Title: The role of proteolytic processing and the stable signal peptide in expression of the Old World arenavirus envelope glycoprotein ectodomain.
Authors: Burri DJ, Pasquato A, da Palma JR, Igonet S, Oldstone MB, Kunz S
Journal: Virology
Year: 2013 Feb 5
Volume: 436
Issue: 1
Pages: 127-33
DOI: 10.1016/j.virol.2012.10.038

Published in final edited form as:
Virology. 2013 February 5; 436(1): 127–133. doi:10.1016/j.virol.2012.10.038.

The role of proteolytic processing and the stable signal peptide in expression of the Old World arenavirus envelope glycoprotein ectodomain

Dominique J. Burri[1], **Antonella Pasquato**[1], **Joel Ramos da Palma**[1], **Sebastien Igonet**[2], **Michael B.A. Oldstone**[2], and **Stefan Kunz**[1,*]

[1]Institute of Microbiology, University Hospital Center and University of Lausanne, Lausanne, Switzerland [2]Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, CA 92037

Abstract

Maturation of the arenavirus GP precursor (GPC) involves proteolytic processing by cellular signal peptidase and the proprotein convertase subtilisin kexin isozyme 1 (SKI-1)/site 1 protease (S1P), yielding a tripartite complex comprised of a stable signal peptide (SSP), the receptor-binding GP1, and the fusion-active transmembrane GP2. Here we investigated the roles of SKI-1/S1P processing and SSP in the biosynthesis of the recombinant GP ectodomains of lymphocytic choriomeningitis virus (LCMV) and Lassa virus (LASV). When expressed in mammalian cells, the LCMV and LASV GP ectodomains underwent processing by SKI-1/S1P, followed by dissociation of GP1 from GP2. The GP2 ectodomain spontaneously formed trimers as revealed by chemical cross-linking. The endogenous SSP, known to be crucial for maturation and transport of full-length arenavirus GPC was dispensable for processing and secretion of the soluble GP ectodomain, suggesting a specific role of SSP in the stable prefusion conformation and transport of full-length GPC.

INTRODUCTION

The *Arenaviridae* are a large family of negative strand enveloped emerging viruses comprised of currently 23 recognized viral species, including several causative agents of severe hemorrhagic fevers with high mortality in man (Emonet et al., 2009). Based on serological evidence and phylogenetic relationships, the arenaviruses are classically divided into the Old World arenaviruses including the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) and Lassa virus (LASV) and the New World arenaviruses (Charrel, de Lamballerie, and Emonet, 2008). The prototypic LCMV represents a powerful experimental model for experimental virology and is a prevalent human pathogen that is of concern in pediatric medicine (Bonthius et al., 2007; Bonthius and Perlman, 2007). LCMV has also been associated with severe infections associated with high mortality in transplantation patients (Fischer et al., 2006; Palacios et al., 2008). The most prevalent

*Corresponding author. Mailing address: Institute of Microbiology, University Hospital Center and University of Lausanne, Lausanne CH-1011, Switzerland. Phone: +41-21 314 7743, Fax: +41-21 314 4060, Stefan.Kunz@chuv.ch,.

human pathogenic arenavirus is LASV that currently causes several hundred thousand infections per year with tens of thousands of death and represents a continuing serious public health problem and a potential bioterror risk being placed on the CDC/HIH list of category A viruses (McCormick and Fisher-Hoch, 2002).

Arenaviruses are enveloped bi-segmented negative-strand RNA viruses with a non-lytic cell cycle restricted to the cytoplasm (Buchmeier, de la Torre, and Peters, 2007). Each RNA segment, L, and S, includes two open reading frames separated by a non-coding intergenic region (IGR) (de la Torre, 2009). The L segment codes for the matrix protein (Z) and the RNA-dependent RNA polymerase (L), whereas the S segment contains the envelope glycoprotein precursor (GPC) and the nucleoprotein (NP). The arenavirus GPC is a type I membrane protein synthesized initially as a single polypeptide that undergoes processing by cellular signal peptidases and the proprotein convertase subtilisin kexin isozyme-1(SKI-1)/site-1 protease (S1P) (Beyer et al., 2003; Lenz et al., 2001; Rojek et al., 2008). Processing of GPC by SKI-1/S1P yields the N-terminal GP1 (44 kDa), which is implicated in binding to cellular receptors (Borrow and Oldstone, 1992), and the transmembrane GP2 (35 kDa) that mediates fusion (Eschli et al., 2006; Igonet et al., 2011). In contrast to other viral GPs, arenavirus GPs contain a remarkably long and stable signal peptide (SSP), which forms part of a mature tripartite complex SSP/GP1/GP2, which represents the functional unit of host cell attachment, cell entry, and fusion (Eichler et al., 2003a; Eichler et al., 2003b; Froeschke et al., 2003; York et al., 2004).

The SSP of arenaviruses consists of 58 amino acids and contains two putative transmembrane helices with its N- and C-termini located in the cytosol (Agnihothram et al., 2007). Within mature GP, SSP associates non-covalently with the cytoplasmic GP2 domain (Agnihothram, York, and Nunberg, 2006) involving a Zinc-binding motif (Briknarova et al., 2011; York and Nunberg, 2007). Replacement of SSP by a generic signal peptide prevented transport and processing of GPC (Agnihothram, York, and Nunberg, 2006; Eichler et al., 2004). Interestingly, complementation of SSP in *trans* rescued GPC maturation and cleavage indicating a role of SSP as a trans-acting maturation factor (Agnihothram, York, and Nunberg, 2006; Eichler et al., 2004).

Upon low pH activation, the transmembrane GP2 subunit undergoes a membrane fusogenic conformational change. Biochemical analysis and crystal structure determination at neutral pH (Eschli et al., 2006; Igonet et al., 2011) have demonstrated that the recombinant GP2 spontaneously forms an elongated trimer in which the C-terminal -helices of each protomer associate with the hydrophobic grooves of the trimeric coiled-coil formed by N-terminal helices. The resulting GP2 six-helix bundle represents the post-fusion state typically observed in other class I viral fusion proteins (Harrison, 2008). Interestingly, the fusion pH of arenavirus GP2 is markedly affected by mutations of a conserved lysine residue in SSP (K33), indicating a role of SSP in regulation of fusion (Nunberg and York, 2012; York and Nunberg, 2006).

Here we investigated the roles of proteolytic processing and SSP in the biosynthesis of the recombinant GP ectodomains of LCMV and LASV. We provide evidence that the soluble ectodomains of LCMV and LASV GP are processed by SKI-1/S1P, and that GP1 spontaneously dissociates from GP2 upon secretion. We confirm that the released GP2 subunit adopts a trimeric conformation, likely corresponding to the post-fusion state. The endogenous SSP was found to be dispensable for processing and secretion of the soluble GP ectodomain, suggesting a specific role of SSP in the stable prefusion conformation and transport of full-length GPC.

RESULTS AND DISCUSSION

Expression of the ectodomain of LCMV and LASV GP in mammalian cells

Based on available structural data and molecular modeling studies of LCMV GP (Eschli et al., 2006; Gallaher, DiSimone, and Buchmeier, 2001; Igonet et al., 2011), we constructed a soluble variant of the ectodomains of LCMV GP (LCMV GPed) containing the SSP, GP1, GP2 lacking transmembrane and intracellular domains, followed by a six-histidine (His)-tag (Fig. 1A). LCMV GPed and full-length LCMV GP were expressed in HEK293H cells by transient transfection and protein production performed in serum-free medium. After 48 hours, total protein was isolated from cell supernatants as well as total cell lysates and subjected to Western-blot analysis using a monoclonal antibody (mAb) to a conserved epitope in LCMV GP2 (Weber and Buchmeier, 1988). In supernatants of LCMV GPed transfectants, a GP2-reactive fragment of 28-30 kD was detected corresponding to the expected size of the mature truncated GP2 ectodomain (Fig. 1B). As expected, no GP2 was found in supernatants of cells transfected with full-length LCMV GP. In lysates of LCMV GPed transfectants only the unprocessed precursor was detected, whereas both, GPC and mature GP2 were found in cells expressing wild-type LCMV GP (Fig. 1B). To further characterize the putative GP2 fragment, we performed deglycosylation with PNGase F that removes N-linked glycans. For this purpose, LCMV GPed was precipitated from the conditioned supernatant of transiently transfected HEK293H cells using NiNTA agarose. Upon incubation with PNGaseF and control buffer, proteins were separated via SDS-PAGE and the GP2 fragment of LCMV GPed detected with an antibody to His-tag. Treatment with PNGase F resulted in a shift of the apparent molecular mass from 28-30 kDa to circa 20 kDa (Fig 1C), which was compatible with the expected apparent molecular mass of the deglycosylated truncated GP2 polypeptide of 183 amino acids.

Since mAbs recognizing LCMV GP1 are mainly directed against conformational epitopes, detection of LCMV GP1 in Western blot was not feasible. In order to detect the GP1 and GP2 portions of LCMV GPed, we performed ELISA using specific mAbs anti-GP1 (36.1 and 67.1) and anti-GP2 (33.1 and 83.6). In supernatants of HEK293H cells transfected with LCMV GPed, we were able to detect specific signals for GP1 and GP2 (Fig. 1D). Since mAbs 36.1 and 67.1 to GP1 recognize conformational epitopes (Buchmeier et al., 1981), the data suggest proper folding of the GP1 parts.

In a next step, we generated an analogous soluble variant of LASV GP (LASV GPed) and examined its expression in HEK293H cells. Similar to LCMV GPed, LASV GPed was efficiently processed and secreted (Fig. 1E). When compared to LCMV GPed, more unprocessed LASV GPed was detected in the cell culture supernatant (Fig. 1D), suggesting differences in the efficiency of processing.

The GPC of LASV and LCMV is processed by the protease SKI-1/S1P at the recognition sites RRLL and RRLA, respectively to yield GP1 and GP2 (Beyer et al., 2003; Lenz et al., 2001; Rojek et al., 2008). To investigate this issue in the context of our soluble LCMV and LASV GP ectodomains, we expressed LCMV GPed and LASV GPed in SKI-1/S1P-deficient SRD12B cells (Rawson et al., 1998) and wild-type CHOK1 cells. In SRD12B cells, LCMV GPed and wild-type LCMV GP were not cleaved while correct processing occurred in CHOK1 cells (Fig. 2A). Despite similar expression levels of precursor in SRD12B and CHOK1 cells, secretion of uncleaved LCMV GPed was reduced in SRD12B cells, suggesting impaired transport of LCMV GPed in absence of processing by SKI-1/S1P (Fig. 2A). Expression of LASV GPed in SKI-1/S1P-deficient cells resulted in detection of abundant unprocessed protein in the supernatant (Fig. 2B). In sum, the data indicate that the soluble ectodomain of LCMV and LASV GP undergoes processing by SKI-1/S1P. The reasons for the reduced secretion of unprocessed LCMV GPed, but apparently not LASV

GPed are currently unclear. However, several lines of evidence indicate that LCMV GP and LASV GP undergo SKI-1/S1P processing in distinct intracellular compartments: the ER/cis-Golgi in case of LASV GP (Lenz et al., 2001) and a late Golgi compartment for LCMV GP (Beyer et al., 2003; Wright et al., 1990). Interestingly, the subcellular location of processing of LASV GP and LCMV GP seems to de largely determined by the specific SKI-1/S1P recognition sequence, RRLL and RRLA, respectively (Burri et al., 2012). On might speculate that that processing in different compartments of the secretory pathway may influence the stability and/or transport of the soluble recombinant ectodomains of LASV GP and LCMV GP in a different manner.

The GP2 ectodomain spontaneously forms trimers

Initial studies on the oligomerization of LCMV GP2 performed on synthetic peptides and recombinant GP2 fragments expressed in bacteria provided first evidence for a trimeric structure of the GP2 ectodomain (Eschli et al., 2006). These initial studies have been corroborated by recent X-ray crystallographic studies on a bacterially expressed LCMV GP2 fragment that revealed the characteristic trimeric coiled coil found in class I viral fusion proteins in their post-fusion conformation (Igonet et al., 2011). To extend these findings, we studied the oligomeric state of our soluble full-length LCMV GP ectodomain produced in mammalian cells. For this purpose, we performed chemical cross-linking using the homobifunctional cross-linker disulfosuccinimidyl tartrate (S-DST), which reacts with free amines on proteins and has a short spacer of 0.64 nm, restricting covalent coupling to nearest neighbors. Briefly, cleared conditioned culture supernatants of cells transfected with LCMV GPed were incubated with increasing concentrations of S-DST. After quenching of the reaction, total protein was precipitated and separated by SDS-PAGE under reducing conditions. Cross-linked species were detected in Western blot with mAb 83.6 to GP2. In samples without cross-linker, mostly monomers of the GP2 ectodomain of circa 30 kDa were detected (Fig. 3). Reaction with increasing concentrations of S-DST resulted in the formation of two distinct additional GP2-reactive species with apparent molecular masses of 60 kDa and 90 kDa, respectively, which likely corresponded to dimers and trimers of the GP2 ectodomain. At higher concentrations of the cross-linker, the signals for the putative GP2 monomer and dimer diminished whereas the 90 kDa species became more abundant, suggesting that the apparent GP2 trimer represented the end product of the reaction (Fig. 3). Under no circumstances were GP2-reactive species of > 90 kDa observed, suggesting that the ectodomain of GP2 spontaneously forms trimers in absence of the transmembrane and intracellular domain. Notably, no putative GP1-GP2 heterodimers with a predicted apparent molecular mass of circa 75 kDa (44 kDa + 30 kDa) were detected in our cross-linking experiments. The reasons for this were unclear: either the cross-linked species consisted only of GP2, without containing GP1, or reactive amino functions were absent from the GP1/GP2 interface, preventing cross-linking with S-DST.

The structural and modeling data at hand predicted that the structural elements responsible for trimer formation in Old World arenavirus GPed are entirely localized within the ectodomain of GP2 without a contribution by GP1 (Eschli et al., 2006; Gallaher, DiSimone, and Buchmeier, 2001; Igonet et al., 2011). To confirm this in our system, we purified the GP2 ectodomain. In mature LCMV GP present at the surface of virions, GP1 and GP2 are associated non-covalently, predominantly via ionic interaction (Burns and Buchmeier, 1991). To isolate the GP2 ectodomain, we performed pull-down of LCMV GPed via the C-terminal His-tag using NiNTA affinity matrix, followed by washing under increased ionic strength (Fig. 4A-C). Using ELISA, we monitored the enrichment of GP2 and the loss of GP1 during the purification procedure. We noticed a high proportion of GP1 in the flow-through of the NiNTA column and rapid removal of GP1 already during the initial wash steps (Fig. 4C), suggesting spontaneous dissociation of GP1 from GP2 already under

physiological ionic strength. Purified GP2 ectodomain was then subjected to chemical cross-linking with S-DST, as described above. Similar to cross-linking performed on conditioned supernatants (Fig. 3), reaction with increasing concentrations of S-DST yielded putative GP2 dimers and trimers of 60 kDa and 90 kDa, respectively. This confirmed that the ectodomain of LCMV GP2 contains the necessary and sufficient structural elements for trimer formation, as proposed earlier (Igonet et al., 2011). The similar cross-linking pattern observed with purified GP2 ectodomain (Fig. 4D) and conditioned supernatant (Fig. 3) together with the apparent dissociation of GP1 from GP2 in mature, soluble LCMV GPed (Fig. 4C) further suggests that the GP2 trimers detected here correspond to the post-fusion six-helix bundle (6HB) conformation reported by Igonet and colleagues (Igonet et al., 2011).

Previous studies indicated similarities of arenavirus GPCs to class I viral fusion proteins (Eschli et al., 2006; Gallaher, DiSimone, and Buchmeier, 2001; Igonet et al., 2011). Upon translation in the ER, viral fusion proteins initially fold into a metastable prefusion conformation. Several such fusion proteins have now been studied in great detail, with crystal structures determined for both the form of the protein present on the viral surface before interaction with the cell (‘pre-fusion’ conformation) and the form of the protein after fusion is complete (‘post-fusion’ conformation). Exposure to acidic pH or other triggering signals like co-receptor binding trigger conformational changes in viral fusion glycoproteins, thereby driving membrane fusion (Harrison, 2008). In its post-fusion conformation, LCMV GP2 contains a characteristic trimeric coiled coil present in other class I post-fusion conformations that are stabilized by intrachain salt bridges (Igonet et al., 2011). However no structural information is currently available about the pre-fusion state of arenavirus GPs. High resolution structures for pre-fusion conformations prior and after cleavage have been solved only for influenza hemagglutinin (HA) (Chen et al., 1998; Wilson, Skehel, and Wiley, 1981) and parainfluenza virus 5 (PIV5) F protein (Welch et al., 2012; Yin et al., 2006). In both cases the structures before and after proteolytic processing are largely identical, with exception of polypeptide structures surrounding the cleavage site. Interestingly, ultracentrifugation studies performed on LASV GPC provided evidence that uncleaved and cleaved LASV GP may adopt distinct conformations (Schlie et al., 2010). Structural studies on the secreted, uncleaved ectodomain of PIV3 protein revealed that, in absence of a transmembrane domain, the ectodomain adopts a post-fusion six-helix bundle conformation (Yin et al., 2005). This indicated that the isolated ectodomain tends to spontaneously collapse to the low-energy postfusion form. Accordingly, stabilization of the ectodomain trimer conformation by addition of a trimerization domain at the C-terminus allowed to solve the structure of the metastable prefusion conformation of PIV5 F protein (Yin et al., 2006). A similar instability of the soluble ectodomain of LCMV GP reported here suggests that the transmembrane domain and/or cytosolic tail of GP2, possibly involving the SSP, are likewise crucial for maintaining the metastable pre-fusion conformation of the full-length tripartite SSP/GP1/GP2 complex

The SSP is dispensable for processing and secretion of the soluble ectodomain of LCMV and LASV GP

Several lines of evidence indicate that the SSP is crucial for correct proteolytic maturation and transport of full-length arenavirus GPC (Agnihothram, York, and Nunberg, 2006; Eichler et al., 2003a; Eichler et al., 2003b; Froeschke et al., 2003; York et al., 2004), however, the underlying mechanism is not fully understood. It is conceivable that SSP may be required for correct folding of the ectodomain of the GPC precursor to allow S1P/SKI-1 cleavage at the luminal site of the secretory pathway. Alternatively, SSP may be required for transport of the full-length GPC precursor through the specific Golgi compartment in which the active protease resides. To address the role of SSP in processing and transport of the soluble GP ectodomain, we replaced the SSP of LCMV GP and LASV GP by the generic SP

of Ig light chain (Fig. 5A). Consistent with published data, replacement of the SSP in full-length LCMV GPC and LASV GPC by a generic SP prevented processing (Fig. 5B) and cell surface transport as assessed by flow cytometry (data not shown). However, in the context of the soluble ectodomains of LCMV GP and LASV GP, the endogenous SSP seemed dispensable for SKI-1/S1P processing and secretion (Fig. 5C, D). The data are in line with the previously reported role of SSP in transport of the full-length GPC by masking ER retention signals in the cytoplasmic tail of GP2 of JUNV, which is required for transport through the Golgi, where processing by SKI-1/S1P occurs (Agnihothram, York, and Nunberg, 2006). While the cytoplasmic domain of JUNV GP2 contains two dibasic motifs implicated in ER retention, the C-terminal part of LCMV and LASV contain only one. However, our data support a conserved role for SSP in the regulation of exit from the ER between Old World and New World arenavirus GPCs. Our findings are also consistent with a recent study that was able to separate the roles of SSP in proteolytic processing and transport on the one hand from the role in membrane fusion activity on the other hand (Messina, York, and Nunberg, 2012). Using hybrid GPCs derived from LASV and JUNV, Messina and colleagues demonstrated that heterologous SSPs provided in *trans* can rescue assembly, processing, and transport of hybrid GPCs, but are unable to confer membrane fusion activity (Messina, York, and Nunberg, 2012). The species-specificity of the SSP in membrane fusion could be mapped to the first transmembrane segment of SSP, which is likely crucial for the stable prefusion conformation of the tripartite SSP/GP1/GP2 complex that represents the functional unit of membrane fusion.

MATERIALS AND METHODS

Antibodies and cell culture

Monoclonal antibodies (mAb) 36.1, 67.1 (anti-LCMV GP1), and 33.1, 83.6 (anti-LCMV GP2) have been described (Buchmeier et al., 1981; Weber and Buchmeier, 1988). HRP-conjugated anti-mouse IgG was from Pierce Chemical Co., Rockford, IL. HEK293 cells, chinese hamster ovary (CHO)-K1 cells, and the S1P-deficient CHOK1 derived cell line SRD12B (Rawson et al., 1998) were maintained as described (Rawson et al., 1998).

Construction, expression, and purification of recombinant LCMV GP and LASV GP variants

To construct LCMV GPed, a C-terminal fragment of the LCMV GP ectodomain was amplified by PCR using the forward primer 5′GGA CGT AGA ATC TGC CTT GCA3′ and the backward primer 5′AAT TCC CGG GTC AGT GAT GGT GAT GGT GAT GCA TCA ATG CTA GGG GGG T3′ and pC-LCMV GP as a template. The PCR fragment was digested with KpnI and XmaI, and cloned into pC-LCMV GP. To obtain HALCMV GPed and HALCMV GP, the first 58 N-terminal amino acids comprising the LCMV GP SSP were removed. Briefly, a XmaI restriction site was inserted N-terminal of M59 by PCR amplification of a LCMV GP cDNA fragment using the primers 5′AATT CCC GGG ATG TAC GGT CTT AAG GGA CCC3′ and 5′AAT TCC CGG GTC AGT GAT GGT GAT GGT GAT GCA TCA ATG CTA GGG GGG T3′ and pC-LCMV GP as a template. The resulting PCR fragment was digested with XmaI and NdeI and inserted into either pC-LCMV GP or pC-LCMV GPed, together with a EcoRI-XmaI fragment derived from the vector pDisplay (Invitrogen) containing a signal peptide of the Ig light chain, followed by an HA tag and a XmaI restriction site allowing in-frame fusion of C-terminal fusion peptides. LASV GPED was obtained by amplification of a C-terminal fragment of the ecodomain using the forward primer 5′GGA ATT CCA TAC TGT AAT3′ and the reverse primer 5′AAT TCT CGA GTC AGT GAT GGT GAT GGT GAT GAA CTA GAC CCA ATG GTG T3′, followed by digestion with KpnI and XhoI, and sub-cloning into pC-LASV GP (Kunz et al., 2005). To obtain HALASV GPed and HALASV GP, the first 58 N-terminal

amino acids comprising the LASV GP SSP were removed by insertion of a XmaI restriction site N-terminal of T59 by PCR amplification of a LASV GP cDNA fragment using the primers 5′TTAA CCC GGG ACC AGT CTT TAT AAA GGG GTT3′ and 5′GTT GTA TTT TGG ATT ATC AGA3′. The resulting PCR fragment was digested with XmaI and AseI and inserted into either pC-LASV GP or pC-LASV GPed, together with a EcoRI-XmaI fragment derived from the vector pDisplay. All mutants were verified by double-strand DNA sequencing. The construct pC-EGFP was kindly provided by Juan-Carlos de la Torre (The Scripps Research Institute).

SRD12B, and CHOK1 cells were transfected as described (Kunz et al., 2003) and kept in serum free medium CHO-S-SFMII II (Gibco BRL) for protein expression. HEK293T cells were transfected in serum containing medium using calcium phosphate. After 16 hours, cells were washed and serum free medium 293SFMII (Gibco BRL) was added. After 48 hours, supernatants were cleared by centrifugation for 10 min, 4,000 rpm, 4° C.

For NiNTA affinity purification, supernatants were dialyzed against PBS containing 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$ (CM-PBS). NiNTA agarose (QIAGen) was added (10 μl/ml of supernatant) and incubated in the cold for 12 h on a head-over shaker, transferred to a column and washed five times with 10 matrix volumes of CM-PBS containing 10 mM imidazole, pH 8.0 and 500 mM NaCl. Bound proteins were eluted with five volumes 50 mM EDTA, 150 mM NaCl, pH 8.0. Eluate fractions were analyzed by SDS-PAGE with silver staining, pooled according to purity, concentrated and dialyzed against CM-PBS.

Detection of GP1 and GP2 in ELISA

Protein solutions coated in 96-well EIA/RIA high-bond microtiter plates (Corning) for 12 h at 6°C and non-specific binding was blocked with 1% (w/v) BSA/PBS. MAbs 36.1, 67.1 (anti-LCMV GP1), and 33.1, 83.6 (anti-LCMV GP2) were applied in 1: 100 dilution for 2 h at 6° C an detected with peroxidase-conjugated anti-mouse IgG (1: 5000) in a color reaction using ABTS (2,2′Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid)) substrate. OD (405) was measured with an ELISA reader.

Treatment with PNGaseF

Conditioned supernatants of HEK293 cells transiently transfected with LCMV GPed (10 ml) were dialyzed against CM-PBS and NiNTA agarose added (5 μl/ml of supernatant) for 12 h on a head-over shaker. The NiNTA pellet was washed there times with 20 matrix volumes of CM-PBS containing 10 mM imidazole, pH 8.0 and 150 mM NaCl. After the third wash, liquid was removed and 50 μl PNGaseF reaction buffer added, with or without PNGaseF enzyme (New England Biolabs). Deglycosylation with PNGaseF was carried our according to the manufacturers recommendations. The reaction was stopped by adding 50 μl of hot 2 × SDS-PAGE sample buffer and boiling for 5 minutes. Eluted proteins were subjected to SDS-PAGE and Western-blotting as described below.

Western blotting

Cell culture supernatants were cleared by centrifugation, followed by desalting and concentration as described (Wessel and Flugge, 1984). Cells were washed twice with cold PBS and lysed in SDS-PAGE sample buffer (62.5 mM Tris/HCl, pH 6.8, 20% (wt/vol) glycerol, 2% (wt/vol) SDS, 100 mM DTT) supplemented with Complete® protease inhibitors (Roche). Samples were boiled for 10 min at 95°C and centrifuged 5 min at 13,000 rpm prior to gel loading. Proteins were separated on 10% polyacrylamide gels and blotted on nitrocellulose membrane. Membranes were blocked in blocking solution (PBS, 0.2% (wt/vol) Tween-20, 3% (wt/vol) non-fat milk and then incubated with primary antibody mAb 83.3 to LCMV GP2 1:1,000 at 4°C. After overnight incubation, membranes were washed

thoroughly with PBS, 0.2% (wt/vol) Tween-20 and incubated 1 hour at RT with the HRP-conjugated secondary antibodies (1:3,000). After three washes in PBS 0.2% (wt/vol) Tween-20, membranes were developed with the LiteABlot kit (EuroClone, Pero, Italy).

Chemical cross-linking

Protein solutions were dialyzed against the cross-linking reaction buffer 50 mM Hepes, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.5. A 50 mM stock solution of disulfosuccinimidyl tartrate, S-DST (Pierce) was freshly prepared in reaction buffer. Samples were incubated at 6 °C on a head-over shaker for 1 hour, quenched by addition of 50 mM glycine, pH 8.0 for 15 min. Total protein was precipitated and analyzed by Western-blot using mAb 83.6 as described above.

Acknowledgments

The authors thank Erica Ollmann Saphire (Scripps Research Institute, La Jolla) for her expertise and very helpful comments on the manuscript. We further thank Juan Carlos de la Torre (Scripps Research Institute, La Jolla) for valuable reagents and helpful discussions and Michael S. Brown and Joseph L. Goldstein (University of Texas Southwestern Medical Center, Dallas, TX) for the S1P-deficient SRD12B cells. This research was supported by the Swiss National Science Foundation grant FN 31003A-135536 (S.K.) and US PHS grants AI55540 (M.B.A.O., S.K.) and AI09484 (M.B.A.O.).

REFERENCES

Agnihothram SS, York J, Nunberg JH. Role of the stable signal peptide and cytoplasmic domain of G2 in regulating intracellular transport of the Junin virus envelope glycoprotein complex. J Virol. 2006; 80(11):5189–98. [PubMed: 16698999]

Agnihothram SS, York J, Trahey M, Nunberg JH. Bitopic membrane topology of the stable signal peptide in the tripartite Junin virus GP-C envelope glycoprotein complex. J Virol. 2007; 81(8): 4331–7. Epub 2007 Jan 31. [PubMed: 17267481]

Beyer WR, Popplau D, Garten W, von Laer D, Lenz O. Endoproteolytic processing of the lymphocytic choriomeningitis virus glycoprotein by the subtilase SKI-1/S1P. J Virol. 2003; 77(5):2866–72. [PubMed: 12584310]

Bonthius DJ, Nichols B, Harb H, Mahoney J, Karacay B. Lymphocytic choriomeningitis virus infection of the developing brain: critical role of host age. Ann Neurol. 2007; 62(4):356–74. [PubMed: 17696127]

Bonthius DJ, Perlman S. Congenital viral infections of the brain: lessons learned from lymphocytic choriomeningitis virus in the neonatal rat. PLoS Pathog. 2007; 3(11):e149. [PubMed: 18052527]

Borrow P, Oldstone MB. Characterization of lymphocytic choriomeningitis virus-binding protein(s): a candidate cellular receptor for the virus. J Virol. 1992; 66(12):7270–81. [PubMed: 1331520]

Briknarova K, Thomas CJ, York J, Nunberg JH. Structure of a zinc-binding domain in the Junin virus envelope glycoprotein. J Biol Chem. 2011; 286(2):1528–36. [PubMed: 21068387]

Buchmeier, MJ.; de la Torre, JC.; Peters, CJ. Arenaviridae: the viruses and their replication. In: Knipe, DL.; Howley, PM., editors. Fields Virology. 4th ed.. Lippincott-Raven; Philadelphia: 2007. p. 1791-1828.

Buchmeier MJ, Lewicki HA, Tomori O, Oldstone MB. Monoclonal antibodies to lymphocytic choriomeningitis and pichinde viruses: generation, characterization, and cross-reactivity with other arenaviruses. Virology. 1981; 113(1):73–85. [PubMed: 6267791]

Burns JW, Buchmeier MJ. Protein-protein interactions in lymphocytic choriomeningitis virus. Virology. 1991; 183(2):620–9. [PubMed: 1853564]

Burri DJ, Pasqual G, Rochat C, Seidah NG, Pasquato A, Kunz S. Molecular characterization of the processing of arenavirus envelope glycoprotein precursors by subtilisin kexin isozyme-1/site-1 protease. J Virol. 2012; 86(9):4935–46. [PubMed: 22357276]

Charrel RN, de Lamballerie X, Emonet S. Phylogeny of the genus Arenavirus. Curr Opin Microbiol. 2008; 11(4):362–8. [PubMed: 18602020]

Chen J, Lee KH, Steinhauer DA, Stevens DJ, Skehel JJ, Wiley DC. Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. Cell. 1998; 95(3):409–17. [PubMed: 9814710]

de la Torre JC. Molecular and cell biology of the prototypic arenavirus LCMV: implications for understanding and combating hemorrhagic fever arenaviruses. Ann N Y Acad Sci. 2009; 1171(Suppl 1):E57–64. [PubMed: 19751403]

Eichler R, Lenz O, Strecker T, Eickmann M, Klenk HD, Garten W. Identification of Lassa virus glycoprotein signal peptide as a trans-acting maturation factor. EMBO Rep. 2003a; 4(11):1084–8. [PubMed: 14555961]

Eichler R, Lenz O, Strecker T, Garten W. Signal peptide of Lassa virus glycoprotein GP-C exhibits an unusual length. FEBS Lett. 2003b; 538(1-3):203–6. [PubMed: 12633879]

Eichler R, Strecker T, Kolesnikova L, ter Meulen J, Weissenhorn W, Becker S, Klenk HD, Garten W, Lenz O. Characterization of the Lassa virus matrix protein Z: electron microscopic study of virus-like particles and interaction with the nucleoprotein (NP). Virus Res. 2004; 100(2):249–55. [PubMed: 15019244]

Emonet SF, de la Torre JC, Domingo E, Sevilla N. Arenavirus genetic diversity and its biological implications. Infect Genet Evol. 2009; 9(4):417–29. [PubMed: 19460307]

Eschli B, Quirin K, Wepf A, Weber J, Zinkernagel R, Hengartner H. Identification of an N-terminal trimeric coiled-coil core within arenavirus glycoprotein 2 permits assignment to class I viral fusion proteins. J Virol. 2006; 80(12):5897–907. [PubMed: 16731928]

Fischer SA, Graham MB, Kuehnert MJ, Kotton CN, Srinivasan A, Marty FM, Comer JA, Guarner J, Paddock CD, DeMeo DL, Shieh WJ, Erickson BR, Bandy U, DeMaria A Jr. Davis JP, Delmonico FL, Pavlin B, Likos A, Vincent MJ, Sealy TK, Goldsmith CS, Jernigan DB, Rollin PE, Packard MM, Patel M, Rowland C, Helfand RF, Nichol ST, Fishman JA, Ksiazek T, Zaki SR. Transmission of lymphocytic choriomeningitis virus by organ transplantation. N Engl J Med. 2006; 354(21):2235–49. [PubMed: 16723615]

Froeschke M, Basler M, Groettrup M, Dobberstein B. Long-lived signal peptide of lymphocytic choriomeningitis virus glycoprotein pGP-C. J Biol Chem. 2003; 278(43):41914–20. Epub 2003 Aug 12. [PubMed: 12917426]

Gallaher WR, DiSimone C, Buchmeier MJ. The viral transmembrane superfamily: possible divergence of Arenavirus and Filovirus glycoproteins from a common RNA virus ancestor. BMC Microbiol. 2001; 1(1):1. [PubMed: 11208257]

Harrison SC. Viral membrane fusion. Nat Struct Mol Biol. 2008; 15(7):690–8. [PubMed: 18596815]

Igonet S, Vaney MC, Vonhrein C, Bricogne G, Stura EA, Hengartner H, Eschli B, Rey FA. X-ray structure of the arenavirus glycoprotein GP2 in its postfusion hairpin conformation. Proc Natl Acad Sci U S A. 2011; 108(50):19967–72. [PubMed: 22123988]

Kunz S, Rojek J, Perez M, Spiropoulou C, MB O. Characterization of the interaction of Lassa fever virus with its cellular receptor α-dystroglycan. J. Virol. 2005; 79:5979–5987. [PubMed: 15857984]

Lenz O, ter Meulen J, Klenk HD, Seidah NG, Garten W. The Lassa virus glycoprotein precursor GP-C is proteolytically processed by subtilase SKI-1/S1P. Proc Natl Acad Sci U S A. 2001; 98(22): 12701–5. [PubMed: 11606739]

McCormick JB, Fisher-Hoch SP. Lassa fever. Curr Top Microbiol Immunol. 2002; 262:75–109. [PubMed: 11987809]

Messina EL, York J, Nunberg JH. Dissection of the role of the stable signal peptide of the arenavirus envelope glycoprotein in membrane fusion. J Virol. 2012; 86(11):6138–45. [PubMed: 22438561]

Nunberg JH, York J. The curious case of arenavirus entry, and its inhibition. Viruses. 2012; 4(1):83–101. [PubMed: 22355453]

Palacios G, Druce J, Du L, Tran T, Birch C, Briese T, Conlan S, Quan PL, Hui J, Marshall J, Simons JF, Egholm M, Paddock CD, Shieh WJ, Goldsmith CS, Zaki SR, Catton M, Lipkin WI. A new arenavirus in a cluster of fatal transplant-associated diseases. N Engl J Med. 2008; 358(10):991–8. [PubMed: 18256387]

Rawson RB, Cheng D, Brown MS, Goldstein JL. Isolation of cholesterol-requiring mutant Chinese hamster ovary cells with defects in cleavage of sterol regulatory element-binding proteins at site 1. J Biol Chem. 1998; 273(43):28261–9. [PubMed: 9774448]

Rojek JM, Lee AM, Nguyen N, Spiropoulou CF, Kunz S. Site 1 protease is required for proteolytic processing of the glycoproteins of the South American hemorrhagic fever viruses Junin, Machupo, and Guanarito. J Virol. 2008; 82(12):6045–51. [PubMed: 18400865]

Schlie K, Maisa A, Lennartz F, Stroher U, Garten W, Strecker T. Characterization of Lassa virus glycoprotein oligomerization and influence of cholesterol on virus replication. J Virol. 2010; 84(2):983–92. [PubMed: 19889753]

Weber EL, Buchmeier MJ. Fine mapping of a peptide sequence containing an antigenic site conserved among arenaviruses. Virology. 1988; 164(1):30–8. [PubMed: 2452517]

Welch BD, Liu Y, Kors CA, Leser GP, Jardetzky TS, Lamb RA. Structure of the cleavage-activated prefusion form of the parainfluenza virus 5 fusion protein. Proc Natl Acad Sci U S A. 2012

Wessel D, Flugge UI. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem. 1984; 138(1):141–3. [PubMed: 6731838]

Wilson IA, Skehel JJ, Wiley DC. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution. Nature. 1981; 289(5796):366–73. [PubMed: 7464906]

Wright KE, Spiro RC, Burns JW, Buchmeier MJ. Post-translational processing of the glycoproteins of lymphocytic choriomeningitis virus. Virology. 1990; 177(1):175–83. [PubMed: 2141203]

Yin HS, Paterson RG, Wen X, Lamb RA, Jardetzky TS. Structure of the uncleaved ectodomain of the paramyxovirus (hPIV3) fusion protein. Proc Natl Acad Sci U S A. 2005; 102(26):9288–93. [PubMed: 15964978]

Yin HS, Wen X, Paterson RG, Lamb RA, Jardetzky TS. Structure of the parainfluenza virus 5 F protein in its metastable, prefusion conformation. Nature. 2006; 439(7072):38–44. [PubMed: 16397490]

York J, Nunberg JH. Role of the stable signal peptide of Junin arenavirus envelope glycoprotein in pH-dependent membrane fusion. J Virol. 2006; 80(15):7775–80. [PubMed: 16840359]

York J, Nunberg JH. A Novel Zinc-Binding Domain Is Essential For Formation of the Functional Junin Virus Envelope Glycoprotein Complex. J Virol. 2007; 10:10.

York J, Romanowski V, Lu M, Nunberg JH. The signal peptide of the Junin arenavirus envelope glycoprotein is myristoylated and forms an essential subunit of the mature G1-G2 complex. J Virol. 2004; 78(19):10783–92. [PubMed: 15367645]

HIGHLIGHTS

- We investigated the biosynthesis of recombinant ectodomains of arenavirus glycoproteins (GPs).
- The arenavirus ectodomains underwent processing, followed by dissociation of GP1 from GP2.
- The GP2 ectodomain spontaneously formed post-fusion trimers.
- The SSP was dispensable for processing and secretion of the GP ectodomain.

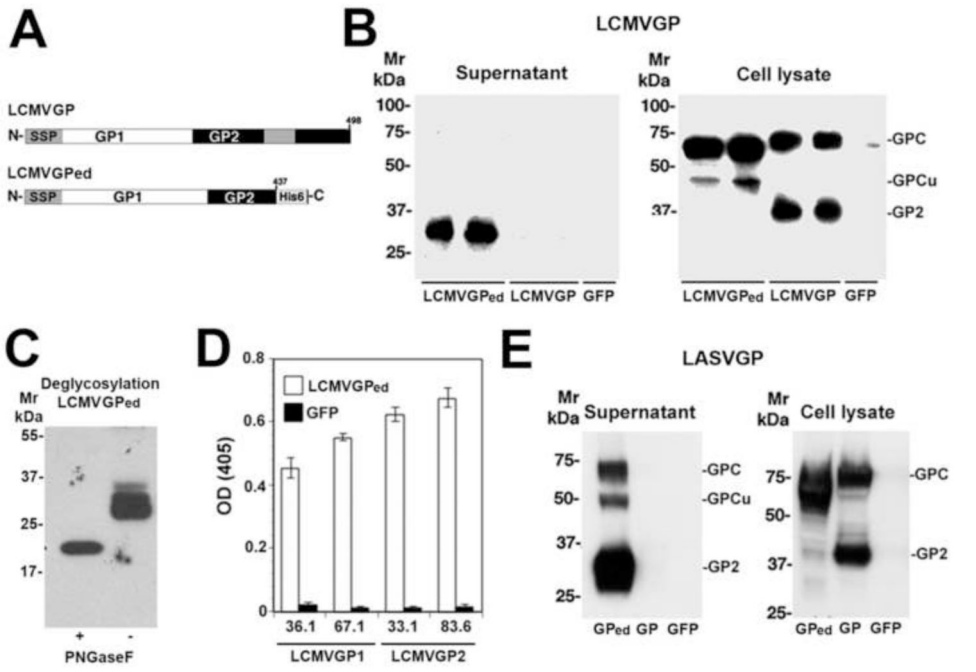

Figure 1. Expression and characterization of the soluble variant LCMV GPed

(A) Schematic representation of the recombinant LCMV GP variants: LCMV GP1 and GP2 are in white and black respectively. The trans-membrane domain of wild-type GP2 is indicated as a gray box. The stable signal peptide (SSP) and the C-terminal His-tag are indicated. (B) Expression of LCMV GPed and wild-type LCMV GP: LCMV GPed (LCMV GPed), wild-type LCMV GP (LCMV GP), and GFP were transiently expressed in HEK293H cells in serum-free medium (duplicates). After 48 h of expression in serum-free conditions, total protein was isolated from cell culture supernatant (supernatant) and cell lysates (cells). Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were probed with mouse mAb 83.6 (anti-LCMV GP2) and a peroxidase-conjugated anti-mouse IgG using ECL for detection. Molecular masses and the expected positions of the precursors (GPC) and mature GP2 are indicated. The additional band at circa 45 kDa likely corresponds to an under-glycosylated form of the GPC (GPCu). (C) Deglycosylation with PNGaseF. LCMV GPed was expressed in HEK293H cells as in (B) and subjected to pull-down with NiNTA agarose. After washing, precipitated proteins were incubated with PNGaseF (+) or reaction buffer only. Proteins were separated by SDS-PAGE (long-run) and GP2 fragments detected in Western blot using an antibody to His6-tag. Apparent molecular masses are indicated. (D) Detection of GP1 and GP2 in supernatants by ELISA: LCMV GPed, and GFP were transiently expressed in HEK293H cells in serum-free medium. Conditioned supernatants were immobilized in microtiter plates and probed with monoclonal antibodies anti-GP1 (36.1 and 67.1) and anti-GP2 (33.1 and 83.6). Primary antibodies were detected with anti-mouse IgG coupled to peroxidase in a color reaction using ABTS substrate. OD (405) was measured using an ELISA reader (data are means ± SD, n = 3). (E) Expression of LASV GPed. LASV GPed (GPed) and wild-type LASV GP (GP), as well as GFP were expressed in HEK293H cells as in (B). The presence of LASV GP2 was detected in cell supernatants and total cell lysates with mAb 83.6 in Western blot as in (B). Molecular masses, GPC, and mature GP2 are indicated. As in (B), the band at circa 50 kDa likely corresponds to under-glycosylated GPC (GPCu).

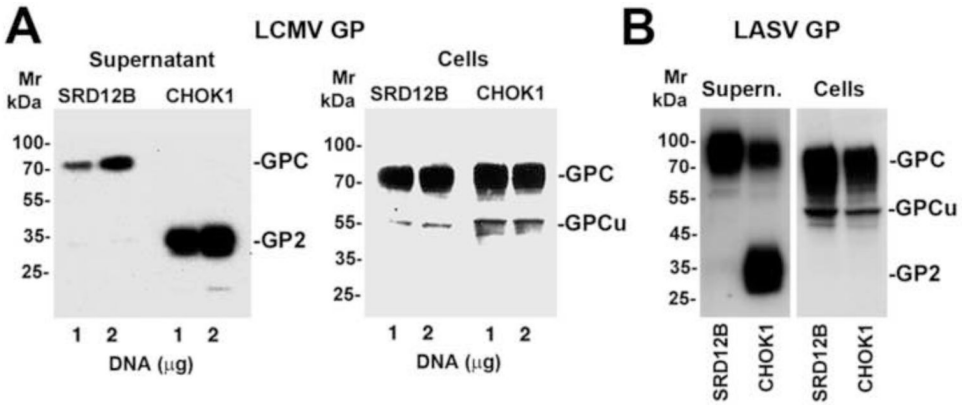

Figure 2. LCMV GPed and LASV GPed are processed by SKI-1/S1P

Expression of LCMV GPed (A) and LASV GPed (B) in SRD12B and CHOK1 cells: SRD12B and CHOK1 cells were transfected with expression plasmids for LCMV GPed and LASV GPed using lipofectamine. After 48 hours of expression in serum-free conditions, total protein was isolated from conditioned cell culture medium (supernatant) and cell lysates (cells). Proteins were separated by SDS-PAGE and Western-blot analysis for LCMV GP2 performed as in Fig. 1. Molecular masses and the position of GPC, GP2, and GPCu are indicated.

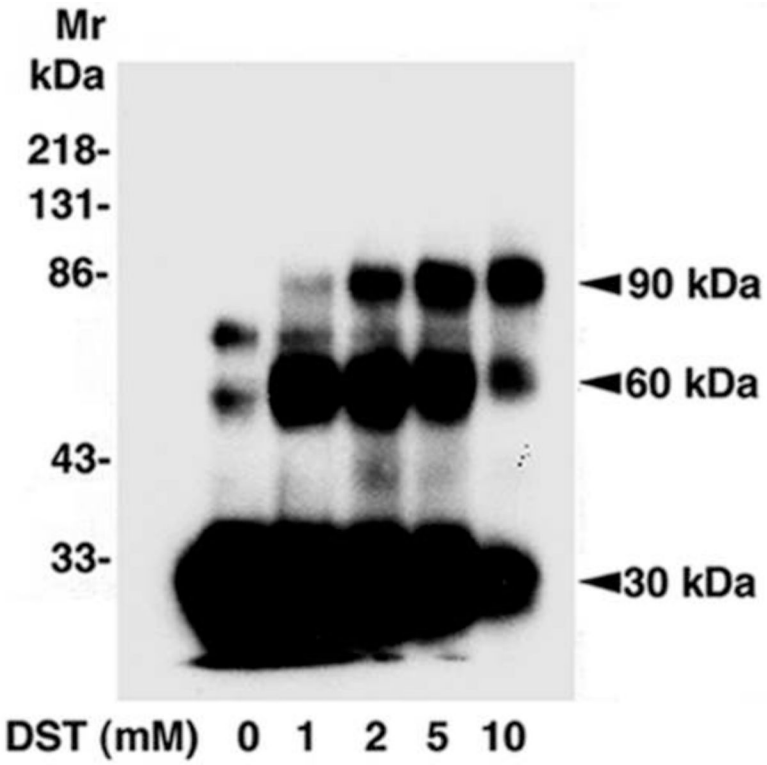

Figure 3. The soluble GP2 domain forms trimers

Recombinant LCMV GPed was expressed in HEK293H cells in serum-free medium. Conditioned supernatants were dialyzed against cross-linking reaction buffer and subjected to chemical cross-linking with the indicated concentrations of S-DST. The reaction was quenched by the addition of 50 mM glycine, total protein isolated, separated by SDS-PAGE, and transferred to nitrocellulose. The blot was probed for GP2 with mAb 83.6. Molecular masses and the calculated apparent molecular weights of the cross-linked GP2 species are indicated.

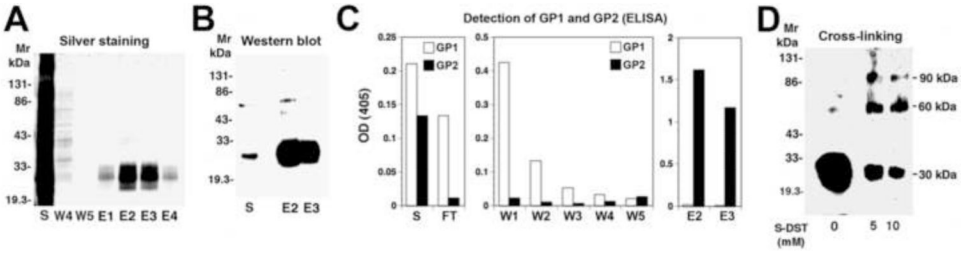

Figure 4. Purified GP2 ectodomain forms trimers

(A-C) Purification of GP2 ectodomain from LCMV GPed: LCMV GPed was expressed in serum-free medium as described in Fig. 1 and purified from concentrated supernatants by NiNTA affinity chromatography. The matrix was washed under high-salt conditions (0.5 M NaCl) and bound GP2 eluted with EDTA. (A) Analysis of the indicated fractions by SDS-PAGE and silver staining (S: supernatant, W4, 5: = late wash fractions, E1-4: eluate fractions). Molecular masses are indicated. (B) Detection of GP2 by Western-blot: Supernatant (S) and eluate fractions E2 and E3 from (B) were subjected to Western blot analysis with mAb 83.6 (anti-LCMV GP2) as described in Fig. 1B. (C) Detection of GP1 and GP2 by ELISA: Supernatant (S), flow-through (FT), the wash fractions W1-W5, as well as the main eluate fractions E2 and E3 were immobilized in microtiter plates and GP1 and GP2 detected by ELISA using mAbs 36.1 (GP1) and 83.6 (GP2), respectively Note the high proportion of GP1 in the flow-through and the early wash fractions. (D) Chemical cross-linking of purified GP2: Eluate fractions E2 and E3 from (B) were pooled, dialyzed against reaction buffer, and chemical cross-linking performed with the indicated concentrations of S-DST as described in Fig. 3. After quenching of the reaction with glycine, total protein was isolated, separated by SDS-PAGE, and transferred to nitrocellulose. The blot was probed for GP2 with mAb 83.6. Molecular masses and the calculated apparent molecular weights of the cross-linked GP2 species are indicated.

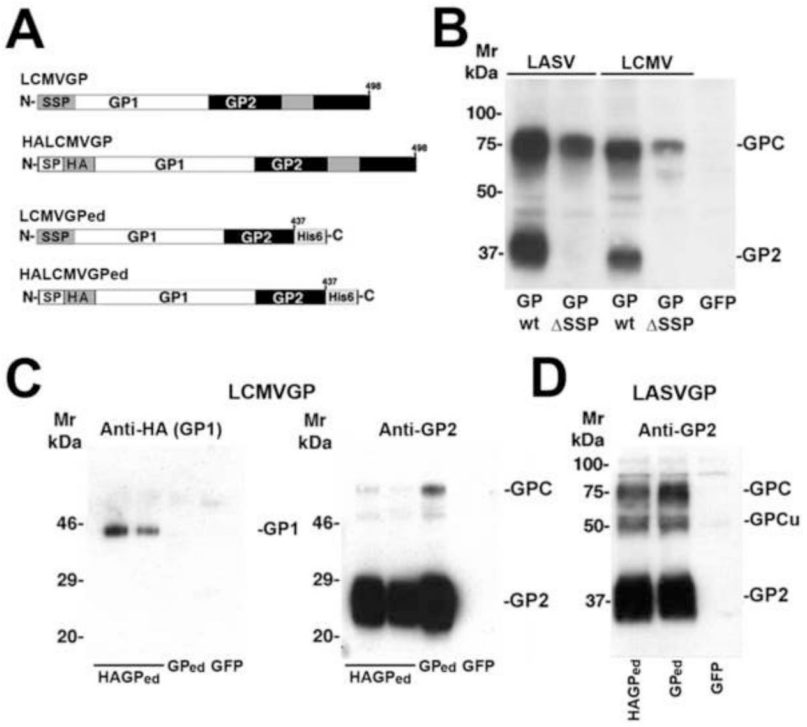

Figure 5. The stable signal peptide (SSP) is required for transport and processing of full-length GPC, but not the GP ectodomain

(A) Schematic representation of the recombinant LCMV GP variants. The SSP and the HA-tag are indicated; SP corresponds to the signal peptide of Ig light chain. (B) The SSP is required for transport and processing of full-length LCMV and LASV GPC. Wild-type LCMV GP and LASV GP (GP wt) and HA-tagged LCMV GP and LASV GP containing the signal peptide of Ig light chain instead of the endogenous SSP (GP SSP) were transfected in HEK293T cells and protein processing detected in Western-blot with antibodies to GP2. (C) The SSP is dispensable for transport and processing of the soluble LCMV GP ectodomain. HALCMV GPed (HAGPed), wild-type LCMV GPed (GPed), and GFP were expressed in HEK293T cells. Recombinant proteins in the supernatant were detected by Western-blot using an antibody to HA (detection of GP1) and to GP2 as in (B). Apparent molecular masses and the positions of GP1, GPC, and GP2 are indicated. (D) Processing of LASV GPed containing the signal peptide of Ig light chain. LASV GPed containing the Ig light chain signal peptide (HAGPed), wild type LASV GP with the authentic SSP (GPed), and GFP were expressed in HEK293T cells. Processing of soluble LASV GP2 in the supernatant was detected by Western-blot as in (B). Molecular masses and the positions of GPC, GPCu and GP2 are indicated.