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Sudhakar S. Agnihothram

Joanne York

Meg Trahey

Jack H. Nunberg
University of Montana - Missoula, jack.nunberg@umontana.edu

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NOTES

Bitopic Membrane Topology of the Stable Signal Peptide in the Tripartite Junín Virus GP-C Envelope Glycoprotein Complex[▽]

Sudhakar S. Agnihothram,[1,2] Joanne York,[1] Meg Trahey,[1] and Jack H. Nunberg[1]*

Montana Biotechnology Center[1] and Division of Biological Sciences,[2] The University of Montana, Missoula, Montana 59812

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The stable signal peptide (SSP) of the GP-C envelope glycoprotein of the Junín arenavirus plays a critical role in trafficking of the GP-C complex to the cell surface and in its membrane fusion activity. SSP therefore may function on both sides of the lipid membrane. In this study, we have investigated the membrane topology of SSP by confocal microscopy of cells treated with the detergent digitonin to selectively permeabilize the plasma membrane. By using an affinity tag to mark the termini of SSP in the properly assembled GP-C complex, we find that both the N and C termini reside in the cytosol. Thus, SSP adopts a bitopic topology in which the C terminus is translocated from the lumen of the endoplasmic reticulum to the cytoplasm. This model is supported by (i) the presence of two conserved hydrophobic regions in SSP (hϕ1 and hϕ2) and (ii) our previous demonstration that lysine-33 in the ectodomain loop is essential for pH-dependent membrane fusion. Moreover, we demonstrate that the introduction of a charged side chain or single amino acid deletion in the membrane-spanning hϕ2 region significantly diminishes SSP association in the GP-C complex and abolishes membrane fusion activity. Taken together, our results suggest that bitopic membrane insertion of SSP is centrally important in the assembly and function of the tripartite GP-C complex.

Arenaviruses are found worldwide, each with their respective rodent hosts (11, 41). Infection in humans occurs through contact with rodents and can cause severe acute hemorrhagic fevers (31, 40). In Africa, up to 300,000 infections by the Lassa fever virus occur annually (32), and outbreaks of Junín, Machupo, and Guanarito viruses arise sporadically in South America (40). Transplant-associated infections by lymphocytic choriomeningitis virus (LCMV) were recently reported in the United States (10). Without effective treatment or immunization, the hemorrhagic fever arenaviruses remain an urgent public health and biodefense concern.

The arenaviruses are enveloped viruses whose genomes consist of two single-stranded RNA molecules that encode ambisense expression of four viral proteins (6, 12). The envelope glycoprotein (GP-C) mediates entry of the virus into the host cell and is the primary target for virus-neutralizing antibodies (21, 42). In contrast to other viral envelope glycoproteins, the arenavirus GP-C retains its cleaved, stable signal peptide (SSP) as an essential element of the mature complex, in addition to the conventional receptor-binding (G1) and transmembrane fusion (G2) subunits (19, 22, 51). In the nascent GP-C protein, the signal sequence acts to direct polypeptide synthesis to the endoplasmic reticulum (ER), where it is cleaved from the G1-G2 precursor by the cellular signal peptidase (SPase) in the ER lumen (5, 19, 49). The mature G1 and G2 subunits are generated through cleavage of the G1-G2 precursor glycoprotein by the cellular SKI-1/S1P protease (2, 26, 28) in the early Golgi compartment (7, 14, 20). The tripartite GP-C complex is ultimately transported to the cell surface for virion assembly and budding (39, 44).

During virion entry, the G1 subunit interacts with cell surface receptors (8, 43) and the virion is endocytosed into smooth vesicles (4). GP-C-mediated fusion of the viral and cellular membranes is activated upon acidification of the maturing endosome to initiate viral replication (4, 9, 15, 16). Membrane fusion is promoted by a series of structural rearrangements in the ectodomain of the G2 subunit to form a highly stable six-helix bundle typical of the so-called class I viral fusion proteins (24, 48).

SSP is distinguished from conventional signal peptides by its length (58 amino acids) (19) and by myristate addition at its N terminus (51). Upon coexpression of a stand-alone SSP with a recombinant G1-G2 precursor containing a conventional signal sequence, the components are able to associate in *trans* to reconstitute a functional GP-C complex (1, 17, 51). Recent studies in our laboratory have demonstrated that SSP is specifically required for GP-C transport from the ER and to the cell surface (1), as well as for the pH-dependent membrane fusion activity of the mature GP-C complex (50). SSP association overcomes endogenous ER localization signals in the cytoplasmic domain of G2 so as to permit transit of the complex through the Golgi and proteolytic maturation of the G1-G2 precursor (1). By contrast, a positively charged side chain in the central region of SSP (K33) is likely exposed on the extracellular face of the membrane to modulate the pH at which membrane fusion is activated (50). Thus, SSP appears to interact with both the cytoplasmic tail and the ectodomain of the G2 transmembrane fusion protein. To investigate the struc-

* Corresponding author. Mailing address: Montana Biotechnology Center, The University of Montana, Science Complex, Room 221, Missoula, MT 59812. Phone: (406) 243-6421. Fax: (406) 243-6425. E-mail: jack.nunberg@umontana.edu.

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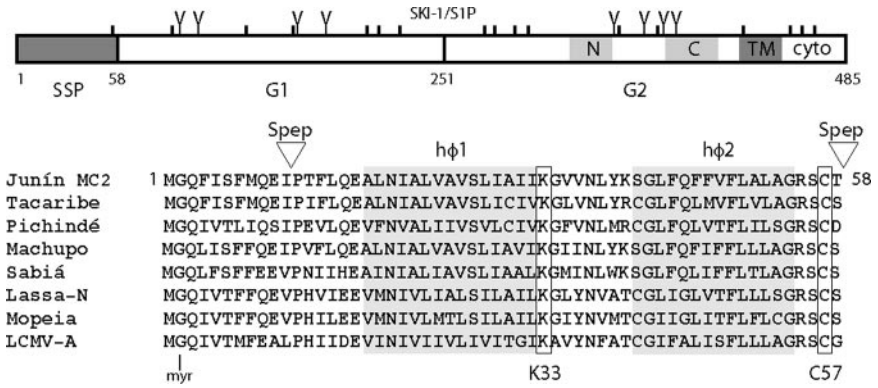

FIG. 1. Schematic representation of the Junín virus GP-C glycoprotein and SSP sequences. Amino acids of the Junín virus envelope glycoprotein are numbered from the initiating methionine, and cysteine residues (|) and potential glycosylation sites (Y) are marked. The SPase and SKI-1/S1P cleavage sites and the resulting 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 SSP sequences among arenavirus species is detailed below. Sequences include the New World isolates Junín (D10072), Tacaribe (M20304), Pichindé (U77601), Machupo (AY129248), and Sabiá (YP_089665) and the Old World isolates Lassa-Nigeria (Lassa-N) (X52400), Mopeia (M33879), and LCMV-Armstrong (LCMV-A) (M20869). The two hydrophobic regions (hϕ1 and hϕ2) are highlighted in gray, and critical K33 (50) and C57 (49) residues are boxed. The N- and C-terminal sites for the insertion of the 15-amino-acid Spep are indicated.

ture and function of this unique subunit in GP-C, we sought to define the topology of SSP in the membrane.

Sequence analysis of the SSP among New World and Old World arenaviruses (Fig. 1) suggests two hydrophobic regions (hϕ1 or hϕ2) (18, 22) that may potentially be inserted in the lipid bilayer. The N terminus of SSP is myristoylated in the cytosol, whereas the C terminus is generated by SPase cleavage in the lumen of the ER (5, 19, 49). Although the C-terminal region of SSP in the GP-C precursor obeys well-documented rules for recognition by SPase (45), the sequence requirements for SSP function in the mature complex are quite different. Specifically, the invariably conserved cysteine residue at position −2 of the SPase cleavage site (C57) is dispensable for SPase cleavage but is absolutely essential for *trans*-complementation by the SSP (49). The requirement at C57 does not arise through disulfide bond formation, as the SSP subunit is noncovalently associated in the mature GP-C complex (49, 51). This observation has led us to speculate that the penultimate C-terminal C57 side chain may lie in the reducing environment of the cytoplasm. Here, we demonstrate that SSP of the New World Junín arenavirus GP-C displays bitopic membrane topology with both the N and C termini residing in the cytosol. This model will guide further investigations of the requirements for SSP association in the tripartite GP-C complex and the interactions that modulate pH-dependent membrane fusion.

We have used digitonin to selectively permeabilize cells expressing Junín virus GP-C in order to examine the intracellular disposition of the N and C termini of SSP. Low concentrations of digitonin permeabilize the plasma membrane (due to its higher cholesterol content) while leaving intracellular membranes intact (27). Protein epitopes that lie in the cytosol are thus accessible in digitonin-permeabilized cells, whereas luminal targets are protected. To validate this methodology, we confirmed the luminal localization of the G1 subunit in wild-type GP-C using a monoclonal antibody (MAb) directed to G1 (MAb BE08) (42). As illustrated in Fig. 2A (top panel), the G1 subunit was found on the surface of intact cells using MAb BE08 and an Alexa Fluor 488-conjugated (green) secondary $F(ab')_2$ antibody. Upon complete solubilization of the cell membranes with 0.1% Triton X-100 detergent, G1 was also detected intracellularly in the ER and Golgi compartments (Fig. 2A) (1). On the other hand, in cells treated with 5 μg/ml digitonin, the G1 subunit was detected only at the plasma membrane and not intracellularly. This pattern is in accordance with the localization of G1 on the outside of the cell and its protection from staining in the lumen of the internal membranes.

As a positive control for permeabilization of the plasma membrane, digitonin-treated cells were also stained using an antibody directed to the cytoplasmic domain of giantin, an integral Golgi protein (29). The cytosolic epitope was visualized with a rabbit polyclonal antibody (PRB-114C; Covance Research Products) and an Alexa Fluor 568-conjugated secondary antibody. This red staining confirms the disruption of the plasma membrane. The green (anti-G1) and red fluorescence signals in digitonin-treated cells expressing wild-type GP-C (Fig. 2A) were spatially distinct and nonoverlapping, in keeping with their respective cell surface and cytosolic locations. Taken together, these studies confirm the utility of digitonin treatment to distinguish between cytosolic and luminal domains of transmembrane proteins.

N- and C-terminally Spep-tagged SSPs reveal bitopic membrane topology. A 15-amino-acid S-peptide (Spep) affinity tag (25) was introduced into the recombinant SSP to examine the localization of the N and C termini of SSP. We have previously shown that Spep could be appended to the C terminus of SSP without affecting the ability of the SSP subunit to *trans*-complement a G1-G2 precursor bearing the conventional signal peptide of CD4 (CD4sp-GPC) (49). This C-terminally tagged SSP construct containing a T58R mutation (to prevent SPase cleavage [49]) is termed C-term SSP-Spep. The Spep tag can also be appended at the cytosolic C terminus of G2 in CD4sp-GPC without detriment (48, 51). Both tagged molecules, C-term SSP-Spep and CD4sp-GPC/Spep, can promote pH-de-

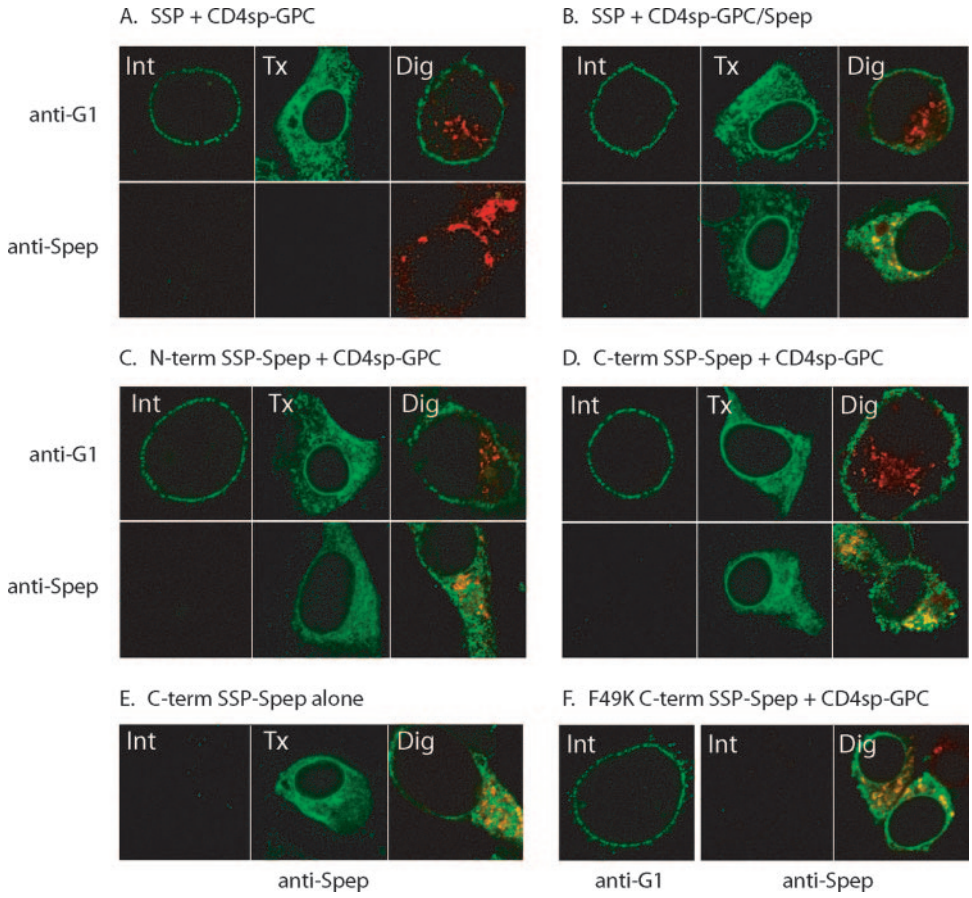

FIG. 2. Confocal microscopy of digitonin-permeabilized cells. Vero cells on two-well chambered coverglasses (Lab Tek II) were infected with the recombinant vaccinia virus vTF7-3 expressing T7 polymerase (23), transfected to express the indicated GP-C proteins, and grown for 6 h in growth medium containing 10 μM araC (1). Intact cells (Int) were incubated in the cold with anti-G1 MAb BE08 (anti-G1) or anti-Spep MAb MA1-198 (anti-Spep) and an Alexa Fluor 488-conjugated (green) anti-mouse immunoglobulin secondary $F(ab')_2$ fragment (Molecular Probes) prior to fixation with 2% formaldehyde. For staining of cells treated with 0.1% Triton X-100 (Tx), cultures were fixed prior to permeabilization. Selective permeabilization with 5 μg/ml digitonin (Dig) was done in the cold using live cells, prior to incubation with primary and secondary antibodies and fixation. Intact and digitonin-treated cells were also incubated with a rabbit polyclonal antibody directed against the cytoplasmic domain of giantin (PRB-114C; Covance Research Products) and an Alexa Fluor 568-conjugated (red) secondary antibody (Molecular Probes) in parallel with the respective anti-G1 and anti-Spep antibodies to detect permeabilization of the plasma membrane. 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). Note that the leftmost image in panel F was captured at a greater laser power than the others to enhance visibility; the intensity of cell surface anti-G1 staining in the F49K mutant was approximately 25% of wild-type levels. The images omitted in the layout of panel F were all unremarkable.

pendent membrane fusion when *trans*-complemented by their respective untagged partners (49, 51).

Here, we engineered Spep into the N-terminal region of SSP (Fig. 1), between residues I11 and P12 (N-term SSP-Spep). This tagged SSP associated with CD4sp-GPC in *trans* comparably to C-term SSP-Spep (Fig. 3A). N-term SSP-Spep also supported SKI-1/S1P maturation of the G1-G2 precursor in the Golgi (Fig. 3A, bottom panel) and transport of the GP-C complex to the cell surface (Fig. 3B). Interestingly, the GP-C complex containing N-term SSP-Spep was unable to mediate pH-dependent cell-cell fusion (not shown). Nonetheless, both N- and C-terminally tagged SSPs allow for the assembly of the tripartite GP-C complex and its transit to the cell surface and therefore provide biologically relevant structures for the determination of SSP membrane topology.

GP-C complexes containing the N-term SSP-Spep and C-term SSP-Spep subunits were readily detected on the surface of intact cells with the G1-directed MAb BE08 (Fig. 2B and C, respectively), reflecting their wild-type assembly and transport. In contrast, a MAb raised against the S peptide (MA1-198; ABR) was unable to detect Spep on the surface of cells expressing the *trans*-complemented complexes, as indicated by the lack of green fluorescence (Fig. 2B and C, lower panels). The cytoplasmic tag at the C terminus of G2 in CD4sp-GPC/Spep was likewise not detected on the cell surface upon *trans*-complementation (Fig. 2B). With complete solubilization of the cell membranes by using 0.1% Triton X-100, both G1 and Spep were visualized intracellularly by their respective MAbs. Importantly, the Spep tag was detected inside cells selectively permeabilized with 5 μg/ml digitonin. These cells expressing *trans*-complemented N-term SSP-Spep or C-term SSP-Spep (Fig. 2B and C) showed intracellular staining of Spep compa-

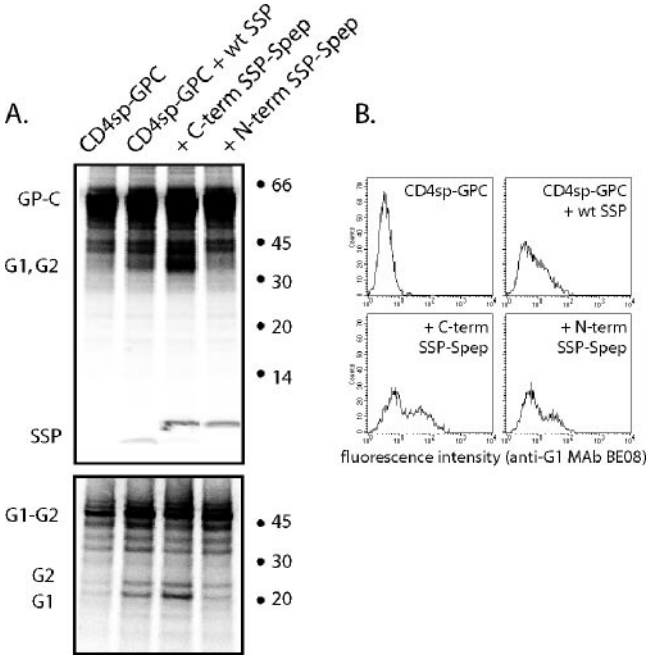

FIG. 3. Expression of the GP-C complex containing terminally tagged SSP. (A) Vero cells were transfected to express CD4sp-GPC alone or in *trans* with wild-type (wt) SSP, C-term SSP-Spep, or N-term SSP-Spep (50). In all cases, transcription was directed by the T7 polymerase of vTF7-3 (23, 51). Metabolically labeled glycoproteins were immunoprecipitated using a G1-specific MAb, BF11 (42), and separated on NuPAGE (Invitrogen) 4 to 12% bis-Tris gels under denaturing and reducing conditions (top panel). The G1 glycoprotein migrates heterogeneously with the discrete G2 subunit, and together, they are labeled G1,G2. In the bottom panel, the glycoproteins were first treated with PNGase F to resolve the G1 and G2 polypeptides (51). [^{14}C]-labeled protein markers (Amersham Biosciences) are indicated (in kilodaltons). (B) Cell surface expression of the GP-C complex was determined by flow cytometry using MAb BE08 (1, 50). The cell population was subsequently stained using propidium iodide (1 μg/ml) to exclude dead cells. Formaldehyde-fixed cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences).

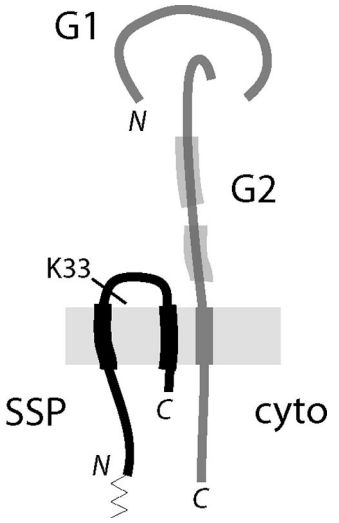

FIG. 4. Model for bitopic topology of SSP in the GP-C complex. In this drawing, the insertion of the hϕ1 and hϕ2 regions of SSP in the membrane results in both N and C termini of SSP residing in the cytosol (cyto). The intervening ectodomain of SSP includes the K33 side chain that is critical for pH-dependent membrane fusion (50), perhaps through interaction with the membrane-proximal or heptad repeat (thicker lines) region of the G2 ectodomain. The drawing is not to scale.

rable to that of *trans*-complemented CD4sp-GPC/Spep (Fig. 2D), indicating cytosolic localization of the Spep tags. Colocalization of some of the Spep tag (in green) with the Golgi marker giantin (red) is indicated by the orange-yellow color (Fig. 2D).

Collectively, these results suggest that SSP assumes a bitopic topology in the membrane with both the N and C termini in the cytosol (Fig. 4). In this model, hϕ1 and hϕ2 span the membrane in opposite orientations. The intervening central region of SSP forms a short ectodomain loop that includes the K33 residue critical for pH-dependent membrane fusion (50).

Bitopic topology of SSP is independent of G1-G2 expression. Our model for membrane insertion of SSP requires that the C terminus of SSP be translocated across the membrane following SPase cleavage. To determine whether this translocation is dependent on SSP interaction with the G1-G2 precursor, we examined the intracellular localization of the C terminus of C-term SSP-Spep upon expression without CD4sp-GPC. As shown in Fig. 2E, the pattern of Spep staining in digitonin-permeabilized cells was indistinguishable in the presence and absence of the G1-G2 precursor. The SSP amino acid sequence alone is sufficient for the translocation of the C terminus of SSP into the cytosol. Because our methods do not specifically detect Spep in the lumen, we cannot exclude the possibility that the SSP C terminus is distributed on both sides of the membrane. If so, small effects of the G1-G2 precursor on this balance may be difficult to visualize.

The orientation of membrane-spanning protein segments is thought to be determined cotranslationally during passage of the nascent protein through the channel of the translocon machinery (38, 47). In membrane proteins with type II topology, the N terminus generated by SPase cleavage is likely translocated to the cytosol prior to the insertion of the transmembrane domain into the lipid bilayer. Similarly, the C termini of the signal sequences of the hepatitis C virus envelope glycoproteins are reoriented into the cytosol upon SPase cleavage (13). In some polytopic proteins, transmembrane segments can be reoriented posttranslationally (30, 37). This dynamic flexibility in membrane insertion allows certain proteins to assume two distinct membrane topologies (30, 33, 35, 37). We surmise that the short cytoplasmic C terminus of SSP is translocated to the cytosol prior to SSP insertion in the membrane.

Genetic analysis of the hϕ2 amino acid sequence. We utilized site-directed mutagenesis to further investigate the role of hϕ2 as a membrane-spanning region and to identify sequence determinants of SSP association in the GP-C complex. Previous studies have shown that charged residues flanking hϕ2 (K40 and R55) are dispensable for SSP function (50). In this study, we individually replaced positions F44, Q45, F46, F47, and F49 at the center of hϕ2 with alanine in order to examine the effects of sequence alterations. In all five mutants, SSP associated with the GP-C complex (Fig. 5A, left) and supported wild-type levels of pH-dependent membrane fusion (Fig. 5B). We subsequently replaced these residues in blocks of

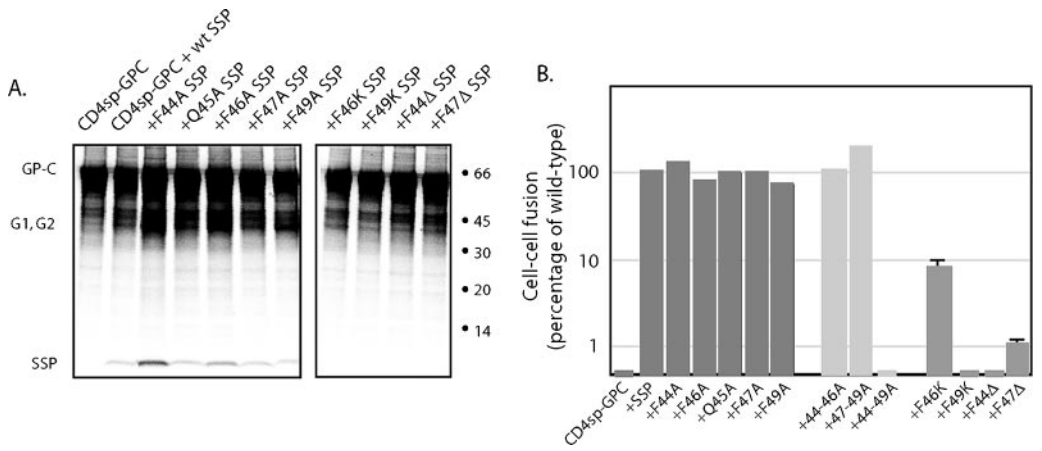

FIG. 5. Genetic analysis of the hϕ2 region of SSP. (A) SSP mutants without Spep tags were expressed in *trans* with CD4sp-GPC, and the radiolabeled GP-C complex was immunoprecipitated using the anti-G1 MAb BF11 as described in the legend of Fig. 3. The stable association of SSP in the GP-C complex is demonstrated by coprecipitation of the SSP subunit. The right and left panels were imaged at comparable settings; excessive darkening of the right panel reveals low levels of SSP (see text). (B) pH-dependent cell-cell fusion by the *trans*-complemented GP-C complex was initiated by a pulse of medium at pH 5.0 and detected using a recombinant vaccinia virus-based β-galactosidase reporter assay (36) as previously described (50, 51). The β-galactosidase expression induced upon syncytium formation was quantitated using the chemiluminescence substrate GalactoLite Plus (Tropix), and the percentage of pH-dependent fusion relative to that of the wild-type (wt) GP-C complex is indicated. Error bars (±1 standard deviation) are drawn where discernible on the scale of the graph.

three (44FQF46 and 47FVF49) with alanine and again did not observe a defect in SSP function (Fig. 5B). Only when all six residues in SSP were changed to alanine (44-49A) did the mutant show a deficiency in SSP association and abrogation of GP-C-mediated cell fusion activity. We conclude that the side chain requirements in hϕ2 for SSP association in the GP-C complex and membrane fusion are minimal, consistent with hϕ2 insertion in the lipid bilayer.

Introducing a charged residue within the hϕ2 region of SSP would, however, be expected to be disruptive. In fact, F46K and F49K mutants of SSP were markedly reduced in their ability to associate with GP-C (Fig. 5A, right). Nonetheless, the lysine side chain did not prevent the insertion of hϕ2 into the membrane, as judged by the retention of bitopic topology in digitonin-permeabilized cells expressing a C-terminally tagged F49K mutant of SSP (Fig. 2F, anti-Spep). Positively charged residues have been reported to be accommodated in other naturally occurring and model transmembrane helices (34, 46).

Interestingly, SSP association in GP-C was not completely abrogated by the F46K and F49K mutations and could be detected in overly darkened images from Fig. 5A (not shown). Notably, the level of F49K SSP association was sufficient to enable limited transport of the assembled GP-C complex to the cell surface (Fig. 2F, anti-G1). Residual cell surface expression was approximately 25% of wild-type levels and was also observed in complexes containing 44-49A SSP (not shown). By comparison, no G1-G2 glycoprotein is detected on the surface of intact cells in the absence of SSP (1). Despite transit of the complex to the cell surface, the F46K and F49K mutants were largely unable to support membrane fusion activity (Fig. 5B). F46K SSP allowed fusion at 10% of wild-type levels, whereas the complex containing F49K SSP was entirely defective. The elimination of membrane fusion activity by the F49K mutation is likely not due to the low level of GP-C on the cell surface, as cell-cell fusion by the wild-type complex is retained at far lower levels of expression (48; unpublished data). Although the mutation at F49K is compatible with a bitopic topology of SSP and with limited assembly and transport of the GP-C complex, we infer that the placement of the mutant SSP in the membrane is sufficiently perturbed to abolish membrane fusion activity.

Further evidence that hϕ2 spans the membrane was obtained by examining the effects of single amino acid deletions. These changes would shorten the putative transmembrane domain and may preclude proper positioning in the membrane. Additionally, the deletions will affect the register of any transmembrane helical regions. Single amino acid deletions at F44 and F47 (F44Δ and F47Δ) markedly reduced SSP association with GP-C (Fig. 5A, left) and ablated its membrane fusion activity (Fig. 5B). Taken together, these results are consistent with the hϕ2 region spanning the membrane to bring the C terminus of SSP to the cytosol and suggest an important role for this region in the assembly and function of the GP-C complex.

SSP topology in the Old World arenaviruses. Previous attempts to determine the membrane topology of the SSP of the Old World LCMV and Lassa fever virus have yielded different and mutually conflicting results (18, 22). Our model for a bitopic topology in the New World Junín arenavirus SSP differs from both previous suggestions. These differences may reflect the phylogenetic division between New World and Old World arenaviruses (11) or the use of different recombinant SSP constructs. In our studies of the Junín virus SSP, we have confirmed the functional integrity of the Spep-tagged N-terminal and C-terminal SSPs in assembly and transport and thus the biological relevance of their membrane disposition. However, our studies do not assess whether termini of SSP also reside in the ER lumen. Because membrane insertion can be dynamic, it remains possible that the hydrophobic regions in SSP can display mixed orientations, some of which give rise to the luminal C terminus proposed for the Old World viruses (18, 22). If so, none of these alternative topologies are found

on the surface of cells expressing the GP-C complex of Junín virus.

Role of bitopic topology in the stable association of SSP. Although the C terminus of SSP is able to translocate to the cytosol in the absence of the G1-G2 precursor, it is plausible that interactions in the GP-C complex may stabilize the bitopic form of SSP under natural conditions. The cytoplasmic domain of G2 is itself required for SSP association (1). Here, we demonstrate that SSP mutations that likely perturb the placement of hϕ2 in the membrane (F46K, F49K, F44Δ, and F47Δ) greatly reduce SSP association in the GP-C complex. Stable association of SSP in GP-C is also dependent on the penultimate C-terminal residue in SSP, C57 (49). Although C57 does not participate in disulfide bond formation in the mature GP-C complex, the requirement for the thiol side chain at this cytosolic position is absolute. The C57S mutant of SSP, for instance, is unable to associate with the G1-G2 precursor (49). In the absence of precedents from other viral envelope glycoproteins, the structure and function of SSP remain to be fully defined. It is possible that the critical C57 residue interacts noncovalently with the cytoplasmic domain of G2 to stabilize the bitopic form of SSP and thus position the ectodomain loop for its role in pH-dependent membrane fusion. The unique organization of the arenavirus GP-C complex may also present novel opportunities for antiviral intervention (3).

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