Published in final edited form as:

Methods Mol Biol. 2018 ; 1604: 157–167. doi:10.1007/978-1-4939-6981-4_10.

A cell-cell fusion assay to assess arenavirus envelope glycoprotein membrane-fusion activity

Joanne York and Jack H. Nunberg*

Montana Biotechnology Center, The University of Montana, Science Complex Room 221, Missoula, MT 59812

Abstract

For many viruses that enter their target cells through pH-dependent fusion of the viral and endosomal membranes, cell-cell fusion assays can provide an experimental platform for investigating the structure-function relationships that promote envelope glycoprotein membrane-fusion activity. Typically, these assays employ effector cells expressing the recombinant envelope glycoprotein on the cell surface and target cells engineered to quantitatively report fusion with the effector cell. In the protocol described here, Vero cells are transfected with a plasmid encoding the arenavirus envelope glycoprotein complex GPC and infected with the vTF7-3 vaccinia virus expressing the bacteriophage T7 RNA polymerase. These effector cells are mixed with target cells infected with the vCB21R-lacZ vaccinia virus encoding a β -galactosidase reporter under the control of the T7 promoter. Cell-cell fusion is induced upon exposure to low-pH medium (pH 5.0), and the resultant expression of the β -galactosidase reporter is quantitated using a chemiluminescent substrate. We have utilized this robust microplate cell-cell fusion assay extensively to study arenavirus entry and its inhibition by small-molecule fusion inhibitors.

Keywords

arenavirus; envelope glycoprotein; membrane fusion; syncytium formation; cell-cell fusion; endosome; fusion inhibitor

1 Introduction

The defining function of the viral envelope glycoprotein is to orchestrate fusion of the viral and cellular membranes, thereby promoting release of the virion core into the cell to initiate viral replication. Classically, enveloped viruses have been subdivided into those that fuse at the plasma membrane in response to receptor binding (such as HIV-1 and the paramyxoviruses) and those that undergo receptor-mediated endocytosis and fuse upon exposure to acidic pH in the maturing endosome. Many of the hemorrhagic fever viruses surveyed in this volume belong to this latter group, with membrane fusion taking place in endosomal and lysosomal compartments, removed from facile experimental manipulation. For the majority of these viruses whose envelope glycoproteins normally traffic to the cell surface for virion assembly, this process of pH-induced endosomal fusion can be readily

*Corresponding author: jack.nunberg@umontana.edu.

studied using the surrogate of cell-cell fusion. First noted as syncytium formation among Sindbis virus-infected cells exposed to acidic pH (“fusion-from-within” [1]), these systems have evolved with emerging technology to serve as robust platforms for structure-function studies of membrane-fusion activity. Modern assays typically utilize effector cells expressing the envelope glycoprotein and target cells engineered to quantitatively report fusion of the two. It is important to bear in mind that these assays do not necessarily recapitulate all aspects of virus entry within the endocytic pathway. Specifically, the possible role of secondary receptors and endolysosomal proteases need be considered. Nonetheless, these simple biosafe assays can provide a reliable quantitative read-out of membrane-fusion activity.

We have utilized cell-cell fusion assays extensively to study arenavirus entry and its inhibition by small-molecule fusion inhibitors. Arenaviruses enter the host cell through pH-dependent fusion of the viral and endosomal membranes, a process mediated by the viral envelope glycoprotein complex GPC [2]. GPC is synthesized as a precursor that trimerizes and is cleaved by the cellular S1P/SKI-1 protease [3-5] to generate the mature receptor-binding (GP1) [6,7] and transmembrane fusion (GP2) [8-11] subunits. Unlike other class I fusion proteins, GPC retains a 58 amino-acid residue signal peptide as a third, noncovalently associated subunit in the mature complex [12-14]. This stable signal peptide (SSP) contains two hydrophobic regions that span the membrane to form a hairpin structure [15], with a central external loop that interacts with the membrane-proximal ectodomain of GP2 to sense acidic pH and trigger membrane fusion [16,17]. SSP association is also required for transit of the GP1-GP2 precursor through the Golgi, and thus for proteolytic maturation and transport to the cell surface for virion assembly [18]. Several chemically distinct classes of small-molecule fusion inhibitors have been demonstrated to act through the pH-sensing SSP-GP2 interface to antagonize the effect of acidic pH in activating GPC membrane fusion [19].

The GPC-mediated cell-cell fusion assay used in our laboratory was developed through modification of the recombinant vaccinia virus-based fusion reporter assay described by Nussbaum, Broder and Berger in 1994 to study HIV-1 Env [20]. In brief, Vero cells are infected with a recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase and transfected with a pcDNA-based plasmid encoding GPC. These cells are mixed with target cells infected with another recombinant vaccinia virus encoding a β -galactosidase reporter under the control of the T7 promoter. Antiviral drugs are used to block production of progeny vaccinia viruses and thereby prevent cross-infection of effector and target cells. Cell-cell fusion is induced upon exposure of the co-culture to acidic medium (pH 5.0), and the resultant expression of the β -galactosidase reporter is quantitated using a chemiluminescent substrate. A version of this assay that eliminates the use of BSL2 recombinant vaccinia viruses has also been described [21].

2 Materials

2.1 Cell culture

1. Vero-76 African green monkey (*Cercopithecus aethiops*) kidney cells (ATCC CRL-1587).

2. Recombinant vaccinia virus vTF7-3 (NIH AIDS Reagent Program, Division of AIDS, NIAID, catalog #356, contributed to the AIDS Reagent Program by Dr. Thomas R. Fuerst and Dr. Bernard Moss; *see Note*¹).
3. Recombinant vaccinia virus vCB21R Lac-Z (NIH AIDS Reagent Program, Division of AIDS, NIAID, catalog # 3365, contributed to the AIDS Reagent Program by Dr. Christopher C. Broder, Paul E. Kennedy, and Dr. Edward A. Berger; *see Note*¹).
4. Falcon™ 96-well clear flat-bottom cell-culture microplates (Corning).
5. Dulbecco's Modified Eagle Medium (DMEM), high glucose, pyruvate (Gibco) is used in a variety of formulations. Growth Medium for the Vero cells is DMEM with 10% heat-inactivated fetal bovine serum (FBS) and 1X L-glutamine. For routine cell passage, we also include 1X Pen Strep. Infection Medium is DMEM with 2% FBS and 1X L-glutamine. Serum-free medium (SFM) contains only the additional 1X L-glutamine. The Post-Transfection Addition is prepared using Infection Medium made to 20% FBS and 10 µM araC. Low-pH Pulse Medium is prepared within 3 hours of use and consists of DMEM with 5% FBS, 1X L-glutamine, 10 µM araC, 100 µg/ml rifampicin plus 10 mM PIPES and 10 mM HEPES, pre-warmed and titrated to pH 5.0 using concentrated HCl.
6. 10,000 U/ml (100X Pen-Strep) Penicillin-Streptomycin (Gibco).
7. 200 mM (100X) L-Glutamine (Gibco).
8. 0.05% trypsin-EDTA (Gibco).
9. 1 mg/ml poly-D-lysine in H₂O (10X stock) (Sigma)
10. 10X Dulbecco's phosphate-buffered saline (DPBS), no calcium or magnesium (Gibco). Diluted this to make a 1X solution.
11. 10 mM (1000 X stock) cytosine β-D-arabinofuranoside (araC) in H₂O (Sigma).
12. 100 mg/ml rifampicin in DMSO (1000X stock) (Sigma).
13. Opti-MEM Reduced Serum Medium (Gibco).
14. Lipofectamine 2000 Transfection Reagent (Invitrogen).
15. 0.5 M PIPES (1,4-piperazinediethanesulfonic acid) brought to pH~7 by titration with concentrated NaOH (Sigma).
16. 1M HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (Gibco).
17. Plasmid encoding expression of envelope glycoprotein: For expression of GPC, we typically utilize the minimal T7 promoter present in pcDNA3.1 vectors (Invitrogen), (*see Notes*² and ³).

¹The recombinant vaccinia viruses vTF7-3 and vCB21R Lac-Z are derived from the non-highly-attenuated WR strain of vaccinia virus. These viruses are handled using Biosafety Level 2 facilities and practices, and prior vaccination is recommended for laboratory workers [23]. We have investigated a version of this assay that does not utilize recombinant vaccinia viruses, using instead T7 polymerase-expressing BHK-21 cells (BSR-T7 [24]) and a T7 promoter-based β-galactosidase expression plasmid [21]. This assay is less robust than the vaccinia virus-based assay and may benefit from further optimization.

18. Monoclonal antibodies to Junin GPC [22] are available through BEI Resources, an NIH/NIAID-funded repository of reagents for biodefense and emerging infectious disease research (www.beiresources.org). We typically use monoclonal antibody QC03-BF11 for immunohistochemical staining of GPC (catalogue # NR-43775).

2.2 Quantitation of β -galactosidase fusion reporter

1. Galacto-Light Plus™ β -Galactosidase Reporter Gene Assay System (Applied Biosystems T1007). This kit contains Lysis Solution, Galacton-Plus substrate, Reaction Buffer Diluent and light emission Accelerator-II.
2. Costar™ 96-Well White Opaque Plates (Corning).
3. Protease inhibitors (Roche) for use with Galacto-Light Plus Lysis Solution:
1 mg/ml Leupeptin in H₂O (1000X stock).
1 mg/ml Pepstatin in MeOH (1000X stock) .
1 mg/ml Aprotinin in H₂O (1000X stock). These three protease inhibitors are added to Lysis Solution just before use to make 1X in each.
- 3 SpectraMax L microplate Luminometer; 1 channel and at least one injector (Molecular Devices).

3 Methods

3.1 Generation of GPC-expressing effector cells

1. Vero cells are typically split 1:4, and given fresh Growth Medium, one day prior to use in the assay.
2. On Day 1, harvest cells with trypsin-EDTA. Centrifuge and resuspend in Growth Medium without Pen Strep.
3. Seed 1.5×10^6 cells in 5 ml to a 6-cm culture dish and incubate overnight at 37°C in a CO₂ incubator. With losses sustained in subsequent steps, one 6-cm dish provides enough effector cells for ~20 co-cultures in the 96-well microplate used in the assay. Set up and combine multiple 6-cm dishes if more wells are required for any effector-cell population.
4. Reseed extra cells to a flask for generating target cells on the following day (section 3.2).

²GPC is very poorly expressed using the human cytomegalovirus immediate-early promoter and enhancer engineered into pcDNA plasmids. By using the vTF7-3 vaccinia virus-encoded T7 polymerase, we take advantage of the strong T7 promoter and the mRNA capping and polyadenylation activities of vaccinia virus for rapid and high-level production of GPC mRNA in the cytoplasm of transfected cells. Any promoter that results in adequate envelope glycoprotein expression should suffice for the assay, although timing may need to be modified to accommodate nuclear expression of the transfected plasmid.

³SSP can be co-expressed with the GP1-GP2 precursor (directed to the ER via a heterologous signal peptide) to reconstitute the tripartite GPC complex [13,25]. We utilize the conventional signal peptide of human CD4. This approach obviates concerns regarding incomplete cleavage of SSP in wild-type and mutant GPCs [12,14,26] and generally results in slightly higher levels of membrane-fusion activity than the full-length GPC construct (unpublished).

5. On Day 2, pre-treat effector cells with 10 μ M araC for 2 hr at 37°C to block DNA replication and late-gene expression of the vTF7-3 vaccinia virus (*see Note 4*). To each 6-cm dish containing 5 ml Growth Medium, add 5 μ l of the 1000X araC stock.
6. vTF7-3 is used at a multiplicity of infection (moi) of 2 to infect effector cells. Assuming that the cells have doubled overnight: 3×10^6 cells $\times$ 2 = 6×10^6 pfu vTF7-3 per 6-cm dish. Quickly thaw pre-titered vTF7-3 stock in 37°C water bath and vortex vigorously to disaggregate.
7. From each 6-cm dish, remove Growth Medium and replace with 750 μ l Infection Medium containing additional 10 μ M araC and 6×10^6 pfu vTF7-3. Incubate 30 min in CO₂ incubator at 37°C, swirling every 10 min.
8. During incubation with vTF7-3, prepare for plasmid transfection. With our pcDNA3.1-based GPC expression plasmids, we use a total of 2 μ g DNA per transfection (2 μ g plasmid expressing full-length GPC or a 3:1 mixture of GP1-GP2 and SSP plasmids – *see Note 3*). Dilute 2 μ g DNA in 500 μ l Opti-MEM. Separately, dilute 20 μ l Lipofectamine 2000 in 500 μ l Opti-MEM and let sit for 5 min at room temperature. Combine DNA and Lipofectamine 2000 solutions and incubate for 20 min at room temperature.
9. Aspirate vaccinia virus-containing medium from cells and wash twice with serum-free medium. Refeed with 2 ml SFM containing 10 μ M araC. Add DNA-Lipofectamine 2000 complex dropwise to culture; rock dish to distribute evenly. Return to incubator for 3 hr.
10. After 3 hr, add 2 ml of Post-Transfection Addition medium to each dish to bring the final concentration of FBS to 8% and araC to 8 μ M. Incubate overnight.

3.2 Generation of target cells

1. During the 3-hr incubation in transfection medium (3.1.9), prepare vCB21R Lac-Z-infected target cells using cells reserved the previous day (3.1.4).
2. Harvest cells with trypsin-EDTA, wash with DPBS and resuspend in SFM to count. Add 5×10^6 cells for each full 96-well microplate to be seeded to a 50 ml conical tube and pellet.
3. Infect cells with vCB21R Lac-Z using an moi of 2: 5×10^6 cells $\times$ 2 = 10^7 pfu. Quickly thaw pre-titered vCB21R Lac-Z stock in 37°C water bath and vortex vigorously to disaggregate. Pellet cells in a conical tube and resuspend in 1 ml of Infection Medium containing 10^7 pfu vCB21R Lac-Z. Incubate 90 min in CO₂ incubator at 37°C, swirling every 20 min.

⁴AraC is used with the effector cells to block replication of vaccinia virus DNA, thereby preventing the production of progeny viruses. Similarly, rifampicin is used with target cells to inhibit formation of mature virion particles [27]. Together, these reagents prevent cross-infection between effector and target cells, and hence unwanted fusion-independent expression of the β -galactosidase reporter. Inadequate control of vaccinia virus production is manifest in an elevated background level of β -galactosidase expression. Histochemical staining of test co-cultures using X-gal substrate [13] would reveal individual cells with intense color.

4. Fill conical tube with SFM and wash twice by centrifugation to remove free virus. Resuspend cell pellet and adjust to 0.4×10^6 cells/ml in Growth Medium with 100 $\mu\text{g/ml}$ Rifampicin. Seed 100 μl (40,000 cells) per well in a 96-well flat-bottom microplate and culture overnight. Possibly use poly-D-lysine to pre-coat wells (*see Note*⁵).

3.3 Co-culture of effector and target cells

1. On Day 3, harvest effector cells from 6-cm dish (3.1.10). Remove medium and wash once with 1 ml DPBS. Cells could exhibit significant cytopathic effect and may not adhere strongly to the dish (*see Note*⁵). Use caution when washing; pipet using the walls of the dish. If some cells are inadvertently dislodged in the DPBS wash, save wash into collection tube.
2. Remove cells from dish by incubating with 2 ml of pre-warmed 0.5 mM EDTA in DPBS for ~5 min at 37°C. Gently pipet up and down to disrupt cell clumps and save to 15 ml conical tube. Rinse dish with DPBS and combine to fill collection tube.
3. Pellet cells and wash in 10 ml SFM before resuspending in 2 ml Growth Medium containing 10 μM araC and 100 $\mu\text{g/ml}$ rifampicin. Adjust to 0.4×10^6 cells/ml.
4. Carefully remove medium from 96-well microcultures containing vCB21R Lac-Z-infected target cells, and add 100 μl of the resuspended effector cells per well (40,000 cells; 1:1 effector cells to target cells), (*see Note*⁶).
5. Reseed additional effector cells in a separate microplate to determine transfection efficiency by immunochemical staining. Monoclonal antibodies directed to JUNV GPC typically detect GPC expression in ~70% of the cells. Poor or variable transfection efficiencies will reduce the reliability of the assay.
6. Centrifuge microplate at 300 rpm ($\sim 16 \times g$) for 3 min to facilitate cell settling and cell-cell contact. Incubate for 3 hr at 37°C to allow cells to adhere.

3.4 Exposure of co-culture to acidic pH medium to induce cell-cell fusion

1. During the 3-hr incubation (3.3.6), prepare the Low-pH Pulse Medium (*see Notes*⁷ and ⁸).

⁵Vaccinia-infected effector and target cells typically display significant cytopathic effect that can compromise adhesion to the culture substrate. In some cases, pre-coating the 96-well microculture dish with poly-D-lysine can help. In general, extreme care must be taken in all medium changes of the 96-well microcultures to minimize cell loss. Uneven cell loss between wells will render quantitation of cell-cell fusion difficult.

⁶To assess inhibition of cell-cell fusion by small-molecule fusion inhibitors or monoclonal antibodies, serial dilutions of these reagents can be added to the co-culture just prior to or with the effector cells. Appropriate dilutions are prepared in separate round-bottom microplates and transferred to the adhered target cells or mixed with effector cells. For tightly bound inhibitors and monoclonal antibodies that target the prefusion form of GPC, the reagent need not be present in the Pulse Medium or subsequently to achieve maximal effect [19]. By contrast, a monoclonal antibody directed to the fusion peptide of JUNV GPC, which is only exposed upon pH-induced fusion activation, needs be present in the Pulse Medium in order to inhibit cell-cell fusion [28].

⁷The Low-pH Pulse Medium is not strongly buffered and should be prepared just prior to use. Care should be taken to remove all neutral medium from wells prior to the addition of the Pulse Medium in order to maintain the pre-determined pH, while working quickly to prevent cells from drying out.

⁸In studies to determine the pH profile for optimal fusion activation, the Pulse Medium can be adjusted within the range of pH 4.0 to pH 7.0. We typically use increments of 0.5 pH units. The point of maximal cell-cell fusion by JUNV GPC varies from 5.0 and 5.5 in independent assays. In contrast to published reports [29], we find that LASV GPC displays the same optimal pH as JUNV GPC [30].

2. Pipet all medium from co-cultures and replace with 100 μ l of Pulse Medium, pre-warmed to 37°C. Tilt plate to ensure all of the neutral medium is removed prior to the addition of the Pulse Medium, and work carefully so that the cell monolayer is neither disturbed nor allowed to dry out.
3. Incubate 20 min at 37°C in a CO₂ incubator. In general, maximal cell-cell fusion can be induced using significantly shorter exposures (~ 5 min).
4. Carefully remove Pulse Medium and replace with 100 μ l of Growth Medium containing 10 μ M araC and 100 μ g/ml rifampicin.
5. Incubate 5 hr to allow for mixing of cell contents and induction of the β -galactosidase fusion reporter. In some instances, syncytium formation can be detected microscopically within 15 min (*see Note* ⁹).

3.5 Chemiluminescence detection of the β -galactosidase fusion reporter

1. The Galacto-Light Plus™ β -Galactosidase Reporter Gene Assay System is used to detect β -galactosidase expression, as described by the manufacturer.
2. Carefully remove medium from wells and add 60 μ l of the kit Lysis Solution to which protease inhibitors have been added.
3. Incubate at room temperature for 10 min, and pipet up and down to ensure complete solubilization.
4. Remove 20 μ l to wells in a Corning 3912 96-well white opaque microplate. The remaining lysates can be frozen for back-up analysis. To each well, add 70 μ l of the Reaction Buffer Diluent with Galacton-Plus substrate. Incubate in the dark at room temperature for 1 hr.
5. Turn on SpectraMax L luminometer and program the following parameters for injection of the kit Accelerator-II enhancer and luminescence detection: room temperature, no shaking, endpoint integration time 1.6 sec; P-injector for Accelerator-II, volume 100 μ l, delay 1.3 sec, speed 270 μ l/sec.
6. Place white opaque microplate into luminometer and record luminescence. Output relative light units (RLUs) can be exported to Excel or GraphPad Prism for subsequent analysis.

3.6 Considerations on controls, replicates and expected results

1. A variety of negative controls can be used to establish a “no fusion” background: i) mock transfection/no envelope glycoprotein, ii) envelope glycoprotein but mock-pulsed with neutral medium, iii) if SSP and the GP1-GP2 precursor are to be co-expressed to reconstitute GPC, SSP can be omitted to block maturation and transport to the cell surface. As illustrated in the example below (Fig. 1),

However, cell-cell fusion by LASV GPC appears less robust than that by JUNV GPC, with a ~20-fold dynamic range in this assay relative to the ~100-fold range typically seen with JUNV GPC. This may reflect a requirement by LASV GPC for interaction with LAMP1 in the endolysosome [31].

⁹If the RLU signal is low when assayed after 5 hr, extending the co-culture overnight may increase β -galactosidase accumulation without an undue increase in background.

background is typically $\sim 1 \times 10^5$ RLU relative to the JUNV GPC signal of $\sim 1 \times 10^7$ RLU. The absolute number of RLUs is dependent on the instrument and assay system used. In our hands, the signal from LASV GPC is generally lower ($\sim 2 \times 10^6$ RLU; see Note 8).

2. We typically prefer to run 6-8 replicate wells, although reliable preliminary information can be obtained using as few as 2-3 replicates. The range of replicate RLUs should be within 2-fold (see Fig. 1).

Acknowledgments

Our studies of arenavirus membrane fusion and its inhibition have been funded over the past decade by the National Institutes of Health through the following research grants: R21 AI059355, U54 AI065357 (Rocky Mountain Center for Excellence in Biodefense and Emerging Infectious Diseases; John Belisle, Colorado State University), R01 AI074818, R01 AI093387 (Partnerships for Biodefense; Sean Amberg, SIGA Technologies), and R21 AI120490.

References

1. Mann E, Edwards J, Brown DT. Polycaryocyte formation mediated by Sindbis virus glycoproteins. *J Virol.* 1983; 45:1083–1089. [PubMed: 6834476]
2. Di Simone C, Zandonatti MA, Buchmeier MJ. Acidic pH triggers LCMV membrane fusion activity and conformational change in the glycoprotein spike. *Virology.* 1994; 198:455–465. [PubMed: 8291229]
3. Lenz O, ter Meulen J, Klenk H-D, Seidah NG, Garten W. The Lassa virus glycoprotein precursor GP-C is proteolytically processed by subtilase SKI-1/S1P. *Proc Natl Acad Sci U S A.* 2001; 98:12701–12705. [PubMed: 11606739]
4. Beyer WR, Popplau D, Garten W, von Laer D, Lenz O. Endoproteolytic processing of the lymphocytic choriomeningitis virus glycoprotein by the subtilase SKI-1/S1P. *J Virol.* 2003; 77:2866–2872. [PubMed: 12584310]
5. Kunz S, Edelmann KH, de la Torre J-C, Gorney R, Oldstone MBA. Mechanisms for lymphocytic choriomeningitis virus glycoprotein cleavage, transport, and incorporation into virions. *Virology.* 2003; 314:168–178. [PubMed: 14517070]
6. Radoshitzky SR, Abraham J, Spiropoulou CF, Kuhn JH, Nguyen D, et al. Transferrin receptor 1 is a cellular receptor for New World haemorrhagic fever arenaviruses. *Nature.* 2007; 446:92–96. [PubMed: 17287727]
7. Cao W, Henry MD, Borrow P, Yamada H, Elder JH, et al. Identification of alpha-dystroglycan as a receptor for lymphocytic choriomeningitis virus and Lassa fever virus. *Science.* 1998; 282:2079–2081. [PubMed: 9851928]
8. York J, Agnihothram SS, Romanowski V, Nunberg JH. Genetic analysis of heptad-repeat regions in the G2 fusion subunit of the Junin arenavirus envelope glycoprotein. *Virology.* 2005; 343:267–279. [PubMed: 16169032]
9. Eschli B, Quirin K, Wepf A, Weber J, Zinkernagel R, et al. Identification of an N-terminal trimeric coiled-coil core within arenavirus glycoprotein 2 permits assignment to class I viral fusion proteins. *J Virol.* 2006; 80:5897–5907. [PubMed: 16731928]
10. Igonet S, Vaney MC, Vohnrein C, Bricogne G, Stura EA, et al. X-ray structure of the arenavirus glycoprotein GP2 in its postfusion hairpin conformation. *Proc Natl Acad Sci U S A.* 2011; 108
11. Parsy ML, Harlos K, Huiskonen JT, Bowden TA. Crystal structure of Venezuelan hemorrhagic fever virus fusion glycoprotein reveals a class I postfusion architecture with extensive glycosylation. *J Virol.* 2013; 87:13070–13075. [PubMed: 24049182]
12. Eichler R, Lenz O, Strecker T, Garten W. Signal peptide of Lassa virus glycoprotein GP-C exhibits an unusual length. *FEBS Lett.* 2003; 538:203–206. [PubMed: 12633879]

13. York J, Romanowski V, Lu M, Nunberg JH. The signal peptide of the Junín arenavirus envelope glycoprotein is myristoylated and forms an essential subunit of the mature G1-G2 complex. *J Virol.* 2004; 78:10783–10792. [PubMed: 15367645]
14. York J, Nunberg JH. Distinct requirements for signal peptidase processing and function of the stable signal peptide (SSP) subunit in the Junin virus envelope glycoprotein. *Virology.* 2007; 359:72–81. [PubMed: 17045626]
15. Agnihothram SS, York J, Trahey M, Nunberg JH. Bitopic membrane topology of the stable signal peptide in the tripartite Junín virus GP-C envelope glycoprotein complex. *J Virology.* 2007; 81:4331–4337. [PubMed: 17267481]
16. York J, Nunberg JH. Role of the stable signal peptide of the Junín arenavirus envelope glycoprotein in pH-dependent membrane fusion. *J Virol.* 2006; 80:7775–7780. [PubMed: 16840359]
17. York J, Nunberg JH. Intersubunit interactions modulate pH-induced activation of membrane fusion by the Junin virus envelope glycoprotein GPC. *J Virol.* 2009; 83:4121–4126. [PubMed: 19224989]
18. Agnihothram SS, York J, Nunberg JH. Role of the stable signal peptide and cytoplasmic domain of G2 in regulating intracellular transport of the Junin virus envelope glycoprotein complex. *J Virol.* 2006; 80:5189–5198. [PubMed: 16698999]
19. York J, Dai D, Amberg SA, Nunberg JH. pH-induced activation of arenavirus membrane fusion is antagonized by small-molecule inhibitors. *J Virol.* 2008; 82:10932–10939. [PubMed: 18768973]
20. Nussbaum O, Broder CC, Berger EA. Fusogenic mechanisms of enveloped-virus glycoproteins analyzed by a novel recombinant vaccinia virus-based assay quantitating cell fusion-dependent reporter gene activation. *J Virol.* 1994; 68:5411–5422. [PubMed: 8057423]
21. Messina EL, York J, Nunberg JH. Dissection of the role of the stable signal peptide of the arenavirus envelope glycoprotein in membrane fusion. *J Virol.* 2012; 86:6138–6145. [PubMed: 22438561]
22. Sanchez A, Pifat DY, Kenyon RH, J PC, McCormick JB, et al. Junin virus monoclonal antibodies: characterization and cross-reactivity with other arenaviruses. *J Gen Virol.* 1989; 70:1125–1132. [PubMed: 2471803]
23. Centers for Disease Control and Prevention. Biosafety in Microbiological and Biomedical Laboratories. In: Wilson, DE., Chosewood, LC., editors. HHS. 2009.
24. Buchholz UJ, Finke S, Conzelmann KK. Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. *J Virol.* 1999; 73:251–259. [PubMed: 9847328]
25. Eichler R, Lenz O, Strecker T, Eickmann M, Klenk HD, et al. Identification of Lassa virus glycoprotein signal peptide as a trans-acting maturation factor. *EMBO Rep.* 2003; 4:1084–1088. [PubMed: 14555961]
26. Froeschke M, Basler M, Groettrup M, Dobberstein B. Long-lived signal peptide of lymphocytic choriomeningitis virus glycoprotein pGP-C. *J Biol Chem.* 2003; 278:41914–41920. [PubMed: 12917426]
27. Hruby DE, Lynn DL, Kates JR. Identification of a virus-specified protein in the nucleus of vaccinia virus-infected cells. *J Gen Virol.* 1980; 47:293–299. [PubMed: 7365469]
28. York J, Berry JD, Ströher U, Li Q, Feldmann H, et al. An antibody directed against the fusion peptide of Junin virus envelope glycoprotein GPC inhibits pH-induced membrane fusion. *J Virol.* 2010; 84:6119–6129. [PubMed: 20392854]
29. Klewitz C, Klenk HD, ter Meulen J. Amino acids from both N-terminal hydrophobic regions of the Lassa virus envelope glycoprotein GP-2 are critical for pH-dependent membrane fusion and infectivity. *J Gen Virol.* 2007; 88:2320–2328. [PubMed: 17622638]
30. Lee AM, Rojek JM, Spiropoulou CF, Gundersen AT, Jin W, et al. Unique small molecule entry inhibitors of hemorrhagic fever arenaviruses. *J Biol Chem.* 2008; 283:18734–18742. [PubMed: 18474596]
31. Jae LT, Raaben M, Herbert AS, Kuehne AI, Wirchnianski AS, et al. Virus entry. Lassa virus entry requires a trigger-induced receptor switch. *Science.* 2014; 344:1506–1510. [PubMed: 24970085]

no SSP													
- control		wt	mut 1	mut 2	mut 3	mut 4	mut 5	mut 6	mut 7	mut 8	mut 9	mut 10	
		1	2	3	4	5	6	7	8	9	10	11	12
A	103091	1.14e 7	125915	159998	1.97e 6	385103	134015	137826	123516	1.30e 7	159887	194677	
B	94635	1.19e 7	125700	101773	2.04e 6	395058	83192	141647	144701	1.21e 7	215790	138235	
C	111676	1.18e 7	124831	132428	1.39e 6	208968	116455	132124	134421	1.10e 7	205349	153328	
D	109570	1.14e 7	222897	102430	1.69e 6	133523	142616	138907	158410	1.20e 7	189504	164127	
E	97605	1.12e 7	138323	100044	2.52e 6	169368	137398	143662	110580	1.13e 7	209058	189703	
F	90098	8.51e 6	136721	92916	1.87e 6	257223	95156	116019	121002	1.20e 7	309684	168715	
G	151363	1.18e 7	113362	124743	1.67e 6	382431	115296	103888	148108	1.08e 7	205508	134792	
H	98545	1.31e 7	132575	186031	2.74e 6	330082	140820	126147	118214	1.42e 7	170075	189177	
Mean	107,073	11,390,000	140,041	125,045	1,986,000	282,719	120,619	130,027	132,369	12,060,000	208,107	166,594	
SEM	6,827	459,978	12,162	12,162	159,045	36,957	7,847	4,927	5,923	397,714	16,097	8,275	

Figure 1. Representative results from cell-cell fusion assays of wild-type (wt) JUNV GPC and 10 SSP mutants (mut 1-10)

JUNV GPC was reconstituted by co-expression of SSP and the GP1-GP2 precursor (with CD4 signal peptide), and the assay background was determined by omitting SSP (no SSP, - control). Each column contains eight replicates. Mean values and standard errors of the mean (SEM) are indicated below.