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NOTES

Bitopic Membrane Topology of the Stable Signal Peptide in the Tripartite Junin Virus GP-C Envelope Glycoprotein Complex

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The stable signal peptide (SSP) of the GP-C envelope glycoprotein of the Junin 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 (h1R and h2R) 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 h2R 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 Junin, 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/SIP 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-
**FIG. 1.** Schematic representation of the Junin virus GP-C glycoprotein and SSP sequences. Amino acids of the Junin virus envelope glycoprotein are numbered from the initiating methionine, and cysteine residues (\( \text{C} \)) and potential glycosylation sites (\( \text{Y} \)) 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 Junin (D10072), Tacaribe (M20304), Machupo (AY129248), and Sabia (YP_089665) and the Old World isolates Lassa-Nigeria (Lassa-N) (X52400), Mopeia (M33879), and LCMV-Armstrong (LCMV-A) (M20869). The two hydrophobic regions (\( h_1 \)) are boxed. The N- and C-terminal sites for the insertion of the 15-amino-acid Spep are indicated.

We have used digitonin to selectively permeabilize cells expressing Junin 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 \( \mu \)g/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 gialtin, 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-Sspep. 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-
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/SIP 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. 2). 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-
nable 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, h/H92781 and h/H92782 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 h/H92782 amino acid sequence. We utilized site-directed mutagenesis to further investigate the role of h/H92782 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 h/H92782 (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 h/H92782 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...
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 hδ2 for SSP association in the GP-C complex and membrane fusion are minimal, consistent with hδ2 insertion in the lipid bilayer.

Introducing a charged residue within the hδ2 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 hδ2 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 over lysed 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 hδ2 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 hδ2 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 Junin 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 Junin 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 Junin 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 h62 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 thylid 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 on the surface of cells expressing the GP-C complex of Junin virus. Arch. Virol. 2006. Identification and characterization of potent small molecule inhibitor of hemorrhagic fever New World arenaviruses. Bleam, and D. E. Hruby. 1993. Ribonuclease S-peptide as a carrier in fusion proteins. Protein Sci. 2:348–356.

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REFERENCES


