GB Virus Type C E2 Protein Inhibits Human Immunodeficiency Virus Type 1 Assembly Through Interference With HIV-1 Gag Plasma Membrane Targeting

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GB virus type C (GBV-C) is a single-stranded positive-sense RNA virus classified in the Flaviviridae family. Persistent coinfection with GBV-C is associated with lower human immunodeficiency virus type 1 (HIV-1) load, higher CD4+ T-cell count, and prolonged survival in HIV-1 coinfected patients. The GBV-C envelope glycoprotein E2 has been reported to interfere with HIV-1 entry. In this study, we showed that the expression of GBV-C E2 inhibited HIV-1 Gag assembly and release. Expression of glycosylated GBV-C E2 inhibited HIV-1 Gag precursor processing, resulting in lower production of CAp24 and MAP17, while the overall expression level of the Gag precursor Pr55 remained unchanged. Membrane floatation gradient and indirect immunofluorescence confocal microscopy analysis showed that glycosylated E2 disrupted HIV-1 Gag trafficking to the plasma membrane, resulting in Gag accumulation in subcellular compartments. This interference in HIV-1 Gag trafficking led to diminished HIV-1 particle production, which is a critical step for HIV-1 to infect new host cells. These findings shed light on a novel mechanism used by GBV-C E2 to inhibit HIV-1 replication and may provide insight into new approaches for suppressing HIV-1 replication.

Keywords. GBV-C; GBV-C E2; HIV-1; HIV-1 assembly; HIV-1 Gag; plasma membrane targeting.
of interleukin 2–stimulated peripheral blood mononuclear cells with secretable truncated E2 blocks HIV-1 entry [18]. In addition, synthetic peptides of E2 were shown to inhibit gp41-mediated liposome fusion or strain-specific inhibition of HIV-1 entry [19, 20]. Furthermore, Mohr et al showed that E2 also elicits antibodies that react with a cellular antigen on HIV-1 particles, resulting in neutralization of diverse HIV-1 isolates and inhibition of HIV-1 attachment to target cells [21]. Recently, GBV-C NS3 and E2 were also shown to play a role in inhibiting HIV-1 replication in a T-cell line through an unknown mechanism [22, 23]. Although GBV-C and HIV-1 target the same cells, the effect of GBV-C on the late stages of the HIV-1 lifecycle is yet to be defined.

HIV-1 Gag proteins play a major role in virus assembly and release [24, 25]. Gag is expressed as the Pr55Gag precursor polyprotein, which is processed into matrix (p17), capsid (p24), nucleocapsid (p7), and p6 proteins by the viral protease [24]. During HIV-1 replication, Pr55Gag is targeted to the plasma membrane via regions within the matrix domain [26–28]. Plasma membrane targeting of HIV-1 Gag is an important initiation step of Gag processing. It has been shown that a Gag matrix mutant that was not able to target to the plasma membrane failed to be processed [29, 30]. During viral budding from the plasma membrane, the viral protease within Gag-Pol complexes autocatalyzes its release from the Pol domain and subsequently processes Pol and Gag proteins into their mature constituent proteins. This process leads to reassembly of the immature virus particle into a mature infectious virion [31]. In this study, we analyzed the effect of GBV-C E2 expression on HIV-1 assembly and release. We found that the expression of GBV-C E2 protein inhibited HIV-1 assembly through interference with HIV-1 Gag intracellular trafficking. These observations may offer insights on novel approaches for the development of antiretroviral therapies.

**METHODS**

**Plasmids, Antibodies, and Reagents**

pAF121950 [3], the full-length cDNA of GBV-C (GenBank accession number AF121950), was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (NIH-ARRRP). E2 gene was amplified from pAF121950, using the forward primer 5′-ATCTAGAGCGCCATG GCCCCCGCCTCCG-3′ and the reverse primer 5′-TGGAT CCTTAAAAGGTCTCTTCTGAGATGTTTTGTCTGGC CACGGCCAGCG-3′, with the cMyc sequence incorporated into the C-terminus of the E2 open reading frame. The E2-cMyc gene was subcloned into the VR1012 expression vector [32] (generously provided by Vical San Diego, CA), using XbaI and BamHI restriction sites, to generate the E2 expression vector. The E2 gene was also amplified from pAF121950, using the forward primer 5′-AGGATCCGCCCGCCTCCG-3′ and the same reverse primer described above. The polymerase chain reaction (PCR) product was subcloned into the VR1020 vector (Vical, San Diego, CA) downstream of the tissue plasminogen activator (tPA) secretory signal sequence at the BamHI restriction site to generate the tPA-E2 expression vector. To establish the immunoglobulin G(igG)–E2 construct, the signal sequence of IgG κ was fused to the N-terminus of E2-cMyc using PCR with 5′-AGTCGACATGGAGACAGACACTCCTGCTATGGGTA CTGCTGCTCGTGGTCTAAGTTCACCTGTTGACGCC CGGGCTCGG-3′ as a forward primer and the same reverse primer. The PCR product was cloned into VR1012 using XbaI and BamHI restriction sites. The HIV-1 Gag-Pol expression construct pGPCINS was a generous gift from Xiaofang Yu (Johns Hopkins University, Baltimore, MD) [33]. The proviral DNA constructs HIV-1 pNL4-3 [34], simian immunodeficiency virus (SIV) pSIvagmTan-1 [35], and xenotropic murine leukemia virus–related virus (XMRV) VP62 [36] were from NIH-ARRRP. Deletions were introduced into IgG–E2 by PCR-mediated mutagenesis, using the following primer sets: forward primer 5′-AACCATGGGCCCTCATC-3′ and reverse primer 5′-GCATGACTGCCACTTTCAAC-3′ for E2DN1, forward primer 5′-TGGCCCGAAAACCGG-3′ and reverse primer 5′-TTGTCCGGTGGCTCAAT-3′ for E2 DN2, forward primer 5′-CIAGCCAGGAAATGTCTG-3′ and reverse primer 5′-GAGTCCCGACAGAC-3′ for E2DC2, and forward primer 5′-AACCAGTTGGCCTTCA-3′ and reverse primer 5′-CATGCACTATTGACGCGC-3′ for E2DMID. For this method, each primer was synthesized with a 5′-phosphate modification. The PCR products were self-ligated using the DNA Quick Ligation Kit (New England BioLabs) and were cloned to form the E2 deletion mutants. Anti-cMyc monoclonal antibody (4A6) was purchased from Millipore. The anti-p24 monoclonal antibody (183-H12-5C) [37], polyclonal anti-HIV-1 p17 (VU47) [38], and HIV immunoglobulin (HIV-1g) were obtained from NIH-ARRRP. Alexa 546–conjugated goat anti-rabbit antibody and Alexa 488–conjugated goat anti-mouse antibody were purchased from Molecular Probes. Anti-MuLV antiserum was purchased from the American Type Culture Collection.

**Cell Culture, Western Blot Analysis, HIV-1 Preparation, and Viral Infectivity (MAGI) Assay**

The human embryonic kidney 293T, HeLa, and TZM-bl cell lines [39] (NIH-ARRRP) were grown in Dulbecco’s modified essential medium supplemented with 10% fetal calf serum at 37°C in a 5% CO2 atmosphere. DNA transfections were performed with polyethylenimine for 293T cells, as described previously [40], or with Lipofectamine 2000 (Invitrogen) for HeLa cells, following the manufacturer’s instructions. Western blot analysis, preparation of HIV-1, and the viral infectivity assay (ie, the MAGI assay) were performed as described previously [40, 41].
**PNGase F Treatment**

Cell lysates from 293T cells transfected with tPA-E2 or IgG-E2 were denatured with glycoprotein denaturing buffer (5% sodium dodecyl sulfate and 10% 2-mercaptoethanol) for 10 minutes at 100°C. The reaction was then activated by adding G7 reaction buffer (0.5 M sodium phosphate, pH 7.5), 10% NP-40, and PNGase F (New England BioLabs) at a 1:10 ratio and incubating the preparation at 37°C for 1 hour. Samples were then analyzed by Western blot with an anti-Myc antibody. All analyses were performed at least 3 times independently.

**HIV-1 p24 Enzyme-Linked Immunosorbent Assay (ELISA)**

The HIV-1 p24 antigen capture assay kit was purchased from Biological Products Core, AIDS and Cancer Virus Program, SAIC-Frederick, National Cancer Institute (Frederick, MD). The lower limit of detection for this kit is around 100 pg. The ELISA was performed as detailed in the kit instructions. All analyses were performed at least 3 times for each experiment.

**Membrane-Binding Analysis**

Membrane floatation analysis of cell lysates was performed as detailed by Ono et al [42]. In brief, 2 days after transfection, 293T cells were washed with phosphate-buffered saline (PBS) and disrupted with homogenization buffer containing 10% sucrose, 10 mM Tris-HCl (pH 7.5), and 1 mM Tris-ethylenediaminetetraacetic acid (TE), with Complete Protease Inhibitor Cocktail (Roche). Lysis was performed by sonication (twice for 15 seconds each time). Nuclear fractions and unlysed cells were removed by adjusting the samples to 150 mM NaCl–1 mM MgCl₂ prior to centrifugation at 1000 × g for 10 minutes at 4°C. Subsequently, 250-µL postnuclear supernatants were mixed with 1.25 mL of 85.5%...
sucrose in TE to adjust the sample to 73% sucrose. The sample was then placed at the bottom of a centrifuge tube and layered with 7 mL of 65% sucrose in TE and 3.25 mL of 10% sucrose in TE. The sucrose step gradient was centrifuged at 35,000 rpm for 18 hours at 4°C, using a Beckman SW41 rotor.

**Immunofluorescence Microscopy**

Confocal analysis was performed as described previously [43]. In brief, HeLa cells were plated on glass coverslips in a 6-well plate and grown overnight. Cells were then transfected using Lipofectamine 2000. Twenty-three hours after transfection, cells were fixed with 4% paraformaldehyde in PBS solution at room temperature for 10 minutes, permeabilized with 0.1% Triton X-100 for 10 minutes, and blocked with 5% bovine serum albumin for 1 hour. Immunofluorescent staining was performed using polyclonal anti-HIV-1 p17 (VU47) and anti-cMyc monoclonal antibody (4A6) as primary antibodies, which were detected via goat anti-rabbit Alexa 546–conjugated and goat anti-mouse Alexa 488–conjugated secondary antibodies, respectively. Images were captured using a Nikon A1R confocal microscope.

**RESULTS**

**Expression of Glycosylated GBV-C E2 Inhibits Proteolytic Processing of HIV-1 Gag and Virus-Like Particle (VLP) Release**

E2 is predicted to be expressed in a glycosylated form and targeted to the endoplasmic reticulum during GBV-C replication. To express E2 in its glycosylated state, the secretory signal
IgG-E2 (Figure 3) lysates of Gag-Pol cotransfected vector (Figure 3A), E2 (Figure 3B), or IgG-E2 (Figure 3C) were analyzed by Western blot.

Figure 3. Expression of glycosylated E2 inhibits HIV-1 Gag membrane targeting. The Gag-Pol expression vector was cotransfected with empty vector or with E2 or immunoglobulin G (IgG)–E2 expression vector. Two days after transfection, 293T cells were sonicated. Nuclear fractions and unlysed cells were removed by centrifugation. The clear postnuclear supernatants were mixed with 85.5% sucrose to adjust the sample to 2 mL with 73% sucrose. The sample was then placed at the bottom of a centrifuge tube and layered with 7 mL of 65% sucrose in Tris–ethylene-diaminetetraacetic acid (TE) and 3.25 mL of 10% sucrose in TE. The sucrose step gradient was centrifuged at 35,000 rpm for 18 hours at 4°C in a Beckman SW41 rotor. Eight fractions were collected from each sample after ultracentrifugation. The fraction samples and total cell lysates of Gag-Pol cotransfected vector (Figure 3A), E2 (Figure 3B), or IgG-E2 (Figure 3C) were analyzed by Western blot.

Peptide of tPA and IgG were fused to the N terminus of E2 to create tPA-E2 and IgG-E2, respectively. 293T cells were transfected with empty vector or E2, tPA-E2, or IgG-E2 expression constructs. Forty-eight hours after transfection, Western blot analysis of the cell lysates showed that the tPA and IgG leader sequence caused E2 to have an electrophoretic banding pattern commonly observed for glycosylated proteins, whereas the unglycosylated form of E2 migrated as a single band (Figure 1A). To confirm that sugar moieties were responsible for the banding pattern observed with tPA-E2 and IgG-E2, 293T cells lysates expressing tPA-E2 and IgG-E2 were digested using a protein deglycosylation mixture containing PNGase F, which deaminates asparagine to aspartic acid, to remove asparagine-linked oligosaccharide chains. Western blot analysis showed that the deglycosidase treatment caused tPA-E2 and IgG-E2 to mobilize as a 42-kD band that was indistinguishable from the band of the unglycosylated E2 control (Figure 1B). To determine whether E2 affects proteolytic processing of HIV-1 Gag, the HIV-1 Gag-Pol expression construct pGPCINS was cotransfected into 293T cells with empty vector, E2, IgG-E2, or tPA-E2. As shown by Western blot analysis in Figure 1C, when HIV-1 Gag-Pol was coexpressed with empty vector or E2, the Gag precursor was efficiently processed into p24 and p17 (Figure 1C). However, when Gag-Pol was coexpressed with tPA-E2 or IgG-E2, the p24 and p17 productions were dramatically inhibited, with no noticeable change in Gag precursor production (Figure 1C), suggesting that the expression of tPA-E2 or IgG-E2 inhibited HIV-1 Gag precursor processing. We also cotransfected the p24 expression construct with empty vector or with E2, tPA-E2, or IgG-E2 expression vectors. Western blot analysis showed that the expression of E2, tPA-E2, or IgG-E2 did not alter the p24 expression level (data not shown). These results ruled out the possibility that tPA-E2 or IgG-E2 affected p24 stability. They further demonstrated that the glycosylated E2 (designated “glycol-E2”) inhibited HIV-1 Gag processing. To investigate whether glycol-E2 affects Gag VLP release, the Gag-Pol expression construct was cotransfected with empty vector, E2, or the tPA-E2 expression construct. Forty-eight hours after transfection, the culture supernatants were harvested for collection of VLPs by ultracentrifugation, as described in Materials and Methods. Figure 1D shows that glycol-E2 dramatically inhibited Gag-Pol VLP release, as determined by densitometry analysis (Figure 1E). Cell viability and vitality analysis showed that E2 and IgG-E2 expression did not alter cell viability (Supplementary Figure S1).

Glycol-E2 Inhibits HIV-1 Release but Not Maturation

To determine whether glycol-E2 inhibits HIV-1 release, the HIV-1 proviral construct NL4-3 was cotransfected with empty vector or with E2 or IgG-E2 expression constructs. Cell lysates and viral pellets were analyzed by Western blotting. For an unknown reason, overall expression of NL4-3 Gag was reduced when the IgG-E2 and NL4-3 plasmids were cotransfected at a 1:1 ratio, using 2 µg of each plasmid (Figure 2A). To assess Gag processing, the expression of Pr55 has to be the same among all samples. Therefore, 4 µg of the NL4-3 plasmid were used to increase the overall Gag expression level (Figure 2A) to levels seen with 2 µg NL4-3 in cotransfections with vector or E2 (Figure 2A). As shown in Figure 2A, when similar levels of Gag precursors were expressed, glycol-E2 inhibited intracellular p24 production and NL4-3 viral release (Figure 2A). Densitometry analysis of the Western blot bands of Figure 2A showed that the NL4-3 viral release was reduced approximately 5-fold by the expression of glycol-E2 (Figure 2B). As seen in Supplementary Figure S2, similar results were
obtained in more physiologically relevant Jurkat and SupT1 T cells. Viral supernatants were also collected for measuring infectivity by the MAGI assay. After the input viral amount was normalized by HIV-1 p24 ELISA, there was no significant change in viral infectivity among vector, E2, and IgG-E2 transfected samples (Figure 2C). These data suggest that the expression of glyco-E2 did not affect HIV-1 maturation.

Expression of Glycol-E2 Inhibits HIV-1 Gag Membrane Targeting

Gag association with cellular membranes is a critical step in HIV-1 assembly and release. To examine whether glyco-E2 expression alters Gag membrane targeting, the Gag-Pol expression construct was cotransfected into 293T cells with empty vector or with E2 or IgG-E2 expression vectors. Forty-eight hours after transfection, cell lysates were subjected to membrane flotation centrifugation and Western blot analysis, as described in Materials and Methods. In this assay, membrane-associated proteins migrate to the interface between 65% and 10% sucrose (Figure 3). When Gag-Pol was expressed alone with empty vector or with E2 or IgG-E2 expression vectors, significant amounts of Gag were localized in fraction 3, representing the membrane-associated protein (Figure 3A and 3B). By contrast, in cells coexpressing glycol-E2, most of the Gag was localized in fractions corresponding to cytosolic Gag. Only residual amounts of Gag were targeted to the membrane-associated fraction (Figure 3C). This result suggests that the expression of glycol-E2 inhibited HIV-1 Gag membrane targeting. We next sought to confirm the membrane targeting effect of glycol-E2 by immunofluorescence microscopy. The Gag-Pol expression construct was transfected into HeLa cells along with empty vector or with E2 or IgG-E2 expression vectors. Other experiments confirmed that the inhibitory effect of IgG-E2 on Gag assembly and release could be replicated in the HeLa cell line (data not shown). The cells were stained with antibodies directed against Gag-p17 for analysis of Gag subcellular localization. E2 and IgG-E2 were stained with a monoclonal anti-cMyc antibody. Gag is known to have 3 types of subcellular distribution patterns that can be observed through fluorescent microscopy: diffused, intracellular punctate, or plasma membrane localization. Twenty-three hours after transfection, the localization of Gag was assessed by confocal microscopy. As seen in Figure 4, in the absence of E2 or glycol-E2, Gag was shown to be punctate and primarily localized toward the periphery along the plasma membrane. A similar distribution pattern was also seen with cells containing a plasmid encoding unglycosylated E2. However, in the presence of glycol-E2, Gag was predominantly accumulated throughout the cytoplasm. These data indicate that the expression of glyco-E2 disrupted Gag trafficking to the plasma membrane, resulting in intracellular accumulation of Gag.
Membrane Interaction Domain (MID) Is Essential for the Inhibitory Effect of Glycol-E2 on HIV-1 Gag Processing and Release

To determine the essential domain for the inhibitory effect of glycol-E2, a series of internal deletion mutants of IgG-E2 were generated, as shown in Figure 5A. The Gag-Pol expression vector was cotransfected with empty vector, E2, IgG-E2, and the IgG-E2 internal deletion mutants. Forty-eight hours after transfection, cell lysates were analyzed by Western blot analysis. As shown in Figure 5B, among the 4 internal deletion mutants, E2DMID lost the inhibitory effect on Gag processing (Figure 5B). Moreover, the inhibitory effect of E2DN2 was partially lost (Figure 5B). However, when increasing amounts of E2 plasmids were cotransfected with Gag-Pol, E2DMID continued to have no effect on Gag precursor processing, whereas E2DN2 behaved more like wild-type IgG-E2 (data not shown). This result indicates that the MID of E2 was essential for its inhibitory effect. The Gag-Pol expression vector was also cotransfected with empty vector and with E2, IgG-E2, and E2DMID expression vectors. Forty-eight hours after transfection, cell and viral lysates were analyzed by Western blot. E2DMID showed no inhibition in Gag processing and viral release (Figure 5C). The results confirmed that the MID was essential for the inhibitory effect. The MID comprises 2 membrane interaction domains (aa 267–298 and aa 347–364) [44–46] and the putative transmembrane domain (aa 336–387) [47].

Figure 5. The membrane interaction domains (MIDs) of GB virus type C E2 are essential for its inhibitory effect on human immunodeficiency virus type 1 (HIV-1) Gag processing and release. A, Schematic of immunoglobulin G (IgG)–E2 and mutant constructs. B, Gag-Pol was expressed in the presence of E2, IgG-E2, or truncated IgG-E2 constructs (E2DN1, E2DN2, E2DC2, and E2DMID) by transfection of 293T cells. Cells were harvested and lysed 2 days after transfection to analyze processing of the Gag-Pol precursor by Western blot analysis. C, E2, IgG-E2, or the E2DMID mutant was cotransfected with Gag-Pol into 293T cells. Two days after transfection, Western blotting was used to assess processing of HIV-1 Gag and virus-like particle release.
Expression of Glycol-E2 Also Inhibits SIV and XMRV Gag Processing

To test whether glycol-E2 also has an inhibitory effect on the processing of other retroviral Gag proteins, the SIVagm.tan proviral construct and XMRV proviral construct XMRV VP62 were cotransfected with vector and E2 and IgG-E2 expression vectors. Forty-eight hours after transfection, cell lysates were analyzed by Western blot analysis. IgG-E2 efficiently inhibited both SIV and XMRV Gag processing (Figure 6A and 6B). These data suggest that GBV-C glycol-E2 inhibits not only HIV-1 Gag processing and release, but also Gag expressed from other retroviruses, such as SIV and XMRV.

DISCUSSION

GBV-C and HIV-1 coinfection has been associated with prolonged survival among HIV-1–infected patients in clinical studies [48]. It has been shown that E2, NS5A, and NS3 have inhibitory effects on HIV-1 replication. In this study, we showed that the expression of E2 was able to inhibit HIV-1 Gag processing and viral release. The inhibitory effects were glycosylation dependent because they were eliminated when E2 was expressed in the unglycosylated form (Figure 1C and 1D). Plasma membrane targeting of HIV-1 Gag is an important initiation step of Gag processing and release. A Gag matrix mutant has been shown to be unable to target to the plasma membrane, failing to be processed and resulting in a lower level of particle release [29, 30]. In this study, the membrane floatation and confocal microscopy analysis showed that the expression of glycol-E2 disrupted targeting of HIV-1 Gag to the plasma membrane (Figures 3 and 4), eventually inhibiting Gag processing and release. Furthermore, this inhibitory effect did not appear to be limited to HIV-1, as glycol-E2 was also able to disrupt processing of SIVagm.tan and XMRV Gag proteins. The inhibitory effect was not observed with E2 lacking a signal sequence, indicating the importance of glycosylation and/or trafficking for E2 to exert an inhibitory effect. An recent study by Xiang et al showed that expression of the glycosylated form of an E2 fragment in a CD4+ T-cell line inhibited HIV-1 replication [22]. More interestingly, when E2 expressing Jurkat cells and naive MT-2 cells were cocultured, E2 significantly inhibited HIV-1 replication in MT-2 cells. However, the inhibitory effect was not observed when Jurkat/E2 and MT-2 cells were separated using a transwell system. This suggests that the inhibitory effect of E2 is dependent on cell-to-cell contact. It would be interesting to see whether the inhibitory effect is due to the defect of Gag membrane targeting.

The observed defect in Gag plasma membrane targeting in the presence of glycol-E2 expression may be attributed to several factors. One possibility is that glycol-E2 may redirect Gag trafficking to intracellular compartments. As illustrated in Figure 4, HIV-1 Gag was found to primarily localize on the plasma membrane or intracellularly, in a punctate pattern within the perinuclear region. In contrast, coexpression with glycol-E2 markedly changed Gag distribution to a pattern
within the cytoplasm. This observation suggests that glycol-E2 mislocalizes Gag trafficking to other compartments, thereby preventing it from trafficking to the plasma membrane. The observation that E2 lacking a signal sequence did not alter Gag trafficking reveals that E2 trafficking through the endoplasmic reticulum and Golgi apparatus is important for its inhibitory effect on Gag trafficking. It is well known that cellular factors, such as phospholipid phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P2], clathrin adaptor protein 3 (AP-3), Golgi-localized γ-ear containing Arf-binding (GGA), and ADP ribosylation factor (Arf), play important roles in HIV-1 Gag plasma membrane targeting [43, 49, 50]. The expression of glycol-E2 may render the cellular factor(s) unavailable to assist in Gag targeting to the plasma membrane. This warrants further investigation.

Because of the ability of HIV-1 to develop drug-resistant mutations, there is a constant need for the development of new therapeutic options for HIV-1–infected patients. In this study, we showed that E2 was able to inhibit HIV-1 replication by interfering with Gag trafficking to the plasma membrane. It suggests that when glycol-E2 is introduced into HIV-1–susceptible cells, these cells will be converted to nonsusceptible cells by blocking viral release. Importantly, glycol-E2 did not dramatically alter HIV-1 Pr55 precursor expression (Figures 1 and 2) but, instead, caused it to be retained intracellularly. This observation suggests that the accumulated viral proteins may eventually be presented to the host immune system to boost host immune responses against HIV-1 infection.

GBV-C E2 has been documented as an HIV-1–entry inhibitor in a number of studies. Our study suggests that E2 blocks Gag targeting to the plasma membrane, resulting in the disruption of virus release. These results and those of previous studies support the hypothesis that E2 can inhibit multiple stages of HIV-1 replication. Taken together, along with the inhibitory effects of GBV-C NS5A, NS3, and other advantages of GBV-C, such as its nonpathogenicity, its ability to replicate in CD4+ T cells, and its shared transmission pathway with HIV-1, the inhibitory effects of E2 render GBV-C an attractive source for the development of novel anti-HIV/AIDS therapies.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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**Potential conflict of interest.** All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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