pH-Induced Activation of Arenavirus Membrane Fusion is antagonized by Small-Molecule Inhibitors

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The arenavirus envelope glycoprotein (GPC) mediates viral entry through pH-induced membrane fusion in the endosome. This crucial process in the viral life cycle can be specifically inhibited in the New World arenaviruses by the small-molecule compound ST-294. Here, we show that ST-294 interferes with GPC-mediated membrane fusion by targeting the interaction of the G2 fusion subunit with the stable signal peptide (SSP). We demonstrate that amino acid substitutions at lysine-33 of the Junin virus SSP confer resistance to ST-294 and engender de novo sensitivity of the Old World Lassa fever virus. These compounds, as well as a broadly active inhibitor, ST-193, likely share a molecular target at the SSP-G2 interface. We also show that both ST-294 and ST-193 inhibit pH-induced dissociation of the G1 receptor-binding subunit from GPC, a process concomitant with fusion activation. Interestingly, the inhibitory activity of these molecules can in some cases be overcome by further lowering the pH used for activation. Our results suggest that these small molecules act to stabilize the prefusion GPC complex against acidic pH. The pH-sensitive interaction between SSP and G2 in GPC represents a robust molecular target for the development of antiviral compounds for the treatment of arenavirus hemorrhagic fevers.

Arenaviridae comprise a large family of enveloped, negative-strand RNA viruses whose species have coevolved and diversified with their respective rodent hosts (10, 45). Many arenavirus species are nonpathogenic to humans, but several can be transmitted to cause severe acute hemorrhagic fevers. In the so-called Old World group of arenaviruses, Lassa fever virus (LASV) is responsible for up to 300,000 infections annually in western Africa (36). Although arenavirus disease is less prevalent in the Americas (40), four distinct species of New World arenaviruses are recognized to cause fatal hemorrhagic fevers: the Junin (JUNV), Machupo, Guanarito, and Sabia viruses. New species of disease-associated arenaviruses continue to be identified (8, 13). Without effective treatment or immunization, the hemorrhagic fever arenaviruses remain an urgent public health and biodefense concern.

Arenavirus entry into its host cell is promoted by the virus envelope glycoprotein (GPC) and provides a potential target for therapeutic intervention. The G1 subunit of the GPC complex initiates infection by binding to a cell surface receptor. The pathogenic New World arenaviruses utilize transferrin receptor 1 for entry (41, 42), whereas nonpathogenic New World viruses and Old World viruses bind α-dystroglycan or an unknown receptor (6, 20, 49). Upon receptor binding, the virion is endocytosed (4), and fusion of the viral and cellular membranes is subsequently actuated to drive membrane fusion (19, 24, 53).

GPC is unusual among class I envelope glycoproteins in that the mature complex retains its cleaved signal peptide as an essential subunit (5, 18, 58) (Fig. 1). This stable signal peptide (SSP) contains 58 amino acids and spans the membrane twice, with both N and C termini in the cytosol (1). SSP is likely retained in the mature GPC complex by formation of an inter-subunit zinc-finger structure with the cytoplasmic domain of G2 (55). Interestingly, amino acid substitutions at a lysine in the short ectodomain loop of SSP (K33) have been shown to modulate the pH at which membrane fusion is activated (57). Because the charge at K33 is itself not altered by acidic pH, we reasoned that the lysine side chain might respond to a titratable pocket in the ectodomain of G2. Thus, SSP and G2 may interact to mediate the pH-induced transition from the metastable prefusion GPC complex to the activated fusion-competent form, whereupon the structural reorganization in G2 is actuated to drive membrane fusion. In this report, we demonstrate that the recently reported small-molecule inhibitor of arenavirus entry, ST-294 (3), prevents membrane fusion by targeting the SSP-G2 interface and stabilizing the prefusion state of GPC against acidic pH.

MATERIALS AND METHODS

Molecular and chemical reagents and monoclonal antibodies (MAbs). GPC from the pathogenic JUNV strain MC2 (25) was expressed in Vero cells by transfection using a pcDNA3.1-based plasmid carrying the complete GPC open reading frame or by cotransfection using plasmids encoding 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) (57). These two components associate in trans and reconstitute the native GPC complex (18). The GPC open reading frame of LASV-Josiah (33) and that of the vesicular stomatitis virus (VSV) G glycoprotein (kindly provided by John K. Rose, Yale University) were also expressed in pcDNA3.1 plasmids. Transient
expression utilized a recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3) (23). Mutations in GPC were introduced by QuikChange mutagenesis (Stratagene), and all constructs were verified by DNA sequencing.

The small-molecule fusion inhibitors ST-294, ST-336 (3), ST-193 (33), and ST-161 (unpublished data) were dissolved in dimethyl sulfoxide. Chemical structures are shown in Fig. 2. The murine MAb BF11 (46), directed to G1, was contributed by Tom Kiasek and Tony Sanchez (Special Pathogens Branch, CDC, Atlanta, GA) and obtained through the NIH Biodefense and Emerging Infections Research Resources Repository.

Cell-cell fusion assay. A vaccinia virus-based β-galactosidase fusion reporter assay (39) was used to characterize the ability of GPC to mediate pH-dependent cell-cell fusion (57, 58). Briefly, Vero cells infected with vTF-7 and expressing GPC were maintained in 10 μM of 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) (39) and seeded into 96-well microculture dishes (40,000 cells/well) in the presence of 100 μg rifampin/ml to minimize vaccinia virus assembly and cytopathic effect (28). 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 (~25 × g) to ensure cell-cell contact. These cocultures were continued for 5 h in medium containing araC and rifampin. Membrane fusion was initiated by incubation in drug-containing medium that had been adjusted to low pH by using HEPES and PIPES [piperazine-N,N′-bis(2-ethanesulfonic acid)] buffers. After 10 to 30 min at 37°C, the cells were restored to the neutral drug-containing medium and cultured for 5 h to allow for expression of the β-galactosidase fusion reporter gene, which was then quantitated using the chemiluminescent GalactoLite Plus substrate and a Tropix TR717 microplate luminometer. The wide dynamic range of the assay permits robust measurement of ≤1% of wild-type activity. This low level is routinely 10-fold greater than that measured using nonfusogenic GPC molecules (e.g., CD4spGPC in the absence of SSP or a cleavage-defective mutant) (58) or upon treatment of cells expressing wild-type GPC at neutral pH.

For studies of fusion inhibition, cells were incubated with serial dilutions of the compounds developed by SIGA Technologies throughout the initial 3.5-h period of coculture. In these studies, 4 to 6 replicate wells were used for each determination, and control cocultures were treated with the dimethyl sulfoxide solvent (which did not affect the assay at final concentrations of ≤0.25%). In preliminary studies, inhibitor was added prior to, during, or after the pH pulse to show that pretreatment was both necessary and sufficient for inhibition (Fig. 3B).

G1 shedding. pH-induced shedding of the G1 subunit from metabolically labeled cells was detected by immunoprecipitation (57, 58) from the low-pH culture supernatant. Vero cells expressing GPC as described above were suspended and pulsed at the indicated pH for 10 min at 37°C. Cells were also treated in medium adjusted to pH 2.5 in an effort to normalize G1 shedding relative to the total amount of G1 eluted under highly stringent conditions. In studies of fusion inhibition, the cell suspension was pretreated for 30 min with the SIGA compounds. The low-pH medium was recovered and neutralized prior to centrifugation to remove cell debris and shed membranes. G1 was recovered from this supernatant by immunoprecipitation using MAb BF11 (46) and protein A Sepharose (Sigma) in buffers containing 1% Triton X-100. The glycoprotein was
The percentage of fusion relative to that of the control in the absence of ST-294, washed, and then pulsed for 20 min with medium at pH 5.0. (A) Vero cells expressing JUNV GPC (filled circles) or VSV G protein (open squares) were incubated with the indicated concentrations of ST-294, washed, and then pulsed for 20 min with medium at pH 5.0. The percentage of fusion relative to that of the control in the absence of ST-294 is indicated. Error bars representing ±1 standard deviation are calculated for all points and in some cases may not be visible on the scale of the graph. (B) Cells expressing JUNV GPC were incubated with ST-294 prior to (pre), during (pulse) or after (post) the pH pulse (the numbers 10 or 50 represent the μM concentration of ST-294). Cell-cell fusion is reported as relative light units (RLUs) in the assay. Pretreatment with ST-294 was necessary and sufficient for inhibition.

RESULTS

ST-294 targets the prefusion form of GPC to inhibit cell-cell fusion. ST-294 was developed by SIGA Technologies through high-throughput screening for inhibition of cell culture infection with the nonpathogenic New World arenavirus Tacaribe (TCRV) (3). This class of compounds inhibits in vitro infection by the related New World arenaviruses JUNV, Machupo virus, and Guanarito virus at submicromolar concentrations yet is minimally active against the Old World LASV. Studies demonstrated that these inhibitors bind to intact virions and act prior to infection. TCRV isolates selected for in vitro resistance to ST-336 had several mutations in G2 and none in G1 (3). Together, these data suggested that ST-294 acts upon the virion GPC complex prior to the initial stages of cell infection.

To determine whether ST-294 directly inhibits the membrane fusion activity of GPC, we investigated its ability to inhibit syncytium formation among cells expressing a molecularly cloned JUNV GPC (53). In this assay, Vero cells are briefly pulsed with medium adjusted to pH 5.0 and cell-cell fusion is assessed using a β-galactosidase reporter gene (39). As illustrated in the left panel of Fig. 3, we found that the membrane fusion activity of JUNV GPC was sensitive to ST-294, whereas pH-dependent cell-cell fusion by the unrelated G protein of VSV was unaffected. In these studies, cell-cell fusion was reduced to 50% at 4 μM ST-294, whereas previous work had determined a 50% inhibitory concentration (IC_{50}) of 0.3 μM for JUNV plaque reduction (3). The numerical difference in the inhibitory concentrations likely reflects differences inherent in the two assays. Thus, ST-294 prevents arenavirus entry by specifically inhibiting the membrane fusion activity of GPC.

Studies had previously demonstrated that prior exposure of virions to ST-336 was sufficient to block subsequent infection of untreated cells (3). In order to determine the timing requirements for inhibition of cell-cell fusion, GPC-expressing cells were treated with 10 μM ST-294 prior to the pH pulse, during the pH pulse, or upon return to neutrality (Fig. 3, right panel). Inhibition was observed only when ST-294 was included prior to pulsing with pH 5.0. Pretreatment with 10 μM ST-294 reduced cell-cell fusion by 75%, and no additional inhibition was seen if treatment was continued throughout. Further inhibition could be detected upon pretreatment with 50 μM ST-294. These findings support the conclusion that these compounds act prior to infection (3) and demonstrate clearly that ST-294 targets a prefusion form of the GPC complex.

Mutations in G2 and SSP confer resistance to ST-294. ST-336-resistant TCRV isolates encode mutations in the membrane-proximal ectodomain and transmembrane domain of G2 (3). To determine whether the amino acid changes were responsible for viral resistance, we introduced the individual mutations (T416N, I418T, S433I, and F436I) into the homologous positions in JUNV GPC (T418N, L420T, A435I, and F438I). The GPC mutants were expressed, proteolytically matured, and transported to the surface of the cell similarly to the wild type (not shown) (53), and all were able to promote cell-cell fusion, albeit to differing degrees. The relative fusion competence levels of the mutants are indicated on the left panel of Fig. 4A. All four mutants were highly resistant to 10 μM ST-294 (Fig. 4A, right panel). Thus, each of the mutations identified in the membrane-proximal and transmembrane domains of G2 from ST-336-resistant TCRV isolates (3) also rendered JUNV GPC resistant to ST-294.

Alanine-scanning mutagenesis was used to identify additional amino acid determinants of ST-294 resistance in the membrane-proximal ectodomain of G2. We focused on the large number of charged residues in this region (Fig. 1), some of which may be positioned to interact with the critical K33 side chain in the ectodomain loop of SSP (57; unpublished data). We also included an existing collection of mutations spanning the N- and C-terminal heptad-repeat regions of the G2 ectodomain (53). As in other class I fusion proteins, the N- and C-terminal heptad repeats in the prefusion GPC complex are sequestered in an unknown conformation and refold to form the six-helix bundle structure during membrane fusion.

Of over 30 new and existing G2 mutants examined, 22 were able to mediate cell-cell fusion at levels of ≥10% that of the wild-type GPC (Fig. 4B, left panel). Three additional determinants of ST-294 resistance were identified among these: I347A, D400A, and F427A (Fig. 4B, right panel). I347 lies in the N-terminal heptad repeat of G2, at an interhelical a-position in the postfusion six-helix bundle, and the alanine mutation reduces membrane fusion to ~40% of wild-type levels (53). D400 is located in the C-terminal heptad repeat (53) and at a position expected to reside on the outer surface of the six-helix bundle. D400A reduces cell-cell fusion to ~75% of the wild type. F427 is located near the ectodomain face of the predicted...
transmembrane domain, between the L420T and A435I mutations identified in ST-336-resistant TCRV, and F427A is comparable to the wild-type GPC in its membrane-fusion activity.

Although the seven resistance mutations span much of the G2 ectodomain, the three-dimensional relationship between these residues in the prefusion GPC complex is unknown.

Six of the ST-294 resistance mutations (D400A, T418N, L420T, F427A, A435I, and F438I), however, lie close in sequence to the nominal ectodomain face of the membrane, where they may be positioned to interact with SSP (57). We therefore extended our analysis of ST-294 resistance to include residues in SSP (1, 56). For the 15 fusion-competent mutants examined, only mutations at N37 and K33 in the ectodomain loop of SSP were able to render GPC resistant to ST-294 (Fig. 4C). The K33R and K33H mutations, which have been shown to reduce the pH threshold for membrane fusion (57), both generated resistance to ST-294 (as did K33Q and K33E [not shown]). Amino acid changes in the second membrane-spanning region of SSP (positions 41 through 54) did not significantly affect membrane fusion activity (1) or sensitivity to ST-294. Mutations in the two cytosolic regions of SSP—G2A (58), E17A and R55A (57), and T58R (54)—did not appreciably alter sensitivity. Thus, the ectodomain loop of SSP that is critical in modulating the pH of fusion activation is also an important determinant of inhibition by ST-294. The K40 residue at the nominal C terminus of the ectodomain loop (57) appears to not participate in determining sensitivity.

The K33H mutation confers sensitivity to Old World-specific inhibitors. SIGA Technologies has independently identified two distinct chemical classes of Old World arenavirus fusion inhibitors (Fig. 2) through high-throughput screening for molecules that prevent cell entry by pseudotyped retroviruses bearing the LASV GPC (33). We characterized the inhibitory activity of these new compounds, ST-161 and ST-193, by using JUNV and LASV GPCs in cell-cell fusion assays. ST-161 was found to be specific to LASV GPC and did not inhibit cell-cell fusion by JUNV GPC (Fig. 5, left panel), while the broadly active ST-193 molecule (33) was shown to inhibit cell-cell fusion by both LASV and JUNV GPCs (Fig. 5, right panel).

In order to probe the molecular basis for the inhibitory activities of ST-161 and ST-193, we first investigated whether these new inhibitors shared determinants of resistance with ST-294. Of the four resistance mutations identified in TCRV, we focused on L420T because of its good fusion activity and its localization in the G2 ectodomain, the three-dimensional relationship between these residues in the prefusion GPC complex is unknown.

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inhibitor ST-161 but was now resistant to the broadly active molecule ST-193 (Fig. 5, left panel). We also examined the effect of ST-294 resistance mutations at K33. Remarkably, we found that the K33H mutant JUNV GPC now exhibited de novo sensitivity to the LASV-specific inhibitor ST-161 (Fig. 5, left panel). Both K33H and K33R mutations also enhanced sensitivity to ST-193 (Fig. 5, right panel).

Together, these results suggest that the three independently identified chemical classes of arenavirus fusion inhibitors share a common molecular target involving both SSP and G2. Sensitivity among the different classes of inhibitors may be determined in part by SSP. However, because K33 is uniformly conserved among naturally occurring arenaviruses (57), species specificity is also determined by the amino acid diversity in G2.

**Inhibitors prevent pH-induced G1 shedding from the GPC complex.** We have previously shown that amino acid substitutions at K33 that decrease positive polarity (K33R, K33H, K33Q, and K33E) systematically depress the pH needed for activation of cell-cell fusion (57). Substitution of a nonpolar alanine side chain at this position abolishes all evidence of membrane fusion. In these studies, we also noted that K33A mutant GPC retains much of its G1 subunit during biosynthesis, as opposed to wild-type GPC in which the loss of G1 from the complex is readily discerned (57). This observation suggested that the K33A mutation may limit spontaneous dissociation of the G1 subunit by stabilizing the GPC complex and thereby prevent membrane fusion. Stabilization of the prefusion GPC complex might likewise represent a possible mechanism of action of the SIGA inhibitors.

To investigate this notion, we made use of the observation in class I envelope glycoproteins that the receptor-binding subunit can be shed in response to the activation of membrane fusion (32, 38). Partial dissociation of the receptor-binding subunit is considered an early event in the conformational cascade to membrane fusion in all class I proteins (16, 17, 26, 29, 48). In the absence of a covalent linkage between the subunits, the receptor-binding subunit can be physically shed from the complex concomitant with activation. Although the functional relationship between shedding and membrane fusion is uncertain (22, 37, 51), the degree of dissociation can serve as a useful marker for both the stability of the prefusion complex and its activation toward fusion (21, 32, 47, 52).

To determine whether G1 shedding is related to pH-induced activation of GPC, we quantitated the amount of metabolically labeled G1 shed from the cell surface during a 10-min incubation in low-pH media. Soluble G1 was collected by immunoprecipitation and deglycosylated by pretreatment with either 50 μM ST-294 or 25 μM ST-193 (Fig. 6B). Each compound reduced shedding by ≥20-fold, consistent with the complete inhibition of membrane fusion (Fig. 3 and 5). By contrast, ST-161 is a very poor inhibitor of JUNV membrane fusion activity (20% inhibition at 25 μM [Fig. 5]). Accordingly, pretreatment of JUNV GPC with 50 μM ST-161 reduced G1 shedding minimally, to 50% of the untreated level (Fig. 6B). These findings extend the correlation between G1 shedding and membrane fusion activity and suggest that the SIGA fusion inhibitors act to stabilize the prefusion GPC complex against low pH.

**Fusion inhibition can be overcome by reduced pH.** Stabilization of the prefusion GPC complex can be accomplished by mutation at K33 or by inhibitor binding. Substitutions at K33 lower the pH at which membrane fusion is activated (57), and we wanted to determine whether inhibition by these small molecules might be functionally linked to this pH-responsive mechanism in GPC. If the metastable prefusion state of GPC were stabilized by inhibitor binding in opposition to pH-in-
Membrane fusion must be appropriately controlled to enable productive virus entry. The generally accepted model for activation and progression to membrane fusion by class I viral fusion proteins has been developed over several decades of...
now-classic research (reviewed in references 16, 17, 26, 29, and 48). The class I envelope glycoproteins are assembled as stable but inert precursors that require proteolytic cleavage for fusion activity. The fusion-competent envelope glycoprotein complex is thought to exist in a metastable state, established on proteolytic maturation and maintained by a balance of stabilizing and destabilizing forces within the protein. For viruses such as human immunodeficiency virus type 1 that enter through the plasma membrane, the membrane fusion activity is subsequently triggered by binding to cell surface receptor(s) at neutral pHs. Other viruses, including the arenaviruses and orthomyxoviruses, are endocytosed and exposed to acidic pH in order to activate the fusion process. Upon activation, all class I envelope glycoproteins undergo a structural reorganization that follows a thermodynamically determined path toward formation of the stable six-helix bundle and fusion of the virus and cell membranes. Our results suggest that the SIGA inhibitors prevent membrane fusion by stabilizing the prefusion GPC complex against activation at low pH.

The molecular basis for pH-induced activation of envelope glycoprotein-mediated membrane fusion is largely undefined. Despite detailed structural knowledge of membrane fusion in the best-studied pH-dependent envelope glycoprotein, influenza virus hemagglutinin (HA), key elements of the activation process remain to be completely elucidated (9, 43, 50). Studies of tert-butyl hydroquinone HA fusion inhibitors have demonstrated the metastable nature of the prefusion HA complex and the ability of small molecules to stabilize against acidic pHs (2, 27). In accordance with this mechanism of action, tert-butyl hydroquinone-resistant mutants were found to display an opposing increase in their pH of fusion (27). When mapped onto the atomic structure of the neutral-pH form of HA, these mutations cluster at the HA1-HA2 interface and proximate to the fusion peptide at the N terminus of HA2 (27). The possible role of the fusion peptide and surrounding structures in stabilizing and destabilizing the prefusion HA complex was independently highlighted by directed mutagenesis in this region and in studies of the pH-associated effects of cell-culture adaptation and amantadine resistance (11, 35, 52). It remains uncertain, however, whether the determinant for pH-induced activation of HA is distributed or localized to a specific region.

Our current model of the GPC complex proposes a pH-sensitive interaction between the ectodomains of SSP and G2. This interaction is stable at neutral pHs and thereby maintains the GPC complex in its prefusion state. The interaction is destabilized upon protonation at low pH to initiate the conformational cascade leading to class I type membrane fusion. We now demonstrate that amino acid determinants for inhibition by the SIGA compounds likewise reside in SSP and G2. Together, our observations suggest that these novel fusion inhibitors act by interfering with the ability of the SSP-G2 interface to sense acidic pH or, subsequently, to respond productively. This provides a starting point in efforts to characterize the molecular basis for pH-induced activation in GPC. The abundance of charged residues in the membrane-proximal ectodomain of G2 raises the possibility that the pH-sensing interface may comprise an ensemble of titratable interactions with K33 in SSP, similar perhaps to that recently described in the acid-sensing ion channel 1 (30). Although atomic-resolution structure of the prefusion GPC complex is far in the distance, localization of an element of the pH sensor to SSP may facilitate efforts to characterize the molecular determinants for pH-induced activation and its inhibition by small-molecule compounds.

The unique subunit organization of GPC clearly provides a robust molecular pocket for binding small molecules that are capable of stabilizing the complex against acidic pH. At least three chemically distinct classes of inhibitors, independently identified in different arenavirus species, appear to target a common site at the SSP-G2 interface. As with the chemically diverse nonnucleoside analog inhibitors of human immunodeficiency virus type 1 reverse transcriptase, all of which bind a common hydrophobic pocket (12, 31, 44), it is possible that the bound forms of the SIGA inhibitors share spatial and chemical characteristics. It will surely be of interest to determine whether the chemically distinct GPC-directed fusion inhibitors described by Lee and colleagues (34) bind similarly. If identified, this pharmacophore core may provide scaffolding in the design of potent and broadly active second-generation inhibitors. To date, both ST-294 and ST-193 have shown promise in small-animal efficacy studies (3, 33). Our observation that inhibition by these compounds is profoundly sensitive to pH suggests that coadministration with drugs that reduce acidification in the endosome might further enhance their therapeutic efficacy.

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