).
N-glycan modifications function to prevent protein aggregation of hydrophobic motifs by increasing the hydrophilic properties of nascent proteins. Interactions between the nascent protein and the calnexin/calreticulin chaperone proteins, in addition to glycan trimming by glycosidase and mannosidase enzymes are required for proper protein structure formation and protein transport (187-189). Terminally misfolded or misglycosylated proteins undergo ER-associated degradation (ERAD) and are retrotranslocated to the cytoplasm for degradation via the proteasome complex (190, 191).

Proteins destined to be embedded within membranes or to be secreted from cells are targeted to the cellular secretory pathway by a portion of amino acids located at the amino-terminus of these nascent proteins. These signal sequences are generally short segments of variable length that contain common features. The amino terminal region, averaging 15-50 amino acids, is the most variable in terms of it amino acid composition though consists of positively charged, polar residues (192). The central hydrophobic region ranges from 6-15 amino acids and is the most essential portion of signal peptides due to its function for targeting the nascent protein to the ER membrane (193). The carboxy terminal region, comprised of 3-7 amino acids, contains polar amino acids, along with proline or glycine residues, and is recognized by cellular signal peptidase to cleave the signal peptide from the rest of the newly synthesized protein (194).

The arenavirus glycoprotein encodes a non-canonical signal peptide that is highly conserved across both serogroups. This 58 amino acid polypeptide is retained upon signal peptidase cleavage and is an essential subunit of the glycoprotein complex. Limited resources exist for the direct study of SSP, as modifications to this compact GP subunit interfere with glycoprotein
expression. Here, we discuss the development of a full-length glycoprotein containing the influenza HA epitope within the SSP region of the open reading frame. This HA SSP GPC is expressed at wild type GPC levels and allows for the direct study of SSP without requiring transfection of multiple plasmids.

Results

Trimeric glycoprotein complex

The rodent arenavirus glycoprotein complex is expressed as a polyprotein precursor that requires processing by two cellular proteases for proper expression and function. The pre-precursor glycoprotein (pGPC) undergoes processing by cellular signal peptidases to cleave, though retain, the viral signal peptide. Since this cleaved signal peptide is retained for downstream functions, it is termed the ‘stable’ signal peptide (SSP). The glycoprotein precursor (GPC) is post-translationally glycosylated within the ER and traffics, along with SSP, to the Golgi apparatus for further glycan trimming and proteolytic processing by the cellular enzyme SKI-1/S1P (Figure 3.1A and Figure 1.5) (66). This non-covalently interacting subunit trimer traffics to the plasma membrane for packaging within nascent virions (Fig 3.1B).

Potential SSP orientations with lipid membranes

The SSP is 58 amino acids in length and has a peculiar structure given it encodes two predicted hydrophobic domains, whereas canonical signal peptides contain one hydrophobic domain (194). Several independent research laboratories have discussed the proposed SSP topology with respect to both the ER membrane and the plasma membrane (Fig. 3.2). The hairpin orientation with both the N-terminus and the C-terminus residing within the cytoplasm is currently the most
referenced topology used to depict the SSP structure (Orientation A, Fig. 3.2). This data is supported by the myristoylation modification of the amino terminal SSP region in addition to the putative contribution of the carboxyl terminus cysteine residue to the coordination of a zinc molecule with amino acids within the C-terminal domain of the GP2 subunit (195). The SSP is strongly embedded within membranes, as the SSP was unaffected by carbonate extraction and protease digestion, affording varied options for membrane orientation (75, 196, 197).

**SSP is highly conserved**

All rodent arenaviruses encode a SSP with a high degree of amino acid conservation (Fig. 3.3). This implies conservation in functions pertaining to glycoprotein expression and function. The snake arenavirus sequences are excluded from this alignment due to the replacement of an arenavirus-like glycoprotein with that of a filovirus-like glycoprotein (115).

**SSP is required for GPC processing**

Due to the high degree of sequence conservation, we focused our interest on the SSP and its role in glycoprotein maturation. Since there currently does not exist a native anti-SSP antibody for confocal microscopy, as the SP7 antibody only detected SSP under denatured and reduced conditions we generated a carboxyl-terminus epitope-tagged SSP to aid in our studies (SSP-HA) (Figure 3.4A) (75, 79). Additionally, we generated a GPC plasmid construct lacking SSP (GP1/2). Transfection of BHK-21 cells with a panel of glycoprotein plasmids resulted in varied expression. Western blot detection of transfected cell lysates expressing the wild type GPC plasmid yielded the detection of SSP as well as the glycosylated precursor (GPC) and the cleaved GP2 subunit (Figure 3.4B). Cells transfected with a plasmid encoding only SSP failed to
produce a signal via western blot, likely due to ER degradation. Cells transfected with the GP1/2 plasmid lacking its cognate signal peptide, but rather containing a signal sequence from the Influenza A virus hemagglutinin protein for delivery to the secretory pathway, expressed the glycosylated precursor protein but failed to produce a cleaved GP2 subunit. Previous studies from several labs focusing on SSP topology within cellular membranes, as well as the role of SSP in glycoprotein maturation, employed epitope-tagged signal peptides (196, 197). When a tagged version of SSP, expressing the HA epitope at the carboxy-terminus (SSP-HA) was provided in trans with the GP1/2 plasmid, mature GP2 subunit was not detected, indicating the carboxyl terminus of SSP is critical for precursor GPC processing. Mature GP2 detection was rescued upon wild type SSP in trans with GP1/2, indicating SSP is required for proteolytic processing of the GPC precursor (Figure 3.4).

**Insertion of an epitope tag within the SSP region of the GPC open reading frame**

Previous SSP studies made use of epitope tagged constructs with in trans glycoprotein expression (58, 196, 197). We used the full-length SSP-tagged LCMV glycoprotein open reading frame (HA SSP GPC) to detect SSP expression from the same gene as the rest of the GP complex (Figure 3.5A). The HA epitope was inserted within the variable amino-terminus region of SSP, downstream of the myristoylation motif. Previous in trans studies using the New World Junín virus SSP proved amenable to foreign epitope insertions (197). Our HA SSP GPC construct mirrored wild-type glycoprotein expression and GP2 processing (Figure 3.5B). Transfection of the SKI-1/S1P deficient cell line SRD-12B did not yield a cleaved GP2 subunit, indicating the addition of this foreign HA epitope did not alter GPC trafficking through the secretory pathway (Figure 3.6).
SSP required for secretory pathway and plasma membrane trafficking

Using a series of glycoprotein plasmid transfections with secretory pathway markers and confocal microscopy, we were able to follow intracellular glycoprotein transport. The GP1/2 and GP1/2 + SSP-HA samples resulted in glycoprotein accumulation within the ER, as detected by co-staining using the ER marker Grp78 (Figure 3.7). The remaining glycoprotein samples (WT GPC, GP1/2 + WT SSP, HA SSP GPC) resulted in positive Grp78 staining in addition to a defined plasma membrane signal. We also transfected cells with these plasmids and stained cells to detect the Golgi apparatus (Mannosidase II – MannII) and the trans Golgi network (TGN46). Only the WT GPC, GP1/2 + WT SSP, HA SSP GPC samples localized with the Golgi marker (Figure 3.8) and the trans Golgi marker (Figure 3.9), indicating SSP is required for the glycoprotein complex to exit the ER.

SSP detection on the exterior surface of the plasma membrane

In order to determine if our confocal microscopy results were indeed identifying plasma membrane expression, and not the result of a fixation technique artifact, we used flow cytometry to detect glycoprotein expression on the surface of live cells (Figure 3.10). We were able to detect the GP1 subunit, in addition to SSP via the HA epitope, on the surface of transfected HEK 293T cells. The samples which did not result in a signal above background, GP1/2 and GP1/2 + SSP-HA, were the same samples that showed ER accumulation in our microscopy studies and lacked the detection of processed GP2 via western blot analysis. WT GPC, GP1/2 + WT SSP, and HA SSP GPC all yielded comparable levels of surface GP1 subunit expression. Since HA SSP GPC provided both SSP and GP1 detection, this modified glycoprotein is an essential tool to determine SSP functions in terms of GP complex maturation and trafficking.
Discussion

The arenavirus glycoprotein complex, like myriad transmembrane or secreted proteins, requires the cellular secretory pathway for expression, maturation, and transport to the membrane surface for viral egress. The rodent arenaviruses encode a conserved and unorthodox signal peptide that remains stably associated with the remaining portions of the glycoprotein complex after signal peptidase cleavage. The extensive SSP conservation and non-canonical features demonstrate this glycoprotein subunit contains multiple functions.

Generally, signal peptides are short extensions of newly synthesized proteins that are degraded during, or upon, translocation of the holo-protein into the ER lumen (194). Several virus families express glycoproteins with unusual signal peptides. Alphaviruses such as the Semliki Forest Virus and Sinbis Virus encode a 6-kDa peptide (6K) at the Carboxy-terminus region of the envelope precursor protein (198, 199). Deletion of 6K affects virus assembly and budding, glycoprotein-lipid interactions, as well as an unstable spike structure defective for acidic fusion (200, 201). Retroviruses encode an exceptionally long (> 95 amino acids) signal/leader peptide. The Foamy Virus signal peptide mediates envelope protein – capsid protein interactions as well as virus particle release whereas the signal/leader peptide from the Mouse Mammary Tumor Virus leader peptide, Rem, or the Jaagsiekte Sheep Retrovirus is required for nuclear export of unspliced viral transcripts (202-205).

The arenavirus SSP differs from canonical signal peptides in many ways. SSP is retained upon SPase cleavage and becomes an essential glycoprotein subunit. SSP is myristoylated, thus increasing the affinity of this subunit to membranes (206). Additionally, SSP encodes two
regions of hydrophobic residues instead of the traditional one hydrophobic core that plays a
critical role in protein translation. One lysine residue ($K_{33}$) resides between the hydrophobic
motifs and has been experimentally shown to play a critical role in pH-dependent membrane
fusion during viral entry (73). The presence of these two hydrophobic domains affords several
suggestions regarding the manner in which SSP associates with membranes. Using $K_{33}$ as a
reference residue, complementary mutagenic analysis of the GP2 subunit, upstream of and
including the transmembrane domain, provides plausible evidence that the loop region between
the hydrophobic domains resides within the lumen and the exterior surface of virions (207).
Depending on the arenavirus used for each study, various interpretations exist for how SSP is
positioned within membranes (79, 196, 208).

In order to better understand the role SSP plays in the glycoprotein life cycle, we created
epitope-tagged SSP and GPC plasmid constructs. Previous experiments using the carboxy-
terminus tagged SSP with in trans Z protein expression indicated a role for this tagged SSP in
inter-arenavirus protein interactions (58). This SSP-HA, when co-expressed with the remaining
GP1/2 portion of the GP complex, interfered with downstream glycoprotein cleavage. GP1/2 was
post-translationally modified by the addition of N-linked glycans in the absence of SSP,
indicating N-glycan modification does not require the presence of the signal peptide. N-linked
glycans are required for proper GPC folding, which occurs prior to processing within the Golgi
stacks (68, 179).

We sought to develop of a tool that would eliminate the necessity for co-transfection
experiments. Previous studies by Nunberg et al. used an internally tagged SSP plasmid to study
membrane orientation (197). We extended this preexisting concept and incorporated the epitope tag within the context of the entire LCMV Armstrong strain glycoprotein open reading frame. We inserted the epitope at various sites within SSP though the only region that allowed detection of a processed GP2 subunit was the polar amino-terminus. This SP region is the most variable in terms of its amino acid composition, charge, and length – characteristics conducive to allowing modifications (209, 210).

The first site we chose for epitope insertion was near the carboxyl-terminus SPase site, though we did not want to insert an epitope directly at the SPase recognition site due to disruption of the ‘-3, -1’ rule for amino acid composition for SPase recognition (211). Residues at these positions relative to the SPase cleavage site are small with uncharged side chains (Ala, Cys, Gln, Gly, Ser, Thr). Insertion at this first site produced a glycoprotein defective in N-linked glycan modifications. The second epitope insertion site was located upstream of a highly conserved FLLL motif, within the second hydrophobic motif, allowed for a glycosylated, unprocessed precursor GPC. This similar unprocessed GPC phenotype was also produced when the epitope was inserted near the K$_{33}$ residue in the loop region flanking both hydrophobic domains. The third modified GPC encoding the HA epitope within the amino-terminal region of SSP, HA SSP GPC, provided GPC processing and GP2 subunit detection via western blot. We were able to detect the modified SSP from this plasmid using both the SSP antibody (SP7) as well as with anti-HA antibodies.

We tested the expression of HA SSP GPC using the Chinese Hamster Ovary-derived cell line (SRD-12B) defective in the cellular enzyme that cleaves the arenavirus GPC, the SKI-1/S1P.
This cell line was originally developed to study cholesterol regulation, as the SKI-1/S1P enzyme processes the sterol regulatory element-binding protein (SREBP) transcription factor that regulates fatty acid and triglyceride biosynthesis (94). Transfection of SRD-12B cells with the HA SSP GPC failed to cleave GPC without plasmid-driven supplementation of a functional SKI-1/S1P enzyme, indicating insertion of the HA epitope within the GPC gene did not alter the glycoprotein’s maturation pathway. The HA SSP GPC is defective in our syncytium-forming pH-dependent fusion assay, indicating this modified glycoprotein does not support the development of HA epitope-tagged recombinant virus production.

We validated HA SSP GPC expression and trafficking by comparing this protein’s secretory pathway maturation with a panel of other glycoprotein constructs and with flow cytometry for surface detection. HA SSP GPC behaved at wild-type levels in terms of secretory pathway localization as well as SSP and GP1 subunit surface expression. Our flow cytometry data detected the HA epitope on the surface of live HEK 293T cells. These results indicate the SSP amino-terminal region and the first hydrophobic domain are exposed on the extracellular face of the plasma membrane, in disagreement with the most strongly defended hairpin orientation for SSP. Given that HA SSP GPC traffics to the plasma membrane though lacks acidic fusion in our in vitro pH-dependent fusion assay, these results suggest SSP utilizes more than one membrane orientation and the hairpin structure might occur at a late-stage in glycoprotein complex assembly. It is possible the HA epitope, though not interfering with glycoprotein complex maturation and trafficking, might obstruct a late-stage structural rearrangement for SSP in order to produce a fully functional glycoprotein complex.
HA SSP GPC reacts with conformation-specific, neutralizing antibodies, suggesting the HA epitope does not interfere with downstream protein folding. Given this modified glycoprotein has comparable expression patterns with wild type GPC; HA SSP GPC provides a valuable tool to address SSP-related experiments in the context of the equimolar glycoprotein expression and the elimination of in trans transfection experiments.
Figure 3.1. Glycoprotein complex organization
(A) All rodent arenaviruses encode a glycoprotein precursor that undergoes two proteolytic cleavage events for maturation and function. Cellular signal peptidases (SPase) cleave the signal peptide from the remaining precursor, GPC, within the endoplasmic reticulum (ER). The cellular enzyme Subtilisin/Kexin Isoenzyme-1/Site-1 Protease (SKI-1/S1P) cleaves the precursor GPC into GP1 and GP2 subunits within the Golgi apparatus. Note ‘TM’ represents the GP2 transmembrane domain. (B) All three glycoprotein subunits non-covalently interact at the surface of both the plasma membrane and within intact virions. Note the K\textsubscript{33} residue within the SSP.
All rodent arenaviruses share conserved SSP features including myristoylation and the presence of two predicted hydrophobic motifs. The lysine at residue 33 is 100% conserved and plays a critical role in pH-dependent membrane fusion. Included is a summary of the various proposed topologies SSP may employ for membrane arrangement.
Figure 3.3 Arenavirus SSP conservation

Multiple sequence alignment of all current rodent arenavirus SSP amino acid sequences. Viral sequences are grouped according to serocomplex, then further grouped into clades. Red stars indicate human pathogens. Alignment performed using CLC Sequence Viewer 6. ‘X’ indicates ambiguous sequence alignment.
Figure 3.4 Carboxyl-terminus epitope within SSP inhibits precursor GPC processing
(A) Schematic representation of each plasmid construct. The GP1/2 contains the signal sequence from the Influenza A virus hemagglutinin protein. (B) Western blot analysis of transfected cell lysates probed for either the SSP (SP7 antibody) or GP2 (83.6 antibody) subunits. Processed GP2 detection was absent when the SSP contained the HA epitope at its carboxy-terminus. ‘X’ indicates where the nitrocellulose membrane was sliced for antibody incubation. The membrane was reassembled for chemiluminescence processing and image acquisition. WT SSP expression was not detected, indicating its rapid intracellular degradation when not stably expressed with the rest of the glycoprotein complex.
Figure 3.5 Epitope insertion sites affect glycoprotein precursor processing

(A) Stable signal peptide (SSP) amino acid sequence where each star depicts each HA epitope insertion site. The conserved lysine (K_{33}) residue plays a critical role in pH-dependent membrane fusion for viral entry and is located on the exterior face of the virion or within the secretory pathway lumen is highlighted in blue. The schematic indicates the epitope-tagged SSP in the context of the entire glycoprotein open reading frame. (B) Western blot analysis processed GP2 subunit detection from epitope-tagged glycoprotein complex expression. The Site1 GPC resulted in a glycoprotein precursor unable to undergo post-translational N-linked glycan modifications. Site 2 and Site 4 GPC allowed for post-translational modifications though failed yielding GP precursor cleavage. Only the Site 3 GPC (HA SSP GPC) allowed glycoprotein processing, as detected using the GP2-specific antibody 83.6.
Figure 3.6 Insertion of the HA epitope within the SSP region of the glycoprotein open reading frame does not alter glycoprotein processing

(A) SRD-12B (SKI-1/S1P deficient) cells were transfected with a plasmid expressing the functional enzyme (pS1P). After 24h incubation, cells were transfected again with either the HA tagged SSP or the full-length HA SSP GPC, or infected with LCMV Ar4. Cell lysates were collected after additional 24h incubation, separated by SDS-PAGE and probed for the processed GP2 and the SSP subunits. (B) Densitometry analysis of western blot detection of the cleaved GP2 subunit. Each sample was normalized to its respective expression level in the presence of supplemented S1P.
Figure 3.7 ER accumulation of the LCMV glycoprotein lacking a functional SSP
DBT cells were transfected with the indicated plasmid(s). After 48h incubation, cells were stained for the ER (Grp78) and the GP1 subunit. With the two samples allowing SSP detection, cells were stained with Grp78 and HA, followed by an additional overnight staining for GP1 using a GP1 antibody directly conjugated to Alexa fluor 488.
Figure 3.8 LCMV glycoprotein lacking a functional SSP blocks efficient transport to the Golgi apparatus

DBT cells were transfected with the indicated plasmid(s). After 48h incubation, cells were fixed with paraformaldehyde prior to staining for the Golgi stacks (MannII – 594; red) and the GP1 (+ 488; green) subunit. With the two samples allowing SSP detection, cells were stained with MannII (+ 594; red) and HA (+ 405; blue), followed by an additional overnight staining for GP1 using a GP1 antibody directly conjugated to Alexa fluor 488.
Figure 3.9 Lack of \textit{trans} Golgi network trafficking of the LCMV glycoprotein lacking a functional SSP

DBT cells were transfected with the indicated plasmid(s). After 48h incubation, cells were stained for the \textit{trans} Golgi network (TGN46 – 594; red) and the GP1 (+ 488; green) subunit. With the two samples allowing SSP detection, cells were stained with TGN46 (+ 594) and HA (+ 405; blue), followed by an additional overnight staining for GP1 using the directly conjugated GP1 – 488 antibody.
Figure 3.10 Surface expression of LCMV glycoprotein subunits
293T cells were transfected with the indicated plasmid(s) and allowed to incubate for 48h. Live cells were collected for flow cytometry to detect either the GP1 or SSP subunits. Representative histograms from three independent experiments. The values in each upper right quadrant represent the MFI of the noted epitope expression level.
Chapter 4

Controlling Motif for Stable Signal Peptide-Mediated Glycoprotein Complex

Maturation and Assembly

The experimental data presented in this chapter were produced, collected, and analyzed by Lydia H. Bederka. Ms Emily Ling assisted in the development of several plasmid DNA constructs. Dr. Nori Ueno assisted with the flow cytometry experimental setup and data analysis. Data within this chapter is presented in the manuscript by Bederka et al., mBio 2014 Oct 28;5(6).
Abstract

The rodent arenaviruses contain a viral glycoprotein that encodes a highly conserved and multifunctional signal peptide. SSP is the driving force for intracellular and plasma membrane transport of the glycoprotein complex. Mutational analysis of the highly conserved, putative FLLL motif near the SSP carboxy terminus confirms the importance of SSP for GPC processing within the Golgi stacks, surface expression, and pH-dependent membrane fusion. Using immunoprecipitation assays targeting SSP, we confirmed an interaction between SSP and the GP2 subunit. Immunoprecipitation assays directed at the GP1 subunit resulted in the lack of glycoprotein subunit interactions when SSP expressed a mutated FLLL motif. We determined the SSP-encoded FLLL motif functions as an endoplasmic reticulum (ER) exit motif while additionally coordinating mature glycoprotein complex organization at the plasma membrane. Insight into the intracellular mechanism utilized by arenaviruses to produce infectious glycoprotein-encapsulated virus particles will allow for targeted antiviral drug therapeutics directed at various steps within the glycoprotein maturation pathway.
Introduction

Arenaviruses are simple in their design yet complex in the execution of their cytoplasmic life cycle. Both segments of ssRNA express ambisense-encoded viral proteins in a well-coordinated manner. Each of the four viral open reading frames borrow cellular machinery to direct the synthesis of viral gene products which ultimately reconvene at the plasma membrane for virion assembly and viral egress (212). The polymerase and nucleoprotein function in cytoplasmic replication-transcription complexes while NP combats the innate immune response. The matrix-like Z protein controls the level of viral gene expression while packaging each component into budding virions. The glycoprotein complex, freshly modified through the secretory pathway, joins its viral partners at the plasma membrane to ornament the surface of newly assembled virions.

The arenavirus glycoprotein retains a 58-amino acid stable signal peptide for functions downstream of directing the nascent polypeptide to the secretory pathway (74, 206, 213). Specifically, SSP traffics with immature GPC, which undergoes cleavage by the SKI-1/S1P enzyme within the Golgi stacks to yield the GP1 and GP2 subunits (67, 68). The three independent subunits, consisting of SSP, GP1, and GP2 traffic to the plasma membrane for viral assembly and egress (130, 175, 176). These three subunits interact non-covalently at the virion surface to interact with host cell receptors and membranes to initiate infectivity. The GP1 subunit interacts with the known cellular receptors, α-Dystroglycan (α-DG) for the OW arenaviruses or the human transferrin receptor 1 (hTfR1) for the NW viruses (37, 38). The acidification of the ensuing endocytic vesicle induces both the dissociation of GP1 from the remaining subunits followed by a conformational change within the GP2 subunit to reveal its fusion domain. In
concert with SSP, the GP2 subunit induces fusion between the virion and the host membrane to deliver the virion components into newly infected cell cytoplasm (214).

Several virus families utilize the envelope/glycoprotein’s signal peptide for functions aside from directing protein biosynthesis via the secretory pathway. The Ebola virus glycoprotein signal peptide moderates the addition of high-mannose glycans, thereby determining the binding efficiency of the glycoprotein to surface proteins DC-SIGN/R (215). The signal peptide of the HIV-1 envelope protein has been suggested to detain gp160 folding and transport, along with gp160 interactions with the CD4 protein as a method of shielding against immune surveillance or as a means to trigger cell death by protein accumulation within the ER (216). Further, the Jaagsiekte sheep retrovirus envelope signal peptide regulates post-transcriptional nuclear export of full-length viral RNA, gag protein expression, and viral particle release (217-219).

Our current studies focus on the contribution of the signal peptide in terms of nascent glycoprotein expression, maturation, and function. Our study counters previous work with both NW Junín virus and OW LCMV in terms of the importance of the signal peptide for cleavage of GPC into GP1 and GP2 subunits. Further, we focused on the role of a conserved FLLL motif located near the COOH terminus of SSP, upstream from the SPase cleavage site. Using western blot analysis and confocal microscopy, we show that LCMV GP harboring mutations within this FLLL motif accumulate within the endoplasmic reticulum. Two mutants, FALA GPC and YALL GPC, resulted in plasma membrane localization of mature GP complexes, though at levels 50% lower than wild type GP. Further, our studies show LCMV SSP is the driving force for full length and functional GP complex maturation and GP complex organization.
Results

SSP is essential for GPC processing

Previous studies with the LCMV and Junín virus glycoproteins, as well as studies in non-viral systems, have demonstrated a role of basic amino acid motifs for the intracellular transport and processing of pro-proteins into their mature forms (213, 220, 221). Moreover, results from these works showed the importance of dibasic residues within the Junín GP2 cytoplasmic domain for cell surface transport (213, 222). The LCMV GP2 cytoplasmic domain encodes one such predicted dibasic motif. We introduced doublet alanine point mutations to disrupt this motif and assessed the ability of the various constructs to produce a cleaved GP2 subunit, since GPC cleavage into GP1 and GP2 precedes membrane transport. Wild type GPC with the RRAA mutation did not show a significant decrease in protein expression when compared to the non-mutated protein (Figure 4.1). The GP1/2 RRAA mutant protein lacking its native SSP did not produce the cleaved GP2 subunit. When we added wild type SSP in trans with GP1/2 RRAA, cleavage and detection of GP2 was restored, indicating LCMV employs SSP for trafficking and not the dibasic motif.

Based on the western blot results we obtained regarding GP2 processing, we assessed the surface expression of these glycoprotein variants via flow cytometry (Figure 4.1B). The WT GPC RRAA variant resulted in a slight decrease in GP1 expression levels, which were restored to WT GPC levels with the co-expression of wild type SSP. GP1/2 and GP1/2 RRAA lacking an arenavirus SSP, resulted in mock levels of GP1 expression. A partial complementation for surface GP1 expression resulted when wild type SSP was supplemented in trans, indicated SSP and not basic residues within GP2 allow glycoprotein processing and plasma membrane
trafficking. It is possible wild-type surface levels were not achieved with this GP1/2RRAA plus WT SSP condition due to unequal ratios of expression for each glycoprotein subunit. Our results demonstrate SSP plays a dominant role in ER exit for S1P-mediated GPC processing and not the GP2-based motif.

**FLLL motif affects glycoprotein complex cleavage**

The signal peptide is the most heavily conserved protein throughout the *Arenaviridae* family (Figure 3.3) though is partnered with the GP2 subunit for phylogenetic analyses (171). Our laboratory previously showed single point mutations within this region affect glycoprotein infectivity (78). To further investigate the significance of the SSP COOH region for GPC maturation, we focused on the highly conserved hydrophobic putative motif (FLLL) upstream of the signal peptidase recognition signal.

Both single and double point mutations were introduced within this FLLL motif to confirm its importance for downstream GPC maturation (Figure 4.2A, B). Individual F49 amino acid substitutions produced a processed GP2 subunit when substituted with a conservative aromatic tyrosine or with the charged histidine residues. Substitutions with hydrophobic alanine or isoleucine residues resulted in the lack of GPC processing. For both the leucine residues at position L50 and L52, substitutions with phenylalanine resulted in wild type levels of processed GP2. The 50L position was not affected by an alanine substitution though this amino acid at 52L resulted in a processing defect. For both L50 and L52, replacement of the leucine residue with either an aspartic acid or a lysine decreased GPC expression and lacked processed GP2, indicating these positions do not tolerate charged/hydrophilic amino acids. Due to the lack of
sequence conservation in the amino acid occupying position 51, we did not subject this residue to mutagenic analyses.

Double point mutations within this FLLL motif further confirmed our single point mutation results, reinforcing the observation that not one residue within this FLLL motif serves as the major determinant for glycoprotein maturation. We subjected each of the double point mutant glycoproteins to PNGase F digestion in order to confirm N-linked glycan modifications and to assess if mutating this putative FLLL motif might effect this post-translational modification. The AALL GPC was defective in allowing post-translational N-linked glycan modifications. The doublet bands in the AALL GPC lane represent full-length, un-glycosylated GP with our without the SPase-processed SSP. YLAL GPC was defective in expression and was cytotoxic to transfected cells. The ALLA, FALA, and YALL GPC variants all allowed glycosylation of the precursor GPC. ALLA GPC failed to produce a cleaved GP2 subunit, which was observed at varied levels with FALA GPC and YALL GPC (Figure 4.2C).

**FLLL motif mutations affect fusion activity**

We subjected of FLLL motif double point mutations to co-transfections with wild type SSP in order to assess if supplemental SSP would rescue the processing levels of these mutant GPC constructs (Figure 4.3A). The N-linked glycan defective AALL GPC revealed a dominant negative phenotype since supplementation with wild type SSP was unable to rescue neither glycoprotein post-translational modification nor GP2 cleavage. The ALLA glycoprotein supported cleavage and detection of GP2 upon *in trans* addition of wild type SSP. Co-transfection of FALA glycoprotein mutant with wild type SSP, where FALA GPC retaining the
conserved phenylalanine encoded by all known rodent arenaviruses, resulted in a reduced level of GP2 cleavage from the precursor GPC. The same observation was true for the YALL glycoprotein. Consistently, we were not able to detect the YLAL and co-transfection with wild type SSP did not rescue expression of this mutant glycoprotein, indicating this variant also produced a dominant negative phenotype.

**FLLL motif variants are defective in fusion activity**

In order to assess function with these FLLL mutants, we performed a pH-dependent fusion assay, as previously described (78, 185). Transfected cells were subjected to pH = 5 DMEM growth media to induce syncytium formation via conformational changes in the surface-expressed glycoprotein complex. The mutants that allowed for GP2 detection, FALA and YALL GPC, produced various levels of fusion activity. The FALA glycoprotein resulted in one-third the fusion activity compared to the wild type glycoprotein whereas the YALL glycoprotein allowed for about 50% the level of fusion activity (Figure 4.3B). The AALL GPC, YLAL GPC, and GP1/2 variants did not produce fusion activity above mock levels due to lack of GPC cleavage. GP1/2, when supplemented with SSP rescued fusion activity. The ALLA GPC, unable to process and express GP2 unless supplied wild type SSP *in trans*, allowed for a slight increase in fusion activity, though this increase was not significantly above mock background levels.

**FLLL motif variants do not support sustained rLCMV production**

We, and others, have used a plasmid-based technique for the development of recombinant arenavirus production. This technique requires four plasmids: one each encoding both full-length genome segments, including plasmids driving expression of the L polymerase and NP; the
minimum viral factors required for arenavirus genome transcription (Figure 4.4). Only the YALL GPC supported limited recombinant LCMV (rLCMV) production. One-step growth analysis of the rYALL virus compared to wild type rLCMV revealed an initial lag in secreted virus before obtaining viral titers comparable to wild type rLCMV (Figure 4.4). We isolated RNA from each serial passage to monitor the stability of the glycoprotein mutant and discovered the rYALL glycoprotein tyrosine residue reverted to the original phenylalanine by the fourth serial passage to produce a SSP motif consisting of the FALL amino acid sequence. This indicated conserved amino acid substitutions within SSP were not tolerated within this putative motif, giving credence to the importance of the carboxyl portion of SSP for downstream glycoprotein functions.

**Intracellular accumulation of FLLL glycoprotein variants**

We analyzed each double FLLL GPC mutant by confocal microscopy in order to determine the correlation between GPC processing defects and intracellular glycoprotein expression. Co-localization between the FLLL GPC variants and the cellular Grp78 protein indicated the FLLL mutants accumulated within the endoplasmic reticulum (Figure 4.5). WT GPC expression revealed extensive plasma membrane definition, a phenotype shared by only the YALL GPC. YALL GPC also resulted in considerable Grp78 co-localization, indicating a delay in GPC trafficking to the plasma membrane. The FALA GPC variant, which also yielded processed GP2 detection and some fusion activity, resulted in more Grp78 co-localization than plasma membrane detection. The AALL, ALLA, and YLAL GPC samples also yielded ER-retention, though the AALL and the YLAL samples were more difficult to detect (~ 2-3 positive cells total per coverslip).
We further analyzed the intracellular trafficking of these glycoprotein mutants by detecting glycoprotein expression in relation to the Golgi apparatus marker mannosidase II. We were unable to detect AALL GPC expression, while YLAL GPC yielded a faint GP1 signal (Figure 4.6). AALL GPC lacked N-glycan modifications and the appropriate protein folding for GP1 epitope recognition. ALLA GPC produced no Golgi co-localization while FALA GPC resulted in slight Golgi localization. YALL GPC, though resulting in cytoplasmic expression also showed vast peri-nuclear Golgi staining, indicating this GPC variant was able to exit the ER.

**Mutations within SSP affect glycoprotein expression on the plasma membrane**

We used flow cytometry to assess the surface expression of each FLLL GPC variant by flow cytometry using live, transfected HEK 293T cells. In agreement with the microscopy observations, YALL GPC resulted in GP1 surface detection at roughly 50% less than WT GPC levels (Figure 4.7). FALA GPC revealed a shift in fluorescence intensity and produced a three-fold decrease in surface detection compared to WT GPC. The lack of surface staining for the AALL, ALLA, and YLAL GPC samples was consistent with the ER localization and the lack of GPC processing, further supporting the importance of SSP in multiple aspects of the glycoprotein complex life cycle

**SSP is vital for glycoprotein complex association**

We performed immunoprecipitation (IP) assays to investigate the interaction between SSP and the rest of the GP complex, since mutations within SSP result in fusion activity deficiencies and impaired surface transport. Previous studies using GP2-targeted IP showed an interaction between the GP2 subunit and SSP (74, 79). We subjected a panel of point mutations within the
SSP FLLL motif, as well as FALA GPC and YALL GPC double point mutants, to immunoprecipitation assays using both the HA and the GP1-specific antibodies (Figure 4.8A). Cells transfected with wild type GPC, SSP + GP1/2, and HA SSP GPC resulted in properly processed GP2 subunit detection (Figure 4.8B, lanes 2, 4, 6). The GP1/2 missing its native SSP, as well as the co-transfected SSP-HA plus GP1/2, did not result in processed GP2 (Figure 4.8B, lanes 3, 5). In order to directly assess SSP interactions by IP, we introduced single FLLL motif point mutations within the context of the HA SSP GPC open reading frame. The HA SSP 49A GPC point mutant did not result in processed GP2 (Figure 4.8B, lane 7). The HA SSP GPC 49Y, 50A, and 52A point mutants, as well as FALA GPC and YALL GPC double point mutants all allowed GPC processing and GP2 detection (Figure 4.8B, lanes 8-12).

Immunoprecipitation of SSP using HA agarose beads retained its association with the GP2 subunit. The wild type HA SSP GPC immunoprecipitation results, containing only the inserted HA epitope with no FLLL point mutations, revealed the interaction with the GP2 subunit (Figure 4.8C, lane 6). HA SSP 49A GPC lacked glycoprotein processing and GP2 detection (Figure 4.8C, lane 7). HA SSP 49Y GPC and HA SSP 50A GPC point mutants also resulted in varied GP2 subunit immunoprecipitation (Figure 4.8C, lanes 8, 9). HA SSP 52A GPC sample, as well as FALA GPC and YALL GPC, did not reveal any GP2 pull-down product (Figure 4.8C, lanes 10-12). FALA GPC and YALL GPC did not contain HA epitope expression for successful immunoprecipitation and served as additional agarose bead controls. We are not able to confirm if the SSP subunit pulls down the GPC precursor as this GPC band may represent full length, uncleaved SP precursor GPC.
The GP1 subunit was immunoprecipitated using the 2.11-15 conformation specific monoclonal antibody. The only samples to precipitate the cleaved GP2 subunit were the WT GPC, the \textit{in trans} reconstituted GPC, and the HA SSP GPC (Figure 4.8D, lanes 2, 4, 6). None of the SSP single or double point mutant glycoprotein constructs allowed for glycoprotein subunit associations. In no sample did GP1 immunoprecipitate SSP (Figure 4.8D). Further, FALA GPC and YALL GPC did not result in GP2 subunit detection, indicating mutations within the SSP affect proper glycoprotein complex association. For both the HA and the GP1 IP assays, we did not interpret GPC pull-down results as positive IP interactions since the antibodies are capable of interacting with the nascent, full-length, uncleaved precursor glycoprotein.

**Discussion**

Given that the arenavirus signal peptide is an essential component of the glycoprotein complex, our goal was to dissect this subunit’s role in glycoprotein maturation. SSP was not degraded upon translocation into the secretory pathway but rather was retained and trafficked with the rest of the glycoprotein complex to the plasma membrane for viral packaging and egress. The requirement for SSP packaging within virions demonstrates two critical roles for this 58 amino acid polypeptide. First, SSP acts as the leader protein for ER exit and GPC processing within the Golgi stacks, as deletion of either hydrophobic domain inhibits downstream GPC processing (78, 79). Second, SSP is an important structural component within virions as SSP itself matures into a membrane-bound subunit alongside the spike GP1 and transmembrane GP2 subunits as a GP3-like subunit.
Using truncated glycoprotein constructs, previous authors reported SSP is dispensable for GP ectodomain expression (223). The presence of the signal peptide was required for proper, full-length glycoprotein ectodomain processing (224). The results we presented here contrast previous findings as we used, or reconstituted, full-length glycoprotein expression vectors and not truncated plasmid constructs. Our results indicate the LCMV Armstrong (Arm-4) glycoprotein does not significantly depend on a GP2-based basic motif for ER exit, as has been suggested previously (213, 222). Our mutational analysis of the GP1/2 containing a mutated basic motif at the ultimate portion of the GP2 cytoplasmic domain did not result in any cleaved GP2 subunit, indicating this glycoprotein lacking its SSP was not capable of exiting the ER for proteolytic processing. Additionally, our results contrasted with Lassa glycoprotein studies which indicated the GP2 cytoplasmic domain played a role in GP maturation cleavage, though these authors did not use a full-length Lassa glycoprotein in their characterizations and did acknowledge possible SSP contributions (225).

The SSP and the GP2 cytoplasmic domain have been shown to both play critical roles in maturation. Recent studies using reverse genetics permitted infectious, chimeric Lassa/Junín-Candid1 virus production when the glycoprotein SSP and GP2 cytoplasmic domain were homologous and not of mismatched viral origin (226). This observation, together with our flow cytometry and immunoprecipitation experiments, highlights the critical interactions between the SSP and GP2 subunits. Since we only used LCMV glycoprotein constructs for our experiments, it is possible the differing results we observed may be due to slight differences in arenavirus protein expression mechanisms.
In order to determine whether the carboxy-terminal region of SSP is genuinely mediating the intracellular trafficking of the glycoprotein complex, and not a function of the inserted, foreign epitope, we focused on a highly conserved hydrophobic motif (FLLL) upstream of the signal peptidase recognition signal in the context of the full-length glycoprotein open reading frame. As stated previously, SSP is the most heavily conserved protein throughout the *Arenaviridae* family, though is partnered with the GP2 subunit for phylogenetic analyses (171). Results from the expression of the FLLL GPC mutants indicate each residue within this motif plays an equal role in glycoprotein maturation cleavage.

Previous studies reported that single point mutations within this FLLL region, specifically the phenylalanine at position 49, affect both pH-dependent membrane fusion and glycoprotein infectivity (78, 197). Additionally, SSP residues at SSP residues 5 and 50, the later of which resides within this conserved FLLL motif, have been implicated to play roles in LCMV pathogenicity and virus propagation (227). Our microscopy results from the FALA GPC and YALL GPC mutants revealed defects in progression through the secretory pathway. Levels of processed GP2 from the FALA GPC increased with the supplemented wild type SSP, though the presence of additional SSP failed to enhance the fusion activity of the glycoprotein. The reduction in membrane localization exhibited by these mutants at 48h post-transfection indicates an impaired maturation process, as the intracellular staining patterns observed were merely nascent glycoprotein at intermediate maturation stages as transfection efficiencies were comparable in all conditions. Defects observed by mutating the FLLL motif indicate this SSP functions as a sorting signal for secretory pathway trafficking.
With regards to the AALL GPC and the YLAL GPC, these two mutants lacked the ability to produce a glycosylated precursor GPC, thus limiting detection of the GP1 subunit by the conformation-dependent GP1 antibody. Results from the GP1/2 expression, where the glycoprotein completely lacks its native SSP demonstrated SSP was not required for the post-translational N-linked glycan modification. AALL GPC and YLAL GPC produced a defective, non-glycosylated, and non-functional protein whereby the presence of \textit{in trans} wild type SSP was unable to rescue downstream cleavage and transport. These two GPC variants, especially the YLAL GPC, were cytotoxic, likely due to the induction of ER stress responses as these two mutant GPCs lacked the ability to traffic to the Golgi stacks for precursor processing.

\textit{In trans} SSP expression was able to moderately rescue ALLA GPC processing since this mutant GP did produce a glycosylated precursor, allowing SSP to provide some chaperone function to an otherwise able (glycosylated) precursor. Based on our fusion assay results, the levels of processed GP2 from the co-transfection of wild type SSP with the ALLA GPC were not enough to rescue a significant amount of functional glycoprotein. For the FALA GPC and the YALL GPC mutants, the decrease in fusion activity with \textit{in trans} wild type SSP indicated a defect in SSP-mediated transport. This fusion activity defect was likely mediated by ER stress due to unequal ratios and overabundance of SSP relative to the rest of the glycoprotein complex. Insertion of the FLLL double point mutations within the HA SSP GPC construct was detrimental to GPC expression though single point mutations within this motif were tolerated. These mutations, while allowing SSP to retain its association with the transmembrane-spanning GP2 subunit, obstructed the interaction with the remaining extracellular spike GP1 subunit.
Proteins secreted from the ER require proper quality control approval for protein folding, as ER chaperone proteins and incompletely folded proteins are excluded from COPII-coated vesicle exit sites for anterograde transport (228). ER-retention due to improperly folded proteins results in the degradation of an estimated 30 – 50% of newly synthesized proteins, with some protein degradation occurring within 20-30 minutes of newly synthesized or still nascent polypeptide chains (228-231). This degradation allows cell surface presentation of peptide antigens in the context of major histocompatibility complex-I (MHC-I) during normal cell homeostasis as well as with viral-infected cells (232). This protein degradation phenomenon applies to LCMV, as various levels of GPC degradation provide the potent SSP – derived CTL epitope gp33 (SSP residues 33-41), packaged with MHC-I via the transporter associated with antigen presentation (TAP) – dependent pathway (64). Though we did not use proteasome inhibitor treatment with our FLLL GPC experiments, the lack of GPC expression observed by some of these variants may indicate protein degradation as an option for the lack of GPC processing and trafficking.

If SSP uses the hairpin topology for membrane orientation, the FLLL motif would localize either within, or juxtapose with, the membrane. The mutational analysis we show here indicates this motif functions as a non-canonical sorting signal for ER exit. Further, this FLLL motif resembles a combination of two different sorting motifs, the tyrosine-based motif consisting of YXXΦ sequence (where Φ is any hydrophobic residue), and the di-leucine motif (E/D-XXX-LL/I). These motifs generally reside within the cytoplasmic domains of integral/transmembrane proteins and mediate endocytic or lysosomal trafficking via the clathrin network (233, 234). Arenaviruses, being elegant opportunists, may have acquired their own use of these components of ER exit motifs for their advantage.
The severe acute respiratory syndrome (SARS) coronavirus 3a protein encodes an YXXΦ motif within its cytoplasmic tail that when mutated, results in increased protein accumulation within the Golgi and lipid droplets (235, 236). The measles virus glycoproteins, the hemagglutinin and the fusion protein, as well as the HIV-1 envelope glycoprotein, use YXXΦ motifs for basolateral transport and viral spread (237-239). Moreover, the HIV-1 Nef protein uses di-leucine motifs, which preferentially direct proteins to the lysosome, to connect the adaptor protein AP-1 with the tail of the CD4 protein in order to down-regulate surface expression of CD4 (240). Our FLLL GPC experiments were based on cell culture-based transient transfections, meaning the translated glycoproteins were not present for the entry and endocytosis steps where the YXXΦ and di-leucine motifs typically function. Instead, the level at which this LCMV FLLL motif functions was downstream of entry and within the secretory pathway for ER exit. There are always exceptions to rules and variations to themes. A non-canonical di-leucine motif example is the cellular NKCC2 protein. This Na-K-2Cl co-transporter regulates sodium homeostasis and blood pressure, and a naturally occurring cytosolic tail domain deletion leads to the kidney disease Bartter Syndrome (241). NKCC2 encodes several di-leucine motifs that when disrupted present ER exit defects as well as disruption in the protein’s glycosylation and cell surface expression. As with LCMV, the mechanism used by NKCC2 to exit the ER is not fully understood.

We have shown that mutations within SSP affect the downstream GPC cleavage by the cellular SKI-1/S1P enzyme, a processing event that occurs within the Golgi stacks. Cargo vesicles originating at ER exit sites, laden with COPII coat proteins, must recognize properly folded proteins before appropriate secretory pathway trafficking may ensue. A possible explanation for the altered trafficking we observed with the FLLL GPC variants is the inability of COPII
machinery to recognize the mutated FLLL motif, since exit cargo proteins harbor conformation-dependent epitopes for efficient packaging into ER-Golgi transport vesicles (242, 243). If anterograde transport via COPII vesicles plays a role in LCMV GPC maturation, inhibition or expression of dominant negative versions of COPII subunits (the small GTPase Sar1, Sec23/24, Sec13/31) would demonstrate a block in transport by measuring the extent of, or absence of, GPC processing.

This study highlights the importance of this often-overlooked 58-amino acid protein in orchestrating the transport and production of functional non-covalently interacting trimeric glycoprotein complexes. Components of all three glycoprotein subunits play a role in the proper glycosylation and proteolytic processing of the glycoprotein complex (224). Without SSP, the glycoprotein precursor is retained within the endoplasmic reticulum and is not capable of continuing its passage through the secretory pathway. Immunoprecipitation of both the SSP and the GP1 subunits revealed SSP is the subunit that stabilizes the organization of the trimeric glycoprotein complex. Whole genome siRNA screens have implicated the involvement of the COPI subunit for LCMV infection (124). Furthermore, proteomic analysis led to the identification of the ERGIC-53 as a host factor involved in the production of infectious particles (125). Future experiments using the tools presented here may allow for the identification of host proteins that interact with SSP. A deeper understanding of the mechanism by which this family of viruses involves host pathways would define targets for antiviral therapeutics.
Figure 4.1 SSP mediates GPC processing and surface transport
Transfected BHK-21 cell lysates were probed with antibodies directed against the GP2 (83.6) and SSP (SP7) subunits. The ‘X’ indicates the nitrocellulose membrane was sliced for antibody incubation and then reassembled for chemiluminescence processing and image acquisition. (B) Detection of the GP1 subunit by flow cytometry using live, transfected HEK 293T cells incubated with the GP1-specific 2.11-10. Gray shaded cells indicated mock IgG control population. The values located in the upper right corner of each histogram represent the mean fluorescence intensity (MFI) of GP1 expression. Shown are representative data from one of three independent experiments.
Figure 4.2 Mutations within SSP affect glycoprotein complex processing

Transfected cell lysates were analyzed for processed GP2 via western blot using denatured and reduced conditions. (A) Individual point mutations within each of the conserved FLLL motif residues. (B) Western blot analysis of GPC constructs encoding double point mutations within the SSP. (C) PNGase F treatment of double point mutant transfection lysates. Note the shift in molecular weight for each glycoprotein, GPC and GP2.
Figure 4.3 Fusion activity defects from FLLL motif-mutant glycoprotein complexes
(A) Western blot analysis of the partial rescue of GPC processing when WT SSP is supplied in trans. (B) Quantification of fusion activity with indicates in trans supplementation of SSP does not rescue FLLL GPC variants’ pH-dependent fusion activity deficits.
Figure 4.4 Modifications to SSP do not support recombinant LCMV production
Schematic representation of the transfection-based technique used for the generation of rLCMV particles. Included is a one-step grown curve comparing viral titers of wild type rLCMV to the YALL viral variant.
Figure 4.5 Intracellular accumulations of FLLL glycoprotein variants
Confocal microscopy detecting the endoplasmic reticulum (Grp78) in relation with the detection of the GP1 subunit from cells transfected with each indicated plasmid.
Mannosidase II

Mock GP1

WT GPC GP1

FALA GPC GP1

ALLA GPC GP1

YALL GPC GP1

YLAL GPC GP1
Figure 4.6 FLLL glycoprotein variants show defects with ER exit
Confocal microscopy detecting the Golgi apparatus protein mannosidase II (MannII) in relation to detection of the GP1 subunit from each transfected glycoprotein sample.

Figure 4.7 Variations in glycoprotein surface expression with FLLL variants
Flow cytometry analysis of GP1 subunit expression on the surface of live HEK 293T cells. The values in the upper right quadrant of each histogram represent the MFI of GP1 subunit expression.
Figure 4.8 Mutations within SSP affect glycoprotein complex association
(A) Schematic representation of the modified GPC constructs used in this immunoprecipitation assay. (B) Lysates from 293T cells transfected with glycoprotein complex plasmids harboring either single or double point mutations within the SSP region of the GP open reading frame were analyzed for GP complex processing and subunit detection via western blot. The single point mutation constructs are in the context of the internally HA-tagged GPC open reading frame while the FALA and the YALL double point mutants are within the untagged GPC open reading frame. (C) Western blot analyses of HA agarose bead immunoprecipitation. (D) Immunoprecipitation results to determine GP1 subunit interactions were analyzed by western blot.
Chapter 5

Future Directions

The experimental data presented in this chapter were produced, collected, and analyzed by Lydia H. Bederka in collaboration with Dr. Giselle Knudsen and Dr. Joseph DeRisi at the University of California, San Francisco. Additional recognition for the UCSF Bio-Organic Biomedical Mass Spectrometry Resource must be acknowledged for this section.
The purpose of this dissertation was to elucidate functions of SSP downstream of secretory pathway translocation. Our studies confirm SSP as a critical component necessary for several aspects of the glycoprotein life cycle. Mutagenic analyses of the highly conserved FLLL motif provide a compelling argument for SSP’s role in mediating the entire glycoprotein complex’s exit from the endoplasmic reticulum. The HA-tagged glycoprotein plasmid (HA SSP GPC) tool we developed allowed us to directly target SSP for experimentation, since SSP reagents are not readily available. This plasmid allows for future glycoprotein studies to build upon the data we presented in this dissertation, as there are questions that remain to be answered, in addition to limitations that must also be acknowledged.

One limitation to the HA SSP GPC tool we developed is its defect in acidic pH fusion, thus not enabling the development of infectious rLCMV particles. Though this glycoprotein complex allowed surface expression of both the SSP and GP1 subunits, it remains to be determined if this glycoprotein organizes into trimeric complexes similar to those observed for the wild type glycoprotein (72). Studies using glutaraldehyde cross-linking and circular dichroism spectral analysis, which were used to determine the trimeric state of the wild type LCMV glycoprotein, would shed light on the ability of HA SSP GPC to oligomerize in a manner akin to the unmodified glycoprotein complex. Additionally, we do not know if HA SSP GPC is capable of forming virions or virus-like particles. It remains to be determined if the HA epitope-induced fusion inhibitory phenotype we observed would interfere with GPC encapsidation or the interaction with the Z matrix protein, either in the context of recombinant virus or virus-like particle production, which is presumed to biochemically interact with SSP (58). If no interaction
between SSP and Z occurs, one may speculate the HA tag interfered with a very late event for the structural rearrangement of SSP.

One unanswered question that remains is the actual membrane topology SSP employs to drive its myriad functions, as SSP structural data is not available. Of the limited arenavirus glycoprotein structures available within the Protein Data Bank, all have been solved using truncated plasmids encoding modified versions of either the GP1 or GP2 subunit (127, 195, 244). One structure of the GP2 subunit in its post-fusion state, though lacking the presence of SSP, was expressed in a HEK 293 variant deficient in GlcNAC transferase and provides in-depth detail regarding the glycocalyx-type shield N-linked glycans employ to surround the GP2 subunit (245). SSP-inclusive structural data would aide in our understanding of the mechanics behind the glycoprotein complex’s assembly in addition to providing insight for the development and fine-tuning of small molecule inhibitor and antibody targets (246).

Though I personally do not have any structural biology background, the information gained from this field would shed light on several biological processes involved in the glycoprotein life cycle. The solution structure of the zinc-binding GP2 domain solved using solution nuclear magnetic resonance (NMR) relied on a 40 amino acid GP2 synthetic peptide (195). One means to attempt structural characterization of the SSP-included glycoprotein complex would be to use the amphipol tool to stably maintain the transmembrane subunits. Amphipols are amphipathic polymers that have the potential to maintain transmembrane proteins in solution without the added inclusion of detergents (247). Research from Dr Melanie Cocco and colleagues used amphipols to solubilize and test the *Chlamydia trachomatis* major outer membrane protein in a
vaccine candidate study (248). It would be interesting to see if this detergent-free approach to stabilizing the glycoprotein complex would prove feasible with techniques such as solution NMR, for which structural analysis of β-barrel proteins has proved successful (247, 249). It remains to be determined if this system work with the LCMV GPC. If the amphipol technology is amenable to arenavirus glycoprotein analysis, this system may provide a rewarding project not so much as a vaccine candidate but in terms of the potential structural information that would be the fruit of this research.

Structural insights as to SSP membrane topology may also reveal the location of the FLLL motif. The current hairpin model for SSP orientation suggests the carboxyl terminus is located within the cytoplasm while the K33 residue is in the ER lumen, or external face of the plasma membrane. Given there are only 16 amino acids separating the K33 residue from the FLLL motif, one might predict the second SSP hydrophobic domain (along with the first hydrophobic domain at ~17 amino acids) does not contain enough amino acids to form a helical transmembrane domain. If the hairpin model were in fact correct, one would predict an antiparallel pair of beta sheets to form the hairpin topology. There also exists the possibility SSP would reveal an unusual/novel fold, as was the case with the analysis of the NW Machupo virus GP1 subunit (244).

An additional unanswered question pertains to how the stable signal peptide’s FLLL motif mediates intracellular trafficking for SKI-1/S1P processing. Proteomic analysis using the NW Junín virus glycoprotein identified the requirement of the ERGIC-53 protein for arenavirus infectivity (125). Interestingly ERGIC-53, which functions as an ER cargo protein receptor, was
not shown to be involved in glycoprotein processing (125). This is curious because arenavirus glycoproteins must traffic from the ER to the Golgi for proteolytic processing, so the lack of ERGIC-53 assistance implies the existence of an unidentified factor for glycoprotein processing.

We attempted to answer the question regarding SSP’s involvement with intracellular trafficking by performing mass spectrometry (MS) analysis from our SSP immunoprecipitation experiments. One MS target we pursued was the surfeit locus protein 4 (Surf-4). This protein functions as a cargo receptor for ER to Golgi trafficking (250). We hypothesized this protein may play a role in LCMV GPC trafficking and performed siRNA knockdown assays targeting human Surf-4. Our knockdown experimental system entailed a 72h knockdown, of which the last 24h involved infection with the LCMV to allow one virus replication cycle. We collected the supernatant to quantify viral titers to determine if knocking down Surf-4 levels affected the amount of virus released into the supernatant. Additionally, we collected the cell lysates to analyze protein expression levels. Viral titer, protein expression levels, in addition to GPC processing were unaffected by Surf-4 siRNA treatment, though we were not able to detect Surf-4 protein expression due to poor antibody quality. We used two anti Surf-4 antibodies, one commercially available and one from a laboratory-produced (a published reagent), for this analysis and our data were inconclusive due to lack of quality reagents.

Our second MS target was the clathrin heavy chain (CHC). Based on electron microscopy images of LCM virions within smooth-welled vesicles, it was accepted that LCMV entry involved a clathrin-independent mechanism, though other arenaviruses employ clathrin-mediated endocytosis (40, 174, 251). We hypothesized the robust mass spectrometry fragment hits we
obtained for CHC would implicate its role in GPC intracellular trafficking within the trans-Golgi network. We performed the same knockdown strategy: 72h siRNA knockdown of the clathrin heavy chain, the last 24h of which the cells were infected with LCMV. Western blot analysis of lysates revealed the total suppression of the GP complex, indicating clathrin heavy chain knockdown blocked expression upstream of our predicted step in the glycoprotein life cycle (Figure 5.1). We expected CHC knockdown to either block GPC processing (indicating a block in ER to Golgi trafficking) or to show no difference in GP2 levels, indicating GP2 expression would not be affected but rather the ability of the GP complex to localize to the plasma membrane would be blocked. Clathrin heavy chain knockdown resulted in a log-fold decrease in viral titer, which was rescued to levels near mock treatment with the addition of plasmid-encoded clathrin heavy chain (Figure 5.2). The ‘global’ suppression in GPC expression indicates GPC translation was inhibited; implying the knockdown of CHC indeed interfered with LCMV entry.

Understanding the mechanism by which arenaviruses exit the ER for proteolytic processing within the Golgi by SKI-1/S1P would provide a broad-spectrum, anti-arenavirus target for novel therapeutics, given that SKI-1/S1P processing is conserved across all rodent arenaviruses. As mentioned previously, understanding how the signal peptide FLLL motif mediates secretory pathway trafficking will clarify this biological process. We tested the effects of glycoprotein expression in the presence of a dominant negative form of the small GTPase Arf1 (T31N) to assess how Golgi disorganization would affect glycoprotein processing. The Golgi protein Arf1 plays an essential role in the recruitment of coatamer (COP) proteins for ER to Golgi export, as well as in maintaining Golgi structure. The dominant negative mutant, T31N, has an affinity for
GDP and inhibits ER export and releases β-COP to the cytoplasm (252). Also, T\textsubscript{31}N expression causes Golgi structures to disappear and to re-localize to ER exit sites (253). We transiently transfected these plasmids, then infected the cells with LCMV, alongside our CHC knockdown experiments. Plaque assay results from the T\textsubscript{31}N-transfected, LCMV-infected sample did not result in a decrease in viral titer when compared to the wild type Arf1 condition. Interestingly, these two samples resulted in a slight increase in viral titer (Figure 5.2). Western blot analysis of lysates from both Arf1 transfected + infected conditions showed no difference in GPC or GP2 expression. If T\textsubscript{31}N expressing cells do not interfere with glycoprotein (and virus) production, the question of how the glycoprotein complex traffics beyond the ER remains to be answered.

Arenaviruses possess several unique features, so it would not seem improbable for the mechanism used by the GP complex to exit the ER to be peculiar. Though the arenavirus glycoprotein precursor is cleaved by the cellular SKI-1/S1P, the Golgi compartment where this processing event occurs is not the same for each viral glycoprotein and is dependent on the pH optimum for each individual substrate (68, 92, 254, 255). Studies comparing the Lassa and LCMV GPCs showed Lassa GPC processing occurred in the cis/medial Golgi whereas LCMV GPC is processed in the late Golgi or in a post-Golgi compartment (68). If proposed experiments looking at SSP-mediated ER exit with Sar1/COP vesicle-mediated transport do not show an interaction, an explanation for such results would suggest, at least for LCMV, this glycoprotein does indeed uses a novel mechanism for bypassing cis Golgi compartments and that this sorting is mediated by SSP with the assistance of unidentified cellular factors.
The interaction between arenavirus glycoproteins and SKI-1/S1P for effective processing remains nebulous. One further unanswered question is if processing of these viral glycoproteins requires an unidentified cellular factor(s). The most well studied cellular substrates processed by the SKI-1/S1P enzyme interact with a cofactor: SREBP + SCAP and ATF6 + BiP (90, 256). We initially hypothesized SSP would fit this cofactor model, though based on immunoprecipitation assays, we were never able show any direct association between SKI-1/S1P and any of the LCMV glycoprotein subunits (data not shown). Moreover, we did not detect any SKI-1/S1P peptide fragments upon analysis of our mass spectrometry results.

Even though my experiments focusing on this last question, on SKI-1/S1P – GPC interactions, were inconclusive this research focus should not be ignored. The most widely used cell line for approaching this question, derived from Chinese Hamster Ovary cells (CHO) and termed SRD-12B, are defective in SKI-1/S1P processing. These cells did yield efficient transfections. Upon examination of transfected SRD-12B cells by immunofluorescence microscopy we regularly detected less than 10 positively transfected cells within one entire well of a 6-well plate (data not shown). This made transfection + infection experiments, where we transfected these cells with a functional and tagged version of SKI-1/S1P followed by LCMV infection, quite difficult and produced unreliable and inconclusive results.

Though these, and our mass spectrometry-based target, results did not yield our desired products, this does not mean SSP-based experiments should be forgotten or discontinued. Sometimes research aims do not produce the desired data though research should progress while noting the progress that has been made. I believe the stable signal peptide is a small, yet mighty, force to be
reckoned with and you should not disregard things based on their small size or presumed insignificance.

The HA-tagged GPC plasmid construct I developed will continue to serve as a means for SSP detection and manipulation and may complement novel reagents for the direct detection of SSP. The use of this modified glycoprotein open reading frame allowed for detection and manipulation of SSP in the context of the entire GPC gene. This plasmid construct will continue to provide a tool for dissecting SSP roles for intracellular trafficking. The mutagenic analyses I performed focusing on the putative FLLL motif solidify SSP as a bona fide contributor for glycoprotein complex trafficking. Remaining questions for SSP roles in this process reside in the protein interactions SSP mediates and the mechanism by which SKI-1/S1P processes the GPC precursor. Based upon my observations, future experiments targeting cellular factors for secretory pathway trafficking, such as those involved in ER exit and vesicle transport, would contribute to our understanding of the mechanisms for intracellular arenavirus glycoprotein maturation.

At 58 amino acids, SSP is truly remarkable. I have yet to be convinced this polypeptide only interacts with the other glycoprotein subunits. Given that SSP controls the ability of its larger GPC partner to properly mature and function, I am under the impression SSP may play a polyfunctional role in terms of the multiple protein interactions that must occur in order for the entire glycoprotein complex to traffic from the ER to the plasma membrane. The interactions that mediate ER to Golgi trafficking for SKI-1/S1P processing may possibly be different from those required for glycoprotein trafficking to the plasma membrane. As new tools, reagents, and
techniques become available, I look forward to reading about and discussing future discoveries with the arenavirus (and the general scientific) community.
Figures

Figure 5.1 Suppression in siRNA-mediated protein expression only with knockdown of clathrin heavy chain
(A) Western blot detection of glycoprotein expression from cells treated with the siRNA pool targeting the human Surf-4 protein and infected with LCMV at MOI = 0.1.  (B) Analysis of clathrin heavy chain siRNA-mediated protein expression knockdown. We also treated cells with siRNAs targeting the clathrin light chain (siCLC) or plasmids encoding the Golgi GTPase Arf1 or its dominant negative mutant T31N.  The lines in between each detected protein indicate where the nitrocellulose membrane was sliced for antibody incubation. Membranes were reassembled for chemiluminescence processing and image acquisition.
**Figure 5.2 siRNA Knockdown of CHC, though not of Surf-4, Reduced Viral Titers**

(A) HEK 293T cells were treated with siRNA pools targeting the human surf-4 protein, or transfected with the plasmid encoding the wild type human Surf-4 protein. Treated cells were infected with LCMV at MOI = 0.1 48h post treatment. (B) siRNA or plasmid treatment targeting the clathrin heavy chain. As controls, we treated cells with siRNAs targeting the clathrin light chain (siCLC) or plasmids encoding the Golgi small GTPase Arf1 or its dominant negative mutant, T31N. Supernatants were collected at 24h post-infection, which totaled a 72h siRNA/plasmid DNA incubation. Viral titers were determined by plaque assay using Vero E6 cells Statistical analyses indicate P<0.05 (*) and P<0.001 (**).
Concluding Remarks

Graduate school has been an equally challenging and rewarding experience. The years I spent at the University of California, Irvine taught me to think critically and to have contingency plans for purposes such as alternative experiments/approaches and data interpretations. The crux of my tenure in the Buchmeier Laboratory was spent on trying to figure out why the glycoprotein’s stable signal peptide is significant for the virus life cycle. We knew SSP was important for the production of a functional glycoprotein and for virion infectivity, but to this day, SSP remains an overlooked and underappreciated viral factor.

My motivation was to figure out how SSP factored into the overall process for the production of a mature glycoprotein complex. For this, I started at the beginning by cloning the full-length GPC gene from infected cell RNA. I then inserted an epitope tag within the SSP region of this ORF to be able to detect SSP using various experimental means. Using genetic, microscopic, and biochemical approaches, the data I have published and presented here demonstrate that the 58-amino acid SSP plays a vital role in several aspects of the glycoprotein’s intracellular maturation pathway. Not only did the tools I develop allow for SSP detection, but also I defined an unorthodox motif that coordinates proper glycoprotein complex maturation.

This data is significant because SSP is highly conserved across the entire viral family, and thus serves as an attractive drug target. I hope the tagged GPC plasmid construct I developed will be used for future structural studies with the hope of obtaining useful information regarding how SSP orients itself within the membrane as well as its orientation in complex with the GP2
subunit. Structure-based rational antiviral development, along with inhibitors targeting the genome terminal promoter element, and the Z protein’s inhibition of RNA synthesis properties, would serve as targets for an anti-arenaviral drug cocktail.

Further, the tools I developed have the potential to unravel more of the unanswered questions regarding the cell biology of the glycoprotein’s maturation. The means by which GPC exits the ER and its intracellular trafficking pathway is still nebulous. We know SSP plays a critical role in this whole process but the specific mechanism remains to be elucidated. One unanswered question is if SSP directly interacts with cellular cargo proteins for ER exit as well as intracellular trafficking. Since SSP-less glycoprotein precursors lack localization with Golgi markers or surface expression, determining the contribution of cellular processes involved in trafficking would help the arenavirus community to define the intracellular pathway used for the production of mature viral glycoproteins.

The carboxyl terminus, and specifically the FLLL motif, of SSP is critically important for the intracellular processing and trafficking of the precursory GPC. An additional remaining question is whether blocking the C-terminus interferes with SSP packaging into virions. Additionally, packaging of HA SSP GPC into virions, or the interactions with the arenavirus matrix-like Z protein, remains to be determined.

Arenaviruses do not cause significant cell damage so the cellular factors that are borrowed for the benefit of viral protein production will allow for a deeper understanding about general cellular functions. In this way, studies focusing on this multifaceted 58 amino acid polypeptide
will contribute to our understanding of biology. Since all rodent arenaviruses share this conserved stable signal peptide, this attractive drug target would contribute to the antiviral arsenal in addition to the maintenance of the public’s health.
Materials and Methods

Cells and Viruses

For the P-PMO studies Vero-E6 cells were maintained in 1× Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 5mM L-glutamine, and 10mM HEPES buffer at 37°C, 5% CO2. For experimental use, Vero-E6 cells were incubated with serum-free media (VP-SFM – Gibco).

For the subsequent experiments, Baby Hamster Kidney-21 (BHK-21) cells, mouse Delayed Brain Tumor (DBT) astrocytoma cells, Human embryonic Kidney 293T (HEK-293T) cells, and African green monkey (Vero E6) cells were maintained in 1X DMEM, 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine at 37°C, 5% CO2. The lymphocytic choriomeningitis virus (LCMV) Armstrong-4 (Arm4) strain was used in this study (101, 257). The LCMV Armstrong strain 53b was used for P-PMO experiments. Viral stocks were propagated using BHK-21 cells and viral titers were determined via plaque assays using Vero E6 cells (143).

The SRD-12B cell line deficient in the S1P (SKI-1/S1P) enzyme, derived from Chinese Hamster Ovary (CHO-K1) cells by random gamma irradiation and rescue by supplemented media, was kindly provided by the Brown & Goldstein laboratory from The University of Texas Southwestern Medical Center (94). Cells were maintained in growth media consisting of 50% DMEM-Hams’ F12 supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, 5% fetal calf serum, 5 µg/mL cholesterol (Sigma), 1mM sodium mevalonate (Sigma), 20 mM sodium oleate (Sigma). Cells were allowed to incubate at 37°C, 8% CO2.
Antisense P-PMO Synthesis

We used modified compounds containing a cell penetrating peptide conjugated to the phosphorodiamidate morpholino oligomers (P-PMO) produced at AVI Biopharma, Inc. (Corvallis, OR) and previously described (150, 258). These P-PMO compounds contain an arginine-rich peptide to enhance cellular uptake and share sequence complementarity to the 5’ terminal 19 nucleotides conserved amongst arenaviruses. One of the P-PMOs we tested also contained an additional cytosine residue, complimentary to the non-template guanine modification for arenavirus genome replication (31, 34).

Cytotoxicity Assay

Vero E6 cells were incubated with VP-SFM (Gibco) supplemented with 1% (v/v) antibiotic/antimycotic (Gibco), 5 mM L-glutamine, and including increasing concentrations of P-PMO. After incubating for 24h, 200uL of a 5mg/mL MTT (3-4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide – Sigma) was added to each sample and allowed to further incubate for 40 min at 37°C, 5% CO₂. Supernatants were removed and cells were solubilized with 1mL DMSO. Triplicate sample aliquots were transferred to 96-well plates and photometric absorbance (560 nm) readings were taken using the Victor³ plate reader (Perkin Elmer).

P-PMO Treatment and Viral Infection

Vero E6 cells were pre-treated with various concentrations (0.5 uM – 20 uM) P-PMO in VP-SFM for 3h prior to virus infection. LCMV Armstrong 53b MOI = 0.01 was allowed to adsorb for 1h, then washed once using 1× PBS before adding fresh VP-SFM supplemented with the
same P-PMO concentrations. At 24h and 48h post infection, supernatants were collected to
determine viral titers while cell lysates were collected to assess viral protein expression.

**Plaque Assay**

Vero E6 cells were plated onto 6-well plates. Supernatants were 10-fold serially diluted using
VP-SFM and 1mL inoculum was added to each well. After 1h adsorption with occasional
rocking, inocula were removed and replaced with 3mL plaque media consisting of equal parts 2×
DMEM, 2% FBS, 1% penicillin/streptomycin, 50mM HEPES mixed with 1.4% SeaKem agarose
(Lonza) for a final 1× DMEM, 0.7% agarose overlay. Plates were incubated for 4 days at 37°C,
5% CO₂ prior to overnight fixation using 10% neutral buffered formaldehyde. Agarose plugs
were removed and cells washed three times with 1× PBS and stained with 0.05% crystal violet.

**Western Blot**

For the P-PMO studies: LCMV-infected Vero E6 cells were harvested with 1× PBS, 1mM
EDTA on ice. Cell pellets were trituated using 1% NP-40 lysis buffer (20mM Tris pH = 7.6,
140mM NaCl, 5mM MgCl₂) containing protease inhibitors (RPI Corp). Protein concentrations
were estimated using a standard BCA kit following the manufacturer’s protocol (Thermo).
Samples were loaded onto pre-cast 12% Tris-glycine SDS-PAGE gels (Life Technologies) and
separated under reduced and denatured conditions. Lysates were transferred to nitrocellulose
membranes and blocked with 5% non-fat milk blocking buffer before overnight incubation with
primary antibodies targeting the LCMV glycoprotein GP2 subunit (mouse anti-83.6) or the
nucleoprotein (mouse anti-1.1-3) (257). Blots were processed using alkaline phosphatase reactive
secondary antibodies and visualized using the Bio-Rad ChemiDoc XRS system. Membranes were then stripped with 0.2M NaOH for 10 minutes, and re-probed for actin (Millipore).

For subsequent studies: Transfected cells were incubated for 48h, washed with 1× PBS + 1mM EDTA, collected (600 × g, 4°C, 15 min) and lysed on ice with 1% NP-40 lysis buffer plus complete protease inhibitors (RPI Corp). Clarified lysates (10 000 × g, 4°C, 15 min) were separated under reduced and denatured SDS-PAGE conditions and transferred to nitrocellulose membranes. For LCMV glycoprotein complex detection the anti-GP2 (83.6) and anti-SSP (SP7) antibodies have been described previously (75, 259). The anti-HA (Sigma), anti-clathrin heavy chain (Abcam), anti-actin (Millipore), goat anti-mouse HRP, and goat anti-rabbit HRP (Jackson ImmunoResearch) antibodies were also used. Blots were processed using the Amersham ECL Prime detection reagents (GE Healthcare) and were developed using the Bio-Rad ChemiDoc™ XRS system.

**qRT-PCR with GAPDH control method to quantify viral RNA**

Total RNA was extracted at 24h post-infection using Trizol® as per manufacturer’s protocol (Invitrogen). RNA samples were treated with DNase1 (NEB) according to the manufacturer’s protocol. cDNA synthesis, using GoScript™ Reverse transcription system (Promega,) was performed using LCMV Armstrong NP-specific or monkey GAPDH-specific forward primers (NP forward primer: CGAAGCTTCCCTGGTCATTTC, NP reverse primer: CAGTTATAGGTGCTTTCCGC; GAPDH forward primer: AGTCAACGGATTTGTCGTA, GAPDH reverse primer: GGGTGGAATCATACTGGAAC). Monkey-specific GAPDH primers were modified from Li et al. (260). qRT-PCR was performed using the Applied Biosystems 7300
Real Time PCR system platform with 250ng template cDNA, 12.5uL 2X Maxima™ SYBR Green/ROX qPCR master mix (Fermentas) and 0.2uM each forward and reverse gene-specific primers in 25uL reaction volumes. Melt curve analysis was performed to confirm PCR product specificity. qRT-PCR analysis was performed by the comparative threshold ($C_T$) method (261). The fold change (FC) in NP cDNA relative to the GAPDH control was determined using the equation $FC = -2^{\Delta\Delta C_T}$ where $\Delta\Delta C_T = (C_T_{NP} - C_T_{GAPDH})$ infection with PPMO treatment - (C_T_{NP} - C_T_{GAPDH}) infection without P-PMO treatment.

**Transfections**

Transfections were performed using Lipofectamine™ 2000 (Invitrogen; Carlsbad, CA) and Opti-MEM media (Gibco) as per the manufacturer’s protocol.

**Plasmid DNA construction**

Total RNA was isolated using Trizol® from BHK-21 cells infected with LCMV Arm-4 at an MOI of 0.1 for 48h at 37°C, 5% CO₂. Viral glycoprotein cDNA was synthesized using a GPC-specific primer (sequence provided upon request) flanked by XhoI recognition sequences using the GoScript™ reverse transcriptase as per the manufacturer’s protocol and cloned into the pTargeT™ vector (Promega). Point mutations were introduced using the site-directed QuikChange Lightning kit (Agilent Technologies), sequence verified using standard automated sequencing methods, and sub-cloned into the pCAGGS vector using the XhoI sites (262). The HA epitope tag (YPYDVPDYA), as well as the replacement of the native stable signal peptide with the signal sequence from the Influenza A hemagglutinin protein (MEKIVLLFAIVSLVKS), were inserted into the GPC open reading frame using the overlap PCR technique (263).
For construction of the HA SSP GPC plasmid, the HA epitope was inserted between amino acids A\textsubscript{10} and L\textsubscript{11} in the SSP region of the full-length glycoprotein open reading frame. The plasmid encoding only the tagged SSP was created by introducing two consecutive stop codons at amino acids 59 and 60; the first two residues of the GP1 subunit and immediately downstream of the SPase recognition site. Both these mutants were initially produced using the pTargeT plasmid and were shuttled in to the pCAGGS backbone as stated above.

The plasmids encoding the clathrin heavy chain and surf-4 were purchased from the Harvard PlasmID database at Harvard Medical School Dana-Farber/Harvard Cancer Center DNA Resource Core. The wild type Arf1 and the dominant negative T31N plasmids were kindly provided by Dr Christine Sütterlin.

**Arenavirus SSP multiple sequence alignment**

Amino acid sequences from the following arenaviruses were used to highlight signal peptide conservation: Allpahuayo virus (AY012687), Amapari virus (AF512834), Bear Canyon virus (AF512833), Big Brushy Tank virus (EF619036), Catarina virus (DQ865245), Chapare virus (EU260463), Cupixi virus (AF512832) Flexal virus (AF512831), Guanarito virus (NC_005077), Ippy virus (DQ328877), Junín virus (D10072), Lassa virus (AY628203), Latino virus (AF512830), Lujo virus (NC_012776), Luna virus (AB697691), LCMV (AY847350), Machupo virus (NC_005078), Mobala virus (AY342390), Mopeia virus (DQ328874), Morogoro virus (EU914030), Oliveros virus (U34248), Parana virus (AF512829), Pichinde virus (NC_006447), Pirital virus (AF277659), Sabia virus (NC(006317), Skinner Tank virus (EU123328), Tacaribe
virus (NC_004293), Tamiami virus (AF512828), Tonto Creek virus (EF619033), Whitewater Arroyo virus (AF228063). Sequences were aligned using CLC Sequence Viewer 6.

Confocal immunofluorescence microscopy

DBT cells were plated onto flame-sterilized glass coverslips and transfected with Lipofectamine 2000 as per the manufacturer’s protocol (Life Technologies). After 48h incubation, cells were fixed using 3.7% paraformaldehyde (PFA) at room temperature, and when relevant, permeabilized with PBS + 0.1% Triton X-100 for 5 minutes. Cells were blocked using 5% BSA in PBS-Tween 20 for 1h prior to overnight primary antibody incubation at 4°C. The Phalloidin-FITC and Alexa Fluor -405, -488, and -594 conjugated secondary antibodies (Life Technologies) were used to detect viral or cellular epitopes using the Nikon Eclipse Ti confocal microscope. Images were processed linearly using the Nikon Elements software and ImageJ and cropped for assembly using Adobe Photoshop. For the triple labeling microscopy, cells were incubated with Phalloidin-FITC overnight, followed by mouse anti-HA (Sigma) for an additional night prior to 1h incubation with Alexa Fluor 405. The Mannosidase II antibody was kindly provided to us by Dr Kelley Moreman (University of Georgia). The TGN46 antibody was purchased from Millipore while the GRP78 antibody was from Cell Signaling. Ultimately, the GP1 antibody (2.11-15) directly conjugated to Alexa Fluor 594 was used (Life Technologies). For the SSP-HA + GP1/2 and the HA SSP GPC samples, coverslips were mounted with a mounting medium lacking DAPI. All other samples used DAPI-containing mounting media (Southern Biotechnologies).
Surface epitope detection using flow cytometry

Transfected HEK 293T cells were collected and disassociated using cold 5 mM EDTA in 1× PBS. Cells were blocked using 5% FBS in 1 for 30 minutes on ice. Cells were passed through a 26-gauge syringe to disrupt cellular aggregates. Cells were incubated with either the anti-HA antibody (Sigma), the directly conjugated GP1 antibody to Alexa fluor-488 (2.11.10-488), or mouse normal IgG (BioLegend) for 30 minutes on ice. Cells were extensively washed with 5% FBS. For the samples stained with either HA or IgG, Alexa fluor-conjugated secondary antibodies were allowed to incubate for 30 minutes, on ice. Cells were washed twice with 5% FBS. Cells were further washed with 1X PBS and transferred to pre-chilled FACS tubes. Cells were detected using a FACSCalibur system (BD Biosciences) and analyzed using FlowJo software.

Fusion Assay

DBT cells were plated onto flame-sterilized glass coverslips and transfected using Lipofectamine 2000. After 48h incubation, cells were washed twice with PBS and were allowed to incubate for 1h with pH = 5 adjusted DMEM growth media. Then, cells were washed twice with 1X PBS and allowed to incubate for an additional hour with pH = 7.4 maintenance media. Cells were fixed with 3.7% paraformaldehyde, washed with PBS, and coverslips were mounted with DAPI-containing mounting media. The extent of fusion activity was determined by counting the number of cells versus the total nuclei, as previously described (78).
Recombinant virus production

Recombinant LCMV strains were created as described previously (23, 44, 81, 82). Point mutations were introduced by QuikChange mutagenesis as per the manufacturer’s protocol (Stratagene). Briefly, BHK-21 cells were transfected with plasmids encoding the LCMV large segment, the small segment, pCAGGs NP, and pCAGGS L using Lipofectamine 2000. After 72h incubation, clarified supernatants were used to infect fresh BHK-21 monolayers. Serial passage viral stocks were collected every 72h, clarified, and stored at -80°C prior to titer determination via plaque assay.

Co-Immunoprecipitation

Co-immunoprecipitation experiments followed the same protocol, though replaced with 0.1% NP-40 lysis buffer with fresh protease inhibitors. Clarified lysates were incubated with Protein G beads (GE Healthcare) for 30min at 4°C, collected by mild centrifugation 8200 x g, 4°C, 30 sec), and incubated overnight with anti-HA beads at 4°C with continuous end-over-end rotation (Sigma). Beads were thrice washed with 0.1% NP-40 wash buffer and one final wash without detergent. Proteins were eluted using 0.1M glycine pH = 2.5 and neutralized with 1M Tris pH = 9.5. For mass spectrometry analysis, eluates were incubated with proteomics-grade PNGase F (Sigma) for 3h at 37°C prior to storage at -80°C.

Peptide Sequencing by Mass Spectrometry

LCMV glycoprotein interactions with human proteins were investigated using co-immunoprecipitation combined with mass spectrometry according to a previously published method, in collaboration with Dr Joe Derisi’s laboratory at the University of California at San
Francisco (264, 265). Protein identification in the hemagglutinin-tag captured eluates was performed by LC-MS/MS analysis of trypsin-digested samples, prepared by in solution digestion. Peptide samples were analyzed on either an LTQ-Orbitrap-XL or LTQ-Orbitrap-Velos mass spectrometer (Thermo), each equipped with 10,000 psi NanoAcuity ultra-high-performance liquid chromatography (UPLC) systems (Waters) for reversed-phase C18 chromatography using identical separation and data acquisition parameters. Data were processed using an in house script called PAVA for generation of peak lists, then analyzed using Protein Prospector software (266). Affinity experiments were performed with N = 5 or 6 replicates. As an additional layer of background subtraction, a mock transfection data set was also analyzed (N = 6) to eliminate any background due to the HA-immunoprecipitation system used in these experiments.

**siRNA Knockdown of Mass Spectrometry Targets**

Human On-TARGETplus siRNA pools targeting the clathrin heavy chain (CHC) or the surfeit locus protein 4(Surf-4) protein were purchased from Dharmacon. Dosage and MTT toxicity assays were performed to determine optimal siRNA pool concentration. HEK 293T cells were incubated with 25mMe each siNON-TARGET (siNON) control pools, siCHC, or siSurf4. After 48h siRNA incubation, cells were infected with LCMV at an MOI = 0.1, using a 1h adsorption. After 24h infection (72h total knockdown incubation), supernatants were collected to determine viral titers by plaque assay using Vero E6 cells as stated above. Additionally, cells were lysed using 1% NP-40 plus complete protease inhibitors. Lysates were separated using 12.5% SDS-PAGE and proteins detected by western blot, as described above. For each experiment, a 12-well plate of siRNA-treated cells was used to determine cellular toxicity at 72h post-knockdown.
Statistical Analysis

All graphs and statistical analyses were performed using the Prism software (GraphPad).

Illustrations

All illustrations were created using the Adobe Illustrator program.
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