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Joanne York

Jack H. Nunberg

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

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Intersubunit Interactions Modulate pH-Induced Activation of Membrane Fusion by the Junín Virus Envelope Glycoprotein GPC[∇]

Joanne York and Jack H. Nunberg*

Montana Biotechnology Center, The University of Montana, Missoula, Montana 59812

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The mature arenavirus envelope glycoprotein GPC is a tripartite complex comprising a stable signal peptide (SSP) in addition to the receptor-binding (G1) and transmembrane fusion (G2) subunits. We have shown previously that SSP is a key element in GPC-mediated membrane fusion, and that GPC sensitivity to acidic pH is modulated in part through the lysine residue at position 33 in the ectodomain loop of SSP (J. York and J. H. Nunberg, *J. Virol.* 80:7775–7780, 2006). A glutamine substitution at this position stabilizes the native GPC complex and thereby prevents the induction of pH-dependent membrane fusion. In efforts to identify the intersubunit interactions of K33, we performed alanine-scanning mutagenesis at charged residues in the membrane-proximal ectodomain of G2 and determined the ability of these mutations to rescue the fusion deficiency in K33Q GPC. Four second-site mutations that specifically complement K33Q were identified (D400A, E410A, R414A, and K417A). Moreover, complementation was also observed at three hydrophobic positions in the membrane-spanning domain of G2 (F427, W428, and F438). Interestingly, all of the complementing mutations restored wild-type pH sensitivity to the K33Q mutant, while none themselves affected the pH of membrane fusion. Our studies demonstrate a specific interaction between SSP and G2 that is involved in priming the native GPC complex for pH-induced membrane fusion. Importantly, this pH-dependent interaction has been shown to be vulnerable to small-molecule compounds that stabilize the native complex and prevent the activation of membrane fusion. A detailed mechanistic understanding of the control of GPC-mediated membrane fusion will be important in guiding the development of effective therapeutics against arenaviral hemorrhagic fever.

Arenaviruses are endemic in rodent populations worldwide (37), and several species of the virus can be transmitted to humans to cause severe hemorrhagic fevers (30, 33). These include Lassa fever virus (LASV) in western Africa and Junín virus (JUNV) in the New World. The arenaviruses are enveloped, negative-strand RNA viruses whose bipartite genomes encode the ambisense expression of the four viral proteins (7, 10). The envelope glycoprotein GPC promotes virus entry into the host cell and thereby provides a viable target for therapeutic intervention (3, 27, 28, 44). GPC is synthesized as a precursor glycoprotein that is proteolytically matured by the cellular SKI-1/S1P protease (2, 26, 29) to generate the receptor-binding (G1) and transmembrane fusion (G2) subunits, which remain noncovalently associated in the mature complex. Arenavirus entry is initiated by G1 binding to a cell surface receptor. The pathogenic New World arenaviruses utilize transferrin receptor-1 for entry (34, 35), whereas Old World viruses bind α -dystroglycan (8, 18, 38). The virion subsequently is endocytosed (4), and fusion of the viral and cellular membranes is initiated by acidification in the maturing endosome (9, 12, 13). The details of the acid-induced activation of GPC-mediated membrane fusion are unknown.

The arenavirus GPC is unique among viral envelope glycoproteins in that the mature complex includes a cleaved and stable signal peptide (SSP) as a third subunit (6, 15, 48) (Fig.

1). The 58-amino-acid SSP spans the membrane twice, with both termini in the cytosol (1), and likely is retained in the complex by the formation of an intersubunit zinc finger structure with the cytoplasmic domain of G2 (46). Importantly, we have shown that the short ectodomain loop of SSP plays a central role in the response of the GPC complex to acidic pH and, thereby, in pH-dependent membrane fusion (44, 47). Despite the unusual involvement of SSP in pH-triggered activation, the subsequent mechanics of GPC-mediated membrane fusion are likely similar to those of other class I viral fusion proteins, such as influenza virus hemagglutinin (HA) and the retrovirus envelope glycoprotein (Env) (17, 20, 43). A generally accepted model for membrane fusion by these proteins (reviewed in references 14, 22, and 42) posits that the native envelope glycoprotein exists in a metastable prefusion state that is established on proteolytic maturation. Destabilization of the native complex by acidic pH in HA (or receptor binding by Env) initiates a sequence of conformational changes that ultimately lead to formation of the now-classical six-helix bundle structure in the ectodomain of the transmembrane fusion subunit. In the process of forming this highly stable postfusion structure, the virus and cell membranes are brought into apposition and membrane fusion ensues. HA is the best-studied pH-dependent class I fusion protein, but even though the prefusion and postfusion structures are known in atomic detail, the molecular basis for the pH-induced activation of the complex remains elusive (14, 22, 42).

Our previous studies of JUNV GPC identified the unusual role of SSP in modulating the response of GPC to acidic pH. We demonstrated that mutations that reduce the positive charge at lysine 33 (K33) in the ectodomain loop of SSP correspondingly

* 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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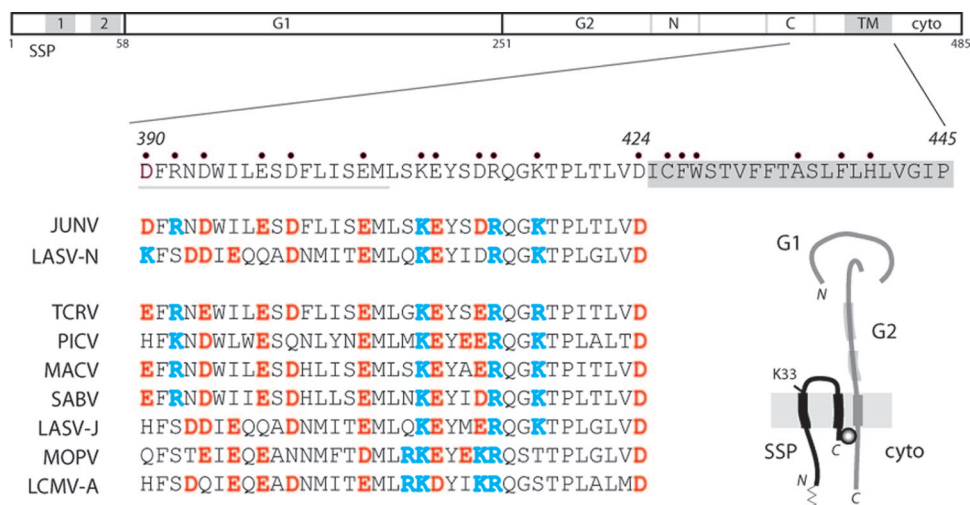


FIG. 1. Arenavirus GPC complex. The JUNV GPC open reading frame is illustrated at the top. Amino acids are numbered from the initiating methionine, and the SSP, G1, and G2 subunits are indicated. Membrane-spanning regions in SSP (h ϕ 1 and h ϕ 2; 1) and in G2 (TM) are shaded dark gray, and the N- and C-terminal heptad repeat regions in G2 (43) are in light gray. The amino acid sequence of the G2 membrane-proximal ectodomain and transmembrane domain (shaded) of JUNV is detailed below; positions studied in this report are indicated by dots. At the lower right is a diagram of the proposed subunit organization in the tripartite GPC complex. Thickened lines represent membrane-spanning domains in SSP and G2 and the heptad repeat regions in G2. The cytosolic N terminus of SSP is myristoylated (thin line) (48), and an intersubunit zinc finger (ball) is thought to link the C terminus of SSP with the cytoplasmic domain of G2 (46). Lysine 33 (K33) in SSP is marked. The drawing is representational and not to scale. In the lower left, we show the alignment of the arenavirus membrane-proximal G2 ectodomain regions. Accession numbers are the following: JUNV, D10072; LASV-Nigeria (LASV-N), P17332; Tacaribe virus (TCRV), NP_694849; Pichinde virus (PICV), AAB58484; Machupo virus (MACV), AAX99337; Sabiá virus (SABV), AAC55091; LASV-Josiah (LASV-J), AAG41802; Mopeia virus (MOPV), AAV54108; and lymphocytic choriomeningitis virus-Armstrong (LCMV-A), NP_694851. Charged residues are highlighted in blue (basic) or red (acidic).

stabilize GPC against pH-induced activation (47). Based on this finding, we sought to identify the pH-responsive elements in GPC with which K33 interacts. In the absence of structural information, we directed our investigation to the membrane-proximal ectodomain and transmembrane regions of G2, which may lie near the membrane-proximal K33 residue in SSP. By using the method of genetic complementation, we now have identified second-site mutations in G2 that rescue the fusion deficiency arising from the substitution of K33 with glutamine. Our findings provide evidence that SSP and G2 interact in controlling the pH-induced activation of GPC-mediated membrane fusion.

MATERIALS AND METHODS

Molecular reagents. GPC from the pathogenic JUNV strain MC2 (21) was expressed in Vero cells by the Lipofectamine 2000 (Invitrogen)-mediated cotransfection of two plasmids: CD4sp-GPC (in which SSP is replaced by the conventional signal peptide of CD4) and SSP-term (in which a stop codon is introduced following the C-terminal SSP amino acid T58) (47). SSP and the G1-G2 precursor associate *in trans* and reconstitute the native GPC complex (15). This experimental system obviates concerns regarding the potential effects of mutation on the signal peptidase cleavage of full-length GPC (45). Mutations in GPC were introduced by QuikChange mutagenesis (Stratagene), and all constructs were verified by DNA sequencing.

Cell-cell fusion assay. A vaccinia virus-based β -galactosidase fusion reporter assay (31) was used to characterize the ability of GPC to mediate pH-dependent cell-cell fusion (47, 48). Briefly, Vero cells expressing GPC and infected by vTF-7 (which expresses bacteriophage T7 polymerase) (19) were maintained in medium containing 10 μ M cytosine arabinoside (araC) to limit vaccinia virus replication. Target cells for syncytium formation were infected with a recombinant vaccinia virus bearing the β -galactosidase gene under the control of a T7 promoter (vCB21R-lacZ) (31) and subsequently were seeded into 96-well microculture dishes (40,000 cells/well) in the presence of 100 μ g rifampin/ml to minimize vaccinia assembly and cytopathic effect (24). Prior to use in the cell-cell fusion

assay, target cells (40,000 cells/well) were added to the microcultures, allowed to settle for 30 min, and subjected to a brief low-speed centrifugation ($\sim 25 \times g$) to ensure cell-cell contact. These cocultures were continued for 3 h in medium containing araC and rifampin. Membrane fusion was initiated by incubation in medium that had been adjusted to low pH using HEPES and piperazine-*N,N'*-bis(2-ethanesulfonic acid) buffers. After 10 min at 37°C, the cells were restored to neutral medium and cultured for 5 h to allow for the expression of the β -galactosidase fusion reporter gene, which then was quantitated using the chemiluminescent GalactoLite Plus substrate (Applied Biosystems) and a Tropic TR717 microplate luminometer.

RESULTS AND DISCUSSION

The pH-induced activation of GPC-mediated membrane fusion is controlled in part by the lysine at position 33 in the short ectodomain loop of SSP. Amino acid substitutions that reduce positive polarity at K33 systematically lower the pH necessary to activate membrane fusion (47). The replacement of lysine with glutamine (K33Q) reduces membrane fusion activity to 6% of the wild-type level (see below) while decreasing the pH of maximal fusion from the wild-type optimum of pH 5.0 to pH 4.5. The apparent stabilization of the mutant complex against acidic pH does not appreciably affect its intrinsic fusion activity, as K33Q GPC retains (at pH 4.5) as much as 40 to 50% of the wild-type GPC activity (at pH 5.0). The replacement of K33 with alanine not only abrogates membrane fusion at any relevant pH but also stabilizes the complex against spontaneous dissociation of the G1 and G2 subunits (44). In order to identify pH-sensitive determinants that might interact with K33, we inspected the amino acid sequence of the membrane-proximal ectodomain of G2. This region, within 35 amino acids of the membrane (D390 to D424 in JUNV), includes 12

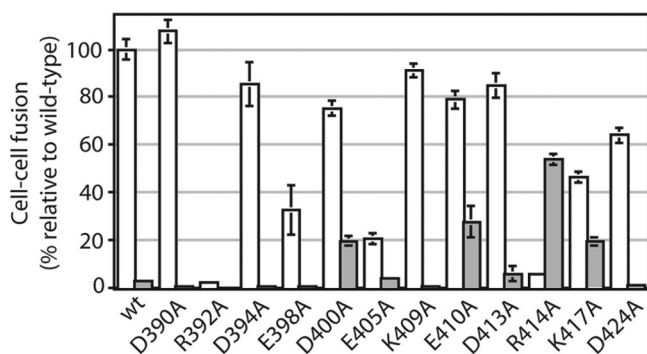


FIG. 2. Membrane fusion activity of G2 mutants. The wild-type (wt) and G2 mutants in CD4sp-GPC were expressed *in trans* with wild-type SSP (open bars) or K33Q SSP (filled bars). The ability of the single and double mutants to mediate cell-cell fusion at pH 5.0 was determined as detailed in Materials and Methods. Error bars representing ± 1 standard deviation are calculated for all points ($n = 6$) and may not be visible on the scale of the graph. These data represent one complete study (of at least three to six complete and partial experiments). All comparisons are reliably reproducible between experiments, although absolute percentages may vary. The average for K33Q GPC ($6.1\% \pm 2.5\%$) differs from that previously cited (47) due to technical changes in the assay. The D400A, E405A, E410A, K417A, and D424A mutants have been reported previously (44).

charged residues in both JUNV and LASV (Fig. 1). Nine of these (D394, D400, E405, K409, E410, D413, R414, K417, and D424) are identical between the two viruses. Other positions vary conservatively among New World and Old World arenaviruses, and all species retain $\geq 30\%$ charged residues (Fig. 1). Only R414 and D424 are invariant among all of the arenaviruses.

The role of these charged residues in membrane fusion was examined by replacing them individually with alanine. In brief, mutations were introduced into a recombinant construct, CD4sp-GPC, encoding the G1-G2 precursor of the pathogenic MC2 isolate of JUNV (21) preceded by the conventional signal peptide of CD4 (48). When transiently coexpressed with SSP (amino acids 1 to 58) in Vero cells, the GPC complex is functionally reconstituted (15, 48). The membrane fusion activity of the mutant GPC was determined using a β -galactosidase fusion reporter assay (31) in which syncytium formation was initiated by exposing the culture of GPC-expressing effector cells and fusion reporter target cells to medium adjusted to pH 5.0 (44, 48). As illustrated in Fig. 2, most of the alanine mutants in G2 were able to mediate pH-dependent cell-cell fusion at levels approaching ($\geq 50\%$) that of the wild type (also see reference 44). Only R392A, E405A, and R414A were significantly diminished in their membrane fusion activity ($\leq 20\%$ of the level of wild-type GPC).

Mutations at charged residues complement fusion defects in SSP. To specifically inquire whether these charged residues in G2 interact with SSP, we screened the panel of mutants for their ability to rescue a fusion deficiency at SSP position 33. This genetic method of complementation is frequently used to study functional interactions between and within viral proteins (5, 39). Of the previously characterized K33 mutants, only K33Q GPC is severely debilitated yet retains sufficient activity at pH 5.0 (6% of the wild type) for experimental measurements of complementation. Mutations in G2 that restored

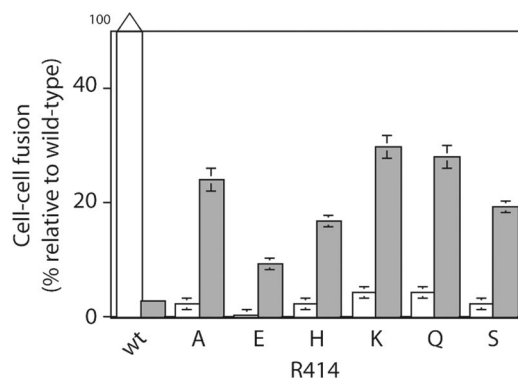


FIG. 3. Membrane fusion activity of charge mutants at R414. These studies were performed in the same fashion as those described in the legend to Fig. 2. The amino acid changes at R414 are indicated by single-letter abbreviations. Note that the vertical axis is truncated. wt, wild type.

fusion activity beyond this 6% baseline level were judged to interact genetically with SSP. We identified four such mutations (Fig. 2). Double mutants with D400A, E410A, R414A, and K417A were able to mediate fusion at 20, 30, 55, and 20% of wild-type levels, respectively. In contrast, little or no increase in fusion activity was observed in double mutants containing D390A, R392A, D394A, E398A, E405A, K409A, D413A, or D424A. The presence or absence of complementation did not correlate with the magnitude of the fusion defect in the single alanine mutant. Nor could we discern significant differences in the extent of SSP association or proteolytic maturation (45) among the mutant GPC complexes (not shown). Selective complementation by the D400A, E410A, R414A, and K417A mutations, all of which cluster toward the membrane-spanning domain of G2, points to specificity in the rescue. These findings indicate a genetic interaction between SSP and the most membrane-proximal region of the G2 ectodomain.

Molecular basis for complementation of K33Q. To investigate whether complementation involves pH-sensitive interactions, we examined a series of charge mutants at the strongly complementing R414 position. As anticipated, R414E was somewhat reduced in membrane fusion activity relative to that of R414A and with respect to R414K, R414H, R414Q, and R414S mutants (Fig. 3). However, none of the charged, polar, or neutral mutations had major effects on the membrane fusion activity or complementation of K33Q. A difference in the extent of rescue by the R414 mutation generally correlated with its effect on fusion activity. In contrast to the systematic effects of mutation at K33, we were unable to discern a charge-dependent interaction between K33Q and the R414 side chain. Thus, complementation by K33Q may arise through effects other than those that are determined primarily by charge.

Compared to that of wild-type GPC, K33Q requires a lower pH to activate membrane fusion (47). With the extraordinary stability of the G1 and G2 association in the K33A mutant (44), we concluded that mutations at K33 stabilize the native GPC complex with respect to pH-induced activation. Therefore, we hypothesized that K33Q-complementing mutations in G2 act in an opposing manner to destabilize the complex and raise the pH for membrane fusion. Initial experiments with three of

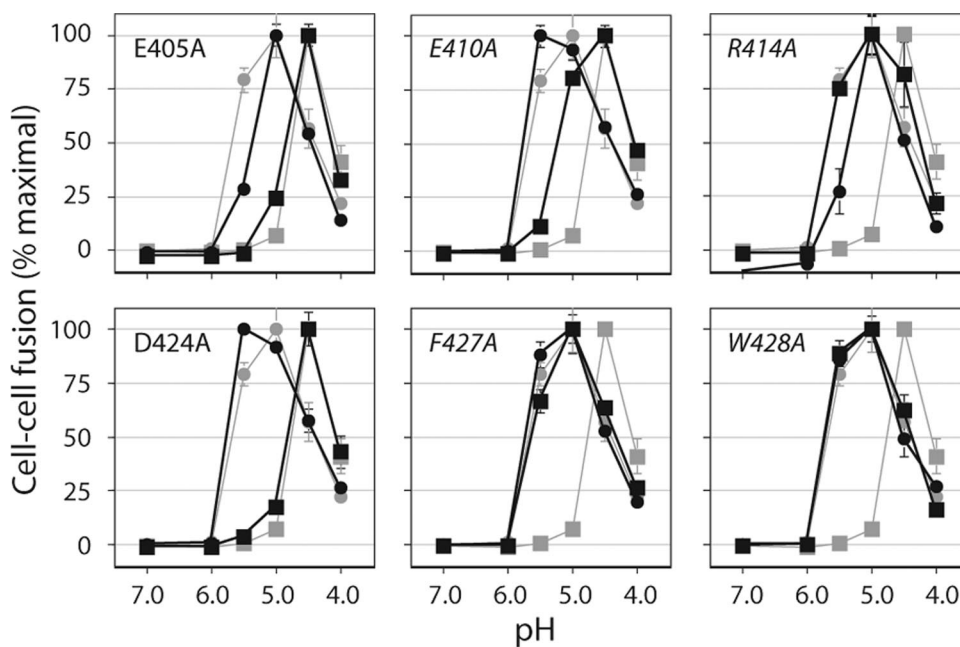


FIG. 4. pH sensitivity of membrane fusion. Cells expressing either wild-type SSP (black circles) or K33Q SSP (black squares) with the G2 mutant were pulsed at the indicated pH. The lightly shaded curves, repeated in all panels, depict fusion of the wild-type CD4sp-GPC with wild-type (gray circles) and K33Q (gray squares) SSP. The results shown in this study are representative of at least three independent experiments. Fusion activity is normalized to the maximum of each mutant for this comparison. Error bars represent ± 1 standard deviation.

these mutations (D400A, E410A, and K417A) had not revealed significant differences (44), so we extended the analysis to include the strongly complementing R414A mutation.

The pH dependence of membrane fusion was determined by varying the pH of the acidic medium used to initiate cell-cell fusion (44, 47). Fusion activity by the wild-type GPC (Fig. 4) was first observed at pH 5.5, attained a maximum level at pH 5.0, and subsequently declined (gray circles). As noted, fusion by K33Q GPC (gray squares) was minimal at pH 5.0 and reached its maximum level at pH 4.5. Single mutants with complementing (E410A and R414A) and noncomplementing (E405A and D424A) mutations behaved much like the wild-type GPC, with maximal fusion activity at pH 5.0 to 5.5 (black circles). Although small differences relative to the wild type could be noted, these did not correlate with the presence or absence of complementation and were not statistically significant in our studies. Thus, the single alanine mutations in the membrane-proximal ectodomain of G2 did not by themselves increase the pH at which membrane fusion is activated.

Complementing mutants abolish pH suppression by K33Q.

However, all of the complementing mutations, and only the complementing mutations, were able to mitigate the K33Q-induced reduction in the pH of activation (Fig. 4). In the highly complementing R414A mutant, maximal membrane fusion was shifted to pH 5.0 (black squares), similar to that of wild-type GPC and distinct from that of the single K33Q mutant (pH 4.5). The weakly complementing E410A mutant showed a partial shift toward pH 5.0. In contrast, optimal fusion by noncomplementing double mutants (E405A and D424A) remained at pH 4.5, similar to K33Q GPC. Thus, the rescue of the membrane fusion activity of K33Q GPC by second-site mutations in G2 can be accounted for entirely by their sup-

pression of the K33Q-induced decrease in the pH of membrane fusion.

Role of the G2 membrane-spanning domain in complementation. Several lines of evidence suggested that interactions between SSP and G2 extend to the membrane-spanning region. In particular, amino acid determinants of resistance to small-molecule fusion inhibitors recently discovered by SIGA Technologies (Corvallis, OR) are localized not only in the ectodomain loop of SSP and the membrane-proximal ectodomain of G2 but also in the adjoining transmembrane domain (3, 27, 44). We therefore extended our complementation analysis to include transmembrane residues in G2 known to affect sensitivity to these inhibitors. As shown in Fig. 5, mutations at two major determinants of resistance (F427A and F438I) were able to rescue the K33Q defect to 50 and 75% of wild-type levels, respectively. A435I, another resistance mutation, did not complement K33Q, whereas the inhibitor-sensitive mutant W428A did. Thus, the interactions responsible for the complementation of K33Q extend into the membrane-spanning domain of G2 and are not restricted to charged residues.

Interestingly, the mechanism for complementation at these hydrophobic positions (F427 and W428) appeared identical to that at charged positions in the membrane-proximal ectodomain. Neither of these uncharged mutations alone affected the pH of membrane fusion, yet both restored the pH of the maximal fusion activity of K33Q GPC to that of the wild type (Fig. 4). Complementation at these hydrophobic positions is unlikely to be mediated by direct interaction with K33. Rather, the restoration of the wild-type phenotype may reflect indirect structural effects emanating from interactions of the membrane-spanning domains of G2 and SSP or from potential oligomerization determinants in G2 (16, 32). Thus, perturba-

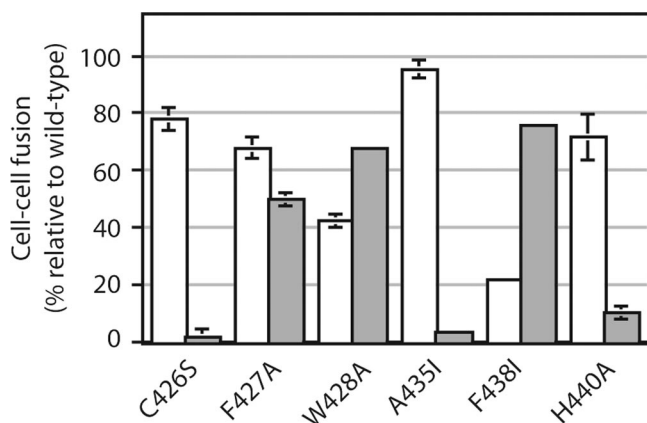


FIG. 5. Complementation by transmembrane domain G2 mutants. The G2 mutants were expressed in *trans* with wild-type SSP (open bars) or K33Q SSP (filled bars), and membrane fusion activity was determined. These data represent one complete study (of at least three complete and partial experiments).

tions in the membrane may couple to the pH-sensing ectodomain.

Additional transmembrane domain residues that might be involved in complementation were also examined. The possibility of a transient disulfide bond at C426 and the potentially ionizable H440 side chain were probed by replacements with serine and alanine, respectively. Neither of these mutations disrupted fusion activity or complemented the K33Q defect (Fig. 5). The H440A mutation eliminated the sole histidine in the membrane and membrane-proximal sequences of G2.

Controlled activation of pH-induced membrane fusion. Our observation that complementing mutations, which alone do not affect the pH of membrane fusion, are able to abrogate the pH effect of the K33Q mutation presents us with a conundrum. In the simplest model for complementation, second-site mutations might generate a corresponding increase in pH sensitivity to counterbalance the reduction by K33Q. Indeed, compensatory changes in pH sensitivity are well described in the influenza virus HA. Fusion inhibition by *tert*-butyl hydroquinone (TBHQ) compounds that stabilize the HA complex against low pH in the endosome can be overcome by second-site mutations that render it more sensitive to pH (23, 36). Similarly, viral resistance to amantadine, an inhibitor of endosomal acidification, arises through mutations in HA that specifically enable fusion at an elevated pH (11, 40). Mutations affecting the pH of HA activation include both charged and uncharged residues that, although widely distributed, tend to cluster near the fusion peptide as well as at monomer interfaces (11, 23, 36, 39–41). Despite atomic resolution knowledge of HA, however, the primary site of pH-induced activation remains unresolved (22, 42). In general, the compact and metastable nature of the native complex makes the dissection of primary and secondary events in the response to pH difficult.

The molecular mechanism whereby mutations in G2 restore wild-type pH sensitivity to K33Q GPC without themselves altering the pH of activation is unclear. We suggest that the original K33Q mutation affects the prefusion complex in two opposing directions. First, by decreasing repulsive charge interactions through K33, the glutamine mutation may directly

stabilize the pH-sensing mechanism against activation. Second, by replacing the natural lysine residue, the mutation also may contribute to destabilization of the complex. In the single G2 mutants, the pH-sensing mechanism remains intact and responds in the same fashion as the wild-type GPC, despite the G2 mutation. However, in the double mutant, the effects of the G2 mutation may combine with the destabilizing component of the K33Q mutation to overcome the otherwise stabilizing effect of the uncharged glutamine. Nonlinear interactions between mutations affecting pH sensitivity in HA also have been documented (39). It remains possible that the primary pH-responsive interaction of K33 includes charged residues elsewhere in G2. As illustrated in the recently published structure of the acid-sensing ion channel ASIC1, pH-sensing sites can be highly distributed networks including multiple-charge, proton-sharing, and hydrogen-bonding interactions (25). It is important to note that the genetic results reported here do not necessarily connote direct chemical interactions. In the absence of the atomic resolution structure, however, our studies provide initial insights into the complex structure-function relationships that promote the transition from the native GPC complex toward membrane fusion.

Without an effective treatment or vaccine, the hemorrhagic fever arenaviruses remain a pressing public health and biodefense concern. In this regard, it is noteworthy that the process of the pH-induced activation of membrane fusion has been shown to offer a viable target for antiviral intervention. In a manner analogous to that of the TBHQ inhibitors of HA (23, 36), small-molecule arenavirus-specific compounds described by SIGA (3, 27) inhibit GPC-mediated fusion by stabilizing the prefusion complex against acidic pH (44). A further understanding of the molecular determinants of inhibition at the SSP-G2 interface may lead to the identification of improved antiviral compounds. The genetic manipulation of pH sensitivity also may generate stable forms of the native GPC complex that can be used to elicit protective immunity as well as in structural studies. The detailed characterization of GPC-mediated membrane fusion will advance the development of effective vaccines and therapeutic agents against arenaviral disease.

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