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Genetic analysis of heptad-repeat regions in the G2 fusion subunit of the Junín arenavirus envelope glycoprotein

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Abstract

The G2 fusion subunit of the Junín virus envelope glycoprotein GP-C contains two hydrophobic heptad-repeat regions that are postulated to form a six-helix bundle structure required for the membrane fusion activity of Class I viral fusion proteins. We have investigated the role of these heptad-repeat regions and, specifically, the importance of the putative interhelical *a* and *d* position sidechains by using alanine-scanning mutagenesis. All the mutant glycoproteins were expressed and transported to the cell surface. Proteolytic maturation at the subtilisin kexin isozyme-1/site-1-protease (SKI-1/S1P) cleavage site was observed in all but two of the mutants. Among the adequately cleaved mutant glycoproteins, four positions in the N-terminal region (I333, L336, L347 and L350) and two positions in the C-terminal region (R392 and W395) were shown to be important determinants of cell–cell fusion. Taken together, our results indicate that α -helical coiled-coil structures are likely critical in promoting arenavirus membrane fusion. These findings support the inclusion of the arenavirus GP-C among the Class I viral fusion proteins and suggest pharmacologic and immunologic strategies for targeting arenavirus infection and hemorrhagic fever.

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Introduction

Arenaviruses are endemic in rodent populations worldwide (Salazar-Bravo et al., 2002) and are transmitted to humans by exposure to infected animals. Infection by Old World arenaviruses such as Lassa virus, or New World species such as the South American group including Junín, Machupo and Guanarito viruses, are responsible for recurring and emerging outbreaks of viral hemorrhagic fevers with high mortality (McCormick and Fisher-Hoch, 2002; Peters, 2002). Prophylactic and therapeutic options to combat arenavirus infection are limited, and the development of effective immunogens and antiviral agents to interfere with arenavirus entry may have broad public health benefits.

The arenaviruses are enveloped bisegmented RNA viruses whose genome consists of two single-stranded RNA molecules (Buchmeier et al., 2001; Clegg et al., 2000). During biogenesis, arenaviral particles assemble and bud at the plasma membrane. The mature envelope glycoprotein complex of the arenavirus consists of three noncovalently associated subunits derived from the GP-C precursor by proteolytic cleavage events: a stable myristoylated 58 amino-acid signal peptide (SSP), the receptor-binding G1 subunit and the transmembrane G2 fusion protein (Buchmeier, 2002; Eichler et al., 2003; York et al., 2004). The initial cleavage to yield SSP is likely mediated by the cellular signal peptidase, and the mature G1 and G2 subunits are subsequently generated by the cellular SKI-1/S1P protease. Both cleavage events, as well as the presence of the myristoylated SSP subunit, are required for envelope glycoprotein-mediated membrane fusion (York et al., 2004). Entry of virion particles into host cells is initiated by G1 binding to cell surface receptors followed by endocytosis of the virion into smooth vesicles (Borrow and Oldstone, 1994). Although α -dystroglycan serves as a binding receptor for the Old World

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arenaviruses (Cao et al., 1998), the receptor utilized by the major New World group of arenaviruses is unknown (Spiropoulou et al., 2002). Membrane fusion is pH-dependent and is activated upon acidification of the maturing endosome (Borrow and Oldstone, 1994; Castilla et al., 1994; Di Simone and Buchmeier, 1995; Di Simone et al., 1994).

The molecular basis for envelope glycoprotein-mediated membrane fusion in the arenaviruses is largely unknown, however, sequence analysis of the G2 ectodomain of Lassa virus and lymphocytic choriomeningitis virus (LCMV) has revealed two heptad-repeat regions that can be represented to form amphipathic helices (Gallaher et al., 2001). This sequence motif is found among the large group of viral envelope glycoproteins that promote membrane fusion through the formation of a fusion-active core structure comprising a stable bundle of six α -helices. These Class I fusion proteins include those of the retroviruses, orthomyxoviruses, paramyxoviruses, filoviruses and coronaviruses. In these proteins, the six-helix bundle structure involves a central coiled coil, formed by N-terminal heptad-repeat regions of the trimeric fusion subunit ectodomain, surrounded by three anti-parallel helices of the C-terminal heptad repeats which bind to the conserved hydrophobic grooves on the coiled-coil surface. Formation of this thermodynamically favored six-helix bundle brings the viral and cell membranes into apposition and is thought to provide the driving energy to initiate membrane fusion (Earp et al., 2005; Eckert and Kim, 2001; Hughson, 1997; Weissenhorn et al., 1999 and references therein). In keeping with the proposal that the arenavirus G2 ectodomain might also form a fusion-active six-helix bundle, Gallaher and colleagues have shown that a synthetic peptide derived from the N-terminal heptad-repeat region of LCMV is able to assume α -helical content (Gallaher et al., 2001).

In this report, we have utilized alanine-scanning mutagenesis of the heptad-repeat regions of the New World Junin arenavirus envelope glycoprotein to obtain genetic evidence in support of this model for membrane fusion in the arenaviruses. We show that mutations at predicted interhelical positions affect the ability of the envelope glycoprotein complex to mediate pH-dependent cell–cell fusion. These findings suggest that membrane fusion by the arenavirus envelope glycoprotein complex is promoted via a refolding into a highly stable six-helix bundle structure characteristic of Class I viral fusion glycoproteins.

Results and discussion

Sequence analysis of the G2 ectodomain of the Junin arenavirus

The amino acid sequences of the heptad repeats of New World and Old World arenaviruses were aligned to demonstrate the high degree of conservation within the family (Fig. 1A). One key consideration in assigning a register to the proposed helical coiled coil of the heptad repeats (i.e., positions *a* through *g*) was to maximize the degree of hydrophobicity at the interhelical *a* and *d* positions. Upon inspection of the

aligned sequences, a unique register of *a* and *d* positions was apparent (Figs. 1A and B). The *a* and *d* positions of the proposed N-terminal helix consist of hydrophobic residues common to the central interface of a trimeric coiled coil (e.g., leucine, isoleucine, methionine and valine). Positions assumed to lie on the exterior face of the coiled coil (*b*, *c* and *f* positions) contain polar or charged residues. This pattern is repeated through the C-terminal heptad-repeat region and appears to end at the C-terminal-most *d* and *a* positions (K409 and D413, respectively). Hydrophilic residues at two *a* positions in the New World viruses (R392 and S399) are replaced by less polar amino acids in the Old World viruses (serine and alanine, respectively). The hydrophilic sidechains, if buried, may impart specificity to the process of coiled-coil folding at the expense of thermal stability (Ji et al., 2000; Lumb and Kim, 1995).

Expression of Junin virus envelope glycoproteins bearing alanine mutations at a and d positions

To investigate the proposed α -helical structures and their role in promoting membrane fusion in the arenaviruses, we subjected the heptad-repeat regions of the Junin virus envelope glycoprotein to scanning mutagenesis. Each *a* and *d* position amino acid was individually changed to alanine, a small residue that is a good helix inducer yet contributes little to the hydrophobic forces that predominantly stabilize coiled-coil structures. Alanine substitutions at these positions are likely to alter the stability of the proposed six-helix bundle, but not to disrupt its overall folding. We anticipated that alanine mutations at interhelical positions within the coiled-coil bundle may selectively affect the ability of the envelope glycoprotein to mediate membrane fusion.

For these studies, we utilized the GP-C gene of the pathogenic Junin virus isolate MC2 (York et al., 2004). The wild-type and mutant plasmids were introduced in Vero 76 cells, and the envelope glycoproteins were expressed using the T7 promoter of the plasmid vector and T7 RNA polymerase provided by infection with the recombinant vaccinia virus vTF7-3 (Fuerst et al., 1986). Cultures were metabolically labeled and subsequently solubilized. The envelope glycoproteins were immunoprecipitated using the mouse MAb BF11 (Sanchez et al., 1989) directed to the G1 subunit (York et al., 2004).

We have previously shown that the Junin virus envelope glycoprotein complex isolated by immunoprecipitation retains the three noncovalently associated subunits: the mature G1 and G2 glycoproteins as well as the stable signal peptide, SSP (York et al., 2004 and see Fig. 2). Cleavage by signal peptidase and the SKI-1/SIP protease are incomplete upon recombinant expression such that two precursor glycoproteins (full-length GP-C and the G1–G2 precursor lacking SSP) are observed. The glycoproteins isolated by immunoprecipitation of the wild-type envelope glycoprotein, and an SKI-1/SIP cleavage-site mutant (cd-JGPC; York et al., 2004), are illustrated in Fig. 2A (far left). The two precursor glycoproteins migrate with molecular weights of 65 kDa and 60 kDa,

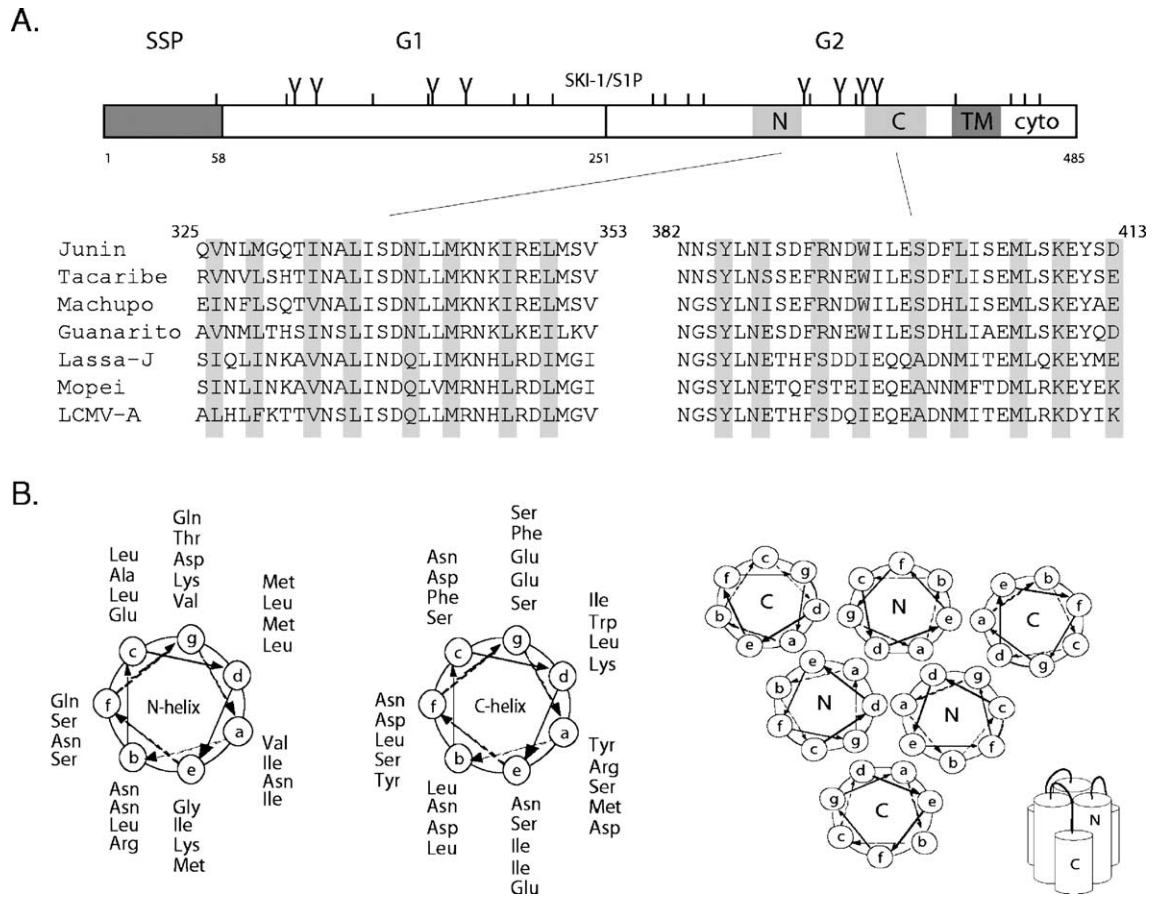


Fig. 1. Schematic representation of the Junin virus GP-C glycoprotein and N- and C-terminal heptad-repeat regions. (A) Amino acids of the Junin virus envelope glycoprotein are numbered from the initiating methionine, and cysteine residues (C) and potential glycosylation sites (Y) are marked. The SSP 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 heptad-repeat sequences among arenavirus species is detailed below. Sequences include the New World isolates Junin (D10072), Tacaribe (M20304), Machupo (AY129248) and Guanarito (AAN05423) and Old World isolates Lassa-Josiah (M15076), Mopeia (M33879), LCMV-Armstrong (M20869). The 3,4 periodicity of *a* and *d* heptad-repeat positions is indicated by shading. (B) Helical wheel projections of proposed heptad-repeat periodicity. Amino acids shown above are listed in numeric order at *a*–*g* positions. A schematic of a six-helix bundle is at the lower right, and the details of the canonical interhelical packing arrangement are illustrated.

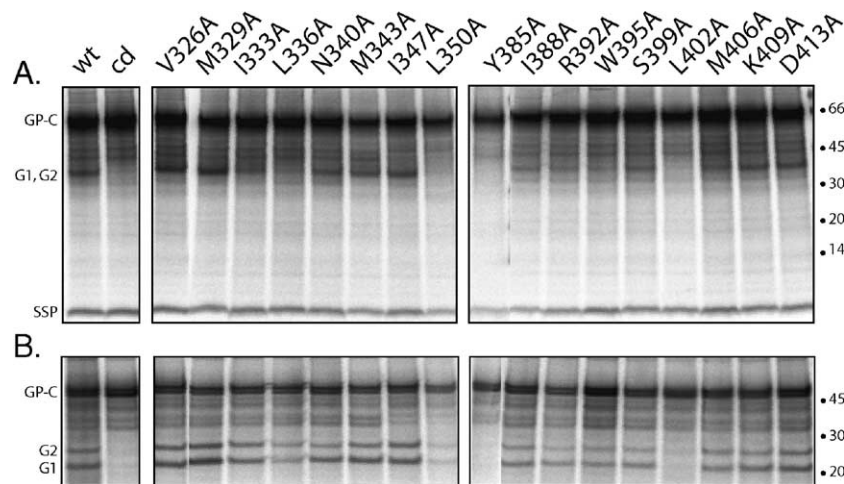


Fig. 2. Expression of wild-type and mutant envelope glycoproteins. (A) Metabolically labeled glycoproteins were immunoprecipitated using the G1-specific MAB BF11 and separated on NuPAGE 4–12% Bis–Tris gels. The wild-type (wt) and SKI-1/S1P cleavage-defective (cd) glycoproteins are shown for comparison with the N- and C-terminal heptad-repeat mutants. V326A indicates the glycoprotein in which V326 has been changed to alanine. The two bands labeled GP-C (i.e., the full-length GP-C and the G1–G2 precursor lacking SSP) as well as the heterodisperse smear of G1 are better resolved following deglycosylation using PNGase F (B).

respectively. The mature G2 glycoprotein appears as a band of ≈ 35 kDa, whereas the heterogeneous smear of the G1 glycoprotein is difficult to discern. These bands are better resolved following deglycosylation using peptide:N-glycosidase F (PNGase F) (Fig. 2B, far left). The G1 and G2 subunits are absent in the cleavage-defective cd-JGPC envelope glycoprotein.

Among the seventeen N- and C-terminal heptad-repeat mutants, all the GP-C glycoproteins were expressed, and all were cleaved by signal peptidase to generate the 5 kDa SSP (Fig. 2A). The relative efficiencies of SKI-1/S1P protease cleavage, however, varied among the mutants (Figs. 2A and B). In some mutants (e.g., Y385A and L402A), SKI-1/S1P cleavage was undetectable, similar to that in the cleavage-site-defective envelope glycoprotein. In other mutants, the amounts of cleavage ranged from markedly reduced to levels greater than those in the wild-type. To quantitate the relative extents of SKI-1/S1P processing, we determined the amount of radioactivity present in the deglycosylated GP-C precursors and in the mature G1 and G2 polypeptides using a Fuji 3000-G phosphorimager and Image Gauge software (Fuji). Our analysis of two independent experiments is shown in Fig. 3.

Several classes of mutants could be defined by this analysis. Proteolytic maturation was essentially absent in the Y385A and L402A mutants and significantly reduced in L350A and W395A (30 and 45% of wild-type levels, respectively). Other mutants were cleaved much as the wild-type glycoprotein (L336A, N340A, I388A, R392A, S399A, M406A and K409A; from 65 to 100% of wild-type levels), whereas several mutations in the N-terminal heptad-repeat

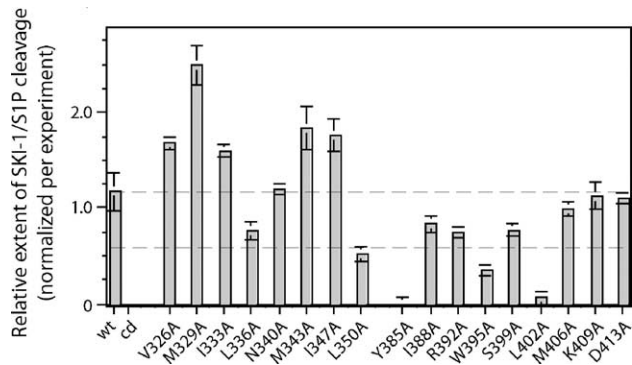


Fig. 3. Quantitation of SKI-1/S1P cleavage and the formation of the mature G1 and G2 subunits. Phosphorimages of the deglycosylated polypeptides represented in Fig. 1B were analyzed using Image Gauge software (Fuji) and the Profile and Background tools to quantitate the radioactivity (PSL units). The efficiency of SKI-1/S1P cleavage was defined as the ratio of PSL units in the sum of the G1 + G2 peaks relative to the total number in both GP-C and G1 + G2 peaks. Results were analyzed for two complete experiments, and the relative conclusions are consistent with those of additional partial studies. Because the absolute efficiency of cleavage varied between experiments, each experiment was normalized to the average extent of cleavage in the entire 19-member panel. This average ratio (16% and 24% in the two complete experiments shown here) was used to determine the relative degree of cleavage in each mutant, and these numbers were averaged to generate the values plotted. By this analysis, the wild-type envelope glycoprotein was cleaved 1.17-fold better than the arbitrary average of the panel; 100% and 50% of the wild-type level are shown as dashed lines. Error bars represent \pm one standard deviation.

region appeared to enhance proteolytic cleavage up to 2-fold (V326A, M329A, I333A, M343A and I347A). Shedding of the mature G1 subunit into the culture supernatant correlated with the extent of SKI-1/S1P cleavage (not shown). Although proteolytic processing varied among the mutants, all but two showed significant cleavage of the G1–G2 precursor.

Transport of the mutant envelope glycoprotein complexes to the cell surface was determined by flow cytometry using the G1-specific MAb BE08 (Sanchez et al., 1989). Cells from cultures transiently transfected to express the wild-type and cd-JGPC glycoproteins displayed two populations: cells expressing GP-C (≥ 30 fluorescence channel) and those that escaped transfection (< 30 fc) (Fig. 4). Proteolytic maturation of the arenavirus envelope glycoprotein complex is clearly not required for transport to the cell surface. In fact, staining of the cleavage-defective mutant cd-JGPC was generally higher and more discrete than that of the wild-type envelope glycoprotein, likely due to shedding of the G1 subunit from the wild-type but not the cleavage-defective complex (York et al., 2004). Among the heptad-repeat mutants (Fig. 4), all patterns of staining were within this range and qualitatively similar. We conclude that transport of the mutant glycoproteins to the cell surface was not markedly affected.

The precise pattern of cell-surface staining is expected to reflect the degree of transport and the relative extent of G1 shedding. Our efforts to further characterize the surface GP-C complex biochemically were limited by the refractory nature of the GP-C complex to covalent modification by standard biotinylation reagents (unpublished). Overall, however, successful entry into the Golgi and transit to the cell surface suggests that the mutations do not globally perturb the structural integrity of the mutant GP-C complexes.

Mutations at interhelical positions affect pH-dependent cell–cell fusion

The effects of the alanine mutations on the ability of the envelope glycoprotein to mediate pH-dependent cell–cell fusion were determined in Vero 76 cells using the recombinant vaccinia virus-based LacZ fusion reporter assay (Nussbaum et al., 1994) as described (York et al., 2004). In this assay, β -galactosidase expression initiated by fusion of the effector and target cells was determined using a chemiluminescent substrate (GalactoLite Plus, Tropix). The extent of pH-dependent cell–cell fusion mediated by the wild-type and mutant envelope glycoproteins is shown in Fig. 5.

Five alanine substitutions at *a* and *d* positions reduced fusogenicity to less than 10% of the wild-type GP-C or comparably to the SKI-1/S1P cleavage-site mutation in cd-JGPC: I333A in the N-terminal heptad repeat and Y385A, R392A, W395A and L402A in the C-terminal heptad repeat (Fig. 5). Although the lack of proteolytic cleavage in Y385A and L402A is likely sufficient to account for the defect in fusion by these mutants, the other glycoproteins (I333A, R392A and W395A) showed significant levels of cleavage. Three additional mutations in the N-terminal region (L336A, I347A and L350A) reduced cell–cell fusion to less than 50%

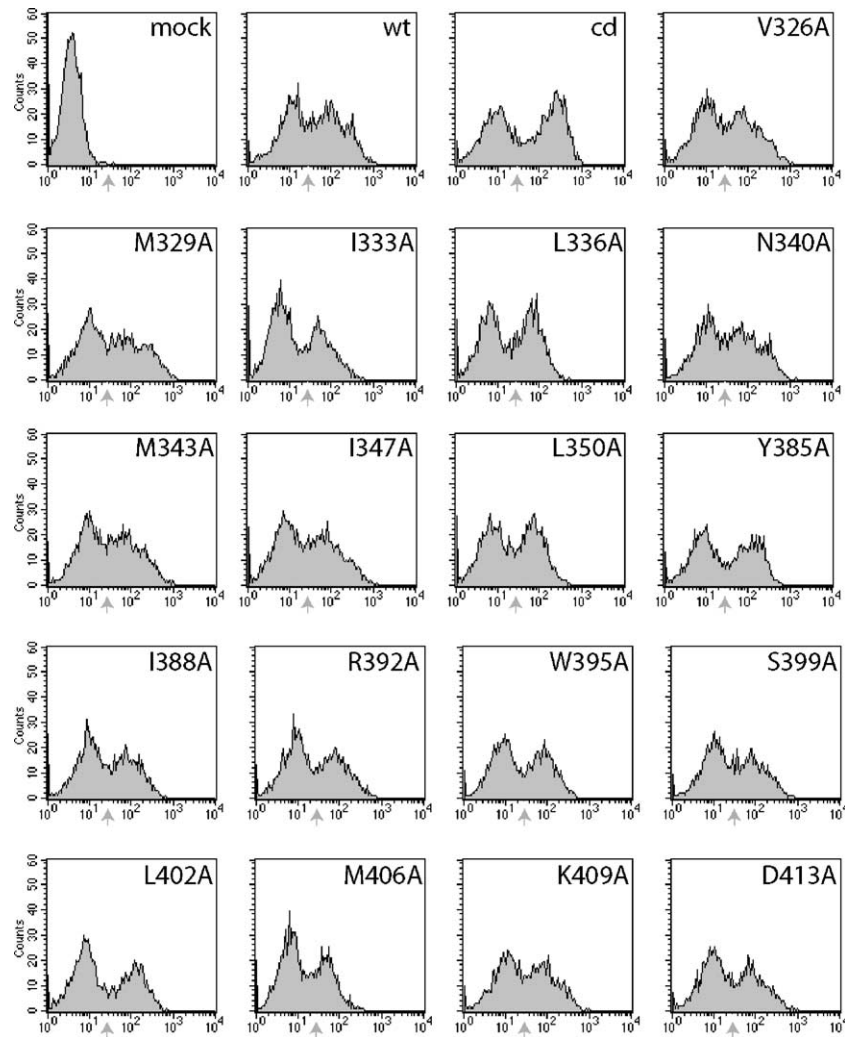


Fig. 4. Flow cytometric analysis of cell-surface envelope glycoprotein. Vero cells expressing the envelope glycoproteins were stained using the G1-specific neutralizing MAb BE08 (Sanchez et al., 1989) and subsequently stained using propidium iodide (1 $\mu\text{g/ml}$) to exclude dead cells in the population. Cells were fixed using 2% formaldehyde and analyzed using a FACSCalibur flow cytometer (BD Biosciences). Background staining of mock-transfected cells is shown in the first panel; the non-expressing cell population within the GP-C transfected cultures (other panels) tend to pick up additional staining, possibly due to membrane exchange within the culture. The expressing cell populations were defined using a gate of ≥ 30 that included $< 0.1\%$ of the mock-transfected population (arrow).

of the wild type. In all cases, cell–cell fusion required exposure of the culture to acidic (pH 5.0) medium.

In some mutants, clear defects in membrane fusion arose despite wild-type levels of SKI-1/S1P cleavage (viz., I333A and I347A). In others, it was difficult to determine whether the deficiencies in cell–cell fusion were directly attributable to the envelope glycoprotein fusion machinery or to defects in SKI-1/S1P proteolytic processing. In several examples, mutants displaying reductions in cleavage efficiency were able to mediate cell–cell fusion much as the wild-type glycoprotein (viz., I388A and S399A), whereas other comparably cleavage-deficient mutants were defective in membrane fusion (viz., L336A, L350A and R392A). These observations suggested that the variable deficiencies in SKI-1/S1P cleavage were not sufficient to account for the majority of the defects in cell–cell fusion under our assay conditions.

In order to more fully examine this point, we reconstructed the variable extents of proteolytic cleavage using mixtures of the wild-type and SKI-1/S1P cleavage-site mutant plasmids. In

these studies, we determined the relative efficiencies of SKI-1/S1P cleavage by PAGE and the extent of pH-dependent membrane fusion in the recombinant vaccinia virus-based LacZ fusion reporter assay (Fig. 6). Although the total amount of envelope glycoprotein remained constant as the wild-type plasmid was diluted 1:3 and 1:9 with cd-JGPC (90% and 85% of the undiluted wild-type level, respectively), the relative amounts of mature G1 and G2 decreased progressively, from 20% to 5% and 2%, respectively. Despite a 90% decrease in overall SKI-1/S1P cleavage, however, cell–cell fusion was unaffected. Within these limits, our measurements of cell–cell fusion appear not to be sensitive to the relative amounts of proteolytically matured G1 and G2 glycoprotein, and we infer that mutants in which SKI-1/S1P cleavage is retained to at least 10% of the wild type level can be informative in defining specific defects in membrane fusion.

Tertiary effects of the heptad-repeat mutations that may indirectly affect both proteolytic cleavage and fusogenicity, such as those that influence overall protein folding, trafficking

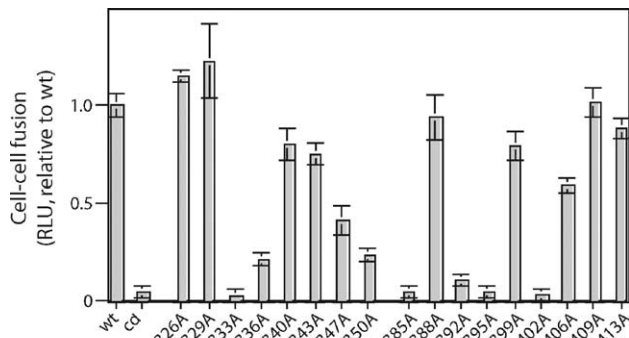


Fig. 5. Fusogenic potential of wild-type and mutant envelope glycoproteins. The ability of the envelope glycoproteins to mediate pH-dependent cell–cell fusion was detected using the recombinant vaccinia virus-based β -galactosidase reporter assay (Nussbaum et al., 1994) as described in Materials and methods. β -galactosidase activity induced upon cell–cell fusion was quantitated using the chemiluminescent substrate GalactoLite Plus (Tropix). Background levels of chemiluminescence obtained from cultures treated at neutral pH were subtracted from the relative light unit (RLU) measurement, and all RLU values were then normalized to that of the wild-type GP-C control. Error bars represent \pm one standard deviation among 6 replicate fusion cultures. All conclusions were replicated using X-gal staining of parallel co-cultures (York et al., 2004).

or oligomer composition, would limit our ability to generalize from our simple reconstitution model. In the case of the cleavage-site defective glycoprotein, these considerations are likely moot as GP-C biogenesis appears to be otherwise blind to the SKI-1/S1P site mutation. Among the heptad-repeat mutants, we also observe no evidence for global defects in folding, assembly or transport and thereby extend the interpretation of the model experiment to these glycoproteins. Thus, we conclude that the following *a* and *d* heptad-repeat positions contribute directly to promoting pH-dependent membrane fusion by the Junin virus envelope glycoprotein: I333, L336, I347 and L350 in the N-terminal region and R392 and W395 in the C-terminal region.

Arenavirus GP-C is likely a member of the Class I fusion proteins

Taken together, our studies identify a series of amino acid residues within the N- and C-terminal heptad-repeat regions of the G2 ectodomain that impair the ability of the Junin virus envelope glycoprotein to mediate pH-dependent cell–cell fusion. The residues chosen for mutagenic analysis were modeled to lie at interhelical *a* and *d* positions, in keeping with the predominant role of these positions for coiled-coil formation. Alanine substitutions at these positions are often benign (Luo and Weiss, 1998), and the rich appearance of fusion-deficient phenotypes among our mutants is consistent with this model and with the important role of the α -helical coiled-coil bundle in promoting membrane fusion in the arenaviruses.

Several of the alanine mutations also affected SKI-1/S1P cleavage of the G1–G2 precursor glycoprotein, presumably through conformational effects that otherwise do not perturb envelope glycoprotein trafficking to the cell surface. These effects on proteolytic cleavage may signal a distinct role of the

heptad-repeat regions in the folding of the native envelope glycoprotein during biogenesis. Importantly, our studies suggest that these effects on proteolytic cleavage per se are unlikely to account for the specific deficiencies in membrane fusion activity.

These genetic studies provide direct support to the proposal, originally based on amino acid sequence analysis (Gallaher et al., 2001), that membrane fusion in the arenaviruses is promoted by the formation of an α -helical coiled-coil structure similar to that described in the Class I viral fusion proteins. Biophysical evidence emerging from the laboratory of M. Lu (Weill Medical College of Cornell University) confirms that peptides derived from the N- and C-terminal heptad repeats of the Junin virus G2 associate to form a six-membered trimeric structure with a high degree of helical content and thermal stability (unpublished). The precise limits of the N- and C-terminal α -helices and the details of the interhelical interactions within the arenavirus six-helix bundle await atomic-level structure determination.

The inclusion of the arenavirus envelope glycoprotein within this class of viral fusion proteins suggests several specific strategies towards the development of vaccines and therapeutic agents against arenavirus infection. Peptides derived from the C-terminal helical region of the HIV fusion protein that are believed to irreversibly interfere with formation of the six-helix bundle are in clinical use in the treatment of HIV disease (Kilby et al., 2002), and small molecule inhibitors may be feasible (Eckert et al., 1999). Similarly, broadly neutralizing antibodies that target the membrane-proximal region of the HIV gp41 ectodomain adjacent to the C-terminal heptad repeat (Muster et al., 1993; Stiegler et al., 2001; Zwick et al., 2001) may likewise interfere in this process (Follis et al., 2002). Further structural and immunochemical studies of the arenavirus envelope glycoprotein complex may point to conserved mechanisms and broadly applicable strategies towards the prevention and treatment of arenaviral hemorrhagic fevers.

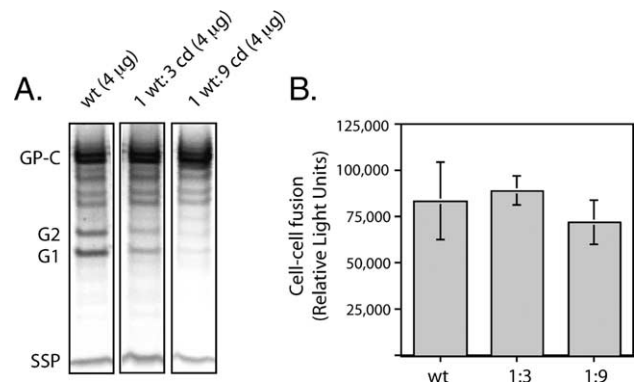


Fig. 6. Reconstruction to determine the effect of variable SKI-1/S1P cleavage efficiency on envelope glycoprotein fusogenic potential. Vero 76 cells were transfected with 4 μ g DNA containing either the wild-type GP-C plasmid (wt), a 1:3 mixture of the wt and cd-JGPC plasmids, respectively, or a 1:9 mixture of the same plasmids. Parallel cultures were metabolically labeled and immunoprecipitated, and PNGase F-treated polypeptides were analyzed as described in Figs. 2 and 3 (A) or subjected to the recombinant vaccinia virus-based fusion assay as described in Fig. 5 (B).

Materials and methods

Molecular reagents, recombinant vaccinia viruses and monoclonal antibodies

The GP-C gene (Ghiringhelli et al., 1991) of the pathogenic MC2 isolate of the Argentine hemorrhagic fever (Junin) virus was expressed using the bacteriophage T7 polymerase promoter in a pcDNA3.1-based (Invitrogen) expression plasmid (York et al., 2004). The GP-C gene was modified to express an innocuous S-peptide (Spep) affinity tag (Kim and Raines, 1993) at the cytoplasmic C-terminus to facilitate manipulations (York et al., 2004). The β -galactosidase fusion reporter assay (Nussbaum et al., 1994) was used to assess the ability of the envelope glycoprotein to mediate receptor-dependent cell–cell fusion. The recombinant vaccinia virus vCB21R-lacZ expressing β -galactosidase under the control of the T7 promoter (Nussbaum et al., 1994) was obtained from C. Broder, P. Kennedy and E. Berger (NIH) through the AIDS Research and Reference Reagent Program. Mouse monoclonal antibodies (MAbs) directed to the G1 subunit of GP-C (Sanchez et al., 1989) were kindly provided by Drs. Tom Ksiasek and Tony Sanchez (Special Pathogens Branch, CDC, Atlanta).

Expression of GP-C

The envelope glycoprotein was transiently expressed in Vero 76 cells using the pcDNA3.1-based T7 promoter and the recombinant vaccinia virus encoding T7 polymerase vTF7-3 (Fuerst et al., 1986). Vero cells were infected with the recombinant vaccinia virus at a multiplicity of two in Dulbecco's Minimal Essential Medium (DMEM) containing 2% fetal bovine serum (FBS). Cytosine arabinoside (araC) was included at 10 μ M to limit vaccinia virus expression (Hruby et al., 1980). After 30 min, the cells were washed and transfected with the GP-C expression plasmid using Lipofectamine 2000 reagent (Invitrogen). Metabolic labeling using 32 μ Ci/ml of [³⁵S]-ProMix (Amersham Pharmacia Biotech) was initiated 6 h post-transfection in methionine- and cysteine-free DMEM containing 10% dialyzed FBS and 10 μ M araC and was continued for 12–16 h. Cultures were then washed in physiological buffered saline (PBS) and lysed using cold Tris–saline buffer (50 mM Tris–HCl and 150 mM NaCl at pH 7.5) containing 1% Triton X-100 nonionic detergent and protease inhibitors (1 μ g/ml each of aprotinin, leupeptin and pepstatin). A soluble fraction was prepared by centrifugation at 15,000 \times g for 30 min at 4 °C. The expressed glycoproteins were isolated either by immunoprecipitation using the G1-directed MAb BF11 (Sanchez et al., 1989) and Protein A–Sepharose (Sigma) or by using the C-terminal (G2) Spep affinity tag and S-protein agarose (Novagen). In some experiments, the isolated glycoproteins were deglycosylated using peptide:N-glycosidase F (PNGase F, New England Biolabs). Glycoproteins and polypeptides were resolved using NuPAGE 4–12% Bis–Tris gels (Invitrogen) and the recommended sample buffer containing lithium dodecyl sulfate and reducing agent. Molecular weight markers included [¹⁴C]-

methylated Rainbow proteins (Amersham Pharmacia Biotech). Radiolabeled proteins were imaged using a Fuji FLA-3000G imager and analyzed using ImageGauge software (Fuji).

Flow cytometry

Vero 76 cells transiently expressing the wild-type and mutant glycoproteins were labeled using the G1-specific MAb BE08 (Sanchez et al., 1989) and a secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Jackson ImmunoResearch). Cells were subsequently stained using propidium iodide (1 μ g/ml) and then fixed in 2% formaldehyde. Populations were analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

Envelope glycoprotein-mediated cell–cell fusion

The β -galactosidase fusion reporter assay (Nussbaum et al., 1994) was used to assess the ability of the envelope glycoproteins to mediate receptor-dependent cell–cell fusion. In these studies, Vero cells infected with vTF7-3 and expressing the envelope glycoprotein were co-cultured with Vero cells infected with vCB21R-lacZ, a recombinant vaccinia virus expressing β -galactosidase under the control of the T7 promoter. These latter (target) cells were obtained by incubating Vero 76 cells with a multiplicity of two vCB21R-lacZ plaque-forming units per cell and allowing the infection to proceed overnight in the presence of 100 μ g/ml of the vaccinia virus assembly inhibitor, rifampicin. After mixing of the envelope glycoprotein-expressing and target cells in medium containing both araC and rifampicin (Hruby et al., 1980; York et al., 2004), the co-cultures were continued for 5 h prior to being subjected to a 30 min pulse of neutral or acidic (pH 5.0) medium as previously described (York et al., 2004). β -galactosidase expression is induced upon fusion of the effector and target 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.

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