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Biochemical Reconstitution of Hemorrhagic-Fever Arenavirus Envelope Glycoprotein-Mediated Membrane Fusion

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Abstract

The membrane-anchored proteins of enveloped viruses form labile spikes on the virion surface, primed to undergo largescale conformational changes culminating in virus-cell membrane fusion and viral entry. The prefusion form of these envelope glycoproteins thus represents an important molecular target for antiviral intervention. A critical roadblock to this endeavor has been our inability to produce the prefusion envelope glycoprotein trimer for biochemical and structural analysis. Through our studies of the GPC envelope glycoprotein of the hemorrhagic fever arenaviruses, we have shown that GPC is unique among class I viral fusion proteins in that the mature complex retains a stable signal peptide (SSP) in addition to the conventional receptor-binding and transmembrane fusion subunits. In this report we show that the recombinant GPC precursor can be produced as a discrete native-like trimer and that its proteolytic cleavage generates the mature glycoprotein. Proteoliposomes containing the cleaved GPC mediate pH-dependent membrane fusion, a characteristic feature of arenavirus entry. This reaction is inhibited by arenavirus-specific monoclonal antibodies and small-molecule fusion inhibitors. The in vitro reconstitution of GPC-mediated membrane-fusion activity offers unprecedented opportunities for biochemical and structural studies of arenavirus entry and its inhibition. To our knowledge, this report is the first to demonstrate functional reconstitution of membrane fusion by a viral envelope glycoprotein.


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Introduction

Entry of enveloped viruses into their host cells requires fusion of the viral and cellular membranes, a process that is mediated by the viral envelope glycoprotein. Class I viral fusion proteins, including those of influenza and human immunodeficiency virus type 1 (HIV-1), are synthesized as inactive precursor glycoproteins that assemble as trimers and are subsequently primed by proteolytic cleavage to generate the mature fusogenic spikes. The membrane-anchored spike is thought to exist in a kinetically trapped metastable state that can be triggered, by engagement with cell-surface receptor or exposure to acidic pH in the endosome, to undergo a series of structural transitions leading to a thermodynamically favored postfusion state and concomitant virus-cell membrane fusion [4]. Interventions strategies that prevent membrane fusion and virus entry thus provide a sound basis for vaccine and drug development. A detailed mechanistic understanding of viral membrane fusion and its inhibition has been hindered by the inherent instability of the prefusion envelope glycoprotein trimer. Solubilization from its membrane-anchored environment invariably causes disassembly and/or refolding to the postfusion conformation. X-ray crystallographic analyses of the most extensively characterized class I envelope glycoproteins— influenza virus hemagglutinin (HA), HIV-1 envelope glycoprotein (Env) and parainfluenza virus 5 F (PIV5 F) - are based on soluble ectodomain fragments. These studies necessarily exclude information regarding the important role of membrane anchorage in envelope glycoprotein assembly, maintenance of the prefusion state and activation of fusogenic conformational changes. The failure of current HIV-1 vaccines to elicit broadly neutralizing antibodies is largely attributed to our inability to produce the trimeric native Env immunogen in a prefusion conformation [5].

Arenaviruses are responsible for severe hemorrhagic fevers worldwide, and Junin (JUNV) and Lassa (LASV) viruses are recognized to pose significant threats to public health and biodefense [4-8]. Arenavirus entry into the host cell is mediated by the viral envelope glycoprotein GPC, a member of the class I viral fusion proteins. The GPC precursor trimerizes and is proteolytically cleaved by the cellular site-1-protease/subtilisinlike kexin isozyme-1 (S1P/SKI-1) [9-11] in the Golgi to generate the receptor-binding (G1) and transmembrane fusion (G2)
subunits. Upon engaging a cell-surface receptor – transferrin receptor 1 (TIR1) for JUNV [12] or alpha-dystroglycan for LASV [13] - the virion is endocytosed and GPC-mediated fusion is triggered by acidic pH in the maturing endosome [14]. The ensuing conformational changes are driven by formation of the stable postfusion trimer-of-hairpins in G2 [15–17]. Unlike other class I fusion proteins, the mature GPC retains its signal peptide as an essential subunit [18,19]. The unusually long (58 amino-acid residues) stable signal peptide (SSP) traverses the membrane twice [20] and binds the cytoplasmic domain of G2 via an intersubunit zinc finger [21,22]. Evidence suggests that SSP interacts with the ectodomain of G2 to maintain the prefusion GPC complex at neutral pH and facilitate its fusogenic response to acidic pH [23]. Importantly, small-molecule fusion inhibitors [24–26] target the pH-sensitive SSP-G2 interface to prevent fusion of the viral and endosomal membranes, and thereby viral entry [23].

Our previous studies showed that the JUNV GPC precursor purified from insect cells exists as a stable trimer and efficiently binds the TIR1 receptor and arenavirus-specific small-molecule fusion inhibitors [27]. The unusual structural integrity of the precursor likely reflects its unique tripartite organization, and suggested the feasibility of generating the mature GPC complex for biochemical analysis. To this end, we have produced the prefusion GPC trimer through in vitro proteolytic cleavage, and demonstrated that proteoliposomes containing this complex are able to mediate pH-dependent membrane fusion that is specifically inhibited by small-molecule fusion inhibitors. Biochemical reconstitution of the fusogenic activity of GPC provides a platform for understanding pH-induced membrane fusion and its inhibition.

Materials and Methods

Monoclonal Antibodies (MAbs) and Small-molecule Fusion Inhibitors

MAbs directed to JUNV G1 (BF11, BF09, BE08 and AG02) and N (BG12) were obtained from the CDC [28] through the NIAID Biodefense and Emerging Infectious Diseases Research Resources Repository. MAb F100G5 recognizes the fusion peptide of JUNV G2 [16] and was provided by the Public Health Agency of Canada. Plasma-derived human soluble furin (sFurin) binds the TfR1 receptor and arenavirus-specific small-molecule fusion inhibitors [27]. A plasmid for baculovirus expression of the recombinant soluble furin C-terminal hexa-histidine tag, was kindly provided by Dr. Alex Strongin (Burnham Institute for Medical Research) and the open-reading frame, including the baculovirus gp67 signal peptide and C-terminal hexa-histidine tag, was transferred to pFastBac1 (Life Technologies) for generating baculovirus. The soluble furin enzyme was purified from the supernatant of infected Spodoptera frugiperda (Sf9) cells using Ni²⁺-affinity chromatography as described [30]. The purified sFurin (~33,000 units/ml [31]) contained no detectable nonspecific protease activity. Cleavage of GPCfur in solution (0.6 mg/ml) was performed using a 3:1 molar ratio of rGPCfur:sFurin at 20°C for 4 hr in buffer containing 50 mM Tris (pH 7.2), 150 mM NaCl, 5 mM MgCl2, 1 mM CaCl2, 100 µM ZnSO4 and 0.05% DDM. Zn²⁺ was present to stabilize the intersubunit zinc-binding domain in GPC and did not appear to reduce the extent of furin cleavage [32]. In preliminary studies, soluble human furin was purchased from New England Biolabs.

Soluble Human Furin (sFurin)

A plasmid for baculovirus expression of the recombinant soluble prodomain of human furin [30] was kindly provided by Dr. Alex Strongin (Burnham Institute for Medical Research) and the open-reading frame, including the baculovirus gp67 signal peptide and C-terminal hexa-histidine tag, was transferred to pFastBac1 (Life Technologies) for generating baculovirus. The soluble furin enzyme was purified from the supernatant of infected Spodoptera frugiperda (Sf9) cells using Ni²⁺-affinity chromatography as described [30]. The purified sFurin (~33,000 units/ml [31]) contained no detectable nonspecific protease activity. Cleavage of GPCfur in solution (0.6 mg/ml) was performed using a 3:1 molar ratio of rGPCfur:sFurin at 20°C for 4 hr in buffer containing 50 mM Tris (pH 7.2), 150 mM NaCl, 5 mM MgCl2, 1 mM CaCl2, 100 µM ZnSO4 and 0.05% DDM. Zn²⁺ was present to stabilize the intersubunit zinc-binding domain in GPC and did not appear to reduce the extent of furin cleavage [32]. In preliminary studies, soluble human furin was purchased from New England Biolabs.

Surface Plasmon Resonance (SPR) Studies

SPR analyses of binding to rGPCCD and rGPCfur were performed using a Bicore T100 instrument (GE Healthcare) as previously described [27]. The proteins are immobilized to the biosensor chip via covalently coupled M2 anti-FLAG MAb. In most experiments, rGPCfur was reconstituted into a lipid bilayer on the chip as previously described [27]. Briefly, a Bicore L1 chip bearing immobilized rGPCfur in DDM-containing running buffer was rinsed with 20 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (Sigma) and the detergent-containing buffer was replaced by liposomes containing a 3:1 mixture of dimyristoylphosphatidylcholine and phosphatidylcholine (DMPC:PC; Avanti Polar Lipids) in running buffer without DDM (non-detergent running buffer). By this procedure, protein-bound detergent is displaced by a lipid bilayer that forms on the hydrophobic chip [33]. At the completion of the SPR experiment, the L1 chip was regenerated with isopropanol/40 mM NaOH (2:3 v/v) to remove lipids and associated proteins, and the M2 MAb was reconditioned using glycine-HCl, pH 3.0.

For proteolytic cleavage of rGPCfur, the L1 chip was incubated with 20,000 units/ml sFurin for 1 hr at 25°C in non-detergent running buffer (containing 1 µM Zn²⁺). The bolus of sFurin was then washed off to achieve a stable baseline. In all binding studies, multiple concentrations of MABs (0.1–2.5 µM), sTIR (0.5–5.0 µM) and small-molecule fusion inhibitors (5–250 µM) were used to
Table 1. Summary of rGPCfur interaction parameters from SPR studies.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$k_a$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_d$ (s$^{-1}$)</th>
<th>$K_d$ (nM)$^a$</th>
<th>rGPCCD $K_d$ (nM)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAb BF11</td>
<td>4.2 × 10$^3$</td>
<td>0.007</td>
<td>16.66 (1.2)</td>
<td>10.1</td>
</tr>
<tr>
<td>MAb BF09</td>
<td>1.8 × 10$^3$</td>
<td>0.062</td>
<td>344.44 (14.5)</td>
<td>219</td>
</tr>
<tr>
<td>MAB B08</td>
<td>1.2 × 10$^3$</td>
<td>0.081</td>
<td>67.25 (20.3)</td>
<td>125</td>
</tr>
<tr>
<td>MAB AG02</td>
<td>0.69 × 10$^3$</td>
<td>0.085</td>
<td>123.01 (93.2)</td>
<td>583</td>
</tr>
<tr>
<td>ST-761</td>
<td>0.29 × 10$^3$</td>
<td>0.078</td>
<td>2.68N (120.5)</td>
<td>1,296</td>
</tr>
</tbody>
</table>

$^a$ $K_d$ values are the means derived by fitting two or three independent biosensor datasets. The number in parenthesis is the difference between the highest and lowest $K_d$ values from individual datasets.

$^b$ $K_d$ values for rGPCCD binding are taken from Thomas et al. [27].

doi:10.1371/journal.pone.0051114.t001

Table 2. Summary of inhibitor-rGPCfur interaction parameters from SPR studies.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$k_a$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_d$ (s$^{-1}$)</th>
<th>$K_d$ (μM)$^a$</th>
<th>rGPCCD $K_d$ (μM)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST-294</td>
<td>7.9 × 10$^3$</td>
<td>0.015</td>
<td>1.89 (0.3)</td>
<td>1.08</td>
</tr>
<tr>
<td>17C8</td>
<td>1.8 × 10$^3$</td>
<td>0.031</td>
<td>17.22 (2.3)</td>
<td>9.50</td>
</tr>
<tr>
<td>ST-375</td>
<td>2.1 × 10$^3$</td>
<td>0.02</td>
<td>9.52 (0.8)</td>
<td>3.45</td>
</tr>
<tr>
<td>ST-193</td>
<td>1.2 × 10$^3$</td>
<td>0.02</td>
<td>16.66 (1.8)</td>
<td>10.52</td>
</tr>
<tr>
<td>ST-761</td>
<td>1.8 × 10$^3$</td>
<td>0.02</td>
<td>11.11 (0.9)</td>
<td>8.67</td>
</tr>
</tbody>
</table>

$^a$ $K_d$ values are the means derived by fitting two or three independent biosensor datasets. The number in parenthesis is the difference between the highest and lowest $K_d$ values from individual datasets.

$^b$ $K_d$ values for rGPCCD binding are taken from Thomas et al. [27].

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50 μM Zn$^{2+}$ (proteoliposomes) or 1 μM of the soluble zinc-sensitive fluorophore FluoZin-1 (Life Technologies) and no Zn$^{2+}$ (target liposomes). Free Zn$^{2+}$ or unincorporated FluoZin-1 was removed by dialysis or separation on a small Sepharose CL4B (Sigma) column.

Protocololiposomes bearing rGPCfur were prepared by mixing rGPCfur (in buffer containing 50 μM Zn$^{2+}$ and 0.05% DDM) with the appropriate LUVs at a protein/lipid molar ratio of ~1:25. After 30 min of incubation at room temperature, the proteoliposomes were desalted by size-exclusion chromatography using Sepharose CL4B, and extruded through a polycarbonate membrane (100-nm pore). Protein incorporation was generally >95%. Protocololiposomal rGPCfur (1.5–3.0 μM protein) was cleaved prior to use in fusion assays by incubation with 0.5 μM sFurin at 20°C for 45 min in buffer containing 10 mM Tris (pH 7.2), 100 mM NaCl.

Liposome Fusion Assays

Lipid mixing: Target liposomes containing rhodamine-PE were added to furin-treated proteoliposomes (~1.5–3.0 μM rGPCfur in 50 μM lipid) at a ratio of 1:10, and lipid mixing was assessed intermittently by fluorescence (excitation 508 nm; emission 600 nm) using a Perkin Elmer LS55 spectrometer. pH was adjusted to 5.0 by the addition of 1 M sodium acetate, and complete dequenching of the fluorophore was determined upon solubilization in 2% Triton X-100. JUNV-specific MAbs and small-molecule fusion inhibitors were pre-incubated with proteoliposomes at a concentration of 5 and 15 μM, respectively.

Content mixing: Target liposomes containing 1 μM FluoZin-1 were added to Zn$^{2+}$-containing furin-treated proteoliposomes (~1.5–3.0 μM protein in 50 μM lipid) at a 1:10 ratio, and content mixing was assessed as described above (excitation 570 nm; emission 485 nm). The fluorescence signal for complete mixing was determined by solubilizing the liposomes in buffer adjusted to 50 μM Zn$^{2+}$.

Expression of GPC in Mammalian Cells

GPC was expressed in Vero cells, with SSP and the G1G2 precursor in trans, using pcDNA-based plasmids and T7 polymerase provided by the recombinant vaccinia virus rTF7-3 [34]. pH-induced cell-cell fusion activity was characterized as previously described [19].

Results

Expression of Recombinant GPC

In previous studies, we produced and characterized the recombinant cleavage-defective JUNV GPC precursor protein [27]. rGPCCD (previously referred to as icd-GPC) was purified from membranes of baculovirus-infected insect cells and found to retain its trimeric structure. We showed that the rGPCCD trimer binds the JUNV TIR1 receptor [12], JUNV-neutralizing MAbs [28], and small-molecule arenavirus-specific fusion inhibitors [27]. Because the rGPCCD mutant is unable to mediate membrane fusion [19], we expressed wild-type rGPC. As previously described [27], our expression strategy takes advantage of the unusual ability of SSP to associate in the native tripartite complex [18,35]. Thus, SSP and the wild-type G1G2 precursor were co-expressed using a pFastBac-Dual dual-promoter baculovirus vector. The G1G2 open-reading frame contains a conventional signal peptide and a FLAG tag is appended at the C-terminus to facilitate purification from solubilized insect-cell membranes.
Despite the existence of an insect S1P/SKI-1 orthologue [36], the wild-type rGPC was refractory to cleavage in insect cells. Serendipitously, several laboratories had previously reported functional GPC mutants in which the S1P/SKI-1 cleavage site (RRSLK[A]) was replaced by that of furin [37,38], a cellular protease used in the maturation of other viral envelope glycoproteins. We therefore introduced a furin recognition site (RRKRKR[A]) into the JUNV G1G2 precursor (GPC\textsuperscript{CD}) and confirmed that the mutant protein was fusogenic when expressed in mammalian cells (Fig. 1). In insect cells, however, rGPC\textsuperscript{fur} remained refractory to cleavage (Fig. 2A), perhaps reflecting the differing specificity of the insect enzyme [39]. Based on these results, we purified the rGPC\textsuperscript{fur} precursor to investigate its susceptibility to in vitro cleavage by human furin.

**Purification of the rGPC\textsuperscript{fur} Precursor**

The rGPC\textsuperscript{fur} precursor was solubilized from insect-cell membranes using 1.5% DDM and purified by affinity chromatography using the C-terminal FLAG tag [27]. SDS-PAGE analysis of the purified protein confirmed SSP association in the rGPC\textsuperscript{fur} complex as well as the lack of proteolytic cleavage in insect cells (Fig. 2A). Similarly to rGPC\textsuperscript{CD} [27], the rGPC\textsuperscript{fur} precursor formed a relatively homogenous oligomer on size-exclusion chromatography, with an apparent molecular size consistent with a trimer (∼220 kDa) (Fig. 2A). The yield of purified rGPC\textsuperscript{fur} by this procedure was ∼1 mg per liter of High-Five\textsuperscript{TM} cell culture.

The furin-site mutation in rGPC\textsuperscript{fur} did not appear to affect recognition of the trimer by virus-neutralizing MAbs or the TIR1 receptor in SPR studies. Briefly, rGPC\textsuperscript{fur} was captured onto a hydrophobic Biacore L1 sensor chip using immobilized FLAG MAbs, and subsequently reconstituted into a lipid membrane [27,33]. Using a panel of four well-characterized virus-neutralizing MAbs directed to JUNV G1 [28], we found that the rGPC\textsuperscript{fur} precursor was antigenically indistinguishable from rGPC\textsuperscript{CD} and bound to soluble TIR1 with comparable affinity [27] (Fig. 3A and Table 1). Together, these results suggest that the purified rGPC\textsuperscript{fur} trimer retains important aspects of the native structure.

**Small-molecule Arenavirus-specific Fusion Inhibitors**

Six chemically distinct classes of small-molecule arenavirus-specific entry inhibitors have been identified through independent high-throughput screening exercises at SIGA Technologies (SIGA) [24–26,29]. These classes differ in their specificities for NW arenaviruses (JUNV and/or OW viruses (LASV) (see Materials and Methods) yet appear to share a common binding site on GPC [26,27]. The compounds are thought to act through the SSP-G2 interface to stabilize prefusion GPC against activation at endosomal pH, thereby inhibiting membrane fusion [26,27].

**Proteolytic Cleavage of Purified rGPC\textsuperscript{fur}**

For proteolytic cleavage of rGPC\textsuperscript{fur}, we expressed recombinant soluble human furin (sFurin) in insect cells and purified the enzyme by Ni\textsuperscript{++}-affinity chromatography [30]. sFurin digestion of the purified rGPC\textsuperscript{fur} precursor in nonionic detergent at 20°C resulted in only partial (∼40%) cleavage (Fig. 2B). We were unable to identify conditions to drive the digestion to completion and speculate that the engineered furin recognition site may not be optimally presented for cleavage [40]. Nonetheless, MALDI mass spectrometric analysis of the G1 and G2 subunits generated by furin digestion at 20°C confirmed cleavage at the predicted site (RRKRKR[A]) and the production of the wild-type G2 subunit (Fig. 2B).

**pH-induced Conformational Changes in Furin-cleaved rGPC\textsuperscript{fur}**

We reasoned that cleaved rGPC\textsuperscript{fur} should differ from the uncleaved protein in its response to acidic pH. Fusion activation in class I fusion proteins typically results in the dissociation of noncovalently associated receptor-binding and transmembrane subunits [41]. Indeed, mammalian cells expressing GPC have been shown to shed G1 on exposure to acidic pH [26]. We therefore examined G1 shedding from cleaved rGPC\textsuperscript{fur} as an indication of pH-induced fusion activation. In these experiments, lipid-reconstituted rGPC\textsuperscript{fur} was incubated on the biosensor chip with a bolus of sFurin (Fig. 4, middle trace, left). On washing, the SPR response units (RU) returned to the pre-digestion baseline, indicating that no protein had been lost from the chip. Following transient exposure to pH 5.0, however, the baseline signal was reduced by ∼800 RU, consistent with pH-induced shedding of G1 (Fig. 4, middle trace, center). By contrast, identical treatment of the rGPC\textsuperscript{CD} precursor resulted in no loss of protein (Fig. 4, top trace). Based on the magnitude of the pH-induced loss from furin-treated rGPC\textsuperscript{fur}, we estimate that cleavage was ∼70% complete under these conditions. Susceptibility of rGPC\textsuperscript{fur} to furin cleavage may be enhanced by membrane anchorage.

To confirm that the pH-induced reduction in baseline RU reflects a loss of G1, we probed the same chip surface with a G1-specific MAb [28]. Binding of MAb BF11 to cleaved rGPC\textsuperscript{fur} was markedly reduced after exposure to pH 5.0 relative to the rGPC\textsuperscript{CD} control (Fig. 4, right), indicative of pH-induced G1 shedding. Furthermore, the loss of G1 from cleaved rGPC\textsuperscript{fur} was found to be inhibited by ST-294 (Fig. 4, lower trace), in agreement with results obtained using native GPC on the surface of mammalian cells.
mammalian cells [26]. We conclude rGPC fur can be functionally matured in vitro and that the prefusion trimer is able to respond to acidic pH in a manner consistent with on-path conformational changes seen in the native GPC complex.

Proteolytic cleavage of the rGPC fur precursor was found to have only a minimal effect on the extent and overall affinity of ST-294 binding ($K_d = 2 \mu M$ vs. $4 \mu M$) (Fig. 5A). However, exposure to acidic pH markedly reduced the extent of subsequent ST-294 binding (Fig. 5A). The $K_d$ of the residual binding was unaffected and is likely attributable to uncleaved rGPC fur remaining on the SPR chip. This notion is supported by observations that the cleavage-defective rGPCCD can be repeatedly cycled at low pH without loss of ST-294 binding activity (Fig. 5B). These latter observations suggest that the trimeric precursor does not undergo significant irreversible change in response to acidic pH. The covalent linkage of the G1 and G2 subunits in the precursor likely constrains pH-induced conformational excursions from the initial state.

Furin-cleaved rGPC fur Mediates pH-dependent Membrane Fusion

In order to determine whether cleaved rGPC fur is able to mediate membrane fusion, we incorporated the recombinant protein into LUVs and examined the ability of the proteoliposomes to fuse with target LUVs in a pH-dependent manner. The target liposomes were doped with a self-quenching concentration of rhodamine-PE, and fusion with the proteoliposome results in dilution of the rhodamine within the merged lipids and dequenching of the fluorophore [42]. At neutral pH, the liposome mixture was stable over the course of 15 min, regardless of whether proteoliposomes had previously been incubated with sFurin (Fig. 6). Proteoliposomes bearing uncleaved rGPC fur were likewise stable when exposed to acidic pH. By contrast, acidification of sFurin-cleaved rGPC fur resulted in a rapid increase in fluorescence over a period of 3 min (Fig. 6). After 5 min, rhodamine-PE fluorescence approached $30\%$ of the maximum obtained on complete dequenching in nonionic detergent. Importantly, pH-induced fusion was inhibited by the prior addition of ST-294, but not by the LASV-specific compound ST-161 (Fig. 6). Thus, reconstituted rGPC fur is proteolytically primed and can be activated by acidic pH to mediate fusion.

Rhodamine dequenching upon pH-induced activation demonstrates mixing of the liposomal lipids. At a minimum, this result indicates that cleaved rGPC fur is capable of inducing hemifusion, an intermediate state in fusion where only the outer leaflets of the two membranes merge. Previous studies have shown that engineered envelope proteins linked to the cell membrane only via a lipid glycoposphatidylinositol (GPI) anchor, or in which the transmembrane domain is truncated, arrest at this hemifusion intermediate [43–46]. To determine whether rGPC fur proteoliposomes can proceed to complete fusion, with merger of both membrane leaflets and opening of a fusion pore, we monitored pH-induced mixing of liposome contents. In these studies, target liposomes containing the Zn$^{++}$-sensitive fluorophore FluoZin-1 were mixed with rGPC fur proteoliposomes containing 50 mM Zn$^{++}$. Complete fusion with content mixing results in an increase in FluoZin-1 fluorescence. Without sFurin-cleavage, only baseline fluorescence was detected over a 20 min period at acidic pH (Fig. 7A). Similarly, furin-treated proteoliposomes were stable at neutral pH. By contrast, acidification of the cleaved rGPC fur proteoliposomes led to a rapid increase in fluorescence that was preceded by a lag period of ~2 min (Fig. 7A). By 10 min, content mixing was ~40% of the maximum. The lag period in content
mixing may indicate a kinetic barrier for resolution of the hemifusion intermediate [47]. Our results indicate that reconstituted rGPC\textsuperscript{fur} mediates complete membrane fusion with content mixing. In addition, we found that rGPC\textsuperscript{fur}-mediated fusion was markedly inhibited by ST-294, but not by the LASV-specific inhibitor ST-161 (Fig. 7A). Taken together, our studies demon-

**Figure 3.** SPR studies of interactions with lipid-reconstituted rGPC\textsuperscript{fur}. The rGPC\textsuperscript{fur} precursor was immobilized onto a Biacore L1 chip and reconstituted in a lipid bilayer as described in Material and Methods. Two or three concentration-dependent data sets were analyzed and sensorgram figures were generated using a five-point smoothing procedure and ORIGIN graphing software. Labels to the right are drawn to coincide with the maximum RUs achieved by the respective ligand. 

A. Binding of G1-directed MAbs (BF11, BF09, BE08, and AG02; 0.5 μM), sTfR (1.5 μM), and a nucleoprotein-directed MAb (aN; BG12).  
B. Binding of SIGA (ST-294, ST-375, ST-193, and ST-761; 150 μM) and TSRI (17C8; 100 μM) small-molecule fusion inhibitors. ST-161 and TSRI 8C1 are specific to the OW LASV and do not inhibit the NW arenavirus JUNV.

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strate that recombinant rGPC\textsuperscript{fur} faithfully reproduces the pH-induced membrane-fusion activity of native GPC.

Distinct Modes of Antibody-mediated Neutralization
Reconstitution of the membrane-fusion reaction provides a platform to investigate the mechanisms of antibody-mediated virus neutralization. For example, MAb F100G5 has been shown to recognize the fusion peptide of JUNV G2 [16] and, as this
region is sequestered in prefusion GPC, does not bind virions and is therefore unable to inhibit entry following endocytic uptake of the particle. Liposomal fusion by rGPCfur proteoliposomes was, however, inhibited when F100G5 was present in the low-pH buffer (Fig. 7B). This result recapitulates previous observations from cell-cell fusion assays [16]. By contrast, the molecular basis for virus neutralization by the G1-directed MAb BF11 [28] is unknown. Interestingly, MAb BF11 did not inhibit proteoliposomal fusion (Fig. 7B). We conclude that this MAb may block TIR binding or virion internalization. This in vitro model of membrane fusion offers unprecedented access for biochemical studies of GPC function and its inhibition.

Discussion

Owing to the inherently labile nature of the prefusion envelope glycoprotein, solubilization of class I fusion proteins from the membrane typically destabilizes the trimeric structure sufficiently to cause disassembly and/or refolding to the stable postfusion state [48–50]. Indeed, this intrinsic instability has greatly hindered biochemical and biophysical studies on the viral fusion machinery, and frustrated efforts to design and develop native envelope glycoproteins as effective vaccine immunogens [51]. We hypothesize that the unique stability of GPC results from interaction among the nine transmembrane domains in the trimeric complex (vs. three in conventional class I fusion proteins), enforced by intersubunit zinc-finger structures. The fortuitous resilience of GPC offers the possibility for investigating the molecular basis of membrane fusion mediated by a membrane-anchored envelope glycoprotein in vitro.

Our current mechanistic understanding of viral membrane fusion was developed in part from now-classical studies of influenza virus HA (reviewed in reference [52]). High-resolution crystallographic structures of the soluble HA ectodomain in its precursor [53], prefusion [54] and postfusion [55] states serve as reference points for the current model [53,56]. Recent studies of PIV5 F [49,50] have reinforced central tenets of the model. These crystallographic structures, as well as those of the stable postfusion core of other class I fusion proteins, have all been determined using soluble forms of the glycoprotein ectodomain and are therefore silent with respect to the transmembrane and cytoplasmic domains. To our knowledge, this report is the first to demonstrate full reconstitution of membrane-fusion activity by a complete virus envelope glycoprotein.

Membrane anchorage is essential for membrane-fusion activity. Envelope glycoprotein ectodomains anchored in the membrane by a GPI lipid or truncated transmembrane domains are unable to drive fusion to completion, arresting at the hemifusion state [43–

![Figure 6](https://example.com/figure6.png)

**Figure 6. pH-induced membrane fusion by rGPCfur proteoliposomes: lipid mixing.** rGPCfur was incorporated into POPG-POPC liposomes and mixed with target POPG-POPC liposomes doped with 1% Rhodamine-PE. In most cases, the rGPCfur proteoliposomes were first treated with sFurin (as indicated by + in the first position of the labels, at right). Exposure to acidic pH at the start of the experiment (time = 0) is indicated by + in the second position of the labels. 15 μM of ST-294 or ST-161 was present prior to and during exposure to acidic pH, where indicated. Lipid mixing and the resulting dequenching of the rhodamine fluorophore were measured at 600 nm (excitation 508 nm). Complete dequenching (100%) was determined by subsequent solubilization in Triton X-100 nonionic detergent. doi:10.1371/journal.pone.0051114.g006

![Figure 7](https://example.com/figure7.png)

**Figure 7. pH-induced membrane fusion by rGPCfur proteoliposomes: content mixing.** A. rGPCfur was incorporated into POPG-POPC-CHS liposomes containing 50 μM Zn⁺⁺ and these proteoliposomes were mixed with target POPG-POPC-CHS liposomes containing 1 μM FluorZin-1. The experimental treatments, labels and colors are as described in the legend of Fig. 6. Content mixing was determined by Zn⁺⁺-induced FluorZin-1 fluorescence measured at 485 nm (excitation 370 nm). Complete mixing was determined by solubilization with Triton X-100 in buffer containing 50 μM Zn⁺⁺. B. pH-induced content mixing was determined in the continued presence of MAbs F100G5, BF11 or an irrelevant IgG (5 μM, 25 μM and 25 μM, respectively). In this experiment, content mixing in the absence of MAb (pH 5.0) was ~50%. doi:10.1371/journal.pone.0051114.g007
46]. Anchorage may be required to transduce forces generated upon protein refolding towards complete fusion of the two bilayers. Lateral contacts within the membrane between several envelope glycoprotein spikes may also be important for formation of the stable fusion pore. Membrane interactions of the membrane-proximal ectodomain of HIV-1 Env are involved in the fusion process and contribute to important determinants for broadly neutralizing antibodies [37,38]. Furthermore, envelope glycoprotein membrane-spanning domains also serve as conduits to transmit information between the internal and external domains for virion assembly and virus entry [59,60]. In GPC, amino-acid changes in the membrane-proximal and transmembrane domains have been shown to affect GPC biosynthesis and intracellular trafficking of the complex [29,35], its sensitivity to acidic pH and small-molecule fusion inhibitors [26,61,62] and, indeed, viral pathogenesis [63,64]. Further analysis of GPC structure and function using this biochemically defined in vitro system will advance our understanding of the conformational transitions that promote viral membrane fusion. This knowledge will directly facilitate efforts to design novel entry inhibitors for the treatment of arenavirus hemorrhagic fevers.

References


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Author Contributions

Conceived and designed the experiments: CJT SS HEC JY. Analyzed the data: CJT SRS JHN. Wrote the paper: CJT SRS JHN.

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