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Role of the Stable Signal Peptide and Cytoplasmic Domain of G2 in Regulating Intracellular Transport of the Junin Virus Envelope Glycoprotein Complex

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Enveloped viruses utilize the membranous compartments of the host cell for the assembly and budding of new virion particles. In this report, we have investigated the biogenesis and trafficking of the envelope glycoprotein (GP-C) of the Junin arenavirus. The mature GP-C complex is unusual in that it retains a stable signal peptide (SSP) as an essential component in association with the typical receptor-binding (G1) and transmembrane fusion (G2) subunits. We demonstrate that, in the absence of SSP, the G1-G2 precursor is restricted to the endoplasmic reticulum (ER). This constraint is relieved by coexpression of SSP in trans, allowing transport of the assembled GP-C complex through the Golgi and to the cell surface, the site of arenavirus budding. Transport of a chimeric CD4 glycoprotein bearing the transmembrane and cytoplasmic domains of G2 is similarly regulated by SSP association. Truncations to the cytoplasmic domain of G2 abrogate SSP association yet now permit transport of the G1-G2 precursor to the cell surface. Thus, the cytoplasmic domain of G2 is an important determinant for both ER localization and its control through SSP binding. Alanine mutations to either of two dibasic amino acid motifs in the G2 cytoplasmic domain can also mobilize the G1-G2 precursor for transit through the Golgi. Taken together, our results suggest that SSP binding masks endogenous ER localization signals in the cytoplasmic domain of G2 to ensure that only the fully assembled, tripartite GP-C complex is transported for virion assembly. This quality control process points to an important role of SSP in the structure and function of the arenavirus envelope glycoprotein.

Arenaviruses are endemic in rodent populations worldwide (53), and infection can be transmitted to humans to cause severe acute hemorrhagic fevers (44, 51). Recurring outbreaks are common in regions of arenavirus endemcity, and therapeutic options to combat arenavirus infection are limited. Phylogenetic analyses divide the arenaviruses into the Old World viruses, and the New World species, such as Junin and Machupo viruses. Up to 300,000 infections with Lassa fever virus occur annually in Africa (45), and outbreaks of New World arenaviruses remain an urgent public health concern.

The arenaviruses are enveloped viruses whose genomes consist of two single-stranded RNA molecules, each of which encodes the ambisense expression of two of the four viral proteins (5, 9). The viral envelope glycoprotein (GP-C) is translated from a genomic-sense mRNA generated from the short (S) genomic RNA, whereas the nucleocapsid protein is translated from the antigenomic-sense mRNA. Similarly, the viral matrix protein (Z) and RNA-dependent RNA polymerase are encoded in an ambisense orientation by the long (L) RNA. During biogenesis, arenaviral particles assemble and bud at the plasma membrane (49, 60). Viral entry into target cells is initiated by GP-C binding to cell surface receptors followed by endocytosis of the virion into smooth vesicles (2). Although α-dystroglycan serves as a binding receptor for the Old World arenaviruses (6), the receptor utilized by the major New World group of arenaviruses is unknown (59). GP-C-mediated membrane fusion is activated upon acidification of the maturing endosome (2, 7, 13, 14) to deposit the virion core into the cell cytoplasm and initiate replication.

The arenavirus envelope glycoprotein complex consists of three noncovalently associated subunits derived from the GP-C precursor: in addition to the typical receptor-binding (G1) and transmembrane fusion (G2) subunits, the complex contains a stable signal peptide (SSP) subunit (4, 18, 65) (Fig. 1). The 58-amino-acid SSP is generated by the cellular signal peptidase and subsequently myristoylated (65). The mature G1 and G2 subunits are generated upon cleavage by the cellular SKI-1/S1P protease (1, 35, 38) in the early Golgi compartment (3). This proteolytic maturation event is essential for membrane fusion activity. The arenavirus G2 is a member of the class I group of viral fusion proteins (25, 64) that orchestrate membrane fusion through the triggered formation of a stable six-helix bundle core (references 16, 17, 32, and 63 and references therein).

A tripartite envelope glycoprotein complex is unusual among viral envelope glycoproteins, and the role of the unique arenavirus SSP subunit has not been fully defined. In the GP-C complex, SSP exists as a transmembrane protein, likely in a type II topology with an extended luminal C terminus (19, 23). The N terminus is modified by myristoylation, which is important for efficient membrane fusion activity (65). Recombinant GP-C constructs in which SSP is replaced by a conventional...
signal peptide do not undergo significant proteolytic matura-
tion by the SKI-1/S1P cleavage site (18). In the Old World
Lassa fever arenavirus, this defect can be rescued by coexpression
of SSP in trans (18).

In the present report, we examine the biogenesis of the
GP-C complex of the Junin virus, a member of the New World
Arenaviridae family that causes severe viral hemorrhagic fever.
We show that SSP association is required for transport of the
G1-G2 precursor from the endoplasmic reticulum (ER) and thereby
for proteolytic maturation in the Golgi. In the absence of SSP, the
G1-G2 precursor is constrained to the ER by dibasic amino acid
sequences in the cytoplasmic domain of G2. Association with SSP
overcomes this block to permit transit of the fully assembled complex through
the Golgi and to the cell surface. Moreover, our studies suggest
that, in addition to modulating trafficking of GP-C, SSP
association may also be important for the membrane fusion activity
of the GP-C complex. The unique roles for SSP in the arenavirus
life cycle may suggest novel strategies towards the preven-
tion and treatment of arenaviral disease.

MATERIALS AND METHODS

Molecular reagents, recombinant vaccinia viruses, and monoclonal antibodies.
The GP-C coding region from the pathogenic Junin virus strain MC2 (28) was
provided by Victor Romanowski (Universidad Nacional de La Plata, Argentina)
and introduced into the mammalian expression vector pCDNA3.1+ as described
previously (65). For trans-complementation studies (18), the CD4sp-GPC
construct in which SSP was replaced by the conventional signal peptide of CD4 (65)
was coexpressed with an SSP construct in which a stop codon was introduced
following the C-terminal SSP amino acid T58 (SSP-term). A chimeric glycopro-
tein (CD4ecto) bearing the CD4 signal peptide and ectodomain fused to the
transmembrane and cytoplasmic domains of G2 was constructed using the hu-
man CD4 cDNA (41) obtained through the National Institutes of Health (NIH)
AIDS Research and Reference Reagent Program. Mutations were introduced by
QuickChange mutagenesis (Stratagene), and PCR was used to generate truncations
and chimeric plasmids. For the cytoplasmic-domain truncation series and in

![Image](58x618 to 286x722)

FIG. 1. Schematic representation of the Junin virus GP-C glyco-
protein and G2 cytoplasmic domain sequences. Amino acids of the
Junin virus envelope glycoprotein are numbered from the initiating
methionine, and cysteine residues (Y) are marked. The SSP and SKI-1/S1P cleavage sites
are indicated by bracket and dibasic amino acid sequences are underlined.

Expression of GP-C and its derivatives. The glycoproteins were expressed and
characterized as previously described (64, 65). Briefly, Vero 76 cells were infected
with the recombinant vaccinia virus vTF7-3 (24) at a multiplicity of 2 in Dulbecco’s minimal essential medium containing 2% fetal bovine serum (FBS)
and 10 μg/ml cytosine arabinoside (araC) (31). After 30 min, the cells were washed
and transfected with the GP-C expression plasmid using Lipofectamine 2000
reagent (Invitrogen). Metabolic labeling using 32 to 50 μCi/ml of [35S]Met-Xen
(Amersham Pharmacia Biotech) was initiated 6 h posttransfection in methio-

teine- and cysteine-free medium containing 10% dialyzed FBS and 10 μM araC
and was continued for 12 to 16 h. Cultures were then washed in physiological
buffer containing PBS and lysed using cold Tris-saline buffer (64) containing
10 mM HEPES, pH 7.4, and 150 mM NaCl (pH 7.5) containing 1% Triton X-100 nonionic detergent and protease inhibitors (1 μg/ml each of aprotinin, leupeptin, and pepstatin). The expressed glycoproteins were isolated from cleared lysates by immunoprecipita-
tion using either the G1-directed MAbs or the CD4-directed MAb SIM.2 and
protease treatment as described above (64, 65).

Flow cytometry. Vero 76 cells expressing GP-C or its derivatives were labeled
using the G1-specific MAb BE08 (54) and a secondary fluorescein isothio-
ycyanate-conjugated goat anti-mouse antibody (Jackson ImmunoResearch). CD4
was detected using a fluorescein isothiocyanate-conjugated mouse anti-CD4
MAb (BD Biosciences). Cells were subsequently stained using propidium iodide
(1 μg/ml) and then fixed in 4% formaldehyde (64). Populations were analyzed
using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

GP-C-mediated cell-cell fusion. The β-galactosidase fusion reporter assay (47)
was used to characterize the ability of the envelope glycoproteins to mediate
photon-dependent cell-cell fusion (64, 65). Briefly, Vero cells infected with vTF7-3
and expressing the envelope glycoprotein were cocultured with reporter cells
infected with vCB21R-lacZ, a recombinant vaccinia virus expressing β-galacto-
sidase under the control of the T7 promoter. The reporter cells were obtained by
incubating Vero 76 cells with vCB21R-lacZ at a multiplicity of 2 and allowing
the infection to proceed overnight in the presence of 100 μg/ml rifampin (31). The
GP-C-expressing cells and reporter cells were cocultured in medium containing
both araC and rifampin for 5 h and then subjected to a 30-min pulse of neutral
or acidic (pH 5.0) medium. β-Galactosidase expression is induced upon fusion of
the effector and reporter cells and was detected, after 5 h of continued cultivation
at neutral pH, in cell lysates (Tropix) using the chemiluminescent substrate
GalactoLite Plus (Tropix). Cell-cell fusion was quantified using a Tropix TR717
microplate luminometer.

Cytokine assay. Cells expressing GP-C glycoproteins were harvested by
trypsinization 6 h after transfection and reseeded to 8-well chambered cover
glasses (Lab Tek II) in medium containing 10 μM araC. After 18 h, cultures were
washed in PBS and fixed with 4% formaldehyde for 10 min at room temperature.
Following washing and quenching with 50 mM Tris (pH 7.4) in PBS, cultures

![Image](58x618 to 286x722)

FIG. 1. Schematic representation of the Junin virus GP-C glyco-
protein and G2 cytoplasmic domain sequences. Amino acids of the
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methionine, and cysteine residues (Y) are marked. The SSP and SKI-1/S1P cleavage sites
are indicated by bracket and dibasic amino acid sequences are underlined.
were either permeabilized in PBS containing 0.1% Triton X-100 and blocked in the same buffer containing 5% FBS (for intracellular staining) or simply blocked in the absence of detergent (for cell surface staining). GP-C glycoproteins were detected using the G1-directed MAb BF11 and an Alexa Fluor 488-conjugated anti-mouse antibody (Molecular Probes) in the appropriate blocking buffer. The Golgi marker giantin was detected using a rabbit polyclonal antisemur (Covance Research Products) and an Alexa Fluor 568-conjugated anti-rabbit antibody (Molecular Probes). Chambers were covered with Slow Fade Gold (Molecular Probes) and visualized using an inverted Nikon TE-300 microscope. Fluorescence was examined using a Bio-Rad Radiance 2000 confocal laser scanning microscope and images were merged using Lasersharp software (Bio-Rad).

RESULTS

SSP association is required for proteolytic maturation. The arenavirus SSP is distinct from conventional signal peptides in that it is retained as an essential subunit of the mature GP-C envelope glycoprotein complex and mediates functions beyond translocation of the nascent polypeptide to the ER (18, 20, 65). We previously showed that a recombinant Junin virus GP-C glycoprotein in which SSP was replaced by the conventional signal peptide of human CD4 (CD4sp-GPC) was unable to undergo efficient maturation by the SKI-1/S1P protease (65), extending similar observations with GP-C of the Old World Lassa fever virus (18). In this Old World virus, the deficiency in proteolytic cleavage in the absence of SSP was reversed by coexpression of SSP in trans (18).

To investigate the role of SSP in the proteolytic maturation of the Junin virus GP-C, we determined whether the coexpression of SSP in trans could likewise rescue cleavage. In these studies, the Junin virus CD4sp-GPC construct was cotransfected with the SSP-term plasmid encoding the 58-amino-acid SSP. Optimal expression in Vero cells was dependent on T7 RNA polymerase provided by the recombinant vaccinia virus vTF7-3 (24). Cells were metabolically labeled, and GP-C glycoproteins were immunoprecipitated using the G1-directed MAb BE08 (54). Baseline studies were performed using the native GP-C glycoprotein that included its endogenous SSP. Expression of the native glycoprotein resulted in the isolation of a 60-kDa G1-G2 precursor glycoprotein and a heterodisulfide smear of G1 and G2 subunits (30 to 35 kDa) (Fig. 2A, top panel). These mature subunits are best resolved following deglycosylation by PNGase F to yield 22- and 27-kDa polypeptides (bottom panel). The deglycosylated GP-C polypeptides reveal both the glycoproteins have been treated with PNGase F to resolve G1 and G2 polypeptides. The deglycosylated GP-C polypeptides reveal both the G1-G2 precursor and, in SSP-containing constructs, the pre-GP-C precursor (65); additional species that migrate more slowly than the G1-G2 precursor and, in SSP-containing constructs, the pre-GP-C precursor (65): additional species that migrate more slowly than the G1-G2 precursor and with the pre-GP-C precursor are likely products of incomplete deglycosylation. cd-GPC contains a C-terminal S-peptide affinity tag and migrates slightly slower than the other G1-G2 precursors. Known GP-C species are labeled at left; minor unidentified bands are also present. The 14C-labeled protein markers (Amersham Biosciences) are indicated (in kilodaltons). (B) Cell surface expression of GP-C in Vero cells was determined by flow cytometry using the G1-specific MAb BE08 (54). The cell population was subsequently stained using propidium iodide (1 mg/ml) to exclude dead cells. Cells were then fixed using 2% formaldehyde and analyzed using a FACSCalibur flow cytometer (BD Biosciences). The histograms plot cell number (counts) versus the fluorescence intensity of MAb binding. Background staining of mock-transfected cells is shown to identify nonexpressing cells in the transfected cell populations.

SSP rescues cell-cell fusion activity in trans. To determine whether the trans-complemented complex was also able to mediate pH-dependent membrane fusion, we cocultured cells expressing GP-C glycoproteins with Vero target cells infected with the fusion reporter vaccinia virus vCB21R-LacZ expressing the β-galactosidase gene under control of the T7 promoter (47). In this assay, activation of GP-C-mediated membrane fusion by acidic pH (5.0) results in syncytium formation between the effector and reporter cells and expression of β-galactosidase; the enzymatic activity is then monitored using a chemiluminescent substrate (64). As shown in Fig. 3, pH-dependent cell-cell fusion is readily detected using the native GP-C glycoprotein and absent in the cleavage-defective cd-GPC mutant. Cells expressing the CD4sp-GPC glycoprotein in the absence of SSP were unable to mediate cell-cell fusion.
FIG. 3. pH-dependent cell-cell fusion activity. pH-dependent fusion was detected using the recombinant vaccinia virus-based β-galactosidase reporter assay (47) as previously described (64, 65). β-Galactosidase activity was quantitated using the chemiluminescent substrate Galfos-Lite Plus (Tropix). Relative light unit (RLU) measurements from cultures treated at pH 5.0 are shown after subtraction of background levels from neutral-pH cultures (average background, 1,500 RLU). Control conditions are shown in the underlined bars at left (mock, wild-type GP-C, and cd-GPC). Note that CD4sp-GPC constructs were bracketed in pairs (below the axis) representing the absence (open bars) and presence (gray bars) of SSP. Some bars are not discernible on the scale of the graph. All conclusions were replicated using X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) staining of parallel cocultures.

(Fig. 3, CD4sp, first of the bracketed pairs of bars). By contrast, coexpression of SSP reconstituted pH-dependent cell-cell fusion activity in the trans-complemented CD4sp-GPC complex (second of bracketed pairs of bars) to levels greater than those seen with the native GP-C glycoprotein. Thus, expression of SSP in trans can fully restore membrane fusion activity to the Junin virus G1-G2 precursor glycoprotein.

SSP association is required for exit from the ER. To investigate the role of SSP in the biogenesis of GP-C, we examined the intracellular localization of the complex by confocal microscopy. In these experiments, Vero cells expressing the wild-type and CD4sp-GPC glycoproteins were fixed, permeabilized, and immunochemically stained using the anti-G1 MAb BF11 (54) and an Alexa Fluor 488-conjugated secondary antibody. Nonpermeabilized cells were similarly stained to detect GP-C accumulation on the cell surface. As shown in Fig. 4, the native GP-C glycoprotein accumulated in the ER and Golgi-like perinuclear structures (GP-C, permeabilized) and on the cell surface (GP-C, surface). Localization to the Golgi apparatus was confirmed using a rabbit polyclonal antibody directed against an integral Golgi membrane protein, giantin (40), and a secondary Alexa Fluor 568-conjugated antibody. Colocalization of GP-C with the Golgi marker is visualized in yellow in the merged images. Expression of CD4sp-GPC in the presence of SSP resulted in a pattern of localization and transport to the cell surface similar to that of native GP-C (Fig. 4, CD4sp, + SSP). These findings highlight the reconstitution of the GP-C complex upon trans complementation with SSP.

In the absence of SSP, however, the G1-G2 precursor of CD4sp-GPC exhibited a diffuse reticulate pattern of intracellular expression consistent with retention in the ER (Fig. 4, CD4sp, − SSP). Notably absent was any concentration of GP-C staining to a morphologically defined Golgi apparatus or specific colocalization with the antigiantin MAb (merged image). The orange in the merged image likely reflects the spatial coincidence of green and red fluorescence rather than specific colocalization to a definable Golgi structure. Also absent was any staining of CD4sp-GPC on the cell surface (surface). The lack of transport to the cell surface is not due to the absence of proteolytic cleavage per se, because the cleavage-site-defective cd-GPC mutant is transported to the cell surface as the wild-type glycoprotein (not shown) (1, 35). Nor did we detect punctate staining in the ER that might suggest misfolding of the G1-G2 precursor in the absence of SSP. The difference in trafficking of the G1-G2 precursor to the Golgi in the presence or absence of SSP likely accounts for the effect of trans complementation on proteolytic cleavage (Fig. 2A), consistent with the activation of SKI-1/SIP protease in the cis-medial Golgi compartment (10, 21).

Next, we examined the role of SSP in the transport of the GP-C complex to the cell surface by using flow cytometry and the G1-specific MAb BE08. In cell cultures transiently expressing the wild-type GP-C glycoprotein, a clear population of GP-C-expressing cells was evident (Fig. 2B, top right). A comparison of cells expressing CD4sp-GPC in the presence or absence of SSP revealed that the GP-C glycoproteins were present on the cell surface only upon coexpression of SSP (bottom panels). Cell surface accumulation of the trans-complemented CD4sp-GPC glycoprotein was comparable to that of the native GP-C glycoprotein. Taken together, these results demonstrate that SSP is essential for GP-C transport to the Golgi and the cell surface. In the absence of SSP, the G1-G2 precursor is localized to the ER.

Transit of a CD4 chimera bearing G2 sequences. To further investigate the role of the G2 subunit in ER localization and the role of SSP in regulating transit to the cell surface, we determined whether control by SSP and the G2 subunit might be transferable to a heterologous cell surface protein. Because the ectodomain of human CD4 forms a soluble and secreted protein (11, 58), we fused the CD4 signal peptide and ectodomain to the transmembrane and cytoplasmic regions of G2. In the CD4ecto construct, the C terminus of soluble CD4 (TPV1773) (11) was spliced at the G2 ectodomain sequence TPV425 (residues upstream of D424) that nominally defines the junction with the transmembrane domain.

Cells expressing the CD4ecto chimera or native CD4 were metabolically labeled, and cell lysates were immunoprecipitated using the anti-CD4 ectodomain MAb SIM.I. The CD4ecto chimera was expressed as a 55-kDa glycoprotein that comigrated with native CD4 (Fig. 5A, left panel). Upon coexpression, SSP was found to coprecipitate with CD4ecto (Fig. 5A, left panel). This association was specific to G2 sequences in the CD4ecto glycoprotein; SSP did not bind to native CD4 (when coexpressed) (not shown). Thus, the transmembrane and cytoplasmic domains of G2 are sufficient for SSP binding.

Importantly, transport of the CD4ecto chimera through the Golgi apparatus and to the cell surface was dependent on coexpression of SSP. As shown by immunofluorescent staining using SIM.2 MAb and confocal microscopy (Fig. 4, CD4ecto, permeabilized), the chimeric glycoprotein was largely con-
strained to the ER in the absence of SSP and failed to colo-
calize with the Golgi apparatus (H11002 SSP). In addition, only trace
amounts of the CD4ecto glycoprotein were detected in the absence of SSP on the cell surface, either through confocal
microscopy (Fig. 4, surface) or flow cytometry (Fig. 5B, H11002 SSP).
Thus, fusion to the G2 transmembrane and cytoplasmic
domains prevented transport of the CD4 ectodomain from
the ER.

By contrast, coexpression with SSP resulted in significant
localization of CD4ecto in the Golgi (Fig. 4, +SSP) and ex-
pression on the cell surface (surface). Mobilization of the chi-
meric glycoprotein by SSP was confirmed by flow cytometry
(Fig. 5B, −SSP). Thus, fusion to the G2 transmembrane and cytoplasmic
domains prevented transport of the CD4 ectodomain from
the ER.

Among transmembrane proteins that are retained in
the ER, specific localization signals are often encoded within
the cytoplasmic domain (references 22, 37, and 62 and refer-
ences therein). In order to define the determinants in G2 that
are required for ER localization, we constructed a series of
C-terminal truncations in the cytoplasmic domain of G2. Three
arginine residues, spaced 4, 7, and 17 amino acids from the
nominal transmembrane domain, were used as endpoints in
the truncations (Fig. 1). These positively charged termini were
chosen to facilitate anchoring of the truncated CD4sp-GPC
glycoprotein in the membrane. The arginine codons were fused
to those encoding an S-peptide affinity tag (34) to facilitate
analysis of the G2 moiety (65). Metabolically labeled glyco-
protein was isolated using the Spep affinity tag and S-protein
agarose (Novagen). The truncated CD4sp-GPC glycoproteins
(R448, R451, and R460) were well expressed in Vero cells
yet failed to coprecipitate significant amounts of SSP (Fig. 6A,
top panel). Nonetheless, all three truncated glycoproteins were

FIG. 4. Intracellular and cell surface visualization of glycoproteins. Confocal images were obtained as described in Materials and Methods.
Permeabilized cells were stained in green using either the MAb BF11 (GP-C) or, for CD4ecto, SIM.2 (CD4). Golgi structures were identified using
a rabbit polyclonal antiserum and stained in red. Merged images (merge) were created using Lasersharp software. Nonpermeabilized cells (surface)
were stained in green using either MAb BF11 or SIM.2. The expressed glycoproteins are indicated in white letters superimposed on the leftmost
images. The top row depicts cells expressing native GP-C or mock-transfected cells (all infected with the recombinant vaccinia virus vTF7-3). In
subsequent rows, the glycoproteins were expressed either in the absence (−SSP) or presence (+SSP) of SSP. In some images, the Golgi apparatus
is vesiculated and dispersed, perhaps due to infection of the cells by vaccinia virus.

Analysis of C-terminal truncations in the G2 cytoplasmic
domain. Among transmembrane proteins that are retained in
the ER, specific localization signals are often encoded within
the cytoplasmic domain (references 22, 37, and 62 and refer-
ences therein). In order to define the determinants in G2 that
are required for ER localization, we constructed a series of
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to those encoding an S-peptide affinity tag (34) to facilitate
analysis of the G2 moiety (65). Metabolically labeled glyco-
protein was isolated using the Spep affinity tag and S-protein
agarose (Novagen). The truncated CD4sp-GPC glycoproteins
(R448Δ, R451Δ, and R460Δ) were well expressed in Vero cells
yet failed to coprecipitate significant amounts of SSP (Fig. 6A,
top panel). Nonetheless, all three truncated glycoproteins were
subjected to SKI-1/S1P cleavage, in the presence or absence of SSP, to produce truncated and affinity-tagged G2 moieties (Fig. 6A, bottom panel). The relative migrations of the truncated G2 polypeptides correspond to their expected molecular weights but cause them to overlap with the intact G1 polypeptide. The association between G1 and the truncated G2 subunits was separately confirmed by communoprecipitation using a MAb directed to G1 (not shown). By contrast, similar truncations in G2 of the Old World LCM virus were reported to prevent SKI-1/S1P cleavage (35).

Flow cytometry was used to determine whether the truncated Junin virus glycoproteins were also transported to the cell surface without SSP. As shown in Fig. 6B, all three truncation mutants were expressed on the cell surface in the absence of SSP, at levels comparable to the trans-complemented CD4sp-GPC glycoprotein (Fig. 2B). Truncations in the context of CD4ecto likewise enabled transport from the ER (not shown). In the LCM virus (35), the truncated GP-C was also expressed on the cell surface. Taken together, these results suggest that amino acid sequences within the cytoplasmic domain of G2 are important in constraining the G1-G2 precursor to the ER. The cytoplasmic region is also important for SSP association.

We have demonstrated that GP-C glycoproteins bearing truncations in the cytoplasmic domain of G2 can be proteolytically processed and transported to the cell surface in the absence of SSP. Surprisingly, however, none of the truncated complexes was able to mediate pH-dependent cell-cell fusion (Fig. 3). It is possible that this failure may be due to insufficient cleavage or transport of the truncated glycoproteins. Alternatively, the failure may reflect a requirement for either SSP or the cytoplasmic domain of G2 for membrane fusion activity.

**Dibasic amino acid sequences participate in ER localization.** Sequence analysis of the G2 cytoplasmic domain revealed conserved motifs that may be involved in protein trafficking and ER localization. In particular, dibasic amino acid sequences such as the canonical KXXX and RRX motifs are widely utilized in the retrieval of transmembrane proteins to the ER (see references 22, 37, and 62 and references therein). The cytoplasmic domain of Junin virus G2 contains two related dibasic sequences: KKPT479 and a C-terminal RRGH485. Variants of these sequences appear in other arenavirus G2 proteins (Fig. 1). To assess the potential role of these sequences in ER localization, we mutated the two basic amino acids at each site to alanines, both individually (KK and RR glycoproteins) and as the double mutant (KK/RR).

Immunoprecipitation studies of metabolically labeled whole-cell lysates revealed that all of the mutant CD4sp-GPC glycoproteins were able to associate with SSP (Fig. 7A, top panel). Neither of the dibasic sequences was essential for SSP binding. trans complementation with SSP enabled wild-type levels of cell surface expression (Fig. 7B, +SSP) and efficient pH-dependent cell-cell fusion (Fig. 3), arguing against significant adverse effects of the mutations on overall protein folding.

In the absence of SSP, importantly, both the single and double mutants were now capable of transport to the cell surface.
surface. This phenotype was evident upon confocal microscopic analysis of nonpermeabilized cells (Fig. 4, surface), although specific localization in the Golgi was difficult to discern (green and merged images). Flow-cytometric studies of cell surface expression indicated that both the single and double mutations provided modest, albeit significant, relief of ER retention (Fig. 7B, /H11002 SSP). Evidence for enhanced SKI-1/S1P cleavage of the mutant glycoproteins was, however, difficult to discern in whole-cell lysates, above the residual level of cleavage in the wild-type glycoprotein (Fig. 7A, bottom panel). It is possible that the cleaved species in the wild-type G1-G2 glycoprotein reflect transient residence in the Golgi, prior to retrieval to the ER. In the Old World Lassa fever virus glycoprotein, where cleaved products are not observed in the absence of SSP (18), retrieval of the G1-G2 precursor may be more rapid. Nonetheless, mobilization of the mutant glycoproteins to the cell surface was consistently observed and distinct from the strict intracellular retention seen with the wild-type glycoprotein. Both KK and RR mutations appeared to be comparably efficacious, and no synergy was observed in the double KK/RR mutant. However, none of the mutant glycoproteins was able to mediate cell-cell fusion in the absence of SSP (Fig. 3). This defect is not attributable to the amino acid substitutions per se, as wild-type levels of fusion were restored upon trans complementation with SSP.

To confirm that the mutations are sufficient for significant mobilization of the G1-G2 precursor in the absence of SSP, we examined the glycoprotein by immunoprecipitation from the cell surface (Fig. 8). These experiments confirmed significant expression of the dibasic sequence mutants on the cell surface and demonstrated a preponderance of the proteolytically processed G1-G2 complex, reflecting access to the SKI-1/S1P protease in the Golgi. The efficiency of cleavage in the mutant glycoproteins was relatively unaffected by the presence or absence of SSP (60% cleaved versus 40% cleaved, respectively).

Taken together, these studies identify the two dibasic amino...
acid sequences (KKPT_{475} and RRGH_{485}) as important determinants of ER localization in the absence of SSP. Alanine mutations at either or both of these sites result in partial relief from ER retention and enable transport to the cell surface in the absence of SSP. On the other hand, these mutations do not completely obviate the requirement for SSP association in transport of the G1-G2 precursor (Fig. 8). Quantitative analysis of the glycoproteins indicated that, whereas the mutations were able to increase cell surface expression at least 10-fold, coexpression of SSP resulted in an additional 10-fold increase in all mutants, to the levels of the wild-type glycoprotein. These findings are consistent with our results from confocal microscopy and flow cytometry studies (Fig. 4 and 7). Thus, constraints on the trafficking of the G1-G2 precursor include the dibasic sequence motifs in the cytoplasmic domain of G2 but also involve additional structural elements provided upon full assembly with SSP.

**DISCUSSION**

The regulation of trafficking through intracellular membranous compartments is central to the biogenesis of membrane glycoproteins (15, 22). Quality control mechanisms for protein folding and assembly are proposed to operate through checkpoints on exit from the ER and through bidirectional transport to and from the Golgi apparatus. Viruses make use of these cellular pathways in the biosynthesis, assembly, and release of new virion particles (15). In our studies, we have characterized the biogenesis of the arenavirus envelope glycoprotein and the requirement for tripartite assembly to enable transport of the GP-C complex from the ER. Without the association of SSP, the wild-type G1-G2 precursor remains localized to the ER. We show that localization is mediated by the cytoplasmic domain of G2 and that the control of trafficking by SSP association is transferable to a chimeric CD4 molecule bearing the G2 transmembrane and cytoplasmic domains. Conversely, regulation of intracellular transport of the GP-C complex does not require G1 or the ectodomain of G2.

Our studies demonstrate that ER localization is mediated in part through dibasic amino acid sequences in the cytoplasmic domain of G2. Alanine mutations to either of two dibasic motifs provide partial relief from ER localization and enable expression of the proteolytically cleaved G1-G2 complex on the cell surface. Upon exit from the ER and transit through the Golgi, the mutant G1-G2 precursor is now fully susceptible to proteolytic maturation by SKI-1/S1P protease. Thus, absent ER localization signals, the arenavirus GP-C precursor can undergo proteolytic maturation much as do the precursor glycoproteins of other class I viral fusion proteins.

Dibasic amino acid sequences are known to mediate ER localization through retrograde transport (retrieval) from the Golgi (references 22, 37, and 62 and references therein). The specific dibasic sequences we have identified as important for ER localization in the Junin virus G2 glycoprotein do not match precisely either of the canonical ER retrieval motifs: the C-terminal KKXX or internal RRXX sequences. Although the internal KK sequence studied here is conserved among the New World arenaviruses, the C-terminal RRXX sequence shows considerable variation (Fig. 1). Among the Old World viruses, only the C-terminal motif is identifiable. However, variants to the canonical motifs are also common in other ER-localized transmembrane proteins (46, 55, 62) and the efficiency of retention by these sequences is often highly context dependent (26, 57, 66). Many details regarding the mechanisms and molecular determinants involved in ER-Golgi trafficking remain unresolved.

It is noteworthy that a viral envelope glycoprotein destined for the cell surface should encode an ER localization signal. For cellular transmembrane proteins that traverse the Golgi and beyond, dibasic ER localization motifs are commonly found to control the assembly and trafficking of heteromultimeric membrane protein complexes (12, 33, 39, 42, 67; reviewed in references 22 and 46). These endogenous signals prevent transport of the individual subunits and are overcome upon assembly of the multimeric complex. This quality control mechanism ensures that only the fully and properly assembled complex is transported from the ER. In the biogenesis of the Junin virus GP-C complex, we propose an analogous role for SSP association—namely, to mask endogenous ER localization signals in the cytoplasmic domain of G2 and thus enable transport of only the fully assembled tripartite complex.

This strategy for assembly-dependent control of viral envelope glycoprotein trafficking is likely not unique to the arenaviruses. The bunyavirus Gc glycoprotein also contains a noncanonical basic amino acid cluster that may be involved in ER localization (29). In these viruses, transport of Gc from the ER requires association with a second envelope glycoprotein, Gn (30, 36), which in turn retains the Gc-Gn complex in the Golgi (27, 29, 56), the site of virus budding. Together, these observations highlight the use of cellular ER-Golgi trafficking mechanisms during the viral life cycle to control the assembly and transport of multimeric envelope glycoprotein complexes.

Despite mutations that enable the transport of the G1-G2 complex in the absence of SSP, wild-type levels of trafficking were not restored by point mutations to the dibasic amino acid sequences or by truncations in the cytoplasmic domain (not shown). It is possible that additional constraints on GP-C transport lie within the transmembrane domain of G2. Moreover, it is likely that the association with SSP remains essential for the integrity of the GP-C complex. The SSP subunit has uniquely evolved within the arenaviruses for purposes other than simply to relieve ER retention of an envelope glycoprotein precursor. It is telling, then, that despite the accumulation of cleaved G1-G2 complex on the cell surface, none of the glycoproteins lacking SSP is able to mediate membrane fusion (Fig. 3). Notably, GP-C glycoproteins bearing mutations at the dibasic amino acid motifs are unable to promote fusion in the absence of SSP yet are restored to full activity by coexpression of SSP. This defect in fusion is likely not due to the lower levels of cell surface glycoprotein in the absence of SSP, as robust fusion is observed with comparably low levels of cleaved wild-type glycoprotein (see Fig. 6 of reference 64). Rather, we suggest that SSP may be directly involved in modulating pH-dependent membrane fusion by the GP-C complex.

In addition, the G1-G2 complex lacking SSP is not myristoylated. GP-C complexes in which myristoylation is blocked by a G2A mutation are less able to mediate cell-cell fusion than the wild-type glycoprotein (65), perhaps due to alterations in trafficking to specific membrane microdomains (52, 61). The G2A glycoprotein, however, retains 30% of the wild-type

fusin activity, significantly more than the present G1-G2 complexes in the absence of SSP. This comparison suggests defects beyond the lack of acylation in G1-G2 complexes lacking SSP. Separately, myristoylation may also be important during virion assembly in facilitating the colocalization of GP-C with the myristoylated Z matrix protein (50).

Further studies will not doubt delineate the additional roles of the unique SSP subunit in the arenavirus life cycle. Unique solutions embodied in the assembly, trafficking, and membrane fusion activity of the arenavirus GP-C complex may suggest novel approaches for intervention towards the prevention and treatment of arenavirus hemorrhagic fevers.

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REFERENCES


