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Sudhakar S. Agnihothram
Joanne York
Jack H. Nunberg
University of Montana - Missoula, jack.nunberg@umontana.edu

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

Sudhakar S. Agnihotram,1,2 Joanne York,1 and Jack H. Nunberg1*

Montana Biotechnology Center1 and Division of Biological Sciences,2 The University of Montana, Missoula, Montana 59812

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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 Junín 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 transit of the assembled GP-C complex through the Golgi and to the cell surface. Our results suggest that the cytoplasmic domain of G2 association yet now permit transport of the G1-G2 precursor to the cell surface. The cytoplasmic domain of G2 is an important determinant for both ER localization and its control through SSP binding. 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). The 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 protease (18, 65). 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
Tacaribe complex of arenaviruses that is responsible for recur-
ring outbreaks of hemorrhagic fever in the pampas grasslands
of Argentina. We show that SSP association is required for
transport of the G1-G2 precursor from the endoplasmic retic-
ulum (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 overcoming 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 asso-
ciation may also be important for the membrane fusion activity
of the GP-C complex. The unique roles for SSP in the arenav-
irus life cycle may suggest novel strategies towards the pre-
vention 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 con-
struct 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
QuikChange mutagenesis (Stratagene), and PCR was used to generate trunca-
tions and chimeric plasmids. For the cytoplasmic-domain truncation series and in

a control cleavage-defective GP-C plasmid (ed-GPC) (65), a C-terminal 15-
amino-acid S-peptide (Spep) affinity tag (34) was introduced to facilitate bio-
chemical analysis (65). All constructs were verified by DNA sequencing, and
three independent clones typically were tested to ensure consistent phenotypes.

Optimal expression of the Junin virus GP-C gene and its derivatives in Vero 76
cells was achieved using the bacteriophage T7 promoter of the pCDNA3.1 vector
and infection by a recombinant vaccinia virus expressing the T7 polymerase
(vT7-3) (24). The vaccinia virus vCB21R-laZ expressing the β-galactosidase
gene under the control of the T7 promoter was used in our analysis of cell-cell fusion
(47). These recombinant vaccinia virus reagents were provided by T.
Fuerst and B. Moss and C. Broder, P. Kennedy, and E. Berger, respectively,
through the NIH AIDS Research and Reference Reagent Program.

Mouse monoclonal antibodies (MAbs) QC05-BF11 (BF11) and GB03-BE08
(BE08) (54), directed against the G1 subunit of GP-C, were kindly provided
by Tom Ksias and Tony Sanchez (Special Pathogens Branch, CDC, Atlanta,
Georgia). The anti-CD4 ectodomain MAb SIM.2 (43, 48) was obtained
through the NIH AIDS Research and Reference Reagent Program.

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 vT7-3 (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). Metalabelling using 32 to 50 μCi/mL of 35S-Met (Amersham Pharmacia Biotech) was initiated 6 h posttransfection in methio-
nine- and cysteine-free medium containing 10% dialyzed FBS and 10 μg/mL
araC and was continued for 12 to 16 h. Cultures were then washed in physiological
buffered saline solution (PBS) and lysed using cold Tris-saline buffer (69 M
Tris-saline buffered saline buffer (69 M Tris-HCl, 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 immunoprecipitation
using either the G1-directed MAbs or the CD4-directed MAbs, and protein A-Sepharose (Sigma). In some experiments, glycoproteins containing the
C-terminaI S-pep affinity tag were isolated using S-protein agarse (Novagen).

Cellular glycoproteins were deubiquitinated by incubating with 10 μM N-0-
ubiquitinyl-L-carboxyphenylethyl-
Rhodo
glycoproteins were deubiquitinated using peptide:N-glycosidase-F (New
England Biolabs). Proteins were analyzed by sodium dodecyl sulfate-
polyacrylamide gel electrophoresis using NuPAGE 4 to 12% bis-Tris gels (In-
vitrogen) and the recommended sample buffer containing lithium dodecyl sulfate
and reducing agent. Molecular size markers included 70-kDa-methylated Rainbow
proteins (Amersham Pharmacia Biotech). Radiolabeled proteins were imaged
using a Fuji FLA-3000G imager and analyzed using ImageGauge software (Fuji).

For immunoprecipitation of cell surface glycoproteins, monolayers of meta-
bolically labeled cells were incubated with MAb BE08 or SIM.2 in ice-cold PBS
containing 2% FBS and 0.1% NaN3 for 2 h. Following extensive washing, cells
were resuspended by scraping in PBS and lysed as described above. Immune
complexes were isolated from cleared lysates using protein A-Sepharose.

Flow cytometry. Vero 76 cells expressing GP-C or its derivatives were labeled
using a FITC-specific MAAb (BF11) and a secondary biotinylated MAAb (V<a rigid
icate-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 2% 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
pH-dependent cell-cell fusion (64, 65). Briefly, Vero cells infected with vT7-3
and expressing the envelope glycoprotein were cocultured with reporter cells
infected with vCB21R-laZ, 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-laZ 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.

Confocal microscopy. Cells expressing GP-C glycoproteins were harvested by
trypsinization 6 h after transfection and resuspended 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

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 (|) and potential glycosylation sites
(Y) are marked. The SSP and SKI-1/S1P cleavage sites and the result-
ing SSP, G1, and G2 subunits are indicated. Within G2, the C-terminal
transmembrane (TM) and cytoplasmic (cyto) domains are shown, as
are the N- and C-terminal heptad repeat regions (light-gray shading).
A comparison of G2 cytoplasmic domain sequences among arenavirus
species is detailed below the schematic. Sequences include the New
World isolates Junin (D10072), Tacaribe (M20304), Pichinde
(U77601), Machupo (AY129248), and Sabia (YP_089665) and Old
World isolates Lassa-Nigeria (X524200), Mopeia (M33879), and
LCMV-Armstrong (M20869). The sites used to generate truncations
in the Junin virus cytoplasmic tail are indicated by angle brackets and
dibasic amino acid sequences are underlined.

LCMV-Armstrong (M20869). The sites used to generate truncations
in the Junin virus cytoplasmic tail are indicated by angle brackets and
dibasic amino acid sequences are underlined.

LCMV-Armstrong (M20869). The sites used to generate truncations
in the Junin virus cytoplasmic tail are indicated by angle brackets 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 antiserum (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 heterodisglycoprotein to produce mature G1 and G2 subunits (30 to 35 kDa) (Fig. 2A, top panel). These mature subunits are best resolved following 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 μg/ml) to exclude dead cells. Cells were 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.

FIG. 2. Coexpression of SSP in trans rescues SKI-1/S1P cleavage and cell surface expression of the G1-G2 precursor. (A) Metabolically labeled glycoproteins were immunoprecipitated using the G1-specific MAb BE08 and separated on NuPAGE 4-to-12% bis-Tris gels. The wild-type (GP-C) and SKI-1/S1P cleavage-defective (cd-GPC) glycoproteins are shown for comparison with the CD4sp-GPC construct encoding the conventional signal peptide of human CD4. CD4sp-GPC was expressed alone (−SSP) or with SSP (+SSP). In the bottom panel, 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 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 μg/ml) to exclude dead cells. Cells were 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
lular 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/S1P 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 (TPV172) (11) was spliced at the G2 ectodomain sequence TPI420, three 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 SIM2. 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 immunocytometric staining using SIM2 MAb and confocal microscopy (Fig. 4, CD4ecto, permeabilized), the chimeric glycoprotein was largely con-
stranded to the ER in the absence of SSP and failed to colocalize with the Golgi apparatus (−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, −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 expression on the cell surface (surface). Mobilization of the chimeric glycoprotein by SSP was confirmed by flow cytometry (Fig. 5B, +SSP). Furthermore, immunoprecipitation studies of CD4ecto expression on the cell surface (Fig. 5A, right panel) identified the surface moiety as the complex of CD4ecto and SSP. Together, these findings demonstrated that the essential elements of ER localization and its control by SSP binding can be recapitulated in a chimeric CD4ecto glycoprotein bearing the transmembrane and cytoplasmic domains of G2.

**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 references 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 glycoprotein 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
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 immunoprecipitation 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 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 KKKX and RXR 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: KKPT$_{479}$ and a C-terminal RRGH$_{485}$. 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 Alanine, 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. (A) The chimeric CD4ecto construct was expressed alone (−SSP) or with SSP (+SSP) and metabolically labeled. Intact cells were incubated with the anti-CD4 MAb SIM.2 (43, 48) and the cell surface glycoproteins were subsequently isolated from cleared cell lysates using protein A-Sepharose (surface). Intracellular CD4ecto glycoprotein was immunoprecipitated from the post-protein A-Sepharose supernatant using additional SIM.2 MAb (lysate). Mock- and SSP coexpression enabled wild-type GP-C glycoproteins 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.
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, $/{\text{H11002}}_{SSP}$). 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 motifs in CD4sp-GPC as essential for mobilization of the G1-G2 precursor from the ER to the cell surface.
acid sequences (KKPT_{479} and RRGH_{485}) as important deter-
minants 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 anal-
ysis 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 micros-
copy and flow cytometry studies (Fig. 4 and 7). Thus, con-
straints 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 membra-
 nous compartments is central to the biogenesis of membrane
glycoproteins (15, 22). Quality control mechanisms for protein
folding and assembly are proposed to operate through check-
points 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 do-
main of G2 and that the control of trafficking by SSP associa-
tion is transferable to a chimeric CD4 molecule bearing the G2
transmembrane and cytoplasmic domains. Conversely, regula-
tion 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 SK1-1/SIP protease. Thus, absent
ER localization signals, the arenavirus GP-C precursor can
undergo proteolytic maturation much as do the precursor gly-
coproteins 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 RRX 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 con-
text dependent (26, 57, 66). Many details regarding the mech-
anisms and molecular determinants involved in ER-Golgi traff-
icking 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 heteromulti-
meric membrane protein complexes (12, 33, 39, 42, 67; re-
viewed 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 trans-
port of only the fully assembled tripartite complex.

This strategy for assembly-dependent control of viral enve-
lope glycoprotein trafficking is likely not unique to the arena-
viruses. The bunyavirus G_c glycoprotein also contains a non-
canonical basic amino acid cluster that may be involved in ER
localization (29). In these viruses, transport of G_c from the ER
requires association with a second envelope glycoprotein, G_N
(30, 36), which in turn retains the G_c-G_N complex in the Golgi
(27, 29, 56), the site of virus budding. Together, these obser-
vations highlight the use of cellular ER-Golgi trafficking mech-
anisms 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. More-
ever, 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 glycopro-
tein 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 myris-
toylated. 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
fusions 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 no 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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