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ORIGINAL ARTICLE

Coagulation factor VIIa binds to herpes simplex virus 1-encoded glycoprotein C forming a factor X-enhanced tenase complex oriented on membranes

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Abstract

Background: The cell membrane-derived initiators of coagulation, tissue factor (TF) and anionic phospholipid (aPL), are constitutive on the herpes simplex virus type 1 (HSV1) surface, bypassing physiological regulation. TF and aPL accelerate proteolytic activation of factor (F) X to FXa by FVIIa to induce clot formation and cell signaling. Thus, infection in vivo is enhanced by virus surface TF. HSV1-encoded glycoprotein C (gC) is implicated in this tenase activity by providing viral FX binding sites and increasing FVIIa function in solution.

Objective: To examine the biochemical influences of gC on FVIIa-dependent FX activation.

Methods: Immunogold electron microscopy (IEM), kinetic chromogenic assays and microscale thermophoresis were used to dissect tenase biochemistry. Recombinant TF and gC were solubilized (s) by substituting the transmembrane domain with poly-histidine, which could be orientated on synthetic unilamellar vesicles containing Ni-chelating lipid (Ni-aPL). These constructs were compared to purified HSV1 TF±/gC ± variants.

Results: IEM confirmed that gC, TF, and aPL are simultaneously expressed on a single HSV1 particle where the contribution of gC to tenase activity required the availability of viral TF. Unlike viral tenase activity, the cofactor effects of sTF and sgC on FVIIa was additive when bound to Ni-aPL. FVIIa was found to bind to sgC and this was enhanced by FX. Orientation of sgC on a lipid membrane was critical for FVIIa-dependent FX activation.

Conclusions: The assembly of gC with FVIIa/FX parallels that of TF and may involve other constituents on the HSV1 envelope with implications in virus infection and pathology.

KEYWORDS
herpesvirus, tissue factor, coagulation factor, enzyme kinetics, enzyme mechanism

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1 | INTRODUCTION

Thrombosis and atherosclerosis are major causes of death worldwide and the incidents are increasing. These diseases have numerous well-established risk factors, which overlap[1] and have common underlying molecular bases. Viral infection is not typically considered within the etiology of thrombosis and atherosclerosis,[2-4] although numerous viruses have been linked to vascular disease[5,6] and are known to affect the hemostatic system.[7-14] As an example, herpesviruses persist as lifelong infections and may contribute to vascular pathology through frequent reactivation promoting leukocyte and platelet adhesion to the vascular wall, lipid accumulation in smooth muscle cells, and thrombin deposition in the periphery.[15-18] Although these examples provide a retrospective clinical correlation, causal evidence was reported using herpes simplex virus 1 (HSV1) as a targeted cytolytic therapeutic for melanoma cells, which resulted in a high propensity for deep vein thrombosis.[19] Case reports have also detailed the presence of thrombi[20,21] and/or bleeding[22,23] in patients with HSV1 encephalitis. In one study examining predictors of outcome in HSV1 encephalitis, one-half of patients experienced acute thrombocytopenia, with ~ 5% reporting cerebral hemorrhage.[23]

To provide a molecular explanation for the clinical evidence, our previous studies have demonstrated that members of the herpesvirus family incorporate host cell-derived coagulation cofactors during assembly of their outer envelope structure.[24-26] These are the transmembrane protein, tissue factor (TF), and anionic phospholipid (aPL), which function to accelerate the initiation and propagation of coagulation protease activation in response to vascular damage. TF is essential for life.[27] It accelerates the factor (F) VIIa-mediated proteolytic activation of FX to FXa by ~100 000-fold.[28,29] FXa subsequently produces the final protease generated during the coagulation pathway: thrombin. aPL plays a key role in accelerating coagulation protease activation by associating with FVIIa and FX via the respective γ-carboxyglutamic acid (Gla)-containing domain to enhance the assembly of the protease/substrate/cofactor complex. The strict physiological control of TF and aPL on the surface of cells is bypassed by their constitutive availability on the virus envelope surface. Coagulation enzyme activation on the virus surface and consequent induction of cell signaling pathways occur, which have pathological implications. An advantage to the virus is enhancement of the early events of infection by coagulation enzyme-mediated cell signaling, as seen in vitro[25] and in vivo.[30] When studied in cell culture, these effects on infection were facilitated by TF and the HSV1-encoded gene product, glycoprotein C (gC), both on the virus envelope.

gC is a multifunctional transmembrane glycoprotein that docks the virus to host cellular glycosaminoglycans, such as heparan sulfate and chondroitin sulfate.[31-33] Evasion of host innate immunity is also aided by gC, which competes with complement protein C5b for binding sites on C3b.[34,35] gC expression on HSV1-infected endothelial cells has been linked to monocyte adhesion, possibly contributing to vascular lesion development.[36] This effect was dependent on thrombin generation and alluded to a role for gC in coagulation protease activation. Further evidence of its coagulation role was exemplified by FX binding to cell-surface gC through transgenic expression of gC in murine cells.[36] Because gC is found on the virus envelope, we investigated FX binding to purified HSV1 and showed that the presence of viral gC created FX binding sites.[37] Although a soluble form of gC contributes to FVIIa-dependent FX activation without being membrane-tethered, the activity was ~1000-fold higher when combined with the virus.[38] To explain how viruses may contribute to pathology, here we addressed the hypothesis that an aPL anchor and interactions with FVIIa and FX are integral to the gC-enhanced FX-activating complex, and that the optimal macromolecular complex involves viral TF.

Essentials

- Host tissue factor (TF) and viral glycoprotein C (gC) combine to trigger clotting on oral herpes.
- Like TF, gC binds FX and enhances FVIIa-dependent FX activation.
- gC also imitates TF by binding FVIIa, which is enhanced by FX.
- gC requires orientation on a lipid membrane to function as a FVIIa cofactor.

2 | MATERIALS AND METHODS

2.1 | Proteins and reagents

Purified human plasma-derived FVIIa, FX, and FXa, as well as corn trypsin inhibitor were purchased from Haematologic Technologies (Essex Junction, VT). Recombinant human FVIIa (NovoSeven) was gratefully obtained from Novo Nordisk (Mississauga, ON) through an unrestricted research grant. Innovin (Dade Behring, Mississauga, ON) was the source of liposome-reconstituted purified recombinant human TF. Soluble TF (sTF-His)[39] was expressed in *Escherichia coli* (strain BL21-DE3) with a His_6 tag and 5-amino acid spacer replacing the transmembrane and cytoplasmic domains, and was purified by nickel affinity chromatography. gC was similarly solubilized (gCΔ457t) by substituting the membrane-spanning domain with His_5 in a baculovirus expression system and was purified using rabbit anti-gCΔ457t polyclonal antibody (R118) as previously reported.[40,41] Biotin-annexin V was purchased from BioLegend. Goat anti-mouse IgG Fc preadsorbed with 6 nm gold particles (ab105285) and goat anti-rabbit IgG H&L preadsorbed with 15 nm gold particles (ab27236) were purchased from Abcam (Toronto, ON). Goat anti-biotin preadsorbed with 10 nm gold particles (BBI Solutions) and acetylated BSA (Aurion) were commercially obtained (Cedarlane, Burlington, ON). Mouse monoclonal anti-TF (TF9-9B4) antibody was produced and purified as before.[42] Frozen pooled normal human plasma (NP) was obtained from George King Bio-Medical. Cyanogen bromide, L-α-phosphatidylserine (brain, porcine, PS), L-α-phosphatidylcholine (egg, chicken, PC) and the nickel salt of 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid) succinyl] (DOGS-NTA-Ni) were purchased from Sigma Aldrich. The FXa-preferred (S-2765) and broad specificity (S-2288) chromogenic substrates were from Diapharma (West Chester, Ohio).

2.2 | Viruses

HSV1 NS strain, a clinical isolate that expresses gC (gC^+) and ns-1 strain, a naturally occurring HSV1 mutant that lacks the extracellular domain of gC (gC^-) were passaged in African green monkey kidney cells (Vero CCL-81; ATCC) to generate initial virus stocks. To obtain viruses containing specific combinations of surface gC and/or TF, NS, or ns-1 were propagated in a human melanoma cell line engineered to express TF after treatment with zeocin (A7/TF), as described previously.[25] Thus, HSV1/TF^+/gC^+, HSV1/TF^+/gC^-, HSV1/TF^-/gC^+, and HSV1/TF^-/gC^- viruses were produced in the same cell type. Virus concentrations were quantified by negative-staining transmission electron microscopy (EM) and the presence or absence of viral gC and TF was confirmed by western blot analysis.[25] The amount of viral TF (~150 molecules/virus particle) was determined antigenically by comparison to a purified sTF standard curve using densitometry (not shown).

2.3 | Small unilamellar vesicles (SUVs)

Compositions of SUVs (mole %) were: PCPS (20% PS and 80% PC); NiPC (15% DOGS-NTA-Ni and 85% PC); and NiPCPS (15% DOGS-NTA-Ni, 20% PS and 65% PC).[43] SUVs were prepared by sonication and differential centrifugation, as described previously.[44] The concentration of lipid was derived after enzymatic hydrolysis using a Phospholipids C kit (Wako, Mountain View, CA). Quantified SUVs were sized by dynamic light scattering using a Beckman Coulter N4 PLUS Particle Size Analyzer and were 41 ± 15 nm average diameter.

2.4 | Chromogenic virus-mediated FX activation

All chromogenic experiments were in HBS (20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 150 mmol/L NaCl, pH 7.4) with BSA (0.1%). Virus was first mixed with FX (100 nmol/L) and FVIIa (1 nmol/L) in 15 µL followed by addition of 5 µL of $CaCl_2$ (calcium, 20 mmol/L) to start the reaction. After 20 minutes at 37°C with shaking every 5 minutes, 10 µL was transferred to a 96-well plate and combined with 90 µL of S-2765 (200 µmol/L) and EDTA (12 mmol/L). The amount of FXa generated was read at 405 nm for 5 minutes at room temperature using a Vmax or Spectramax multiwell plate kinetic spectrophotometer (Molecular Devices, Sunnyvale, CA). The amount of FX activated was derived from a purified FXa standard curve.

2.5 | Immunogold EM

Carbon formvar-coated nickel 400-mesh grids (Electron Microscopy Sciences; Cedarlane) were pretreated with 10 µg/mL human IgG (tested positive for anti-HSV1 antibodies) to attenuate the deposition of HSV1 as aggregates. Purified HSV1 particles (1×10^8 virus particles), sTF-His (10 µg), gCΔ457t (10 µg), PCPS (200 µmol/L), or PC only vesicles (200 µmol/L) were adsorbed to grids for 5 minutes and then blocked with BSA (5%), fish scale gelatin (0.1%), and goat serum (1%) in low-salt HBS (LHBS; 75 mmol/L NaCl, 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4) for 40 minutes. Following 2 washes of LHBS, mixtures of rabbit anti-gC (R118, 1:50), mouse anti-TF (TF9-9B4, 5 µg/mL)[42] and/or biotin-annexin V (1.25 µg/mL) in LHBS and acetylated BSA (0.1%) were layered onto the grid and incubated for 1 hour. Samples probed for aPL using annexin V had $CaCl_2$ (5 mmol/L) included in all buffers and the corresponding control included EDTA (10 mmol/L). After five 3-minute washes in LHBS, secondary antibody solutions of 6 nm gold-conjugated goat anti-mouse IgG (1:40), 15 nm gold-conjugated goat anti-rabbit IgG (1:160) and 10 nm gold-conjugated goat anti-biotin (1:40) in LHBS and acetylated BSA (0.1%) was added and incubated for 45 minutes. Following five 3-minute washes in LHBS, the grids were negatively stained with PTA (2%) stain pH 6.5 for 1 minute. The grids were wick-dried with filter paper and viewed on a 120 kV Hitachi H-7600 transmission electron microscope (Bioimaging Facility, University of British Columbia, Vancouver, Canada). HSV1 particles were defined as particles having gC antigen and a diameter of ~120-300 nm. The proportion of particles that express TF and/or aPL was quantified in 10 squares on the EM grid selected using a random number generator (https://numbergenerator.org/). Within each square, five sections were manually enumerated for a total of ~ 500 virus particles for each replicate.

2.6 | gCΔ457t enhancement of FVIIa-mediated FX activation

The function of gCΔ457t in FVIIa-mediated FX activation was followed by a discontinuous chromogenic assay. Innovin (1:3000) was incubated with gCΔ457t (2 µmol/L), FX (0-100 nmol/L), and FVIIa (0-10 nmol/L). Calcium (5 mmol/L) was added to initiate the reaction and the mixture was incubated at 3°C for 20 minutes. The effect of membrane lipids on FVIIa-mediated FX activation by sTF-His or gCΔ457t was similarly monitored. PCPS, NiPC, or NiPCPS (50 µmol/L) in the presence or absence of gCΔ457t (1.2 nmol/L), sTF-His (1 ρmol/L), FX (100 nmol/L), FVIIa (1 nmol/L), or calcium (5 mmol/L) were incubated for 20 minutes at 37°C. In all cases, the amount of FXa generated was determined by a chromogenic assay.

2.7 | Equilibrium complex formation by microscale thermophoresis

Protein complex formation between gCΔ457t and FVIIa and/or FX was assessed using microscale thermophoresis (MST), by quantifying differential mobility in a small temperature gradient because of complex formation. MST was conducted using a Monolith NT.115Pico instrument (NanoTemper Technologies, Munich, Germany). To detect thermophoretic shifts of a single species in an equilibrium mixture, one protein was covalently labeled with an NHS-linked fluorophore using the manufacturer's kit or labeled reversibly to study solution-phase interactions through association of the poly-His tag with RED-tris-NTA. Fluorophore Alexa Fluor 647 succinimidyl ester was purchased from Invitrogen. His-Tag Labeling Kit RED-tris-NTA and Monolith Protein Labeling Kit RED-N-Hydroxysuccinimide (RED-NHS) 2nd Generation was from NanoTemper Technologies. Based on the protein concentration determined by colorimetric assays (BCA; Thermo Fisher Scientific, Waltham, MA) and the red fluorescent probe extinction coefficient (239 000 and 195 000 cm/(mol/L) for Alexa Fluor 647 and NanoTemper Red at 650 nm, respectively), the labeling stoichiometries of proteins used here were 1.4, 2.1, and 0.9 for Alexa Fluor 647-labeled gCΔ457t, Alexa Fluor 647-labeled sTF-His, and NanoTemper RED-labeled FVIIa (FVIIa-R), respectively. The labeled protein function was comparable to unlabeled in FX chromogenic assays in the presence of sTF-His or gCΔ457t.

Microscale thermophoresis measurements were performed at 25°C with medium MST power and varying LED power to ensure sufficient fluorescence signal. Standard and premium capillaries for MST experiments were from NanoTemper. In addition to using BSA as a carrier, premium capillaries were specially treated to minimize protein adsorption on the capillary wall. Capillaries were cut in half using a zirconium blade and nitrogen gas was purged through the capillaries to remove debris. Cut and uncut capillaries gave identical results. For solution phase binding, gCΔ457t (200 nmol/L) was mixed with the RED-tris-NTA fluorescent probe (3.5 nmol/L) for 30 minutes at room temperature before the addition of FX or FVIIa in HBS/BSA (0.05%)/Tween-20 (0.05%)/calcium (5 mmol/L). To probe for membrane-bound gCΔ457t interactions, FVIIa-R (2 nmol/L) was titrated with gCΔ457t on PCPS, NiPC or NiPCPS (50 μmol/L) vesicles in HBS/BSA (0.1%)/calcium (5 mmol/L). Benzamidine (2 mmol/L) was also included to inhibit protease activity. Equilibrium dissociation constants (K_d values) were derived by fitting data to either a conventional binding isotherm equation based on conservation of mass or the Hill equation.[45,46]

2.8 | sTF-His or gCΔ457t binding to FVIIa and SUV

Binding of sTF-His or gCΔ457t to either FVIIa or SUVs were assessed using continuous chromogenic assays monitoring FXa generation.[43,47] For SUV binding, varying concentrations of SUVs were mixed in HBS containing calcium (5 mmol/L), FVIIa (100 ρmol/L), and either gCΔ457t (120 nmol/L) or sTF-His (100 ρmol/L). For FVIIa binding, sTF-His or gCΔ457t was titrated in HBS with calcium (5 mmol/L), FVIIa (10 ρmol/L), and SUVs (100 μmol/L). After 10 minutes of preincubation at 25°C, FX (30 nmol/L) and S-2765 (200 μmol/L) was added to start the reaction. The amount of FXa generated was immediately monitored spectrophotometrically at 405 nm for 20 minutes.

2.9 | Kinetics of FX activation

To derive Michaelis-Menten kinetic parameters Vmax and Km describing FVIIa function, initial rates of FX activation were measured as reported[43] with minor modifications. Reaction mixtures in HBS contained calcium (5 mmol/L), FVIIa (500 ρmol/L), and either gCΔ457t (10 nmol/L) or sTF-His (10 ρmol/L) with NiPCPS (100 μmol/L). These experiments were conducted at concentrations of cofactor that would saturate the FVIIa. Varying concentrations of FX were added to initiate the reaction at 37°C. Over the course of 10 minutes, 20 μL aliquots of the reaction mixture were added to 50 μL stop buffer (Mes-NaOH (40 mmol/L), pH 5.8, EDTA (12 mmol/L), NaCl (50 mmol/L), Triton X-100 (0.25%), and Antifoam C (0.012%) at 4°C. The stopped reactions were rapidly warmed to room temperature, and the amount of FXa generated was determined by the addition of S-2765 (200 μmol/L), calcium (5 mmol/L), and Tricine-NaOH (0.6 M), pH 8). A standard curve was derived using FXa to convert mOD to nmol/L FXa.

2.10 | Plasma clotting

Virus-induced coagulation was monitored using an ST4 coagulation analyzer (Diagnostica Stago). In a final reaction volume of 75, 12.5 μL of virus and 12.5 μL of HBS was added to 37.5 μL of NP or congenital FVIII-deficient human plasma. Prewarmed magnetic beads were added to the cuvette and incubated for 1 minute at 37°C. Clotting was initiated with 12.5 μL of calcium (60 mmol/L). To inhibit contact pathway initiation, corn trypsin inhibitor was preincubated with the NP for 5 minutes at 37°C. For gCΔ457t-mediated plasma clotting, PCPS or NiPCPS (50 μmol/L final concentration) was incubated with gCΔ457t (1 μmol/L) and calcium (10 mmol/L) for 5 minutes before adding to NP to initiate clotting.

2.11 | Statistical analyses

Data are presented as mean ± standard error of the mean (SEM) when three or more replicates were performed. Otherwise, mean ± SD was used for controls showing evident effect and a n = 2 was reported. The effect of gCΔ457t on virus-mediated FX activation was analyzed by unpaired Student *t*-test. FVIIa amidolytic activity data was analyzed by one-way analysis of variance (ANOVA). Statistical analysis using *t*-tests and one-way ANOVA were performed using Excel software. Fits of MST data and their confidence intervals were derived using quadratic binding isotherm equations written on MathWorks MATLAB® software version 2018b (MathWorks).

3 | RESULTS

3.1 | TF, aPL, and gC are simultaneously available on the HSV1 surface

To investigate the basis of FX activation on HSV1, the assumption that TF, aPL, and gC are simultaneously available on a single virus particle was directly examined by multilabel immunogold EM. Figure 1A shows in representative electron micrographs that single HSV1 particles carry TF, aPL, and gC. Figure 1B confirmed that the gC and TF antibodies, and the aPL probe (i.e., annexin V) were specific to the individual components by using irrelevant isotype controls on HSV1 or by chelating residual calcium for annexin V. Additional controls using recombinant gCΔ457t, sTF-His, PC, and PCPS vesicles further confirmed specificity (Figure S1). Virus particles had various appearances resulting from variable penetrance of negative staining into the envelope, and differential visualization of the virus capsid. Quantification of gold labeling revealed that ~ 10% of gC-bearing HSV1 particles also had detectable TF and aPL (Figure 1C). Of the remaining gC-positive HSV1 particles, ~20% also had detectable TF, whereas another ~ 20% had aPL. These images verify that the virus surface contains combinations of procoagulant cofactors.

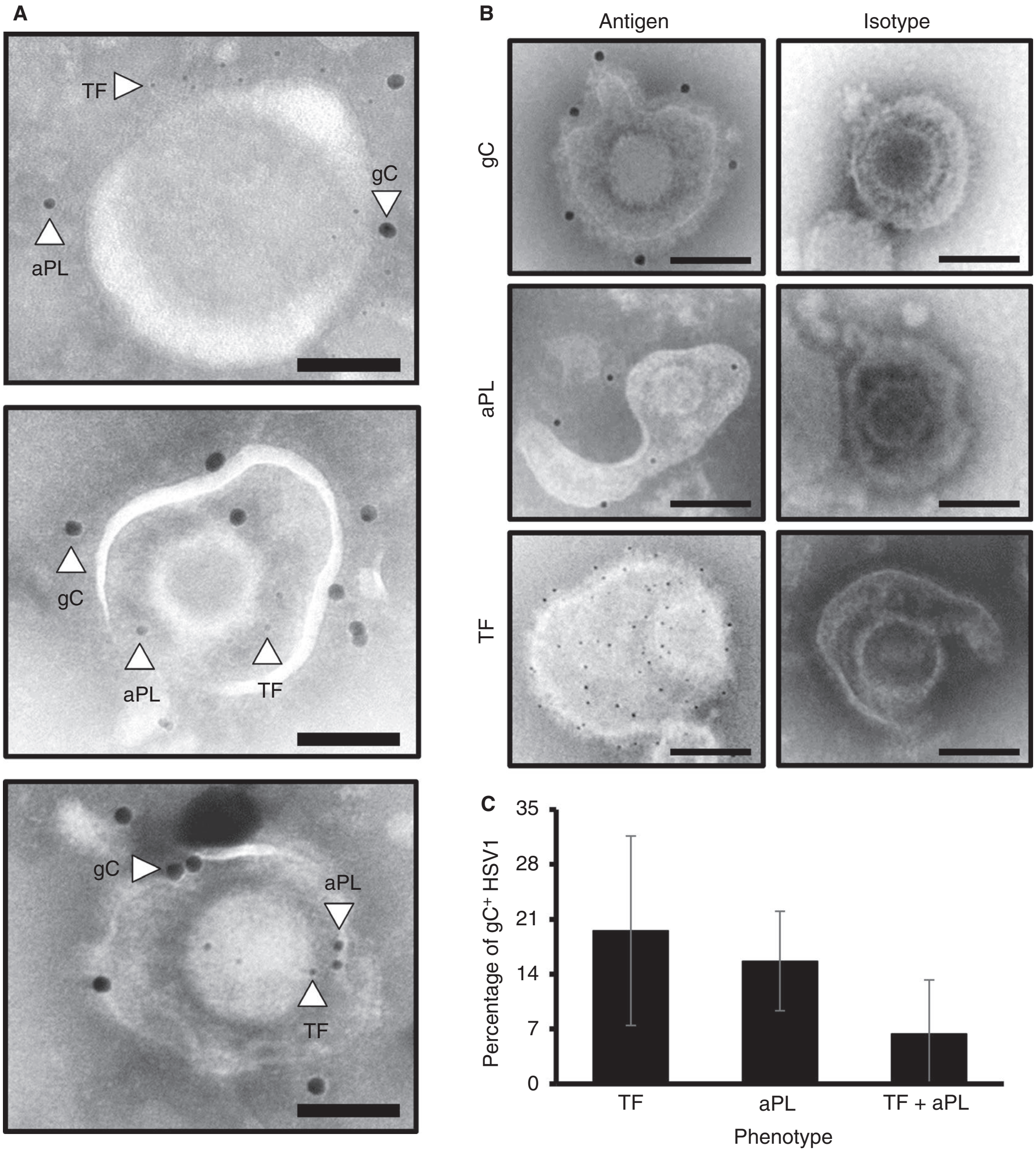

FIGURE 1 TF, aPL, and gC are simultaneously available on the HSV1 surface. (A) Representative immunogold electron micrographs concurrently identifying the HSV1 marker, gC (15 nm gold bead), aPL (10 nm bead), and TF (6 nm gold bead). (B) Individual controls for specificity using gold-labeled irrelevant isotype antibody controls for gC and TF or biotinylated-annexin V in the absence of calcium. (Scale bars = 100 nm. n = 3). (C) The proportion of gC-positive HSV1 particles that express TF, aPL, or TF and aPL was determined by immunogold EM. (n = 2 ± SD)

3.2 | Viral gC enhances HSV1-initiated FX activation when viral TF is present

To dissect the FX-activating mechanism on the HSV1 surface, the effect of purified HSV1/TF$^+$/gC$^+$, HSV1/TF$^+$/gC$^-$, HSV1/TF$^-$/gC$^+$, or HSV1/TF$^-$/gC$^-$ on FVIIa function was compared using a chromogenic assay. Compared with the fully competent HSV1/TF$^+$/gC$^+$ (Figure 2A), both TF-deficient viruses had < 2% FVIIa-mediated FX activation (Figure 2B). The presence of gC on the TF-deficient virus had no measurable enhancement on FX activation (Figure 2B). The small concentration-dependent increase in FX activation resulting from HSV1/TF$^-$/gC$^-$ suggested that additional constituents such as envelope aPL contribute to FX activation. Interestingly, the presence of viral gC and viral TF enhanced the activation of FX ~ 2.5-fold relative to HSV1/TF$^+$/gC$^-$ (Figure 2A). When gCΔ457t was added to 1 × 10^5 vp/µL of purified HSV1 variants, FX activation was further increased on TF-bearing virus (Figure 2C). HSV1/TF$^+$/gC$^+$ exhibited a greater enhancement by gCΔ457t compared with HSV1/TF$^+$/gC$^-$. Addition of gCΔ457t to TF-deficient virus had no measurable effect on FX activation (Figure 2D). Thus, viral TF is required for a contribution of gC or gCΔ457t to FVIIa activity mediated by purified HSV1. FX activation in the absence of FX, FVIIa or calcium was negligible (Figure 2E) demonstrating that purified virus has insignificant intrinsic hydrolytic activity toward S-2765.

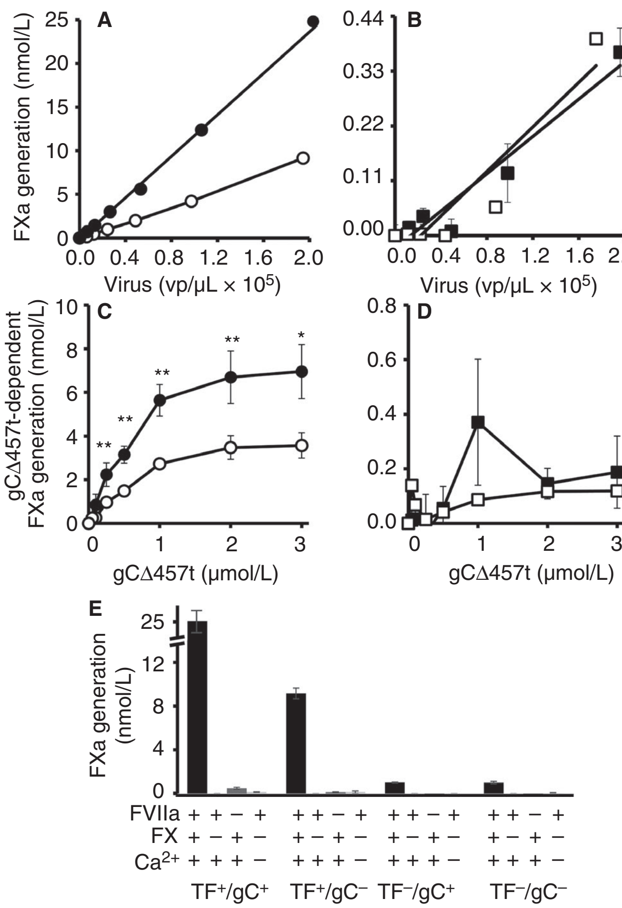

FIGURE 2 gC enhances HSV1-initiated FX activation when viral TF is present. (A, B) Purified FX (100 nmol/L) and FVIIa (10 nmol/L) were combined with HSV1/TF+/gC+ (●), HSV1/TF+/gC− (○), HSV1/TF−/gC+ (■) and HSV1/TF−/gC− (□) viruses in the presence of calcium (5 mmol/L) and FXa generation was followed using the chromogenic substrate S-2765. (C, D) FX activation by HSV1 was followed as in A and B and the effect of adding purified gCΔ457t to the panel of HSV1 (1 × 10^5 vp/µL) was monitored. (E) FX activation with 1 × 10^5 vp/µL HSV1/TF+/gC+, HSV1/TF+/gC−, HSV1/TF−/gC+, and HSV1/TF−/gC− with (first bar) or without FX, FVIIa, or calcium (following bars in order). (A-D: n = 4 ± SEM, $^*P$ < .05, $^{**}P$ < .01; E: virus and no FVIIa: n = 4 ± SEM, no FX or no Ca^{2+}: n = 2 ± SD; error bars may be smaller than the size of symbols.)

3.3 | gCΔ457t enhances FVIIa-mediated FX activation on vesicles

To exclude other virus- or host-encoded factors associated with the envelope of HSV1, gCΔ457t was added to lipidated full-length TF and aPL (Innovin). gCΔ457t enhanced FX activation by FVIIa with saturable dependence on FVIIa (Figure 3A) or FX (Figure 3B). Under these conditions, the addition of gCΔ457t increased maximal activation by ~ two-fold.

sTF-His is known to enhance FX activation by FVIIa optimally when DOGS-NTA-Ni is incorporated into an aPL-containing membrane,[43] where DOGS-NTA-Ni facilitates membrane binding of sTF-His and the aPL enables Gla-domain-dependent membrane-binding of FVIIa and FX. Thus, the respective contributions toward increasing the activity of FVIIa by gCΔ457t were evaluated by comparing vesicles of various lipid composition, PCPS, NiPC, and NiPCPS. Significant FX activation by FVIIa was not observed in the absence of sTF-His or gCΔ457t (Figure 3C, bars 1-3). Expression of gCΔ457t (Figure 3C, bars 4-6) or sTF-His (not shown) activity was dependent on FVIIa, FX, and calcium ions. As a positive control for the anticipated activity of the three vesicle compositions, FX activation was shown to be enhanced by sTF-His in the following order NiPCPS > NiPC» PCPS (Figure 3C, bars 7-9). Interestingly, gCΔ457t also enhanced FVIIa activity and followed the same lipid composition dependence as sTF-His, where combined binding of the His-tagged cofactor and Gla-containing proteins facilitated by NiPCPS was optimal (Figure 3C, bars 10-12). To explore the apparent synergistic effect of gC and TF on the virus (Figure 2), sTF-His was combined with gCΔ457t (Figure 3C, bars 13-15). Unlike the HSV1 variant experiments, the effect of gCΔ457t on sTF-His was additive in these purified protein experiments when the cofactor/enzyme/substrate complex was assembled on NiPCPS (ie, bar 9 + bar 12 = bar 15).

Further demonstrating a gCΔ457t cofactor effect on FVIIa, incubation with NP shortened clotting times in the presence of NiPCPS (Figure 3D, bar 1). With the substitution of NiPCPS for PCPS (Figure 3D, bar 2) the majority of samples did not form a clot during the experimental time (15 minutes). Similarly, gCΔ457t without lipids or the absence of gCΔ457t with either PCPS or NiPCPS yielded sporadic clotting with the majority not forming a clot (Figure 3D, bars 3-5, respectively). Of note, the NP contained antibodies that recognize

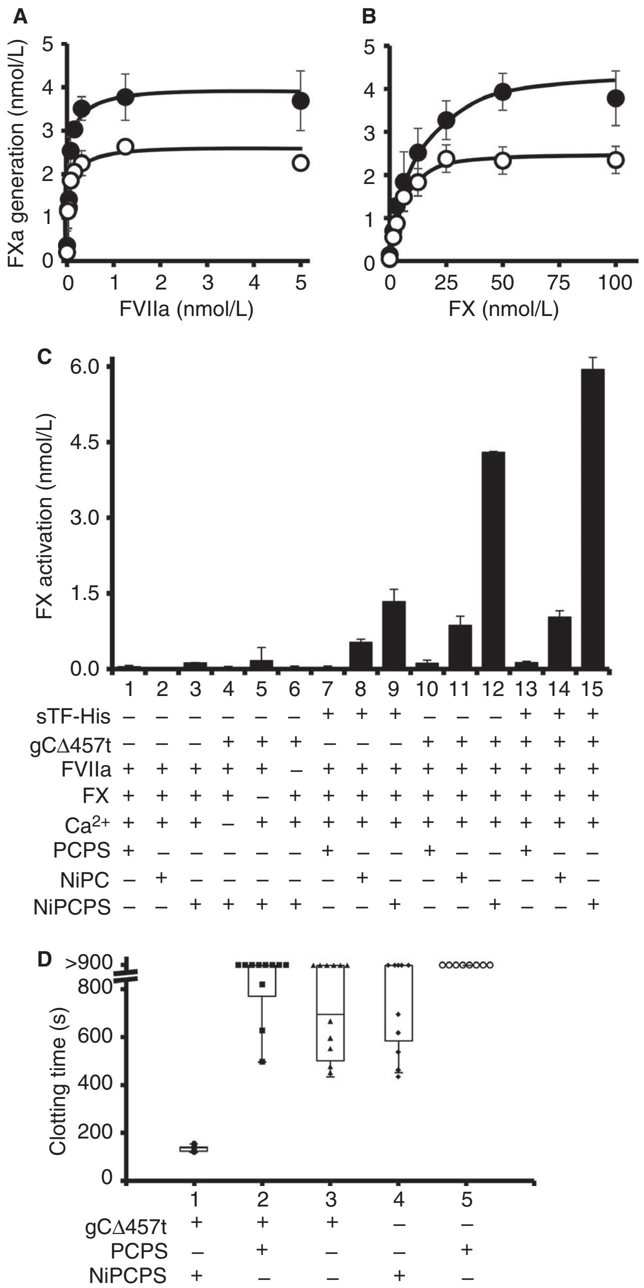

FIGURE 3 gCΔ457t enhances FVIIa-mediated FX activation. (A) FX activation by relipidated full-length TF (1:3000; Innovin) was followed chromogenically in the presence (●) or absence (○) of gCΔ457t (2 μmol/L) at constant FX (100 nmol/L, A) or FVIIa (10 nmol/L, B) and titrating either FVIIa (A) or FX (B). (C) FX activation was monitored by variably assembling tenase constituents on PCPS, NiPC, or NiPCPS (50 μmol/L) in the presence or absence of gCΔ457t (1.2 nmol/L), sTF-His (1 ρmol/L), FX (100 nmol/L), FVIIa (1 nmol/L), or calcium (5 mmol/L) with 20 minutes incubation at 37°C. (D) Normal pooled human plasma clotting time was monitored after initiation by gCΔ457t (1 μmol/L), 50 μmol/L PCPS or NiPCPS, and calcium (10 mmol/L). (A-C: n = 3 ± SEM; D: n = 3, box plot of pooled replicates; error bars may be smaller than the size of symbols.)

HSV1 and gCΔ457t (Figure S2), accredited to the prevalence of HSV1 within the general adult population, which may have affected gCΔ457t clotting activity.

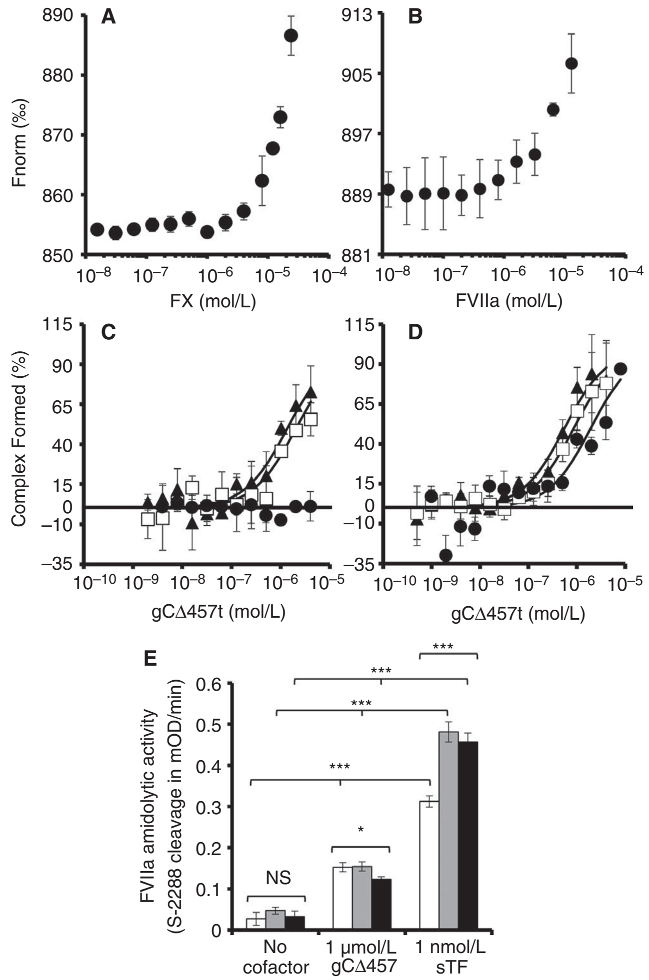

FIGURE 4 FX enhances the interaction between gCΔ457t and FVIIa. MST traces demonstrating weak associations of (A) FX and (B) FVIIa with gCΔ457t noncovalently labeled with RED-tris-NTA in the absence of membrane associations. (C) MST was also used to follow gCΔ457t binding to fluorescent FVIIa-R (2 nmol/L) in the presence of PCPS (●), NiPC (□), or NiPCPS (▲) (50 μmol/L) and benzamidine to inhibit FVIIa. (D) Similar to (C) but with the inclusion of FX (30 nmol/L). (E) Effects on FVIIa catalytic activity was assessed by following FVIIa (2 nmol/L) cleavage of chromogenic substrate S-2288 in the presence of gCΔ457t (1 μmol/L) or sTF-His (1 nmol/L) with calcium (5 mmol/L) and PCPS (white bars), NiPC (gray bars), or NiPCPS (black bars) (50 μmol/L). (All graphs: n ≥ 3 ± SEM). *P* values are provided as determined by 1-way ANOVA. * $P < .05$, *** $P < .001$, $P \geq .05$ was not significant (NS).

3.4 | FX enhances the interaction between gCΔ457t and FVIIa

The His-tag of gCΔ457t was non-covalently conjugated to fluorescent RED-tris-NTA and monitored by MST to follow interactions with FX (Figure 4A) or FVIIa (Figure 4B) in the absence of vesicles. Although an interaction between gCΔ457t and FVIIa or FX was demonstrable, both were relatively weak as the binding isotherms did not approach saturation at the highest available concentration of either ligand. These binding isotherms could not be fit to a binding model with statistical confidence. Although the solution-phase enhancement of FVIIa activity was independent of labelling the gCΔ457t with RED-tris-NTA via the poly-His tag, it could not be used to follow membrane-associated complex assembly because it quantitatively blocked NiPCPS-binding (not shown).

To follow the effects of membrane orientation and the presence of FX on incorporation of FVIIa into the gCΔ457t/FX complex, the FVIIa was fluorophore-labeled via amine coupling (FVIIa-R). As measured by MST, the dissociation constant (K_d) of ~1 nmol/L (Table 1) was ~ 100-fold higher than the reported K_d value for FVIIa binding to sTF-His/NiPCPS that was derived using a FXa product-linked chromogenic assay (~10 ρmol/L).[43] To reconcile this discrepancy, FX was included in the MST studies to mimic the conditions of the reported chromogenic method. In the presence of excess benzamidine to prevent proteolysis, FX enhanced sTF-His/NiPCPS-binding to FVIIa-R by ~ 30-fold (K_d = ~30 ρmol/L; Figure S3), which was consistent with the previous report.

gCΔ457t assembled onto NiPC or NiPCPS exhibited greater affinity for FVIIa-R compared with vesicles without DOGS-NTA-Ni (Figure 4C). This interaction was only moderately affected by the presence of PS, implying that a FVIIa-R-membrane interaction may not be important; the K_d values were 1.6 and 0.8 µmol/L for NiPC or NiPCPS, respectively (Table 1). Binding of gCΔ457t to FVIIa-R in the presence of PCPS was detectable in the absence of DOGS-NTA-Ni but was not quantifiable (K_d > 13 µmol/L) and consistent with the concentration range used for gCΔ457t binding to FVIIa in the absence of vesicles (Figure 4B). In the presence of NiPC or NiPCPS, the addition of FX (Figure 4D) increased the gCΔ457t-FVIIa-R affinity ~ 2-fold (K_d values of 1.0 and 0.4 µmol/L, respectively) indicating that Gla-dependent associations are not required for this enhancement. gCΔ457t emulated the FVIIa-sTF-His binding enhancement by FX, but this was modest in comparison to the ~ 30-fold observed for the latter (Table 1). Omission of DOGS-NTA-Ni reduced the affinity for FVIIa-R binding to gCΔ457t in the presence of FX and PCPS (K_d = 2.7 µmol/L).

Direct functional effects conferred to the FVIIa catalytic site by gCΔ457t was monitored by following chromogenic substrate cleavage (S-2288). Figure 4E shows that at 1 µmol/L gCΔ457t, FVIIa-mediated cleavage of S-2288 was enhanced. This result was independent of DOGS-NTA-Ni or PS availability. sTF-His also enhanced S-2288 cleavage by FVIIa but, unlike gCΔ457t, was further enhanced in the presence of DOGS-NTA-Ni. gCΔ457t was less efficient than sTF-His at directly affecting FVIIa amidolytic activity by ~3700-fold on a molar basis (Figure 4E).

TABLE 1 FVIIa binding to cofactors determined by MST in the presence or absence of FX

Ligand	Lipid	No FX K_d (µmol/L) (95% CI)	With FX K_d (µmol/L) (95% CI)
sTF-His	NiPCPS	8.2×10^{-4} (8.6×10^{-5}-1.5×10^{-3})	3.0×10^{-5} (8.0×10^{-6}-5.2×10^{-5})
gCΔ457t	NiPC	2.0 (1.3-2.8)	0.9 (0.7-1.0)
gCΔ457t	NiPCPS	1.3 (0.8-1.7)	0.6 (0.2-0.9)
gCΔ457t	PCPS	>13.0	2.2 (0.9-3.5)
gCΔ457t	None	>12.0	2.9 (1.5-4.2)

3.5 | gCΔ457t- and sTF-His-binding to Ni-chelating vesicles and FVIIa are similar by kinetics

To understand the mechanism of gCΔ457t cofactor function in our model system further, the His-tag equilibrium with NiPCPS and NiPC was determined by measuring the kinetically linked activation of FX. The availability of PS (Figure 5A) in NiPCPS increased the maximal

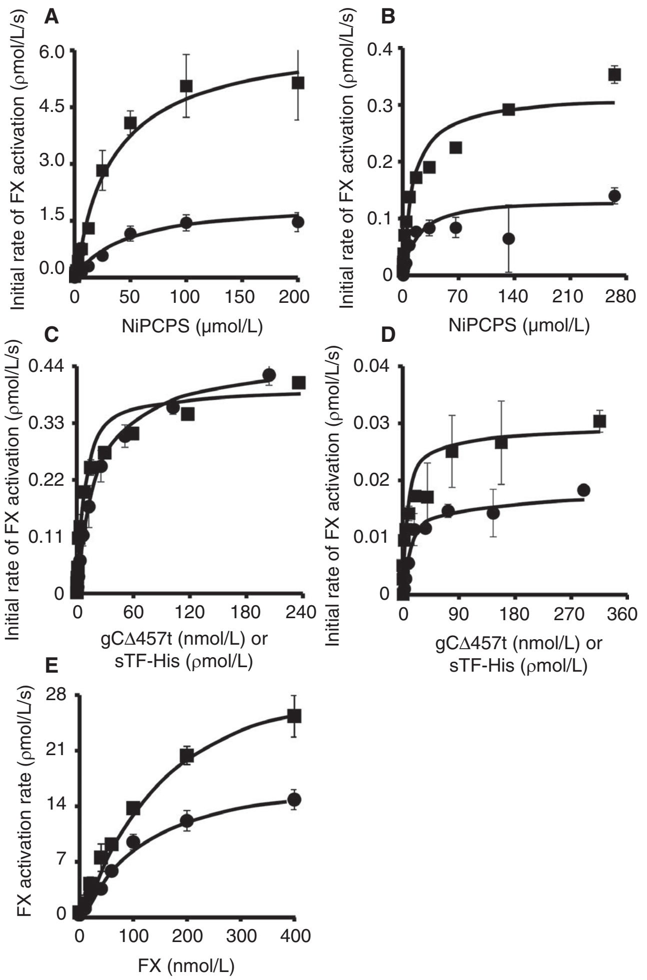

FIGURE 5 gCΔ457t- and sTF-His-binding to Ni-chelating vesicles and FVIIa are similar by kinetics. FX (30 nmol/L) activation by FVIIa (100 ρmol/L) was followed in the presence of gCΔ457t (120 nmol/L, ●) or sTF-His (100 ρmol/L, ■), calcium (5 mmol/L) and titrated with (A) NiPCPS or (B) NiPC. Binding of cofactors to FVIIa was followed in a similar manner except FVIIa (10 ρmol/L), FX (30 nmol/L), calcium (5 mmol/L), and NiPCPS (50 µmol/L, C) or NiPC (50 µmol/L, D), were titrated with gCΔ457t (●) or (■) sTF-His. E. The kinetic parameters of FXa generation by FVIIa (Table 3) was determined by deriving the initial FX activation rate by a limiting concentration of FVIIa (10 ρmol/L) in the presence of gCΔ457t (120 nmol/L, ●) or sTF-His (100 ρmol/L, ■) with FX, calcium (5 mmol/L), and NiPCPS (100 µmol/L). (All graphs: n = 3 ± SEM)

FX activation rate by > 15-fold for both sTF-His and gCΔ457t compared to NiPC. sTF-His and gCΔ457t had similar apparent binding affinities (Figure 5B) for either NiPCPS or NiPC ($K_{d,app}$ = 48 and 34 μmol/L, respectively), as summarized in Table 2. A role for PS in membrane binding of sTF-His and gCΔ457t was implied by an ~ 2.5-fold increase of the $K_{d,app}$ values when PS was omitted from the Ni-chelating vesicle.

The binding of FVIIa to sTF-His or gCΔ457t in the presence of NiPCPS (Figure 5C) or NiPC (Figure 5D) was measured enzymatically by limiting the detection of FXa generation to the concentration of FVIIa. Whereas both the gCΔ457t/NiPCPS and sTF-His/NiPCPS pairs exhibited similar V_{max} values (Figure 5C), reactions involving sTF-His/NiPC yielded a V_{max} value that was two-fold greater than those with gCΔ457t/NiPC (Figure 5D), implying an effect of PS on the number of gCΔ457t-FVIIa binding sites. NiPCPS-tethered sTF-His had a binding affinity (Table 2) consistent with earlier reports addressing FVIIa-binding to membrane-inserted full-length TF ($K_{d,app}$ ~ 10 ρmol/L). This effect was independent of the presence of PS. The $K_{d,app}$ describing the interaction of gCΔ457t with FVIIa was ~20 nmol/L, which was also not influenced by the presence of PS.

With both lipid- and FVIIa-binding affinities known, the concentration of the lipid/cofactor/enzyme complex can be estimated and, consequently, the Michaelis-Menten kinetics were derived (Figure 5E). The K_m for FX activation by FVIIa in the presence of either sTF-His or gCΔ457t was ~0.15 μmol/L (Table 3). The turnover number (k_{cat}) for FX conversion was moderately higher in the presence of sTF-His (3.8 seconds^{-1}) compared with gCΔ457t (2.5 seconds^{-1}). Nonetheless, sTF-His and gCΔ457t had comparable catalytic efficiencies (k_{cat}/K_m) with a nominal 1.4-fold difference. Therefore, a large part of the differential efficacy of sTF-His vs gCΔ457t is attributed to their relative affinity for FVIIa.

TABLE 2 Equilibrium binding constants derived from Figure 5A,B determined by linked chromogenic activity

Ligand	Lipid	Nickel binding $K_{d,app}$ (μmol/L) (95% CI)	FVIIa binding $K_{d,app}$ (nmol/L) (95% CI)
sTF-His	NiPCPS	33.8 (23.0-44.4)	7.0×10^{-3} (4.4×10^{-3}-9.6×10^{-3})
sTF-His	NiPC	13.4 (5.6-21.2)	8.9×10^{-3} (2.9×10^{-3}-1.5×10^{-4})
gCΔ457t	NiPCPS	48.0 (26.0-70.0)	21.5 (18.3-24.7)
gCΔ457t	NiPC	19.1 (7.4-30.7)	18.0 (8.7-27.3)

TABLE 3 Kinetic effects of gCΔ457t compared to sTF on FX activation

Cofactor	Lipid	K_m (μmol/L)	k_{cat} (s^{-1})	k_{cat}/K_m 1/(μmol/L/s)
sTF-His	NiPCPS	0.16 ± 0.02	3.8 ± 0.5	23.6 ± 1.4
gCΔ457t	NiPCPS	0.14 ± 0.02	2.5 ± 0.3	16.6 ± 1.0

4 | DISCUSSION

Here, we demonstrated that the coagulation tenase cofactor function of HSV1-encoded gC is analogous to that of TF using solubilized recombinant forms, gCΔ457t and sTF-His (respectively), in well-defined biochemical studies. Similar to full-length TF and sTF-His,[43,48] gCΔ457t required orientation on a membrane as shown by enhanced activity in the presence of vesicles that contained Ni-chelating lipid to facilitate association of the C-terminal poly-His. Direct binding to gCΔ457t of the tenase substrate, FX, was demonstrated, which confirmed previous studies.[37] The protease subunit of tenase, FVIIa, was shown for the first time to also associate with gCΔ457t, which acted like a receptor for FVIIa. The gCΔ457t-FVIIa interaction was enhanced by FX. Thus, gC is a constituent of a ternary protein complex that is localized to a membrane surface by the availability of substrate, like the TF-dependent tenase.

In addition to the Ni-chelating lipid that anchors gCΔ457t to the membrane, the presence of PS was important for FVIIa-dependent FX activation. The FX-mediated high affinity FVIIa assembly into the gCΔ457t/NiPCPS tenase required PS, which may in part be explained by the intrinsic affinity of FX for PS-containing vesicles being much greater than FVIIa (K_d ~0.2 and ~15 μmol/L, respectively) [29,30] and a consequent pseudo-receptor effect. Thus, the PS contribution to the overall gCΔ457t/FVIIa/FX tenase complex formation is predominantly attributed to FX, presumably via the Gla-domain.[49] In contrast, PS provided minimal contributions to FVIIa binding to gCΔ457t indicating that the Gla-domain-dependent association of FVIIa with PS is a relatively unimportant variable in cofactor/enzyme complex formation. These observations parallel the mechanism of sTF-His.[43,50] gCΔ457t/FVIIa complex formation was enhanced considerably by FX even in the absence of membrane, demonstrating a Gla-independent aspect of viral tenase assembly. Thus, the utility of vesicles comprised of Ni-chelating lipid and PS combinations has revealed congruent characteristics for the various protein-membrane interactions in gCΔ457t/FVIIa/FX and sTF-His/FVIIa/FX.

The mixture of gCΔ457t and sTF-His with saturating FVIIa, FX and NiPCPS resulted in the generation of FXa consistent with the sum of the gCΔ457t and sTF-His individually measured. To evaluate the tenase function of full-length gC and TF within the full complement of the intricate environment of the virus envelope surface, our unique virus panel of HSV1/TF$^+$/gC$^+$, HSV1/TF$^+$/gC$^-$, and HSV1/TF$^-$/gC$^+$ were compared. Interestingly, the additive effect with purified proteins was not observed on the HSV1 surface, where FXa generation was enhanced by gC only when TF or purified sTF-His was also available. This synergistic effect on gC by TF may imply a direct gC-TF interaction. Because four methods (ie, MST, crosslinking, nondenaturing electrophoresis, and ELISA) failed to detect such an association either on the virus or between purified proteins (data not shown), an additional host or virus-encoded constituent(s) may be involved in the macromolecular FX activating complex on the virus surface.

Because FX enhances the gCΔ457t/FVIIa interaction, it follows that the association of FVIIa with gCΔ457t was ~ 10-fold weaker when determined by MST in the absence of FX compared with the kinetically linked FX-activation method. A possible contributor to the higher K_d was the effect of amine modification of FVIIa to produce FVIIa-R. However, this was unlikely a contributor because FVIIa-R retained tenase activity comparable to unlabeled FVIIa where the fluorophore stoichiometry per FVIIa was carefully limited to ~ 1:1. As an additional precaution during MST equilibrium measurements, benzamidine prevented proteolytic activity without altering gCΔ457t binding to FVIIa (data not shown). TF-FVIIa binding measurements by others have reported a wide range of K_d values, because of variable contributions of FX and aPL depending on experimental conditions and the limited sensitivity of some approaches that required protein concentrations in vast excess over K_d.[48] Surface plasmon resonance using relipidated TF yielded a K_d in the ρmol/L range [48] that was very similar to that obtained by enzymatic assays or fluorescence anisotropy.[47,51,52] However, the TF/FVIIa/aPL complex did not dissociate to completion over the course of the measurement, potentially compromising the accuracy of K_d derivation as recognized by the authors. We show a high-affinity recruitment of FVIIa (~30 ρmol/L) into the sTF-His/FX complex on a NiPCPS membrane measured consistently by either MST or enzymatic assays, supporting the importance of membrane orientation- and FX-dependent macromolecular assembly and resolving any literature discrepancy.

Our work has revealed a novel interaction between virus-encoded gC, and the initiating coagulation protease FVIIa, that requires FX and aPL for optimal complex formation. This mechanism parallels that of the physiological hemostatic trigger TF and implies molecular mimicry. The role of gC in enhancing FX activation, even in the absence of TF, is a probable contributor to viral infection [30] and to hemostatic abnormalities correlating to atherosclerotic and thrombotic propensity.[5-6,15-18,21]

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

ADDENDUM

B. Lin designed and conducted experiments, analyzed data, and wrote the manuscript; M. Sutherland prepared specialized reagents, assisted in experimental design, analyzed data, and edited the manuscript; F. Rosell assisted in experimental design; J. Morrissey provided specialized reagents, assisted in experimental design, analyzed data, and edited the manuscript; and E. Pryzdial directed the project, designed experiments, and cowrote the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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