Virion-incorporated PSGL-1 and CD43 inhibit both cell-free infection and transinfection of HIV-1 by preventing virus–cell binding

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HIV-1 particles incorporate various host transmembrane proteins in addition to viral Env glycoprotein during assembly at the plasma membrane. In polarized T cells, HIV-1 structural protein Gag localizes to the plasma membrane of uropod, a rear-end protrusion. Notably, uropod transmembrane proteins PSGL-1 and CD43 cocluster specifically with Gag assembling at the plasma membrane even in cells that do not form uropods. Recent reports have shown that expression of either PSGL-1 or CD43 in virus-producing cells reduces the infectivity of progeny virions and that HIV-1 infection reduces the cell surface expression of these proteins. However, the mechanisms for both processes remain to be determined. In this study, we found that virion incorporation of PSGL-1 and CD43 closely correlates with diminished virion infectivity. PSGL-1 and CD43 inhibited virus attachment to $CD4^+$ cells irrespective of the presence of Env. These proteins also inhibited virion attachment to $CD4^-$ lymphoid organ fibroblastic reticular cells that mediate transinfection of $CD4^+$ T cells. Consistent with the possibility that highly extended extracellular domains of these proteins physically block virus–cell attachment, the inhibitory effect of PSGL-1 required its full-length ectodomain. HIV-1 encoding Gag mutants that are defective in either coclustering with these host proteins or ESCRT-dependent particle release failed to reduce PSGL-1 on surface of infected cells. This study reveals an anti–HIV-1 mechanism that suppresses virus–cell attachment and a previously unappreciated process of HIV-1-mediated down-regulation of host antiviral proteins, both of which likely require virion incorporation of these proteins.

HIV-1 | PSGL-1 | CD43 | virus attachment | virus release

HIV-1 particle assembly, driven by the viral structural protein Gag, takes place at the plasma membrane. In this process, Gag multimerization on the cytoplasmic leaflet of the plasma membrane leads to the formation of virus particles, which incorporate the viral envelope glycoprotein Env before release from the cell surface (1). In addition to Env, some host transmembrane proteins, including restriction factors such as BST-2/tetherin and SERINCs, are incorporated into nascent virions (2–6).

In previous work, we showed that Gag expressed in polarized T cells localizes to uropods, rear-end protrusions (7, 8) where transmembrane proteins PSGL-1, CD43, CD44, ICAM-1, and ICAM-3 also accumulate (9–13). Interestingly, antibody copatching experiments and superresolution localization microscopy studies revealed that even in cells that do not form uropods, Gag specifically associates with plasma membrane microdomains that contain a subset of these uropod proteins (i.e., PSGL-1, CD43, and CD44). In contrast, ICAM-1 and ICAM-3 did not show such specific coclustering with Gag in the same cell types (14). For the efficient coclustering of Gag with PSGL-1, the highly basic region (HBR) within the matrix (MA) domain of Gag is required; Gag derivatives lacking HBR fail to cocluster with PSGL-1 even when they are bound to the plasma membrane (14). Coclustering of PSGL-1, CD43, and CD44 with Gag at the cell surface also requires basic sequences in the juxtamembrane region of these transmembrane proteins (14). Therefore, PSGL-1, CD43, and CD44 are likely to be specifically recruited to the sites of virus assembly via the MA domain and subsequently incorporated into progeny virions.

Our recent study demonstrated that virion-incorporated CD44 is required for transinfection of HIV-1 mediated by $CD4^-$ secondary lymphoid organ stromal cells, known as fibroblastic reticular cells (FRCs) (15). In this transinfection process, virion-incorporated CD44 binds the polysaccharide hyaluronan (HA), which in turn binds CD44 on the FRC surface. HIV-1 particles that are bound to (or "captured by") FRCs can be subsequently efficiently transferred to $CD4^+$ T cells compared to cell-free virions. In contrast to the potential proviral role of CD44, PSGL-1 and CD43 have been reported to reduce the infectivity of progeny virions when expressed in virus-producing cells (16, 17). Down-regulation of these proteins observed with HIV-1–infected cells, as well as evolutionary signatures in their sequences, supports the possibility that PSGL-1 and CD43 are HIV-1 restriction factors (16, 18, 19). However, the molecular mechanisms by which expression of either PSGL-1 or CD43 in virus-producing cells diminishes virion infectivity remain to be determined.

Here we report that virion incorporation of PSGL-1 and CD43 causes a reduction of virion infectivity. Both PSGL-1 and CD43 inhibit the attachment of virus particles to target T cells, a step that has not been known as a target of host antiviral mechanisms. This inhibition of virus–cell attachment occurs regardless of the

Significance

No host-encoded restriction factor identified thus far inhibits the first step of HIV-1 infection: virus–cell attachment. Here we demonstrate that virion-incorporated host transmembrane proteins PSGL-1 and CD43 inhibit attachment of HIV-1 particles to target cells. This inhibition of virus attachment occurs regardless of molecules mediating virus–cell binding and leads to inhibition of both cell-free infection and bystander cell-mediated transinfection. We also show that coclustering of HIV-1 structural protein Gag with PSGL-1 and subsequent progeny virion release contribute to depletion of PSGL-1 from an infected cell surface. Our study reveals an anti–HIV-1 mechanism in which virion-incorporated host transmembrane proteins block virus attachment. In addition, we report a previously unidentified role for Gag in viral down-regulation of antiviral proteins.

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The authors declare no competing interest.

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presence of Env and is dependent on the intact extracellular domains (ECDs) in the case of PSGL-1. We also demonstrate that coclustering of Gag with PSGL-1 and subsequent release of virus particles promote the reduction of surface PSGL-1 in HIV-infected cells, revealing a previously unreported mechanism of viral antagonism against host-encoded antiviral membrane proteins.

Results

Expression of PSGL-1 and CD43 in Virus-Producing Cells Diminishes Infectivity of Progeny Virions. It has been reported that ectopic expression of either PSGL-1 or CD43 in virus-producing 293T cells reduces the infectivity of progeny virions (16, 19). Furthermore, knocking out of PSGL-1 in virus-producing T cells was shown to enhance infectivity of released viruses (16). Consistent with these reports (16, 19), we observed that cotransfection of HeLa cells with an HIV-1 molecular clone pNL4-3 and either PSGL-1 or CD43 expression vector, but not an ICAM-3 expression vector, led to production of viruses with significantly reduced infectivity as measured using an indicator cell line, TZM-bl (Fig. 1*A*). The reduction of virion infectivity due to expression of PSGL-1 or CD43 in virus producer cells was also observed when a $CD4^+$ T cell line, A3.01, was inoculated with these viruses by spin infection (Fig. 1*B*).

Since HIV-1 particles have been observed to incorporate PSGL-1 and CD43 (14, 16, 20–22), we next sought to determine whether virion incorporation of these transmembrane proteins is necessary for the observed reduction in virion infectivity. We previously showed that deletion of the cytoplasmic tail of PSGL-1 (PSGL-1 ΔCT) and basic-to-neutral amino acid substitutions in a juxtamembrane polybasic sequence of the CD43 cytoplasmic tail (CD43 6A) attenuate Gag coclustering with PSGL-1 and CD43, respectively, at the plasma membrane in HeLa cells (14). As expected, in HeLa cells, PSGL-1 ΔCT and CD43 6A mutants were not efficiently incorporated into virions compared with WT PSGL-1 and WT CD43, respectively (Fig. 1*C*). Expression of these variants in virus-producing cells was significantly less suppressive to HIV-1 infection than that of WT proteins (Fig. 1*D*). Taken together, these results strongly suggest that PSGL-1 and CD43 inhibit HIV-1 infection when they can be incorporated into virions.

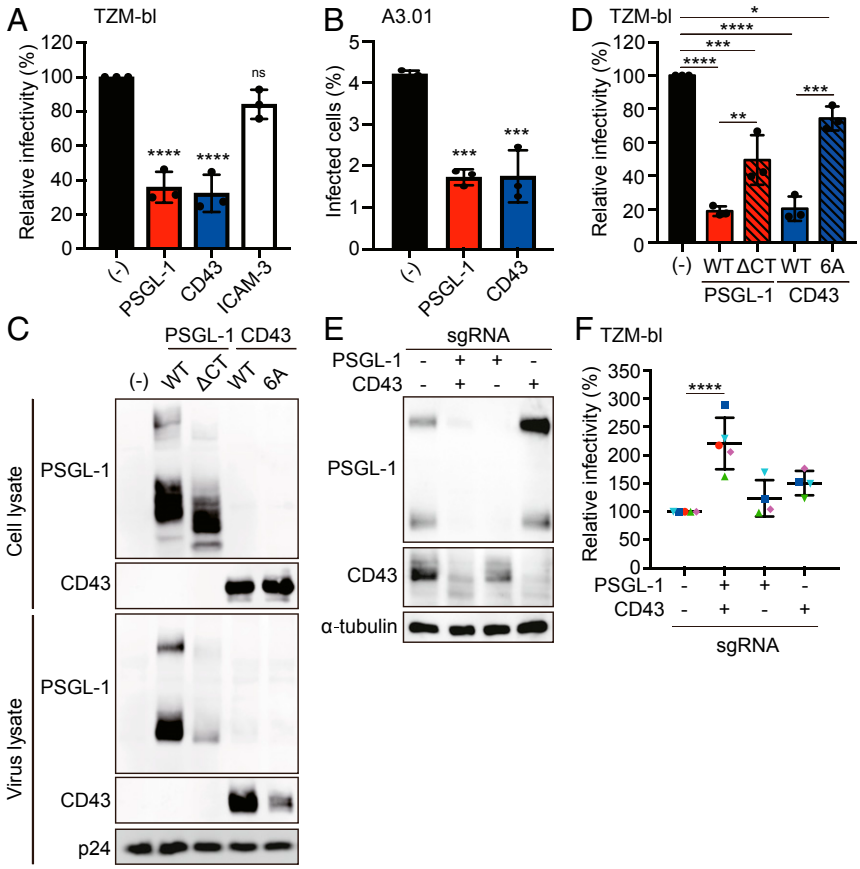

Fig. 1. PSGL-1 and CD43, which can be incorporated into virions, diminish the infectivity of progeny virions. (*A* and *B*) Virion infectivity analysis with TZM-bl cells (*A*) and A3.01 cells (*B*) using HIV-1. Viruses were produced by cotransfection of HeLa cells with pNL4-3 and either empty vector or an expression vector encoding PSGL-1, CD43, or ICAM-3. (*A*) TZM-bl cells were inoculated with the viruses in the presence of saquinavir. At 48 h postinfection, cells were lysed, and luciferase activity was measured using the cell lysates. (*B*) A3.01 cells were spinoculated with the viruses at 25 °C for 2 h. Cells were then cultured for 2 d in the presence of saquinavir and analyzed for Gag expression by flow cytometry. (*C* and *D*) HIV-1 was produced by cotransfection of HeLa cells with pNL4-3 and either empty vector or an expression vector encoding PSGL-1, PSGL-1 ΔCT, CD43, or CD43 6A. (*C*) Western blot analysis of incorporation of indicated proteins into virions. (*D*) Virion infectivity was analyzed by TZM-bl assay as described in *A*. (*E* and *F*) Primary $CD4^+$ T cells were treated with the CRISPR/Cas9 system targeting PSGL-1 and CD43. (*E*) Western blot analysis of PSGL-1 and CD43 expression. A representative result from two independent experiments with a total of four donors is shown. (*F*) Infectivity of virions produced by the CRSPR/Cas9-treated cells was determined by a TZM-bl assay as described in *A*. Three independent experiments with a total of five donors for negative control and double knockout and two independent experiments with a total of four donors for single knockout were performed. For the experiments shown in *A*, *B*, and *D*, the data represent the mean ± SD of three independent experiments. *P* values were determined using Bonferroni's test following one-way ANOVA. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$; ns, not significant.

We found that while virions produced from primary CD4$^+$ T cells contain a comparable amount of CD43 as virions generated from CD43-transfected HeLa cells, the level of endogenous PSGL-1 in T cell-derived virions was substantially lower than that in virions produced by HeLa cells ectopically expressing PSGL-1 (*SI Appendix*, Fig. S1). Therefore, we sought to determine whether endogenous PSGL-1 and CD43 are capable of suppressing virion infectivity. Endogenous PSGL-1 and CD43 in primary CD4$^+$ T cells were knocked out using the nucleofection-based CRISPR/Cas9 system. We found that expression of PSGL-1 and CD43 in the nucleofected cultures was substantially reduced (Fig. 1*E*). We observed an approximate twofold increase in infectivity on knockout of both proteins in virus-producing primary CD4$^+$ T cells (Fig. 1*F*). These results indicate that PSGL-1 and CD43 expressed endogenously in primary T cells are able to suppress the infectivity of progeny virions.

Attachment of Virus Particles to CD4$^+$ Target Cells Is Inhibited by PSGL-1 and CD43. Both PSGL-1 and CD43 are type-1 transmembrane proteins and sialomucins that are highly glycosylated with sialylated *O*-linked glycans (23–25). The ECDs of both PSGL-1 and CD43 are highly extended and estimated to be 45 to 50 nm long (26, 27). These extended ECDs could create a physical barrier that inhibits cell–cell interactions mediated by adhesion molecules. Consistent with this possibility, PSGL-1 and CD43 were shown to attenuate integrin-dependent cell adhesion (28, 29). Since PSGL-1 and CD43 inhibit HIV-1 infection when they are efficiently incorporated into virions (Fig. 1), we hypothesized that PSGL-1 and CD43 on the surface of virions physically block virus attachment to target cells.

To test whether virus attachment to CD4$^+$ target cells is a step susceptible to inhibition by virion-incorporated PSGL-1 and CD43, TZM-bl cells were inoculated with HIV-1 for 2 h at 4 °C, and virus attachment was quantified as the amount of p24 associated with cells after extensive washing. We found that ectopic expression of either PSGL-1 or CD43 in virus-producing cells diminished the binding of released viruses to TZM-bl cells, whereas expression of ICAM-3 did not (Fig. 2*A*).

As described above, ectopic expression of PSGL-1 or CD43 in virus-producing cells reduces infection of the T cell line A3.01 inoculated by spin infection (Fig. 1*B*). To test whether this can also be explained by a defect in virus–cell attachment, we inoculated A3.01 cells by spin infection performed as in Fig. 1 or by inoculation without spin, washed the cells extensively, and measured cell-associated p24. We observed reduced amounts of cell-associated p24 when PSGL-1 or CD43 was expressed in virus-producing cells irrespective of spin during inoculation (Fig. 2 *B* and *C*). Since the inoculation in these experiments was performed at room temperature (RT) as in Fig. 1, it remained possible that virus internalization, rather than attachment, is impaired when PSGL-1 or CD43 is expressed in virus-producing cells. However, we obtained identical results when spin infection was performed at 4 °C, where endocytosis is inhibited (Fig. 2*D*).

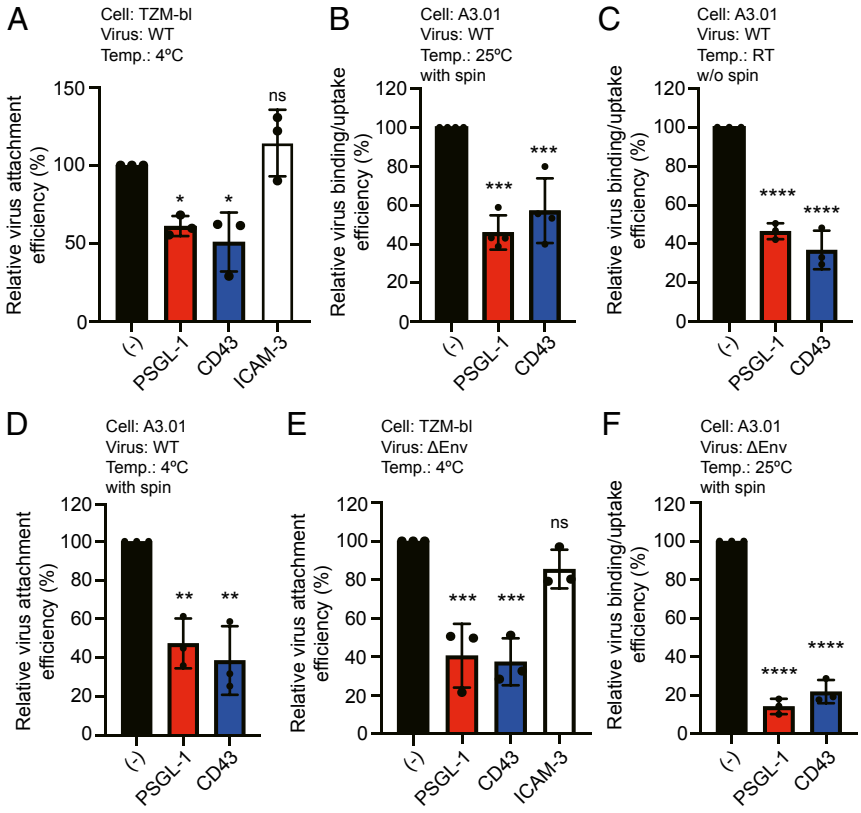

Fig. 2. PSGL-1 and CD43 inhibit virus attachment to target cells regardless of the presence of Env. (*A*–*F*) Cells were inoculated with viruses produced from HeLa cells, washed extensively, and lysed. The amount of cell-associated p24 was determined by p24 ELISA using the cell lysates. (*A*) TZM-bl cells were inoculated with WT viruses produced as in Fig. 1 and incubated for 2 h at 4 °C. (*B*–*D*) A3.01 cells were inoculated with WT viruses produced as in Fig. 1 with (*B* and *D*) or without (*C*) spinning for 2 h at 25 °C (*B*), RT (*C*), or 4 °C (*D*). (*E*) TZM-bl cells were inoculated with ΔEnv viruses produced as in *A* and incubated for 2 h at 4 °C. (*F*) A3.01 cells were inoculated with ΔEnv viruses produced as in *B* with spinning for 2 h at 25 °C. Data are presented as mean ± SD. The experiments were performed three times (*A*, *C*, *D*, *E*, and *F*) and four times (*B*) independently. The *P* values were determined using Bonferroni's test following one-way ANOVA. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; ns, not significant.

Taken together, these results indicate that PSGL-1 and CD43 suppress virus attachment to target cells.

PSGL-1 and CD43 Inhibit Virus–Cell Attachment Regardless of the Molecules Mediating Virus–Cell Attachment. Both Env-dependent and -independent mechanisms mediate HIV-1 attachment to cells (30, 31). To test whether virion-incorporated PSGL-1 and CD43 inhibit virus attachment through an effect on Env, we cotransfected HeLa cells with an HIV-1 molecular clone that does not express Env (ΔEnv) and either empty vector or an expression plasmid encoding PSGL-1, CD43, or ICAM-3. As observed with WT viruses, the expression of either PSGL-1 or CD43 in virus producer cells inhibited stable binding of the ΔEnv virus to TZM-bl cells and A3.01 cells (Fig. 2 *E* and *F*), indicating that PSGL-1 and CD43 do not specifically target Env-mediated attachment.

As described in the introductory paragraph, HA bound to virion-incorporated CD44 interacts with CD44 on the surface of FRCs (Fig. 3*A*) and thereby promotes virus binding to FRCs (virus capture) and subsequent transinfection of T cells (15). We found that expression of either PSGL-1 or CD43 in virus-producing cells also reduces virus capture by FRCs (Fig. 3 *B* and *C*). Of note, virion incorporation of either PSGL-1 or CD43 did not inhibit CD44 incorporation into virions (Fig. 3*D*). These results indicate that PSGL-1 and CD43 can block CD44-dependent binding of virions to FRCs. As reported previously (15), residual binding of virions to FRCs was observed on blocking of CD44 on the surface of FRCs (Fig. 3*B*) or removal of HA from virus particles (Fig. 3*C*). Notably, this residual binding is also susceptible to the expression of PSGL-1 and CD43 (Fig. 3 *B* and *C*). These results suggest that PSGL-1 and CD43 impair both CD44–HA interaction-dependent and -independent virus binding to FRCs. Taken together, the results presented thus far indicate that PSGL-1 and CD43 are capable of inhibiting both cell-free virus infection and transinfection via inhibition of virus-cell binding.

The Intact Extracellular Domain of PSGL-1 Is Necessary for Inhibition of Virus-Cell Attachment. To test whether the intact ECD of PSGL-1 is required for inhibition of virus attachment to target cells, we examined the effect of deletion of the PSGL-1 ECD on the inhibition of virus attachment. To maintain the epitope of an anti–PSGL-1 antibody, we decided to construct a partial deletion mutant of PSGL-1. PSGL-1 is highly O-glycosylated (23, 25), and the glycosylation is thought to extend the ECD (32). Since most of the O-glycosylation sites of PSGL-1 is likely in decameric

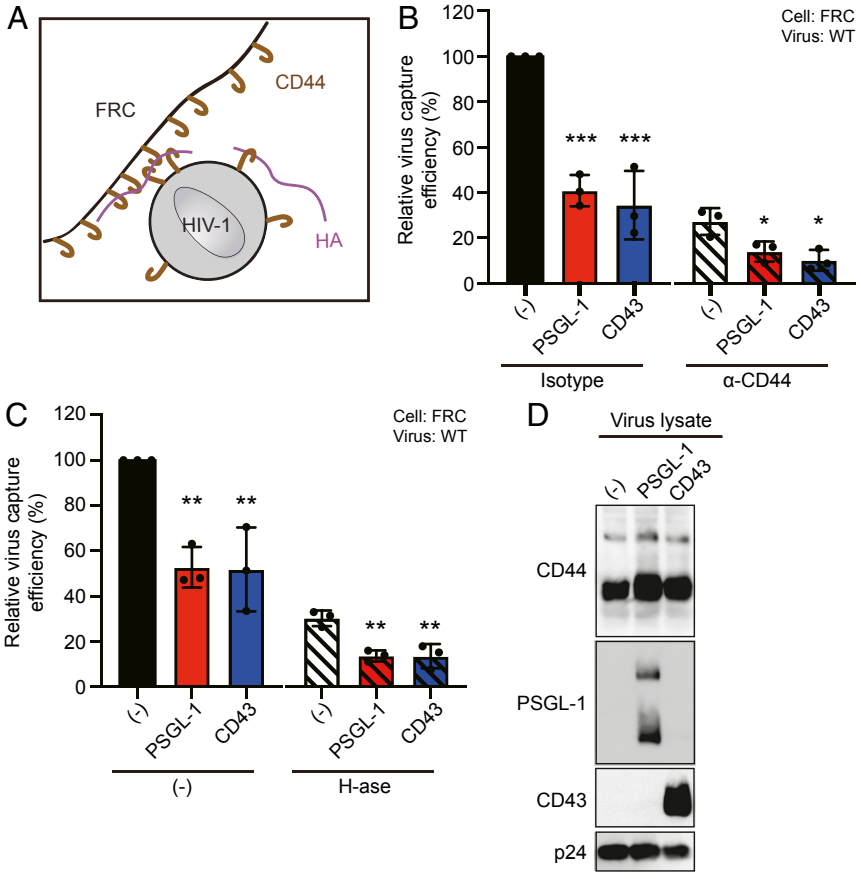

Fig. 3. PSGL-1 and CD43 suppress virus capture by FRCs regardless of molecules mediating virus–FRC binding. (*A*) A schematic model for virus capture by FRCs mediated by CD44–HA interactions. (*B* and *C*) FRCs were pulsed with HIV-1 prepared as in Fig. 1 for 2 h at 37 °C, washed extensively, and lysed. Then virus binding to FRCs (virus capture) was determined as the amount of cell-associated p24 by p24 ELISA. (*B*) FRCs were pretreated with either an anti-CD44 antibody or an isotype control antibody for 1 h at 37 °C before the addition of viruses. (*C*) Viruses were either untreated or pretreated with H-ase for 1 h at 37 °C prior to addition to FRCs, and FRCs were pulsed with the viruses. Data are presented as mean ± SD. The experiments were performed three times independently. FRCs were isolated from three different donors and used for each experiment. The *P* values were determined using Bonferroni's test following one-way ANOVA. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. (*D*) Western blot analysis of incorporation of CD44, PSGL-1, and CD43 into virions. HIV-1 was produced as in Fig. 1 by cotransfecting HeLa cells, which express endogenous CD44, with pNL4-3 and either empty vector (–) or an expression vector encoding PSGL-1 or CD43. The results shown are representative of three independent experiments.

repeats (between residues 118 and 261) (33), we deleted a region encompassing the entire decameric repeats from the ECD of PSGL-1 (PSGL-1 Δ118–277) (Fig. 4*A*). A similar deletion in CD43 caused a defect in stable expression and thus was not examined further. While PSGL-1 Δ118–277 was incorporated into virions at least as efficiently as WT PSGL-1 (Fig. 4*B*), PSGL-1 Δ118–277 was less inhibitory than WT PSGL-1 to attachment of virus to TZM-bl cells (Fig. 4*C*). These results indicate that the intact ECD of PSGL-1 is a determinant for the inhibition of virus attachment to target cells.

Coclustering of Gag with PSGL-1 and Subsequent Virus Release Promote Reduction of PSGL-1 on the Surface of Infected Cells. Previous reports have showed that PSGL-1 and CD43 expressed on cell surface are reduced upon HIV-1 infection and that the down-regulation of PSGL-1 requires Vpu (16, 17). However, expression of Vpu alone was shown to have only minor effects on cell surface levels of PSGL-1 and CD43 in contrast to the effects on tetherin or CD4 (34). Thus, it is possible that other viral factors play a role in this down-regulation of the surface PSGL-1 and CD43 levels. Since Gag coclusters with PSGL-1 and CD43 at the plasma membrane (14), we hypothesized that Gag contributes to down-regulation of PSGL-1 and CD43 via interactions with them at the membrane.

To test this hypothesis, we first examined whether membrane binding of Gag is necessary for depletion of cell surface PSGL-1 and CD43 via comparison of WT Gag and a Gag mutant that lacks the N-terminal myristoylation site (1GA). To monitor the expression of Gag, Gag was C-terminally fused with YFP (Gag-YFP). When A3.01 cells were inoculated with HIV-1 encoding WT Gag-YFP, PSGL-1, and CD43 on the surface of cells expressing Gag at high levels (Gag-YFP High) were significantly decreased compared with cells expressing no Gag-YFP [Gag-YFP(−)] or expressing it at low levels (Gag-YFP Low), confirming previous observations that HIV-1 infection down-regulates PSGL-1 and CD43 (16, 17). In contrast, infection of HIV-1 encoding 1GA Gag-YFP did not show such a decrease in surface PSGL-1 (Fig. 5*A* and *SI Appendix*, Fig. S2*A*). These results indicate that PSGL-1 down-regulation in infected cells requires Gag membrane binding. Likewise, Gag membrane binding contributed to efficient down-regulation of CD43, although a significant reduction in CD43 surface expression was still observed even in cells infected with HIV-1 encoding 1GA Gag (*SI Appendix*, Fig. S2*B*).

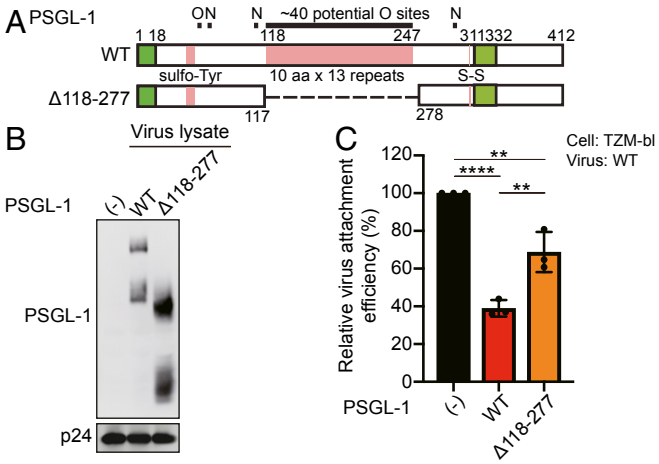

Fig. 4. The intact extracellular domain of PSGL-1 is necessary for inhibition of virus–cell attachment. (*A*) Schematic illustrations of WT and Δ118–277 PSGL-1. *N*- and *O*-glycosylation sites are shown. S-S is the disulfide bond, and green boxes represent the signal peptide and the transmembrane domain. (*B* and *C*) HIV-1 was produced by transfection of HeLa cells as in Fig. 1. (*B*) Western blot analysis of incorporation of indicated proteins into virions. (*C*) TZM-bl cells were inoculated with the viruses and incubated for 2 h at 4 °C. The amount of cell-associated p24 was determined by p24 ELISA. Data are presented as mean ± SD. The experiments were performed three times independently. The *P* values were determined using Bonferroni's test following one-way ANOVA. $^{**}P < 0.01$; $^{****}P < 0.0001$.

Since PSGL-1 down-regulation is entirely dependent on the presence of Gag at the plasma membrane, unlike CD43 down-regulation, we focused on PSGL-1 down-regulation for further study of the role of Gag in this process. In our previous study (14), we examined Gag coclustering with PSGL-1 at the plasma membrane using Gag constructs that have an N-terminal triple acylation sequence [Fyn(10)], which confers membrane binding ability to Gag regardless of the MA sequence. Fyn(10)-modified Gag containing the WT MA sequence [Fyn(10)/Gag] has been shown to localize to the plasma membrane (35). We showed that in the context of Fyn(10)-modified Gag, deletion of the entire MA domain (ΔMA) or substitution of all HBR basic amino acid residues with neutral ones (6A2T) impairs coclustering of Gag with PSGL-1 even though Gag multimers are present at the plasma membrane. These findings led us to conclude that Gag coclustering with PSGL-1 is dependent on the MA domain, especially the HBR (14). To test the role of coclustering between Gag and PSGL-1 in reduction of surface PSGL-1, we compared the Fyn(10)-modified Gag constructs containing the ΔMA [Fyn(10)/ΔMA] and 6A2T [Fyn(10)/6A2T] changes with Fyn(10)/Gag for PSGL-1 down-regulation. We found that HIV-1 encoding Fyn(10)/ΔMA and Fyn(10)/6A2T Gag failed to reduce PSGL-1 on the surface of cells, in contrast to HIV-1 encoding Fyn(10)/Gag (Fig. 5*B* and *SI Appendix*, Fig. S2*C*). These results revealed a clear correlation between coclustering of Gag with PSGL-1 and down-regulation of PSGL-1.

Gag membrane binding and coclustering of Gag with PSGL-1 at the plasma membrane are followed by release of progeny virions. To investigate whether down-regulation of PSGL-1 on surface of infected cells requires virus release, we next examined the effect of substitution of the p6 PTAP sequence ($PTAP^-$ Gag), which is essential for recruitment of the ESCRT machinery and hence robust virus release (36, 37). Compared with WT Gag, $PTAP^-$ Gag was inefficient in promoting reduction of PSGL-1 on the surface of Gag-YFP High cells (Fig. 5*C* and *SI Appendix*, Fig. S2*D*), indicating that virus release contributes to PSGL-1 down-regulation.

The dependence of PSGL-1 down-regulation on Gag membrane binding and efficient release of progeny virions was also observed in HIV-1–infected primary $CD4^+$ T cells. HIV-1 encoding $PTAP^-$ Gag caused only moderate down-regulation of PSGL-1 compared with WT Gag. Strikingly, despite encoding Vpu, HIV-1 encoding 1GA Gag showed a severe defect in PSGL-1 down-regulation, as was observed for Vpu-deleted HIV-1 (Udel) (Fig. 5*D* and *SI Appendix*, Fig. S2*E*), indicating that PSGL-1 down-regulation requires both Vpu and the ability of Gag to bind membrane. Taken together, these results support the possibility that association of PSGL-1 with virus assembly sites followed by release of virions that sequester PSGL-1 leads to down-regulation of PSGL-1 on the surface of virus-producing cells.

Discussion

Although it has been long known that HIV-1 incorporates many host transmembrane proteins, only a limited number of these have been studied for their functions at the surface of HIV-1 particles. In this study, we have demonstrated that PSGL-1 and CD43, which are incorporated into virions, reduce virion infectivity through the inhibition of virus attachment to target cells, a step that has not been known as a target of an anti–HIV-1 restriction mechanism. Interestingly, PSGL-1 and CD43 also

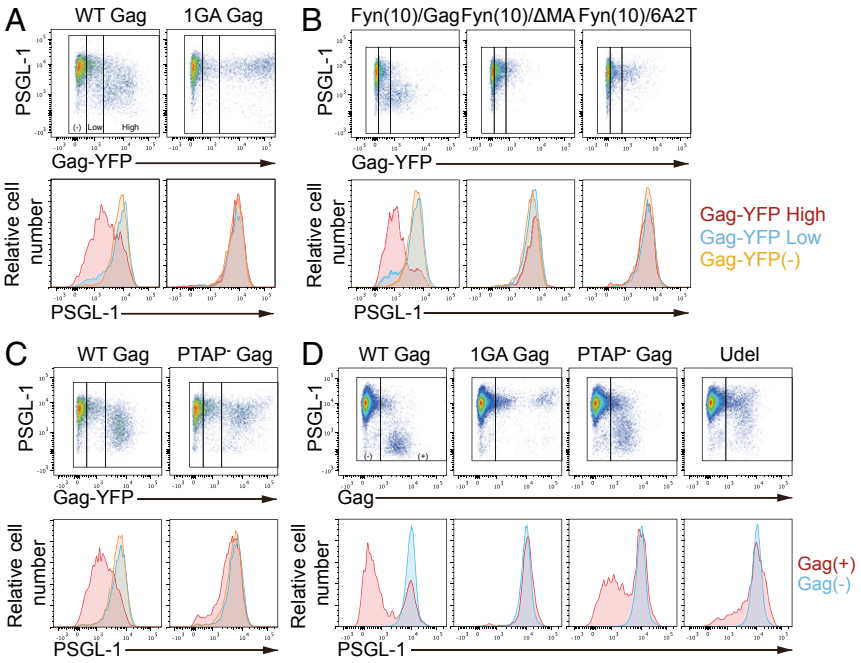

Fig. 5. Coclustering of Gag with PSGL-1 and release of progeny virions promote a reduction in surface expression of PSGL-1 in infected cells. (*A*–*C*) Analyses of surface expression of PSGL-1 in A3.01 cells by flow cytometry. Cells showing no Gag-YFP signal [Gag-YFP(−)], cells expressing Gag-YFP at low levels (Gag-YFP Low), and cells expressing Gag-YFP at high levels (Gag-YFP High) were gated in the flow plots in the top row; histograms of PSGL-1 levels for each Gag-YFP gate are shown in the bottom row. A3.01 cells were spinoculated with HIV-1 encoding either WT Gag-YFP or 1GA Gag-YFP (*A*); YFP-tagged Fyn(10)/Gag, Fyn(10)/ΔMA Gag, or Fyn(10)/6A2T Gag (*B*); and either WT Gag-YFP or PTAP^{-} Gag-YFP (*C*); cultured for 3 d; and analyzed for expression of surface PSGL-1 and Gag-YFP. (*D*) PHA-stimulated PBMCs were spinoculated with HIV-1 encoding WT, 1GA, or PTAP^{-} Gag or Vpu-deleted HIV-1 and cultured for 3 d in the presence of IL-2. The CD3^{+}CD8^{-} cell population was gated and analyzed for expression of surface PSGL-1 and intracellular Gag as shown in the top row. Histograms of PSGL-1 levels for Gag(+) and Gag(−) populations are shown in the bottom row. The flow plots and histograms are representative of three independent experiments. PBMCs were isolated from three different donors and used for each experiment.

suppress Env-independent virus–cell binding, including virus binding to FRCs mediated by interactions between virion-associated HA and CD44 on the surface of FRCs. Therefore, it is likely that virion incorporation of PSGL-1 and CD43 can block both cell-free infection and transinfection mechanisms. We also found that Gag coclustering and release of virus particles likely promote down-regulation of cell surface PSGL-1 in virus-infected cells. Overall, the present study reveals a previously undescribed anti–HIV-1 function of virion-incorporated host transmembrane proteins—inhibition of virus attachment—as well as a mechanism of viral counteraction through Gag functions.

Our results show that inhibition of virion infectivity by PSGL-1 and CD43 closely correlates with virion incorporation of these proteins (Fig. 1), and that these proteins prevent virus–cell binding irrespective of the molecules mediating virus attachment (Figs. 2 and 3). Therefore, while it is formally possible that PSGL-1 or CD43 in virus-producing cells blocks virus–cell attachment without being incorporated into virions, it is most likely that PSGL-1 and CD43 in virus particles exert direct and broad-acting effects on cell-free virus binding to uninfected cells. Nonetheless, it would be interesting to know whether expression of PSGL-1 and CD43 on the surface of virus-producing cells but outside of virus particles affects cell-to-cell transmission of HIV-1. During immunological synapse formation, a cytoskeleton-dependent mechanism excludes CD43 from the cell–cell contact (38, 39). However, this is unlikely the case with the virological synapse, since accumulation of CD43 has been observed at this synaptic structure (8). Therefore, whether these transmembrane proteins modulate the efficiency of virus transfer at cell–cell contacts warrants future investigation.

The lengths of ECDs of PSGL-1 and CD43 are approximately 50 and 45 nm, respectively, and much longer than the combined lengths of the ECDs of Env and CD4, the receptor for Env (26, 27, 40, 41). In addition, the combined lengths of other ligand-receptor pairs implicated in virus attachment, such as the ICAM-1–LFA-1 pair and the Env-integrin α4β7 pair are also shorter than PSGL-1 and CD43 (40, 42, 43) (*SI Appendix*, Fig. S3*A*). It has been proposed that the ECDs of PSGL-1 and CD43 inhibit cell–cell adhesion via steric hindrance (28, 29). In a similar manner, it is quite possible that the tall ECDs of PSGL-1 and CD43 on the surface of virions prevent HIV-1 and cells from getting close enough for interactions between Env and CD4 or between other molecules mediating virus–cell binding (*SI Appendix*, Fig. S3*B*). Indeed, we observed that deletion of a part of the ECD of PSGL-1 dampens the inhibitory effect of this protein on virus attachment (Fig. 4). However, it is also possible that the entity of ECDs of PSGL-1 and CD43 attenuates virus attachment in a manner not directly dependent on the extended structures, for example, via increased negative charge or heavy glycosylation. Further studies are needed to determine the molecular mechanism by which ECDs of PSGL-1 and CD43 inhibit virus attachment. In addition, our study does not rule out the possibility that virion-incorporated PSGL-1 and CD43 attenuate not only virus attachment, but also subsequent steps of HIV life cycle as reported by another group (16).

Down-regulation of many host-encoded restriction factors, such as APOBEC3 family proteins, tetherin, and SERINCs, are facilitated by HIV-1 accessary proteins, such as Vif, Vpu, and Nef (5, 6, 44, 45). A recent study showed that PSGL-1 is also susceptible to Vpu- and proteasome-dependent degradation (16); however, it has also been shown that expression of Vpu alone does not lead to a strong reduction of cell surface PSGL-1,

unlike the cases with CD4 or tetherin (34), suggesting a possible contribution of other viral factors to down-regulation of PSGL-1. Consistent with this possibility, we found that Gag membrane binding and subsequent release of progeny virions, in addition to expression of Vpu, are necessary for efficient reduction of PSGL-1 on the surface of infected cells. Considering the observed correlation between Gag–PSGL-1 coclustering and PSGL-1 down-regulation, it is likely that PSGL-1 on the cell surface is depleted at least partially via virion incorporation. Conceivably, the loss of PSGL-1 via virion incorporation and release is faster than the replenishment of PSGL-1 by de novo synthesis and transport to the plasma membrane, resulting in reduction of PSGL-1 from the surface of infected cells. While Vpu and Nef are well recognized as factors promoting degradation of many host transmembrane proteins (5, 6, 17, 34, 45, 46), our study highlights the importance of Gag and virion incorporation in down-regulation of host transmembrane proteins. Of note, Vpu is essential for efficient release of progeny virions through degradation of tetherin (45, 46). Thus, it is quite conceivable that Vpu plays an important role in depletion of PSGL-1 through two independent mechanisms: directly, by promoting degradation in proteasomal or lysosomal pathway, and indirectly, by enhancing the release of virus particles that have incorporated the transmembrane proteins.

In summary, we have identified a previously undescribed anti-HIV defense mechanism mediated by virion-incorporated host transmembrane proteins PSGL-1 and CD43, which inhibit virus attachment to target cells. We also have shown that the release of progeny virions that incorporate host transmembrane proteins promotes down-regulation of these transmembrane proteins. Based on these observations, we propose that infectivity of progeny HIV-1 produced in the same $CD4^+$ T cell could vary depending on the time point at which they are produced due to varying amounts of antiviral factors, such as PSGL-1 and CD43, on surfaces of the virions.

Materials and Methods

Cell Culture. HeLa cells, 293T cells, and TZM-bl cells (a HeLa-based indicator cell line) were cultured in DMEM (Lonza) containing penicillin-streptomycin (P/S; Gibco) and 5%, 10%, and 10% FBS (HyClone), respectively. A3.01 cells were cultured in RPMI 1640 containing P/S and 10% FBS. FRCs, commercially available as human lymphatic fibroblasts from ScienCell Research Laboratories), were cultured in Fibroblast Medium containing P/S and 2% FBS (all from ScienCell Research Laboratories).

Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats derived from healthy donors (New York Blood Center) and cryopreserved when PBMCs were not used for further experiments immediately. Cryopreserved PBMCs were thawed and cultured in RPMI-10 medium overnight before use in experiments including isolation of $CD4^+$ T cells. $CD4^+$ T cells were isolated by negative selection using $CD4^+$ T cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's protocol. For activation of T cells, PBMCs and isolated $CD4^+$ T cells were cultured in RPMI-10 medium containing 20 U/mL IL-2 (Roche) and 6 μg/mL phytohemagglutinin (PHA; Sigma-Aldrich) for 2 to 3 d.

Plasmids. HIV-1 molecular clones pNL4-3, pNL4-3/Gag-YFP, pNL4-3/1GA/Gag-YFP, pNL4-3/PTAP$^-$/Gag-YFP, pNL4-3/Fyn(10)Gag/Gag-YFP, pNL4-3/Fyn(10)ΔMA/Gag-YFP, pNL4-3/6A2T/Gag-YFP, and pNL4-3/KFS, which does not encode Env (ΔEnv), have been described previously (7, 35, 47–50). A plasmid that expresses HIV-1_{NL4-3} Gag in an HIV-1 Rev-dependent manner, pCMVNLGagPolRRE/φ(–), was constructed by deleting nucleotides 749 to 785 (based on NL4-3) from pCMVNLGagPolRRE (51). A vector that expresses vesicular stomatitis virus G glycoprotein (pHCMV-G) has been described previously (52).

Plasmids encoding full-length cDNA clones of human PSGL-1 (GenBank accession no. NM_003006.3), pCMV6-AC/PSGL-1; human CD43 (NM_003123.2), pCMV6-XL5/CD43; and human ICAM-3 (NM_002162.2), pCMV6-AC/ICAM-3, were obtained from OriGene. pCMV6/empty, pCMV6-AC/PSGL-1 ΔCT, and pCMV6-XL5/CD43 6A were described previously (14). The constructs pCMV6-AC/PSGL-1 Δ118–277, pCMV6-AC/CD43, and pCMV6-AC/CD43 6A were constructed by standard molecular cloning techniques.

Knockout of PSGL-1 and CD43. CRISPR/Cas9-based knockout was performed by nucleofection of the Cas9/sgRNA (RNP) complex using the Nucleofector Kit V (Lonza). To prepare the RNP complexes for single protein knockout, 250 pmol chemically modified synthetic sgRNA (Synthego) was mixed with 80 pmol Cas9 (Synthego) and incubated for 15 min at RT. For double-knockout experiments, 250 pmol each of sgRNAs against PSGL-1 and CD43 were mixed with total 160 pmol Cas9. PHA-stimulated primary $CD4^+$ T cells (1.5×10^6) were mixed with the RNP complex and nucleofected using the X-001 protocol. Cells were cultured in RPMI-10 medium containing 20 U/mL IL-2 for 2 d and then in RPMI-10 medium containing 20 U/mL IL-2 and 6 μg/mL PHA for an additional 2 d. The following sgRNA sequences were used: PSGL-1, 5′-CAGGAGGAGUUGCAGAGGCA-3′; CD43, 5′-CCACCAGCACCCCAAGGAGA-3′; and Negative Control Scrambled#1, 5′-GCACUACCAGAGCUAACUCA-3′.

Virus Stock. To generate HIV-1_{NL4-3} and Env-deleted HIV-1_{NL4-3} in PSGL-1-, CD43-, or ICAM-3-expressing cells, 3.5×10^5 HeLa cells were plated onto six-well plates and cultured for 1 d. The cells were cotransfected with 3.4 μg of pNL4-3 or pNL4-3/KFS and 0.6 μg of pCMV6-AC/PSGL-1, pCMV6-AC/CD43, pCMV6-AC/PSGL-1 ΔCT, pCMV6-AC/CD43 6A, pCMV6-AC/PSGL-1 Δ118-277, or pCMV6-AC/ICAM-3 using Lipofectamine 2000 (Invitrogen). To generate HIV-1_{NL4-3}, 5.25×10^6 293T cells were plated onto 10-cm plates, cultured for 1 d, and then transfected with 20 μg pNL4-3 using Lipofectamine 2000 (Invitrogen). To generate VSV-G–pseudotyped virus stocks, 1.05×10^6 293T cells were plated onto a six-well plates, cultured for 1 d, and cotransfected with 2.0 μg pNL4-3–derived molecular clones, 2.0 μg of pCMVNLGagPolRRE/φ(–), and 0.2 μg of pHCMV-G using Lipofectamine 2000 (Invitrogen). To prepare virus stocks for analysis of PSGL-1 down-regulation in PBMCs, 1.05×10^6 293T cells were plated onto six-well plates, cultured for 1 d, and then cotransfected with 2.5 μg of pNL4-3–derived molecular clones and 2.5 μg of pCMVNLGagPolRRE/φ(–) using Lipofectamine 2000 (Invitrogen). These cells were cultured for 20 h or 48 h after transfection.

To produce HIV-1 from PSGL-1 and/or CD43 KO primary $CD4^+$ T cells, 4 or 5×10^5 cells in a 96-well round-bottom plate were inoculated with 293T cell-derived HIV-1_{NL4-3} (5 μg of p24) by spin infection at 2,500 rpm for 2 h at 25 °C. The cells were washed four times with phosphate-buffered saline (PBS) to remove unbound viruses and then cultured for 3 d in the presence of 20 U/mL IL-2. Virus-containing supernatants were collected and filtered through a 0.45-μm filter. For some experiments, virus-containing supernatants were concentrated by ultracentrifugation (24,000 rpm for 2 h at 4 °C) using a 20% sucrose/PBS cushion or a 100-kDa centrifugal filter unit (Millipore Sigma). To quantify virus amounts, the p24 amount was measured by enzyme-linked immunosorbent assay (ELISA).

p24 ELISA. p24 ELISA was performed as described previously with some modifications (15). In brief, virus-inoculated cells and supernatant containing viral particles were lysed in ELISA lysis buffer (0.05% Tween 20, 0.5% Triton X-100, and 0.5% casein in PBS). An anti–HIV-1 p24 antibody (clone 183-H12-5C; NIH AIDS Research and Reference Reagent Program) was bound to Nunc MaxiSorp plates overnight at 4 °C. Lysed samples were added to the plates and incubated for 2 h, and captured p24 was detected by sequential incubation with a biotinylated anti–HIV-1 p24 antibody (clone 31–90-25; American Type Culture Collection), streptavidin-HRP (Fitzgerald), and 3,3′,5,5′-tetramethylbenzidine substrate (Sigma-Aldrich). Biotinylation of 31–90-25 was performed using the EZ-Link Micro Sulfo-NHS-Biotinylation Kit (Pierce). CAp24 concentrations were determined using recombinant HIV-1 IIIB p24 protein for standards (NIH AIDS Research and Reference Reagent Program).

Flow Cytometry. PBMCs were stained for 15 min at 4 °C using the following antibodies primarily labeled with a fluorophore: anti–CD3-Pacific Blue (clone UCHT-1; BD Biosciences) and anti–CD8-APC/Cy7 (clone HIT8a; BD Biosciences). The stained PBMCs and A3.01 cells were fixed with 4% paraformaldehyde (PFA) in PBS for 30 min at RT. The fixed cells were stained using following primarily labeled antibodies: anti–CD43-PE/Cy7 (clone 1G10; BD Biosciences) and anti–PSGL-1-APC (clone KPL-1; BD Biosciences). To detect intracellular Gag in infected cells, fixed cells were permeabilized by 0.1% Triton X-100 for 5 min at RT and stained using anti-HIV Gag-FITC (clone KC57; Beckman Coulter). The samples were analyzed with a FACSCanto flow cytometer (BD Biosciences).

Immunoblotting. To analyze virion incorporation of PSGL-1, PSGL-1 ΔCT, PSGL-1 Δ118–277, CD43, CD43 6A, or CD44, HeLa cells cotransfected with pNL4-3 and pCMV6-AC/PSGL-1, pCMV6-AC/PSGL-1 ΔCT, pCMV6-AC/PSGL-1 Δ118–277, pCMV6-AC/CD43, pCMV6-AC/CD43 6A, or pCMV6/empty were cultured for 20 h or 48 h. To obtain HIV-1 produced from primary

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$CD4^+$ T cells, 1×10^6 PHA-stimulated cells were inoculated with 293T cell-derived HIV-1_{NL4-3} (5 μg of p24) by spin infection and further cultured as described above. A total of 4×10^6 PHA-stimulated cells were infected for each experiment. To verify the CRISPR/Cas9 knockout of PSGL-1 and CD43, primary $CD4^+$ T cells were nucleofected with the RNP complex and cultured as described above. Cell and virus lysates were prepared as described previously (35).

To compare amounts of PSGL-1 and CD43 in virions produced from primary $CD4^+$ T cells and HeLa cells, the amount of p24 in virus lysates were determined by p24 ELISA, and the lysates containing the same amount of p24 were analyzed. Immunoblotting was performed using anti–PSGL-1 (clone KPL-1; BD Biosciences), anti-CD43 (clone 1G10; BD Biosciences), anti-CD44 (clone 2C5; R&D Systems), anti–α-tubulin (clone B-5–1-2; Sigma-Aldrich), and anti-HIV Ig (NIH AIDS Research and Reference Reagent Program) antibodies in combination with appropriate secondary antibodies conjugated to horseradish peroxidase. Chemiluminescence signals were detected using GeneSys (Syngene) and SuperSignal West Pico chemiluminescent substrate, SuperSignal West Pico PLUS chemiluminescent substrate, or SuperSignal West Femto chemiluminescent substrate (Pierce).

Virion Infectivity Assay. Virion infectivity was measured in a single-cycle assay in which 1×10^4 TZM-bl cells were inoculated with viruses (HeLa cell-derived virus, 50 ng of p24; and primary $CD4^+$ T cell-derived virus, 10 ng of p24) and cultured in the presence of 10 μM saquinavir (NIH AIDS Research Reagent Program). At 48 h postinfection, cells were lysed, and the cell lysates were analyzed for luciferase activity using a commercially available kit (Promega).

To analyze infectivity using A3.01 cells, 2×10^5 A3.01 cells were inoculated with viruses (200 ng of p24) by spinoculation at 2,500 rpm for 2 h, placed at 25 °C. Cells were washed four times with PBS and cultured for 48 h at 37 °C in the presence of 10 μM saquinavir. Cells were fixed with 4% PFA in PBS, permeabilized with 0.1% Triton X-100 in PBS for 5 min at RT, and immunostained with FITC-conjugated anti-Gag monoclonal antibody (clone KC57; Beckman Coulter). The infected cells were then analyzed by flow cytometry.

Virus Attachment Assay. A total of 7.5×10^4 TZM-bl cells were plated onto 24-well plates and cultured for 1 d. Cells were inoculated with viruses of the same amount (200 ng of p24) and cultured for 2 h at 4 °C. Cells were then washed four times with PBS and lysed with ELISA lysis buffer, and the cell lysates were used for p24 ELISA.

To analyze virus attachment to A3.01 cells, 2×10^5 A3.01 cells were inoculated with WT viruses (200 ng of p24) with spin at 2,500 rpm for 2 h, placed at 4 °C. To analyze virus binding to and uptake by A3.01 cells, 2×10^5 A3.01 cells were inoculated with WT viruses (200 ng of p24) and ΔEnv viruses (100 ng of p24) with or without spinning at 2,500 rpm for 2 h, and placed at 25 °C or RT. Cells were then washed four times with PBS and lysed with ELISA lysis buffer, and the cell lysates were used for p24 ELISA.

Virus Capture Assay. A total of 5×10^4 FRCs were plated onto 24-well plates. At 16 to 24 h after plating, at which time the cells were ∼80% confluent, the cells were pulsed with viruses (100 ng of p24) for 2 h at 37 °C. After removal of unbound virus through four washes with PBS, the cells were lysed with ELISA lysis buffer, and the lysates were examined for the amount of captured virus in p24 ELISAs. To test the effect of blocking of cell surface CD44 on virus capture, cells were washed with PBS, preincubated with 10 μg/mL anti-CD44 antibody (clone 515; BD Bioscience) or mouse IgG1 isotype control (BD Biosciences) for 1 h at 37 °C, and then washed twice with PBS, followed by the addition of virus. To test the effect of HA removal from the virus surface, viruses were preincubated with 10 U/mL H-ase for 1 h at 37 °C and used without separation of virus from H-ase.

Statistical Analyses. Statistical analyses were performed with Prism 8.0 (GraphPad Software). For multigroup comparisons, Bonferroni's test following one-way or two-way ANOVA was used. The applied statistical analyses are indicated in the figure legends. Data are presented as mean ± SD and were considered statistically significant at $P < 0.05$.

Data Availability. All data, associated protocols, methods, and sources of materials are provided in the text or *SI Appendix*.

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