Structural Domains of the Herpes Simplex Virus 1 gD Protein That Restrict Human Immunodeficiency Virus Particle Infectivity

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ABSTRACT Previously, we showed that the presence of the herpes simplex virus 1 (HSV-1) gD glycoprotein but not gB potently restricted HIV-1 particle infectivity. This restriction was characterized by incorporation of HSV-1 gD and the exclusion of HIV-1 gp120/gp41 from budding virus particles. To determine the structural domains involved in gD restriction of HIV-1, a series of deletion mutants and chimeric proteins involving combinations between gD and the nonrestrictive gB were generated. Our results show that deletion of the cytoplasmic tail domain (CTD) of gD or replacement of the transmembrane domain (TMD) with the TMD from gB slightly reduced restriction activity. However, replacement of the gD CTD with that of gB resulted in lower cell surface expression, significantly less incorporation into HIV-1 particles, and inefficient restriction of the release of infectious HIV-1. Analysis of gB/gD chimeric proteins revealed that removal of the gB CTD or replacement with gD CTD resulted in enhanced surface expression and an increase in restriction activity. Finally, we show that expression of gD without other HSV-1 proteins resulted in gD fractionation into detergent-resistant membranes (DRM) and that gD colocalized with the raft marker GM1, which may partially explain its incorporation into budding virus particles. Taken together, our results suggest that expression of gD at the cell surface is likely a major factor, but other intrinsic properties are also involved in the gD-mediated restriction of HIV-1 particle infectivity.

IMPORTANCE Previously, we showed that the presence of the gD glycoprotein in virus producer cells but not gB potently restricted HIV-1 particle infectivity. To better understand the relationship between cell surface expression, virus incorporation, and restriction of HIV-1, we analyzed a series of deletion mutants and chimeric proteins in which domains of gD and gB were swapped. Our results indicate that (i) gD/gB chimeras having the cytoplasmic domain (CTD) of gB significantly reduced cell surface expression, release from cells, incorporation into virus, and reduced HIV-1 restriction, (ii) removal of the gB CTD or replacement with the gD CTD resulted in better surface expression, incorporation into HIV-1, and enhanced restriction, and (iii) the transmembrane domain of gB can influence transport and ultimately effect incorporation of gB into HIV-1. Overall, these data support a role for gD surface expression as crucial to restriction of infectious HIV-1 release.

KEYWORDS HIV-1 restriction, HSV-1, chimeric proteins, envelope glycoprotein, gB, gD, gp120/gp41

Previously, we showed that the herpes simplex virus 1 (HSV-1) gD protein potently restricted the infectivity of HIV-1 particle production, while HSV-1 gB had no effect (1). The mechanism of restriction was not related to viral protein synthesis in the cell or release of virus particles but involved the incorporation of HSV-1 gD into virus particles while excluding the HIV-1 Env glycoprotein (gp120/gp41). HSV-1 gD, the receptor binding protein of HSV-1, is synthesized and transported to the cell plasma membrane, as with many viral membrane glycoproteins. However, once at the cell plasma

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membrane, gD is endocytosed to the *trans*-Golgi network (TGN) or an endosomal compartment through the interaction with HSV-1 gM or gK/UL20, which is considered the site for final envelopment of HSV-1 (2–8). HSV-1 gD does not have canonical tyrosine-based or dileucine motifs in its cytoplasmic domain used by many membrane proteins for internalization, which explains why ectopic expression results in gD found predominantly at the cell plasma membrane (8). In contrast, the gB glycoprotein of HSV-1 did not significantly restrict HIV-1 but was incorporated into budding virus particles (1). While ectopic gB expression can be detected at the cell surface, most of the protein expressed is within enlarged intracellular vesicles, which were shown in one study to be multivesicular bodies (MVBs) (9). In a more recent study, investigators showed that gB induced giant vesicle formation due to the homotypic fusion of early endosomes. In this study, gB was found to colocalize with early endosomal antigen 1 (EEA-1) and Rab5 (10). The intracellular accumulation of gB has been associated with the presence of dileucine- and tyrosine-based motifs at positions 871 and 889, respectively, and a phosphorylated threonine at position 887 (11–15). Truncation of gB at amino acid residue 871 results in gB expression at the cell surface, while truncation at amino acid 805 resulted in gB retention in the rough endoplasmic reticulum (12). Studies showed that the tyrosine motif ⁸⁸⁹YTQV⁸⁹² acts as an internalization signal, while the dileucine motif ⁸⁷¹LL⁸⁷² is thought to facilitate retrograde transport to the Golgi complex (13, 16).

In this study, we examined which domains of the gD protein were required to restrict HIV-1 particle infectivity and sought to determine if domain swaps between gD and gB would enhance the ability of gB proteins to restrict HIV-1 infectivity. We constructed gD and gB deletion mutants and gD/gB and gB/gD chimeric proteins. Our results show that more than one domain plays an important role in restriction by gD but that gD/gB chimeric proteins containing the cytoplasmic domain of HSV-1 gB were less efficient at restricting HIV-1 particle infectivity. Our results also show that restriction of virus particle infectivity from producer cells expressing gD deletion mutants or the presence of chimeric proteins containing the gD ectodomain (ED) was correlated with the cell surface expression. Nevertheless, our data also suggest that a second mechanism is involved. We show that ectopic expression of gD can be detected in detergent-resistant membrane (DRM) fractions, which have been associated with microdomains known as lipid rafts. Since HIV-1 has been shown to mature through lipid rafts, these studies may partially explain the incorporation of gD into budding viral particles and the restriction of HIV-1 particle infectivity from virus producer cells.

RESULTS

Expression of the deletion mutants and chimeric proteins. Previously, we showed that virus producer cells expressing gD lacking the transmembrane and cytoplasmic tail (gD[ΔTMCT]) resulted in a secreted protein that lacked restriction activity of HIV-1, while another type I glycoprotein of HSV-1, gB, did not significantly restrict HIV-1 (1). As the gD and gB glycoproteins had different phenotypes with respect to HIV-1 restriction, we constructed a series of gD and gB deletion mutants, chimeric gD/gB and gB/gD proteins, and site-directed mutant proteins of gD and gB to interrogate if a specific domain of gD was responsible for HIV-1 restriction. A schematic summarizing the structure of the gD deletion mutants, chimeric gD/gB proteins, gB deletion mutants, and chimeric gB/gD proteins analyzed in this study is shown in Fig. 1. For simplicity, proteins were designated based on the origin of the ectodomain (ED), the transmembrane domain (TMD), and the cytoplasmic tail domain (CTD). For example, the chimeric protein gDDB would have the ED and TMD of gD fused to the CTD of gB, and the deletion mutant gDD would have the ED and TMD of the gD protein but lacks the CTD.

Restriction of HIV-1 virion production by gD, gD deletion mutants, and gD/gB chimeric proteins. The proteins depicted in Fig. 1 were analyzed for the restriction of HIV-1 virion production. 293 cells were transfected with gD (positive control for restriction), gB (negative control for restriction), truncated gD proteins (gD[ΔTMCT]; gDD), or gD/gB chimeric proteins and pNL4-3. At 48 h, the culture medium was collected and clarified with low-speed centrifugation, and the levels of HIV-1 p24 were determined

FIG 1 Structure of the chimeric proteins and mutants analyzed in this study. The deletion mutants and chimeric proteins were designated based on the origin of the ectodomain (ED), transmembrane (TMD), and cytoplasmic tail domain (CTD). Site-directed mutants were designated based on the amino acid, position, and the amino acid substitution. For gDDB_{LLV}, the dileucine motif at position ⁸⁷¹LL⁸⁷² and the tyrosine motif at position ⁸⁸⁹YTQV⁸⁹² (based on the numbering of gB) were altered to ⁸⁷¹AL⁸⁷² and ⁸⁸⁹ATQV⁸⁹², respectively. For gB_{LLV}, the same amino acids were altered (based on the numbering of gB). The approximate locations of the site-directed changes in gDDB_{LLV} and gB_{LLV} are indicated by stars (★).

using Western blots. The levels of p24 were normalized and equivalent levels used to quantify levels of infectious virus using TZM.bl cell assays. The presence of gD restricted the release of infectious HIV-1 (~0.1%), while the presence of gD[ΔTMCT] did not significantly affect levels of infectious virus released (Fig. 2A). Cotransfection of cells with gDD, gDB, or gDBD and pNL4-3 restricted HIV-1 infectivity to between 2.1 and 7.8% of that of the pcDNA3.1(+) control, respectively. The results indicate that removal of the gD CTD made a small difference in restriction compared with the unmodified gD, although the restriction was statistically very significant ($P < 0.005$) compared with the pcDNA3.1(+) control or gB. Analysis of virus producer cells expressing gD [ΔTMCT] revealed that it did not restrict HIV-1 particle infectivity (81.1%), which was similar to that of gB. Finally, analysis of virus producer cells expressing chimeric proteins gDDB, gDBB, and gDDB_{LLV} revealed that these proteins restricted HIV-1 particle infectivity to between 10.7 and 42.2% of that of the pcDNA3.1(+) control, respectively. These results indicate that the presence of the CTD of gB altered the ability of gD to restrict HIV-1 virion production, and mutagenesis of the internalization motifs (⁸⁷¹LL⁸⁷² to ⁸⁷¹AL⁸⁷² and ⁸⁸⁹YTQV⁸⁹² to ⁸⁸⁹ATQV⁸⁹²) also significantly reduced restriction. The truncated gD and gD chimeric proteins were expressed at comparable levels using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a loading control (Fig. 2B).

Restriction of the release of infectious HIV-1 by gB, gB deletion mutants, and gB/gD chimeric proteins. Previously, we showed that the gB protein did not significantly restrict HIV-1 particle infectivity (1). As previously reported, expression of gD is much higher at the cell surface than that of gB. We determined if (i) deletion of certain domains of gB or (ii) substitution of certain domains of gB with those regions from gD (gB/gD chimeric proteins) would enhance gB cell surface expression and increase the restriction of release of infectious HIV-1. We analyzed the ability of the unmodified gB, gB deletion mutants (gBB), gB/gD chimeric proteins (gBD, gBDB, gBBD, and gBDD), and gB site-directed mutants (gB_{LLV}) to restrict HIV-1 particle infectivity using the same methodology described for the gD constructs (Fig. 2). The unmodified gB did not significantly restrict the release of infectious HIV-1 [70.5% compared with the pcDNA3.1

FIG 2 Restriction of the release of infectious HIV-1 by gD deletion mutant and gD/gB chimeric proteins. 293 cells were cotransfected with either the empty pcDNA3.1(+) vector or vectors expressing the gD deletion mutant and gD/gB chimeric proteins described in Fig. 1. At 48 h, the culture supernatants were collected and subjected to low-speed centrifugation to remove cellular debris, and levels of infectious virus released into the culture supernatants were determined using TZM-bl cell assays. (A, upper graph) Level of infectious virus released into the culture medium from cells cotransfected with pcDNA3.1(+) or vectors expressing gD, gB, gD deletion mutants, or gD/gB chimeric proteins and pNL4-3. (Lower panel) Virus released in the culture medium was normalized for p24 using an antibody directed against p24 and Western blots. (B) Western blots showing expression of gD proteins from infectivity assays in panel A. Expression of GAPDH was used for normalization of gD proteins analyzed in the restriction assay in panel A using Western blots and an antibody directed against gD. All restriction assays were performed at least five times, and statistical differences from the pcDNA3.1(+) HIV-1 control were evaluated using a two-tailed Student's *t* test, with *P* < 0.01 (▲) considered significant.

(+) control], while removal of the CTD (gBB) or replacement of the gB CTD with the same region from gD (gBBD) enhanced the ability to restrict the release of infectious HIV-1 [17.4 and 23.1%, respectively, compared with the pcDNA3.1(+) control] (Fig. 3A). Virus producer cells expressing chimeric gB/gD proteins gBD and gBDD restricted HIV-1 particle infectivity to 18.1 and 50.8%, respectively, compared with the pcDNA3.1(+) control (Fig. 3A). However, the gDBB construct did not restrict HIV-1 particle infectivity (77.9%) compared with the pcDNA3.1(+) control (Fig. 3A). Finally, gB_{LL/YY}, with its

FIG 3 Restriction of release of infectious HIV-1 by gB, gB deletion mutants, and gB/gD chimeric proteins. 293 cells were cotransfected with either the empty pcDNA3.1(+) vector or vectors expressing gB, gD, gB deletion mutants, and gB/gD chimeric proteins described in Fig. 1. At 48 h, the culture supernatants were collected and subjected to low-speed centrifugation to remove cellular debris, and levels of infectious virus released into the culture supernatants were determined using TZM-bl cell assays. (A, upper graph) Level of infectious virus released into the culture medium from cells cotransfected with pcDNA3.1(+) or vectors expressing gB, gD, gB deletion mutants, or gB/gD chimeric proteins and pNL4-3. (Lower panel) Virus released in the culture medium was normalized for p24 using an antibody directed against p24 and Western blots. (B) Western blots showing expression of gB proteins from infectivity assays in panel A. (Lower gel) Expression of GAPDH was used for normalization of gB proteins analyzed in the restriction assay in panel A. All restriction assays were performed at least five times, and statistical differences from the pcDNA3.1(+) HIV-1 control were evaluated using a two-tailed Student's *t* test, with $P < 0.01$ (▲) considered significant.

dileucine- and tyrosine-based internalization motifs altered (⁸⁷¹LL⁸⁷² to ⁸⁷¹AL⁸⁷² and ⁸⁸⁹YTQV⁸⁹² to ⁸⁸⁹ATQV⁸⁹²), restricted HIV-1 particle infectivity at 43.1% compared to the control (Fig. 3A). Analysis of the gB proteins from the restriction assays revealed that the gB and gD proteins were expressed well in cell lysates (Fig. 3B).

Cellular expression of gD deletion mutant proteins and gD/gB chimeric proteins.

We next analyzed the surface expression of gD and various gD mutant proteins by immunofluorescence and confocal microscopy. For these experiments, we used COS-7 cells as this cell line has been previously used to analyze the surface expression of gD and gB proteins (1). COS-7 cells were transfected with vectors expressing each

FIG 4 Expression of gD, gD deletion mutants, and gD/gB chimeric proteins using confocal microscopy. (A) Surface expression of gD proteins. COS-7 cells were transfected with vectors expressing gD, the gD deletion mutants, or gD/gB chimeric proteins. At 24 h, cells on coverslips were washed, fixed, and blocked. The coverslips were reacted with a mouse monoclonal antibody against HSV-1 gD overnight at 4°C followed by an appropriate secondary antibody for 1 h. Cells were washed and counter stained with DAPI (1 μ g/ml) for 5 min as described in Materials and Methods. The coverslips were mounted and examined with a Leica TCS SPE confocal microscope using a 63 $\times$ objective with a 2 $\times$ digital zoom and the Leica Application Suite X (LAS X) software package. A 405-nm filter was used to visualize DAPI, and a 594-nm filter was used to visualize gD staining. (B) Expression of gD proteins following permeabilization. COS-7 cells were transfected with vectors expressing gD, the gD deletion mutants, or gD/gB chimeric proteins. At 24 h, cells on coverslips were washed, fixed, and permeabilized as described in Materials and Methods. The coverslips were reacted with a mouse monoclonal antibody against HSV-1 gD overnight at 4°C followed by an appropriate secondary antibody for 1 h. Cells were washed, counterstained with DAPI (1 μ g/ml) for 5 min, and viewed as described above. For each construct, a minimum of 100 cells were examined, and micrographs are representative of what was observed.

construct, and at 24 h posttransfection, cells were fixed and analyzed for gD cell surface expression by immunofluorescence, as described in Materials and Methods. The results show that gD was readily detected on the cell surface, as were gDD, gDB, and gDBD (Fig. 4A). In contrast, transfection of cells with constructs gDDB, gDDB_{LLY}, and gDBB resulted in little or no gD expression on the cell surface (Fig. 4A). We also examined the same constructs by immunofluorescence following permeabilization with Triton X-100, which showed that gDDB, gDDB_{LLY}, and gDBB were expressed at intracellular compartments of the cells (Fig. 4B). We also quantified cell surface expression in 293 cells using flow cytometry. Cells were transfected with equivalent amounts of each plasmid for 24 h, and the cells were removed from the dish by treatment of cells with Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS) containing EGTA and EDTA. The cell suspensions were washed and reacted with an anti-gD monoclonal antibody followed by an appropriate secondary antibody and analyzed by flow cytometry. The mean fluorescent intensity (MFI) was determined using FlowJo software. The pcDNA3.1(+) control had an MFI of 178, while the unmodified gD protein had an MFI of 11,726 and gD [ΔTMCT] had an MFI of 136, which was expected as gD[ΔTMCT] is readily secreted from cells (Fig. 5A). The MFI of the deletion mutant gDD was slightly lower than that of gD (9,420), suggesting that the CTD had little impact on cell surface expression, while the MFI of the chimeric protein gDB (3,848) was approximately a third of that of gD (11,725), which suggests that the TMD contributes to gD cell surface

FIG 5 Expression of unmodified gD and gB, deletion mutants of gD and gB, and chimeric gD/gB and gB/gD proteins by flow cytometry. (A) Comparison of cell surface expression of gD, gD deletion mutants, and gD/gB chimeric proteins using flow cytometry. 293 cells were transfected with either the empty pcDNA3.1(+) vector or pcDNA3.1(+) expressing the gD deletion mutants and chimeric proteins. At 24 h posttransfection, cells were removed from plates by treatment with Ca²⁺- and Mg²⁺-free PBS containing 1mM EGTA and EDTA. The cells were reacted with an anti-gD monoclonal antibody, followed by reaction with an appropriate secondary antibody. Cells were analyzed with an BD Aria II flow cytometer, and FlowJo software was used to calculate the mean fluorescent intensity (MFI). In the histograms, the gray areas show anti-gD signal and the black areas show the signal of empty vector-stained cells. The table shows the mean and median fluorescent intensity signals of the gD signals minus the background signal of the empty vector-stained cells. (B) Comparison of cell surface expression of gB, gB deletion mutants, and gB/gD chimeric proteins by flow cytometry. 293 cells

(Continued on next page)

expression. The influence of the TMD on cell surface expression was further shown by chimeric gDBD, which had an MFI of 4,380. This suggests that the gD TMD and CTD may somehow interact to stabilize gD cell surface expression. Finally, the three chimeric gD/gB proteins containing the CTD of gB (gDDB, gDDB_{LL/Y}, and gDBB) had much lower MFIs of 2,600, 1,552, and 1,299, respectively. These results indicate that in the context of the gDDB_{LL/Y}, alteration of the dileucine and tyrosine motifs, previously shown to be involved in endocytosis of gB from the cell surface, actually decreased surface expression.

Intracellular expression of gB, the gB deletion mutant, and gB/gD chimeric proteins. We also analyzed the cell surface expression of gB, various gB deletion mutants, and gB/gD chimeric proteins by immunofluorescence. COS-7 cells were transfected with vectors expressing each construct, and at 24 h posttransfection, cells were fixed but not permeabilized and processed for immunofluorescence using anti-gB antibodies. Transfection of cells with vectors expressing gB or gBB, gBD, gBDD, gBBD, or gB_{LL/Y} constructs showed that gB was detectable at the cell surface, except for the gBDB construct, which was not detected at the cell surface (Fig. 6A). We also analyzed gB expression in transfected cells that were fixed and permeabilized. Unlike the cell surface immunostaining, we detected expression of all gB constructs in all cultures transfected with each of the vectors expressing the gB constructs followed by permeabilization (Fig. 6B). Immunostaining of gB-transfected cells showed that gB expression was primarily detected in large intracellular vesicles (Fig. 6B), which has been previously attributed to the CTD and is similar to what has been previously reported (9, 10, 12, 17). Similar results were obtained for constructs gBB, gBBD, and gBDD. In cells transfected with the vector expressing gBDB, the expression pattern was confined to intracellular organelles, which may reflect a lack of processing of N-linked glycans seen in Fig. 3B, while in cells transfected with the vector expressing gB_{LL/Y}, expression appeared confined to smaller vesicles. The cell expression levels of the various gB mutants were also quantified using flow cytometry analysis, as we had done for the various gD mutants (Fig. 5B). The pcDNA3.1(+) control had an MFI of 374, while the unmodified gB protein had an MFI of 3,706 (Fig. 5B). The gB_{LL/Y} mutant had an MFI of 3,106, which was unexpected as the endocytosis signals were removed from the CTD. The MFIs of cells transfected with vectors expressing gBB, gBD, gBBD, and gBDD were 5,255, 9,558, 6,569, and 7,454, respectively. Finally, the cells transfected with the vector expressing gBDB had the lowest MFI of the gB constructs at 176.

Incorporation of gD, gD deletion mutant proteins, and gD/gB chimeric proteins into viral particles. Previously, we showed that gD was incorporated into HIV-1 particles, while the HIV-1 gp120/gp41 glycoprotein was largely excluded (1). We analyzed the gD deletion mutants and chimeric gD/gB proteins for incorporation into viral particles. Cells were cotransfected with vectors expressing the gD deletion mutant proteins or gD/gB chimeric proteins and pNL4-3. At 48 h posttransfection, the culture medium was harvested, and lysates were prepared from the cell pellets. The culture medium was clarified by low-speed centrifugation followed by ultracentrifugation to pellet the virus through a 20% sucrose cushion. HSV-1 gD proteins were immunoprecipitated from cell lysates and culture medium prior to ultracentrifugation and from the virus pellet following ultracentrifugation. Immunoprecipitation of gD proteins from the cell lysates confirmed that all gD proteins were expressed in transfected cells (Fig. 7A). Immunoprecipitation of gD proteins revealed that gD, gB, gD[ΔTMCT], gDD, gDBD, and gDB were easily detected in the culture medium, while gDDB, gDDB_{V/LL}, and gDBB were barely detectable in the medium (Fig. 7B). Immunoprecipitation of HIV-1 proteins

FIG 5 Legend (Continued)

were transfected with either the empty pcDNA3.1(+) vector or pcDNA3.1(+) vector expressing gB, gB deletion mutants, or gB/gD chimeric proteins. At 24 h posttransfection, the cells were processed as described above and reacted with an anti-gB monoclonal antibody, followed by reaction with an appropriate secondary antibody. Cells were analyzed with a BD Aria II flow cytometer. In the histograms, the gray areas show anti-gB signal and the black areas show the signal of empty vector-stained cells. The table shows the mean and median fluorescent intensity signals of the gB signals minus the background signal of the empty vector-stained cells.

FIG 6 Expression of gB, the gB deletion mutants, and gB/gD chimeric proteins using confocal microscopy. (A) Surface expression of gB proteins. COS-7 cells were transfected with vectors expressing gB or each of the gB deletion mutants and gB/gD chimeric proteins. At 24 h, cells on coverslips were washed, fixed, and blocked. The coverslips were reacted with a mouse monoclonal antibody against HSV-1 gB and an appropriate secondary antibody and counterstained with DAPI (1 μ g/ml) for 5 min as described in Materials and Methods. The coverslips were mounted and examined with a Leica TCS SPE confocal microscope using a 63 $\times$ objective with a 2 $\times$ digital zoom using the Leica Application Suite X (LAS X) software package. A 405-nm filter was used to visualize DAPI, and a 594-nm filter was used to visualize gB staining. (B) Expression of gB proteins following permeabilization. COS-7 cells were transfected with vectors expressing gB, the gB deletion mutants, or gB/gD chimeric proteins. At 24 h, cells on coverslips were washed, fixed, and permeabilized as described in Materials and Methods. The coverslips were reacted with a mouse monoclonal antibody against HSV-1 gB overnight at 4°C followed by an appropriate secondary antibody for 1 h. Cells were washed and counterstained with DAPI (1 μ g/ml) for 5 min and viewed as described above. For each construct, a minimum of 100 cells were examined, and micrographs are representative what was observed.

showed that viral proteins were present in the cell lysates and the culture medium (Fig. 7C and D). Following ultracentrifugation through a 20% sucrose cushion, equivalent levels of p24 were immunoprecipitated using an anti-HIV-1 cocktail and the levels of Env (gp120) were determined (Fig. 7E). Our results showed that the constructs that showed no restriction (gB and gD[Δ TMCT]) had larger amounts of gp120 in the pelleted virus (Fig. 7E). Our results show that gD, gD[Δ TMCT], gDD, gDBD, and gDB were detected in virus pellets, while gDDB, gDDB_{YLL}, and gDBB were barely detected in the virus pellets (Fig. 7F). We also examined the incorporation of HIV-1 Env into the virus particles in the presence of truncated and chimeric gD proteins. Using densitometry, we determined the ratio of Env to p24 (minus background) in pelleted virus (Fig. 7G). In the presence of truncated/chimeric gD proteins, the level of Env incorporation was reduced by gD but not in the presence of gD[Δ TMCT] or gB. For other truncated and chimeric gD proteins, the levels of Env incorporation varied but followed a similar trend with Env incorporation and infectivity. To confirm the incorporation of gD, gD[Δ TMCT], gDD, gBBD, gDB into virus, the pelleted virus was loaded onto 20 to 60% sucrose gradients and subjected to ultracentrifugation as described in Materials and Methods (Fig. 8). Fractions were collected and analyzed for gD proteins and HIV-1 proteins, as we have previously described (1). The virus from cells cotransfected with

FIG 7 Incorporation of gD, gD deletion mutants, and gD/gB chimeric proteins into HIV-1 particles. 293 cells were cotransfected with either empty pcDNA3.1(+) vector and pNL4-3, a vector expressing gD and pNL4-3, or vectors expressing different gD deletion mutants or chimeric gD/gB protein and pNL4-3. At 30 h, the cells were starved for methionine/cysteine and radiolabeled, and then the culture medium was harvested at 48 h posttransfection. The cell culture medium was harvested and subjected to low-speed centrifugation, and the culture supernatants were layered onto a 20% sucrose cushion. The virus in the culture supernatants was pelleted by ultracentrifugation. The lysates, culture medium, and viral pellets were analyzed for the presence of gD, gD/gB chimeric proteins, gD mutated proteins, gB (using an anti-gB antibody), and HIV-1 proteins using immunoprecipitation and appropriate antibodies (anti-gD or anti-HIV as described in Materials and Methods). (A) Immunoprecipitation of gD proteins from lysates prepared from cells cotransfected with empty pcDNA3.1(+) or pcDNA3.1(+) expressing gD, gD deletion mutants, or gD/gB chimeric proteins and pNL4-3. (B) Immunoprecipitation of gD proteins from the culture medium of cells cotransfected with empty pcDNA3.1(+) or pcDNA3.1(+) expressing gD, gD deletion mutants, or gD/gB chimeric proteins and pNL4-3. (C) Immunoprecipitation of HIV-1 proteins from the cell lysates of cells cotransfected with empty pcDNA3.1(+) or (Continued on next page)

pcDNA3.1(+) and pNL4-3 was isolated predominantly in fractions 5 to 8 of the sucrose gradients (Fig. 8A), which is what we previously reported (1). Virus producer cells cotransfected with the vector expressing gD and pNL4-3 revealed that p24 and gD were detected primarily in fractions 5 to 8 but that gp120 was largely absent (Fig. 8B and C). Not unexpectedly, the truncated gD (gD[ΔTMCT]) was not detected in virus produced from cells cotransfected with the vector expressing gD[ΔTMCT] and pNL4-3 but rather remained at the top of the gradient (Fig. 8D and E). The results with gDD, gDBD, and gDB indicated that these proteins and p24 were detected in fractions 5 to 8, but gp120 was nearly undetectable (data not shown). As constructs gDDB, gDBB_{LL/YY}, and gDBB were not detected in the viral pellets, they were not analyzed by sucrose density gradients.

Incorporation of gB deletion mutants and gB/gD chimeric proteins into viral particles. We also analyzed whether gB, gB deletion mutants, and gB/gD chimeric proteins were incorporated into virus particles using the same procedures used for the gD proteins. Our results indicate that all proteins were synthesized in cells, although they suggested gBDB may have an altered glycosylation pattern, which may cause its localization to intracellular compartments (Fig. 9A). All the gB proteins with the exception of gBDB were released into the cell culture medium (Fig. 9B). HIV-1 proteins were synthesized in the cells and released into the culture medium (Fig. 9C and D). Our results indicate that ultracentrifugation of the culture medium through a 20% sucrose cushion resulted in all gB proteins being detected in the viral pellet, with the exception of gBDB (Fig. 9E and F). We also analyzed the ratio of HIV-1 Env to p24 in pelleted virus in the presence of the different truncated and chimeric gB proteins as we had done for the gD proteins (Fig. 7G). For those truncated or chimeric proteins that exhibited higher levels of restriction (gBB, gBBD, and gBD), the level of Env incorporation was between 50 and 63% compared with the pcDNA3.1(+) control. Furthermore, those chimeric or mutant gB proteins with lower levels of restriction (gB_{LL/YY}, gBDB, and gBDD) had high levels of HIV-1 Env incorporation (87.5 to 94.5%) compared with the pcDNA3.1(+) control or gB (95.5 to 100%) (Fig. 9G). The incorporation of gB and gB_{LL/YY} into viral particles was confirmed on 20 to 60% sucrose gradients, where the gB proteins colocalized with virus p24 fractions, as previously reported (Fig. 10A to D). Sucrose density gradient analysis of virus particles released into the culture medium revealed that gBB, gBD, gBBD, and gBDD were also incorporated into virus (data not shown). Since gBDB was not released into the culture medium with virus, it was not analyzed on sucrose gradients. These results correlated with cell surface expression of the gB ectodomain proteins.

gD but not gB is found in detergent-resistant membranes and colocalizes with GM1. Previous studies have shown that HIV-1 matures through lipid microdomains known as lipid rafts, which have higher concentrations of cholesterol, glycosphingolipids, and gangliosides (18, 19). In a previous study, it was stated (but not shown) that gD and gH/gL partitioned into detergent-resistant membrane (DRM) fractions in transfected 293T cells (20). However, no studies have examined lipid rafts for the presence of gD when expressed in the absence of other HSV-1 proteins. We hypothesized that ectopic expression of gD would lead to its presence on the cell plasma membrane and incorporation into lipid rafts. We transfected 293 cells with the vector expressing gD, and at 24 h, detergent-resistant membranes were isolated using flotation gradients, as described in Materials and Methods. Internal controls included ganglioside GM1 (a well-studied raft component) and transferrin receptor (a well-studied non-raft protein).

FIG 7 Legend (Continued)

pcDNA3.1(+) expressing gD, gD deletion mutants, or gD/gB chimeric proteins and pNL4-3. (D) Immunoprecipitation of HIV-1 proteins from clarified culture medium prepared from cells cotransfected with empty pcDNA3.1(+) or pcDNA3.1(+) expressing gD, gD deletion mutants, or gD/gB chimeric proteins and pNL4-3. (E) Immunoprecipitation of HIV-1 proteins from the virus pellets following ultracentrifugation of the culture medium from cells cotransfected with empty pcDNA3.1(+) or pcDNA3.1(+) expressing gD, gD deletion mutants, or gD/gB chimeric proteins and pNL4-3. A lane was removed between the lanes for gD and gD[ΔTMCT]. (F) Immunoprecipitation of gD proteins from the virus pellet after the culture supernatants were subjected to ultracentrifugation through a 20% sucrose cushion. (G) The ratio of HIV-1 Env to p24 from pelleted virus analyzed by densitometry of the gel in panel E.

FIG 8 Glycoprotein D but not gD[ΔTMCT] is incorporated into partially purified virus. 293 cells were cotransfected with either empty pcDNA3.1(+) vector and pNL4-3, a vector expressing gD and pNL4-3, or a vector expressing gD [ΔTMCT] and pNL4-3. At 30 h, the cells were starved for methionine/cysteine and radiolabeled, and then the culture medium was harvested at 48 h posttransfection. Following low-speed centrifugation, the culture supernatants were layered onto a 20% sucrose cushion, and virus was pelleted by ultracentrifugation. The pelleted virus was resuspended in DMEM without serum and layered on a discontinuous 20 to 60% sucrose gradient. The virus was subjected to ultracentrifugation for 20 h (76,000 × *g*, SW55Ti rotor), and 12 fractions were collected and subjected to immunoprecipitation analysis using appropriate antibodies against HIV-1 p24 and gp120/gp41, HSV-1 gD, or gD [ΔTMCT]. The immunoprecipitates were collected on protein A-Sepharose, washed, and boiled in sample reducing buffer. The proteins were separated by 10% SDS-PAGE and visualized using standard radiographic techniques. (A) Immunoprecipitation of HIV-1 proteins from gradient fractions of cells cotransfected with empty pcDNA3.1(+) vector and pNL4-3. (B) Immunoprecipitation of HIV-1 proteins from gradient fractions of virus produced from cells cotransfected with a vector expressing gD and pNL4-3. (C) Immunoprecipitation of gD proteins from gradient fractions of virus produced from cells cotransfected with a vector expressing gD and pNL4-3 (from the experiment in panel B). (D) Immunoprecipitation of HIV-1 proteins from gradient fractions of virus produced from cells cotransfected with a vector expressing gD[ΔTMCT] and pNL4-3. (E) Immunoprecipitation of gD[ΔTMCT] proteins from gradient fractions of virus produced from cells cotransfected with a vector expressing gD[ΔTMCT] and pNL4-3 (from the experiment in panel D).

Our results indicate that while most of the gD was observed in non-raft fractions, gD was reproducibly detected in raft fractions 2 and 3 (Fig. 11A). As expected, GM1 was observed in the raft fractions 2 and 3, while transferrin receptor was only observed in the non-raft fractions (10–12) (Fig. 11B and C). We compared these results with those from cells infected with HSV-1 for 24 h, which showed that gD was also found raft fractions (Fig. 11D). Finally, we analyzed cells transfected with the vector expressing gB to determine if it was associated with DRMs. Our results indicate that gB was not associated with DRM fractions (Fig. 11E). In addition to gradient analysis of DRM fractions, we determined if gD colocalized with the lipid-free glycan derived from GM1 (oligo-GM1) (Fig. 12). COS-7 cells were transfected with the vector expressing gD for 48 h, and cells

FIG 9 Incorporation of gB, gB deletion mutants, and gB/gD chimeric proteins into HIV-1 particles. 293 cells were cotransfected with either empty pcDNA3.1(+) vector and pNL4-3, a vector expressing gB, gB deletion mutants, or gB/gD chimeric proteins and pNL4-3, or vectors expressing gB deletion mutants or chimeric gB/gD protein and pNL4-3. At 30 h, the cells were starved and radiolabeled, and then the culture medium was harvested at 48 h posttransfection as described in Fig. 6. Following low-speed centrifugation, the culture supernatants were layered onto a 20% sucrose cushion, and virus was pelleted by ultracentrifugation. The clarified culture medium, cell lysates, and virus pellets were analyzed by immunoprecipitation using appropriate antibodies (anti-HIV, anti-gD, or anti-gB) as described in Materials and Methods. (A) Immunoprecipitation of gB proteins from lysates prepared from cells cotransfected with empty pcDNA3.1(+) or pcDNA3.1(+) expressing gB, gB deletion mutants, or gB/gD chimeric proteins and pNL4-3. (B) Immunoprecipitation of gB proteins from the culture medium of cells cotransfected with empty pcDNA3.1(+) or pcDNA3.1(+) expressing gB, gB deletion mutants, or gB/gD chimeric proteins and pNL4-3. (C) Immunoprecipitation of HIV-1 proteins from the cell lysates prepared from cells cotransfected with empty pcDNA3.1(+) or pcDNA3.1(+) expressing gB, gB deletion mutants, or gB/gD

(Continued on next page)

were washed and reacted with cholera toxin subunit B (CTB) fluorescently conjugated with Alexa Fluor 488 and with an anti-gD mouse monoclonal antibody. CTB is known to bind with the GM1 ganglioside of the membrane microdomains to cause oligomerization (21). Cells were washed with PBS (pH 7.4) and fixed with 4% paraformaldehyde. Cells were washed and reacted with a rabbit anti-mouse secondary antibody conjugated with Alexa Fluor 594 and visualized by laser scanning confocal microscopy. While gD could be observed on the cell plasma membrane, we detected regions on the membrane where gD and CTB colocalized (as evidenced by the yellow color). These results suggest that a fraction of the expressed gD was associated with GM1 (Fig. 12).

DISCUSSION

We previously showed that virus producer cells expressing gD potently restricted HIV-1 particle infectivity (1). While our results showed that gD was incorporated into HIV-1 particles, it also prevented the incorporation of the HIV-1 Env into budding virus particles and was not simply due to the shedding of gp120. Ectopic expression of gD was previously shown to be expressed on the cell surface, with little internalization from the surface (2, 4). In a study with pseudorabies virus (another alphaherpesvirus), gD was fused to the cytoplasmic tails of gB, gE, or gM (22). While the unmodified gD or gD lacking the cytoplasmic tail (gDc) was found predominantly at the cell surface, expression of the chimeric gD proteins with cytoplasmic tails of gB (gDB) or gM (gDM) resulted in expression predominantly within intracellular compartments (22). Our study confirms that ectopic expression of gD resulted in uniform surface expression, while gB resulted in a more punctate staining of the cell surface (1).

An important question that needs to be addressed is, "What is the mechanism used by HSV-1 gD that is responsible for decreased incorporation of HIV-1 Env into budding virus particles and hence decreased infectivity?" We showed that removal of the TMD and CTD (gD[ΔTMCCT]) resulted in a truncated gD that was readily secreted from cells and abolished the ability of gD to restrict the release of infectious HIV-1, suggesting that a membrane-bound protein was essential for restriction activity (1). Here, using a deletion mutant and chimeric protein approach with the gD (restrictive) and gB (non-restrictive) proteins, we have investigated (i) the domains of gD that are responsible for the observed restriction, (ii) if surface expression of deletion mutants and chimeric gD proteins correlated with the level of restriction, and (iii) if a chimeric gB protein could be constructed that potently restricted the release of infectious HIV-1. The chimeric protein approach we utilized involved designing proteins with complete ED, TMD, and CTD domains, the rationale being that this would be less destructive to the overall structure of the protein. One possibility is that expression of gD, which lacks signals for internalization, led to efficient gD expression at the cell surface, which allowed its incorporation over gp120/gp41. However, we believe this is unlikely as the nonrestrictive gB was also incorporated into virus along with HIV-1 Env.

Our results with the gD deletion mutants and gD/gB chimeric proteins indicate the following. First, the gD CTD contributed only marginally to gD restriction of HIV-1 infectivity (mutant gDD that restricted at 2.1% versus gD at ~0.1%). Second, the replacement of the gD TMD with the gB TMD (chimeric proteins gDB and gDBD) reduced the levels of restriction to 7.8 and 3.5%, respectively, suggesting that the gD TMD may slightly contribute to the restriction of HIV-1 particle infectivity. Third, the

FIG 9 Legend (Continued)

chimeric proteins and pNL4-3. (D) Immunoprecipitation of HIV-1 proteins from clarified culture medium from cells cotransfected with empty pcDNA3.1(+) or pcDNA3.1(+) expressing gB, gB deletion mutants, or gB/gD chimeric proteins and pNL4-3. (E) Immunoprecipitation of gB proteins from viral pellets derived from cells cotransfected with empty pcDNA3.1(+) or pcDNA3.1(+) expressing gB, gB deletion mutants, or gD/gB chimeric proteins and pNL4-3. (F) Immunoprecipitation of HIV-1 proteins from viral pellets derived from cells cotransfected with empty pcDNA3.1(+) or pcDNA3.1(+) expressing gB, gB deletion mutants, or gB/gD chimeric proteins and pNL4-3. (G) The ratio of HIV-1 Env to p24 from pelleted virus was analyzed by densitometry of the gel in panel E.

FIG 10 Glycoprotein B but not gBDB is incorporated into partially purified virus. 293 cells were cotransfected with either empty pcDNA3.1(+) vector and pNL4-3, a vector expressing gB and pNL4-3, or a vector expressing gB_{LLV} and pNL4-3. At 30 h, the cells were starved for methionine/cysteine and radiolabeled, and then the culture medium was harvested at 48 h posttransfection. Following low-speed centrifugation, the culture supernatants were layered onto a 20% sucrose cushion, and virus was pelleted by ultracentrifugation. The pelleted virus was resuspended in DMEM without serum and layered on a discontinuous 20 to 60% sucrose gradient. The virus was subjected to ultracentrifugation for 20 h (76,000 × *g*, SW55Ti rotor), and 12 fractions were collected, and subjected to immunoprecipitation analysis using appropriate antibodies against HIV-1 and gB proteins. The immunoprecipitates were collected on protein A-Sepharose, washed, and boiled in sample reducing buffer. The proteins were separated by 10% SDS-PAGE and visualized using standard radiographic techniques. (A) Immunoprecipitation of HIV-1 proteins from gradient fractions of virus produced from cells cotransfected with a vector expressing gB and pNL4-3. (B) Immunoprecipitation of gB proteins from gradient fractions of virus produced from cells cotransfected with a vector expressing gB and pNL4-3 (from the experiment in panel A). (C) Immunoprecipitation of HIV-1 proteins from gradient fractions of virus produced from cells cotransfected with a vector expressing gB_{LLV} and pNL4-3. (D) Immunoprecipitation of gB_{LLV} proteins from gradient fractions of virus produced from cells cotransfected with a vector expressing gB_{LLV} and pNL4-3 (from the experiment in panel C).

biggest change in the restriction came from gD/gB chimeric proteins having the CTD of gB (gDDB, gDDB_{LLV}, and gDBB). While all three had reduced expression at the cell surface and were not incorporated into budding virus particles, they restricted HIV-1 virion production, although at lower levels (gDDB at 10.7%, gDDB_{LLV} at 42.1%, and gDBB at 11.2%), with gDDB and gDBB considered statistically significant. While statistically significant, they were not nearly as efficient as gD. Interestingly, removal of the two of the internalization signals from the gB CTD (gDDB_{LLV}), previously shown to be involved

FIG 11 Glycoprotein D is observed in the detergent-resistant membrane (DRM) fractions in the presence and absence of other herpesvirus proteins. 293 cells were transfected with the vectors expressing gD and transferrin receptor for 48 h, cells were collected, and cell extracts were prepared as described in Materials and Methods. Fractions were collected from the top of the gradient and analyzed by Western blotting for the presence of gD (A) or the presence of GM1 by dot blotting (B), as well as the presence of transferrin receptor (TfR) (C) and gB (E). In a separate experiment, 293 cells were infected with HSV-1 (F) at an MOI of 1 and incubated for 24 h. The cells were collected and DRMs isolated as described in Materials and Methods and analyzed for the presence of gD (D). For all gradients, fraction 1 represents the top of the gradients and fraction 12 the bottom of the gradient.

in internalization of gB, did not enhance cell surface expression and did not lead to incorporation into virus. Thus, while we are unable to identify a specific domain that was responsible for restricting infectivity, these results suggest that some level of gD cell surface expression was required to efficiently restrict HIV-1 particle infectivity. Hence, surface expression plays a role in restricting HIV-1 particle infectivity, but our data also suggest that the mechanism that gD uses to reduce HIV-1 virions is beyond the high expression of gD at the cell surface.

Based on the correlation between gD cell surface expression restriction, we attempted to modify the gB protein (either by creating deletion mutants or gB/gD chi-

FIG 12 Expression of gD results in colocalization with ganglioside GM1. COS-7 cells were transfected with a vector expressing gD for 48 h, and cells were reacted with cholera toxin subunit B (CTB) tagged with Alexa Fluor 488. The cells were fixed (but not permeabilized) and reacted with a mouse monoclonal antibody against gD, then washed and reacted with a secondary goat anti-mouse monoclonal antibody tagged with Alexa Fluor 594. Cells were once again washed, mounted, and examined using a Leica TCS SPE confocal microscope with a 63 $\times$ objective with a 2 $\times$ digital zoom and the Leica Application Suite X (LAS X) software package. A 405-nm filter was used to visualize DAPI, a 488-nm filter was used to visualize CTB, and a 594-nm filter was used to visualize gD staining.

meric proteins) such that it was expressed at higher levels on the cell surface and would reduce HIV-1 particle infectivity. In a previous study, a C-terminal truncation of gB by 33 amino acids (gB871) removed both tyrosine-based and dileucine-based internalization signals and resulted in this protein being transported to the cell surface (12). Our results using gB deletion mutants and chimeric gB/gD chimeric proteins indicate the following. First, our results indicate that the increased cell surface expression of gBB, gBD, gBDD, and gBBD correlated with enhanced restriction, although the level of restriction was <1- to 4-fold, which was far less than the restriction observed with gD. It is unknown why our gBB and gBD proteins trafficked to the cell surface more efficiently than that previously reported in $\Delta 847$ and $\Delta 857$ mutants. However, we used native, untagged proteins, while in the study discussed above, the investigators used a gB in which the gene for enhanced green fluorescent protein (EGFP) was inserted near the N terminus as a marker (12). Second, the mutation of the dileucine- and tyrosine-based internalization signals in the CTD of gB (gB_{LLY}) resulted in slightly decreased cell surface expression and restricted the production of HIV-1 virions at ~43% compared to gB at 70.5%. These results suggest that the presence of the gB CTD increased HIV-1 particle infectivity. The third and most unexpected finding came from the chimeric protein gBDB. The gBDB protein was like the unmodified gB in the level of restriction (77.9% versus 70.5%) but was observed at the cell surface at low levels (4.2% compared to gB) and was not incorporated into virus particles. The low level of surface expression was confirmed by the immunofluorescence and flow cytometry studies. The results with gBDB indicate that the TMD can influence the transport of a glycoprotein to the cell surface. It is possible that the sequence of the gD TMD may have led to alterations in the overall structure of gBDB, which in turn altered its intracellular transport properties.

Lipid rafts are membrane microdomains that are enriched in sterol and sphingolipids, which act as signaling and sorting platforms for numerous molecules (18, 23–26). Many virus families (enveloped and nonenveloped) also depend on plasma membrane cholesterol or lipid rafts for efficient entry or release (27). These include the coronaviruses (28), enteroviruses (29, 30), filoviruses (31, 32), herpesviruses (33–37), orthomyxoviruses (38–43), orthopoxviruses (44), paramyxoviruses (45–49), reoviruses (50), and retroviruses (51). HIV-1 has also been shown to enter and mature through lipid microdomains at the cell surface (27, 52–55). We showed that ectopic expression of gB and gD resulted in the incorporation into HIV-1 particles. We also showed that gD, but not gB, when expressed without other HSV-1 proteins, partitioned with GM1 (a well-characterized raft marker) into DRMs (56). We also analyzed if gD would partition into DRMs during an HSV-1 infection. We found that gD was found in DRMs, similar to transfected cells. In addition to DRM fractionation, we used an alternative approach involving microscopy to analyze gD association with lipid rafts. Cholera toxin subunit B (CTB) can bind up to five copies of its ganglioside receptor (GM1) on the plasma membrane of the host cell (57, 58). The multivalent binding of CTB to ganglioside GM1 results in the reorganization of the membrane structure by cross-linking otherwise small and transient lipid rafts. We found that CTB colocalized with gD in live cells, providing additional support that gD was likely associated with lipid rafts and that gD is incorporated into rafts without other HSV-1 proteins.

Four models have been proposed for the incorporation of the HIV-1 Env into virus particles (59–61). These include the passive model, the cotargeting model, in which Gag and Env are targeted to common sites of the membrane (via lipid rafts) (27, 62–65), the direct interaction model of Gag and Env proteins (66–72), and finally the indirect interaction model, in which Gag and Env interact via a cellular adaptor (73–75). Our data show that an increase in surface expression of gD correlated with a restriction of HIV-1 particle infectivity. However, our data also indicate that gD did not significantly interfere with HIV-1 release. While the chimeric and truncated proteins analyzed in this study show that higher surface expression plays a role in restricting the release of virions, we still do not completely understand the mechanism by which gD, but not gB, prevents the incorporation of HIV-1 Env. HIV-1 assembly is initiated by interaction

of the myristoylated matrix (MA) domain of the Gag precursor with the inner side of the plasma membrane and the interaction of the MA high basic region (HBR) with the phosphatidylinositol 4,5-bisphosphate (PI_{4,5}P₂) (76). There is evidence that the formation of the Gag trimerization and formation of the lattice at the cell plasma membrane are required for Env incorporation. Furthermore, mutations in the Gag MA protein or the cytoplasmic tail of gp41 (gp41CT) can affect Env incorporation, suggesting an interaction between the MA and the gp41CT. It is possible that gD may block the interaction between Gag MA and the gp41CT, favoring its incorporation over the HIV-1 Env. Our results indicate that the cytoplasmic domain of gD is likely not involved. It is possible that the TMD or the ectodomain of gD is involved. Alternatively, the presence of gD may segregate cellular factors involved in Env incorporation from sites of virus assembly. Thus, the use of the gD glycoprotein may facilitate the identification of such factors or sites and assist in the development of novel antiviral therapeutic drugs that inhibit the assembly of infectious HIV-1 particles.

MATERIALS AND METHODS

Cells, viruses, and plasmids. HEK293 cells were used for transfection of vectors expressing the HSV-1 proteins and the HIV-1 genome. The TZM-bl cell line, which was used as an indicator to measure virus infectivity, was obtained from the NIH AIDS Reagent Branch. Both cell lines were maintained in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (R10FBS), 10 mM HEPES buffer (pH 7.3), 100 U/ml penicillin, 100 µg/ml streptomycin, and 5 µg gentamicin. A plasmid with the entire HIV-1 NL4-3 genome (pNL4-3) was obtained from the NIH AIDS Reagent Branch. The plasmid with the HSV-1 gB gene was kindly provided by Richard Longnecker (Northwestern University). Expression of HSV-1 gD and gB constructs was confirmed by transfection with the Turbofect transfection reagent (ThermoFisher) and Western blot analysis using appropriate antibodies. For gD and gB constructs, expression was confirmed using mouse monoclonal antibodies directed against each protein: for gB, Thermo-Fisher, MA1-19265, antibody T111; for gD, Santa Cruz Biotechnology, SC-21719, antibody DL6.

Construction of chimeric proteins and site-directed mutants. We constructed a series of chimeric proteins between HSV-1 gD and gB proteins from HSV-1 strain F. The nomenclature for the chimeric proteins was based on the origin of the ectodomain (ED), transmembrane domain (TMD), and cytoplasmic tail domain (CTD). Thus, chimeric protein gDBD has the ED of gD, the TMD of gB, and the CTD of gD. We grouped these chimeric proteins into two groups based on the origin of the ectodomain. Thus, the gD/gB chimeric proteins included gDBD, gDB, gDDB, gDBB, and gDDB_{LLVY} while the gB/gD chimeric proteins included gBD, gBBD, gBDB, and gBDD. Those proteins with a deletion of either of the three domains described above would have a two-letter designation (i.e., gBB has the ED and TMD of gB but lacks the CTD). These chimeric proteins were synthesized by Symbio Technologies and inserted into the pcDNA3.1(+) vector. The entire gene was sequenced to ensure that no errors were made during its synthesis. Deletion mutants of gD (gD[ΔTMD]; gDD) and gB (gBB) were constructed by site-directed mutagenesis using a QuikChange II site-directed mutagenesis kit (Agilent) according to the manufacturer's protocol. The gD[ΔTMD] and gDD constructs have stop codons inserted at the end of the gD ED and TMD, respectively. Construct gBB has a stop codon inserted at the end of TMD (amino acid position 797). Constructs gDDB_{LLVY} and gB_{LLVY} have the dileucine- and tyrosine-based internalization motifs altered (⁸⁷¹LL⁸⁷² to ⁸⁷¹AL⁸⁷² and ⁸⁸⁹VTQV⁸⁹² to ⁸⁸⁹ATQV⁸⁹²). All plasmid inserts were sequenced to ensure the validity of the mutations and that no other mutations were introduced during the mutagenesis or cloning processes. The expression of the mutant proteins was confirmed using immunoblots and mouse monoclonal antibodies directed against gD or gB.

Surface and intracellular localization studies of gD and gB constructs. We analyzed the surface and intracellular localization of gD, gB, gD deletion mutants, gB mutants, and gD/gB and gB/gD chimeric proteins. COS-7 cells were transfected with either empty pcDNA3.1(+) vector or the vector expressing gD or gB constructs. At 24 h, cells on coverslips were washed, fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and blocked in 5% bovine serum albumin (BSA). The cells on coverslips were reacted with a mouse monoclonal antibody against HSV-1 gD (Santa Cruz; DL6, sc-21719) or against gB (Thermo-Fisher; MA1-19265, antibody T111) overnight at 4°C. The coverslips were washed and then reacted with a secondary Alexa Fluor 594-conjugated rabbit anti-mouse IgG (Abcam 150128) for 2 h and counterstained with DAPI (4',6-diamidino-2-phenylindole; 1 µg/ml) for 5 min. The coverslips were examined using a Leica SP8 confocal microscope with a 100× objective and a 2× digital zoom using the Leica Application Suite X (LAS X) software package. A 405-nm-pore filter was used to visualize the DAPI staining and a 594-nm filter to visualize Alexa Fluor 594. We also analyzed the surface expression localization of various gD and gB proteins. COS-7 cells were transfected with either the empty pcDNA3.1(+) vector or one expressing gD or gB constructs. At 24 h, cells on coverslips were washed, fixed, and blocked. The cells on coverslips were reacted with the same antibodies described above and examined by confocal microscopy as described above. For each construct, a minimum of 100 cells were examined.

Analysis of infectious virus production in the presence of HSV-1 proteins. To analyze the virus restriction properties of the proteins depicted in Fig. 1, 293 cells were transfected with either empty pcDNA3.1(+) vector or a vector expressing one of the HSV-1 proteins (gD, gB, or gD/gB or gB/gD chimeric proteins, or gD or gB mutants) and pNL4-3. For all restriction assays, we used the unmodified gD as the positive control and HSV-1 gB as a negative control for restriction of infectious HIV-1 release. At

48 h posttransfection, the culture medium was collected and then clarified by low-speed centrifugation. Levels of p24 were determined in the collected culture supernatants by Western blots, and samples with equivalent levels of p24 were analyzed for levels of infectious virus by titration on TZM-bl cells (1, 77–80). TZM-bl cells have an integrated copy of a retrovirus vector expressing β -galactosidase under the control of HIV-1 long terminal repeat (LTR). If TZM-bl cells are infected with HIV-1, Tat protein expression will result in the expression of the β -galactosidase and infected cells, as visualized by fixing and staining with X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). All assays were performed at least four times and analyzed for statistical significance using a two-tailed Student's *t* test, with cells cotransfected with the empty pcDNA3.1(+) and pNL4-3 set at 100% infectivity.

Analysis of HIV-1 viral proteins in the presence of gD and gB proteins. We analyzed the synthesis of HIV-1 proteins in the presence of the HSV-1 proteins listed in Fig. 1. 293 cells in 35-mm-diameter dishes were cotransfected with either empty pcDNA3.1(+) or vectors expressing gD, gB, gD/gB or gB/gD chimeric proteins, or gD or gB mutant proteins and pNL4-3. At 30 h posttransfection, cells were washed, starved for methionine/cysteine for 2 h, and radiolabeled with 500 μ Ci of [35 S]methionine/cysteine for 16 h. At 48 h posttransfection, the culture medium was harvested and cell lysates were prepared in 1 $\times$ radioimmunoprecipitation assay (RIPA) buffer. Following centrifugation of the culture medium at 800 $\times g$ for 10 min, an aliquot of the clarified medium was retained, and the remaining medium was layered onto a 20% sucrose cushion and subjected to ultracentrifugation at 110,000 $\times g$ in an SW55Ti rotor for 2 h. The pellet, culture medium before ultracentrifugation, and cell lysates were made 1 $\times$ with respect to RIPA buffer, and HIV-1 gD and gB proteins were immunoprecipitated using anti-HIV, anti-gD, or anti-gB antibodies and protein A-Sepharose. The immunoprecipitated proteins were washed three times with 1 $\times$ RIPA buffer, and the protein A-Sepharose beads were boiled in sample reducing buffer. The proteins were separated using SDS-PAGE, and proteins were visualized using standard radiographic techniques.

Gradient analysis of viruses. We analyzed the incorporation of gp120/gp41 and the proteins depicted in Fig. 1 into virus particles. 293 cells in 35-mm dishes were cotransfected with either empty pcDNA3.1(+) or vector expressing gD, gB, gD/gB or gB/gD chimeric proteins, or gD or gB mutant proteins and pNL4-3. At 30 h posttransfection, cells were washed, starved for methionine/cysteine for 2 h, and radiolabeled with 500 μ Ci of [35 S]methionine/cysteine for 16 h. The culture medium was harvested and centrifuged at 800 $\times g$ for 10 min. The supernatant was then pelleted through a 20% sucrose cushion at 110,000 $\times g$ in an SW55Ti rotor for 2 h. The pellet was resuspended in 200 μ l of serum-free medium (DMEM). The resuspended pellet was loaded into a 20 to 60% discontinuous sucrose gradient and subjected to ultracentrifugation at 76,000 $\times g$ for 20 h in a SW55Ti rotor. At this point, the 12 fractions were collected and HIV-1, gD, and gB proteins were immunoprecipitated as described above.

Flow cytometry to analyze surface expression. 293 cells were transfected with empty vector pcDNA3.1(+) or pcDNA3.1(+) vector expressing the proteins listed in Fig. 1. At 48 h posttransfection, the cells were washed in once Ca^{2+} - and Mg^{2+} -free PBS, and incubated in Ca^{2+} - and Mg^{2+} -free PBS (pH 7.4) containing 1 mM EDTA and EGTA for 10 min on ice. Cells were washed twice with ice-cold PBS and harvested by centrifugation (300 $\times g$ for 5 min). The cells were incubated in DMEM containing FBS and monoclonal antibodies against HSV-1 gD (mouse monoclonal sc-21719; Santa Cruz) or gB (MA1-19265; Invitrogen) for 1 h. The cells were washed three times in ice-cold PBS (pH 7.4) (with centrifugation at 300 $\times g$ for 5 min at 4°C) and incubated with a secondary goat anti-mouse antibody tagged with Alexa Fluor 594 in DMEM containing FBS for 1 h. After another three wash cycles with 1 ml ice-cold PBS each (centrifugation at 250 $\times g$ for 5 min at 4°C), the cells were resuspended in DMEM without FBS and subjected to flow cytometric analysis using a BD LSR II flow cytometer. The negative controls included cells transfected with the empty pcDNA3.1(+) vector and the vector expressing gD[Δ TMCT] (which expresses a secreted gD protein), followed by immunostaining, as described above. The mean and median fluorescent intensities were calculated using the FlowJo software program. The mean and median fluorescent intensities shown in Fig. 4 include the value minus the background, except for the pcDNA3.1(+) control, which shows the MFI background in the text.

Isolation of detergent-resistant membrane fractions on sucrose gradients. For isolation of detergent-resistant membrane (DRM) fractions on sucrose gradients, we used the procedure of Chen and colleagues (81). Briefly, 293 cells were cultured in six-well dishes for 24 h and then cotransfected with the vector expressing gD and one expressing CD71 (transferrin receptor) using Turbofect reagent. At 24 h posttransfection, cells were washed twice with PBS and lysed in ice-cold DRM buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA) containing 1% Triton X-100 on ice for 20 min and pushed through a 22-gauge needle 10 to 15 times. The lysates were briefly centrifuged at 300 $\times g$ for 5 min at 4°C. The supernatants were mixed with an equal volume of 80% sucrose in DRM and placed in the bottom of an ultracentrifuge tube. The cell lysate-sucrose mixture was overlaid with 8 ml 30% sucrose-DRM37 and 2 ml 5.0% sucrose-DRM37. Gradients were spun to equilibrium at 178,500 $\times g$ for 18 h (SW41 rotor; Beckman Coulter) at 4°C. Twelve 1-ml fractions were collected from the top and analyzed for gD and transferrin receptor by Western blotting. All experiments were performed at least three times. The blots shown are representative of all experiments. The ganglioside GM1 was used as a marker for DRM fractions, and transferrin receptor used as a marker for non-DRM fractions (24, 25, 57).

Identification of lipid rafts. COS-7 cells were plated on 13-mm coverslips in 35-mm plates and transiently transfected with empty pcDNA3.1 or pcDNA3.1(+) expressing HSV-1 gD using Turbofect (ThermoFisher) transfection reagent according to the manufacturer's instructions. The cultures were incubated at 37°C for 24 h, and the medium was removed and washed with 1 $\times$ Hanks balanced salt solution with 5% bovine serum albumin (HBSS-BSA). The cells were incubated with cholera toxin subunit B conjugated to CF 488A (Biotium catalog no. 00070) at a concentration of 1 μ g/ml on ice for 30 min.

Following the incubation, the cells were washed with 1× HBSS-BSA and with PBS. The cells were then fixed with 4% paraformaldehyde for 15 min, washed with 1× PBS, and incubated with PBS containing 5% BSA for 1 h. For colocalization of gD with GM1, the coverslips were incubated with a mouse monoclonal antibody against gD for 1 h at room temperature. The coverslips were washed with 1× PBS and incubated with the secondary antibody rabbit anti-mouse Ig conjugated with Alexa 594 (Invitrogen; A11062) for 1 h and counterstained with DAPI (4',6-diamidino-2-phenylindole; 1 µg/ml) for 5 min. The coverslips were washed and mounted in glycerol-containing mounting medium (SlowFade; Invitrogen). The coverslips were then examined using a Leica SP8 confocal microscope with a 100× objective and 2× digital zoom using Lecia Application Suite X (LAS X) software package. The cholera toxin CF 488A was observed using a fluorescein isothiocyanate (FITC) filter, and the HSV-1 gD stained with Alexa 594 was observed using a Texas Red filter.

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