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VIRUS-CELL INTERACTIONS

Simian Immunodeficiency Virus SIVgsn-99CM71 Vpu Employs Different Amino Acids To Antagonize Human and Greater Spot-Nosed Monkey BST-2

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ABSTRACT Viral protein U (Vpu) is an accessory protein encoded by human immunodeficiency virus type 1 (HIV-1) and certain simian immunodeficiency virus (SIV) strains. Some of these viruses were reported to use Vpu to overcome restriction by BST-2 of their natural hosts. Our own recent report revealed that Vpu of SIVgsn-99CM71 (SIVgsn71) antagonizes human BST-2 through two AxxxxxxxW motifs ($A^{22}W^{30}$ and $A^{25}W^{33}$), whereas antagonizing BST-2 of its natural host, greater spot-nosed monkey (GSN), involved only the $A^{22}W^{30}$ motif. Here, we show that residues A^{22}, A^{25}, W^{30}, and W^{33} of SIVgsn71 Vpu are all essential to antagonize human BST-2, whereas a single mutation of either A^{22} or W^{30} did not affect the ability to antagonize GSN BST-2. Similar to A^{18}, which is located in the middle of the A^{14}xxxxxxxW^{22} motif in HIV-1 NL4-3 Vpu and is essential to antagonize human BST-2, A^{29}, located in the middle of the $A^{25}W^{33}$ motif of SIVgsn71 Vpu was found to be necessary for antagonizing human but not GSN BST-2. Further mutational analyses revealed that residues L^{21} and K^{32} of SIVgsn71 Vpu were also essential for antagonizing human BST-2. On the other hand, the ability of SIVgsn71 Vpu to target GSN BST-2 was unaffected by single amino acid substitutions but required multiple mutations to render SIVgsn71 Vpu inactive against GSN BST-2. These results suggest additional requirements for SIVgsn71 Vpu antagonizing human BST-2, implying evolution of the *bst-2* gene under strong selective pressure.

IMPORTANCE Genes related to survival against life-threating pathogens are important determinants of natural selection in animal evolution. For instance, BST-2, a protein showing broad-spectrum antiviral activity, shows polymorphisms entailing different phenotypes even among primate species, suggesting that the *bst-2* gene of primates has been subject to strong selective pressure during evolution. At the same time, viruses readily adapt to these evolutionary changes. Thus, we found that the Vpu of an SIVgsn isolate (SIVgsn-99CM71) can target BST-2 from humans as well as from its natural host, thus potentially facilitating zoonosis. Here, we mapped residues in SIVgsn71 Vpu potentially contributing to cross-species transmission. We found that the requirements for targeting human BST-2 are distinct from and more complex than those for targeting GSN BST-2. Our results suggest that the human *bst-2* gene might have evolved to acquire more restrictive phenotype than GSN *bst-2* against viral proteins after being derived from their common ancestor.

KEYWORDS HIV-1, SIVgsn, Vpu, BST-2, SIV, restriction factor, species specificity, accessary protein, evolution, restriction factors, antagonism, cross-species transmission, flow cytometry, human immunodeficiency virus, simian immunodeficiency virus, virus-host interactions

BST-2 (also known as tetherin, CD317, and HM1.24) is an interferon-inducible type II transmembrane (TM) protein that has been reported to inhibit the release of enveloped virus from infected cells (1–6). It is localized to the cellular membrane with an

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N-terminal transmembrane domain (TMD) and a C-terminal glycophosphatidylinositol (GPI) anchor (7–12). BST-2 is also supposed to be incorporated into virions budding from the cells and retain virus particles at the cell surface, promoting internalization and degradation, so that it prevents virus release from infected cells (1, 2). *bst-2* genes, which have evident genetic diversity among primates, might therefore have evolved under long-term positive selective pressure to block viral cross-species transmission (13–16). The N-terminal cytoplasmic domain of human BST-2 carries a deletion of five amino acids compared to monkeys and the closest nonhuman relative chimpanzee (17, 18). This five-amino-acid deletion was also found in archaic humans Neanderthal and Denisovan, suggesting that this deletion occurred more than 800,000 years ago (19). The TM domain sequences of BST-2 are highly conserved among humans, chimpanzees, and gorillas but different between humans and monkeys (18). To overcome BST-2 of each natural host, primate lentiviruses have evolved several strategies involving different virus-encoded proteins, including viral protein U (Vpu) in HIV-1 and some SIV strains, Nef in some SIV strains, and Env in HIV-2 (15, 17, 20–26).

Vpu is a viral accessory protein, consisting of an N-terminal domain TMD and a C-terminal cytoplasmic domain (CTD) (27). Vpu was shown to interact with human BST-2 via its TMD and to induce its downregulation from the cell surface to facilitate virus release from cells (20–25, 28–32). Vpu is expressed by HIV-1 and some SIV strains isolated from chimpanzees, gorillas, mona monkeys (MON), Dent's mona monkeys, greater spot-nosed monkeys (GSN), and mustached monkeys (MUS) (33–40). Because of the diversity of Vpu and BST-2, successful antagonism or failure to antagonize is determined by the combination of each Vpu and BST-2 (13, 16, 18, 24, 41–43). Vpu of HIV-1 group M can solely antagonize human and chimpanzee BST-2 (13, 18). Except for some Vpus from clinical isolates that can antagonize macaque BST-2 (44), HIV-1 Vpu is thought to be unable to overcome monkey BST-2 (13, 18, 24, 41, 43, 45). Likewise, most SIV Vpu proteins fail to antagonize human BST-2 (18). Our recent study, testing the anti-BST-2 activities of a variety of SIV Vpu variants, found that only SIVgsn-99CM71 (SIVgsn71) Vpu was able to antagonize human BST-2 efficiently (46). Here, we further investigated how SIVgsn71 Vpu differentially antagonizes human and GSN BST-2.

RESULTS

Alanine and tryptophan residues in the AW motifs of SIVgsn71 Vpu work together against human BST-2 but work redundantly against GSN BST-2. We reported (46) that SIVgsn71 Vpu antagonizes human BST-2 with its two AxxxxxxxW motifs ($A^{22}W^{30}$ and $A^{25}W^{33}$), while only the $A^{22}W^{30}$ motif was necessary for inhibition of GSN BST-2 (Fig. 1A). Since human and GSN BST-2 differ in their primary amino acid sequence (Fig. 1B), we hypothesized that the mechanism for antagonizing human BST-2 by SIVgsn71 Vpu might be different from that for GSN BST-2. To clarify what amino acids in the two AW motifs of SIVgsn71 Vpu specifically contribute to the differential modes of antagonism of human or GSN BST-2, we generated single amino acid mutants of SIVgsn71 Vpu (i.e., A22L, A25L, W30A, and W33A in Fig. 1A). We first analyzed the interaction of these Vpu mutants with human BST-2 because Vpu binding to BST-2 serves as a basis of its antagonism (Fig. 1C). Physical interaction between Vpu and BST-2 was assessed by bimolecular fluorescence complementation (BiFC) assay, as reported previously (17, 22, 46, 47). We used SIVgsn71 Vpu as a positive control. The previously characterized Vpu AxxxxxxxW motif double mutants of SIVgsn71, Vpu22/30 (A22L and W30A) and Vpu25/33 (A25L and W33A), were used as negative controls (46). Expression of SIVgsn71 Vpu and human BST-2 was verified by Western blotting (Fig. 1C, bottom). As reported previously, KGN tag alone and SIVgsn71 Vpu double mutants such as Vpu22/30 and Vpu25/33 showed much lower BiFC signal when coexpressed with human BST-2 than SIVgsn71 Vpu WT did (Fig. 1C, top). SIVgsn71 Vpu A22L, A25L, W30A, and W33A single amino acid mutants all showed much weaker BiFC signals with human BST-2 than SIVgsn71 Vpu WT did.

We next assessed whether the four single amino acid mutants downregulate cell surface human BST-2. For this purpose, we generated HIV-1 proviral DNA pNL4-3EGFPΔ*env*Δ*nef*

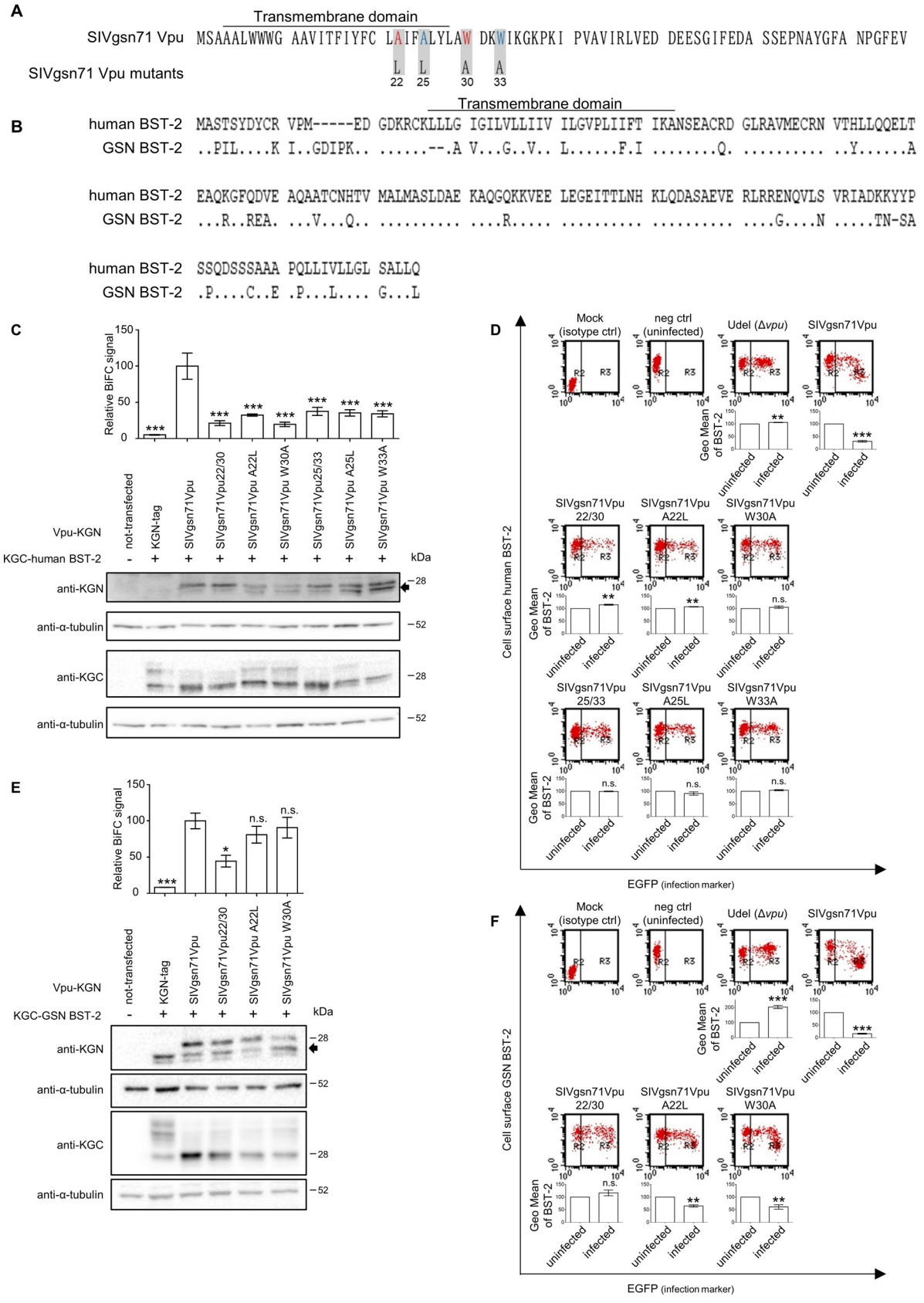

FIG 1 Single-amino-acid substitutions in the AxxxxxxxW motifs of SIVgsn71 Vpu result in different counteractions against human and GSN BST-2. (A) Schematic representation of single-amino-acid substitutions in the two SIVgsn71 Vpu AxxxxxxxW motifs. $A^{22}W^{30}$ and $A^{25}W^{33}$ are

(Continued on next page)

constructs whose *vpu* gene was replaced by each of the SIVgsn71 *vpu* mutant genes and prepared vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped virus, as reported previously (46). A Vpu-null mutant, pNL4-3EGFPΔ*env*Δ*nef*Δ*vpu*, which does not express any Vpu protein (Udel) was used as negative control. HeLa cells were infected with VSV-G-pseudotyped HIV-1 carrying different *vpu* genes, and cell surface human BST-2 expression was evaluated by flow cytometry (Fig. 1D). Cells infected with HIV-1 carrying SIVgsn71 *vpu* WT showed lower cell surface human BST-2 expression (EGFP positive, right gate, R3 in Fig. 1D) compared to that of uninfected cells (EGFP negative, left gate, R2 in Fig. 1D), indicating that SIVgsn71 Vpu downregulated cell surface human BST-2 expression. In contrast, HIV-1 expressing SIVgsn71 Vpu variants 22/30 (A22L and W30A) or 25/33 (A25L and W33A) or lacking the *vpu* gene (Udel) did not affect human BST-2 expression, as shown by similar BST-2 surface expression on EGFP-negative and -positive cells in each panel. None of the single amino acid mutants (A22L, A25L, W30A, or W33A) induced significant downregulation of cell surface human BST-2 in infected cells. Next, we explored the binding and downregulation activity of the same mutants toward BST-2 from its natural host GSN monkey. Since the $A^{22}W^{30}$ motif but not $A^{25}W^{33}$ motif is involved in the SIVgsn71 Vpu antagonism of GSN BST-2 (46), only two single amino acid mutants within the $A^{22}W^{30}$ motif (i.e., A22L and W30A) were tested here. After confirming the expression of KGN-tagged Vpu and KGC-tagged GSN BST-2, the BiFC signals were measured by flow cytometry (Fig. 1E). As expected, the A22L and W30A double mutant (SIVgsn71 Vpu 22/30) and the KGN tag-only control showed a significantly lower BiFC signal compared to SIVgsn71 Vpu WT. However, BiFC signals for the A22L and W30A single amino acid exchange Vpu mutants revealed no significant difference relative to SIVgsn71 Vpu WT (Fig. 1E, upper panel).

We next sought to determine whether these single amino acid changes in SIVgsn71 Vpu affect cell surface GSN BST-2 expression. HeLa cells, in which endogenous human *bst-2* gene was knocked out and replaced with stable expression of GSN BST-2 (HeLa KO-GSN BST-2 cells) (46), were infected with HIV-1 encoding A22L or W30A Vpu mutants (Fig. 1F). SIVgsn71 Vpu but not Udel or SIVgsn71 Vpu22/30 (both A22L and W30A) downregulated cell surface GSN BST-2. In addition, A22L and W30A single amino acid Vpu mutants significantly decreased cell surface GSN BST-2 expression although their downregulation activity was weaker than wild-type SIVgsn71 Vpu. Based on these experiments, we conclude that A^{22}, A^{25}, W^{30}, and W^{33} of SIVgsn71 Vpu function inseparably and are all necessary to antagonize human BST-2, whereas A^{22} and W^{30} of SIVgsn71 Vpu function redundantly to antagonize GSN BST-2. This suggests similar but distinct mechanisms of SIVgsn71 Vpu action on human and GSN BST-2.

A^{29} within the $A^{25}W^{33}$ motif of SIVgsn71 Vpu is essential for antagonizing human BST-2. Besides the $A^{14}W^{22}$ motif, HIV-1 NL4-3 Vpu requires A^{18} located in the center of the $A^{14}W^{22}$ motif for inhibiting human BST-2 (23, 28). SIVgsn71 Vpu has L^{26} and A^{29} in the center of the $A^{22}W^{30}$ and $A^{25}W^{33}$ motifs, respectively. We first evaluated the importance of A^{29} because the amino acid alignment $A^{25}A^{29}W^{33}$ of SIVgsn71 Vpu is similar to $A^{14}A^{18}W^{22}$ of HIV-1 NL4-3 Vpu. To understand the role of A^{29} in the BST-2 downregulation, we constructed HIV-1 proviral vectors capable of expressing the SIVgsn71 Vpu

FIG 1 Legend (Continued)
indicated in red and blue, respectively. The mutated amino acids are highlighted. (B) Amino acid alignments of human and GSN BST-2. Amino acid residues of GSN-BST-2 identical to those of human BST-2 are shown as dots. (C and E) HEK293T cells were transfected with pKGC-huBST-2 (C) or pKGC-GSN BST-2 (E) in combination with KGN-tagged plasmids expressing SIVgsn71 Vpu or its mutants. At 48 h after transfection, the cells were harvested and subjected to flow cytometry for measuring BiFC signal (top). The Geo Mean of BiFC signal in cells transfected with SIVgsn71 Vpu was arbitrarily set as 100 and compared to determine the statistical significance (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; n.s., [not significant], $P > 0.05$). Error bars indicate means ± the SEM from three independent experiments. HEK293T cell lysates were subjected to SDS-PAGE and analyzed with anti-KGN, anti-KGC, and anti-α-tubulin antibodies (bottom). The arrows represent nonspecific bands. From three independent experiments, one representative Western blotting data are shown. (D and F) HeLa cells (D) and HeLa KO-GSN BST-2 cells (F) were infected with VSV-G-pseudotyped HIV-1 virus which carry a deletion (Udel), intact wild type (SIVgsn71 Vpu), or AW motif single-amino-acid mutants of SIVgsn71 *vpu* gene and stained with anti-BST-2 antibody at 48 h after infection to detect cell surface BST-2 expression. From three independent experiments, one representative dot plot is shown. The Geo Mean of BST-2 fluorescence (*y* axis) in the EGFP-negative population (uninfected cells, R2 gate in a dot plot, and left column in a bar graph) and the EGFP-positive population (infected cells, R3 gate in a dot plot, and right column in a bar graph) are shown with the SEM from three independent experiments. The Geo Mean of BST-2 in EGFP-negative cells was normalized as 100 and compared to determine the statistical significance.

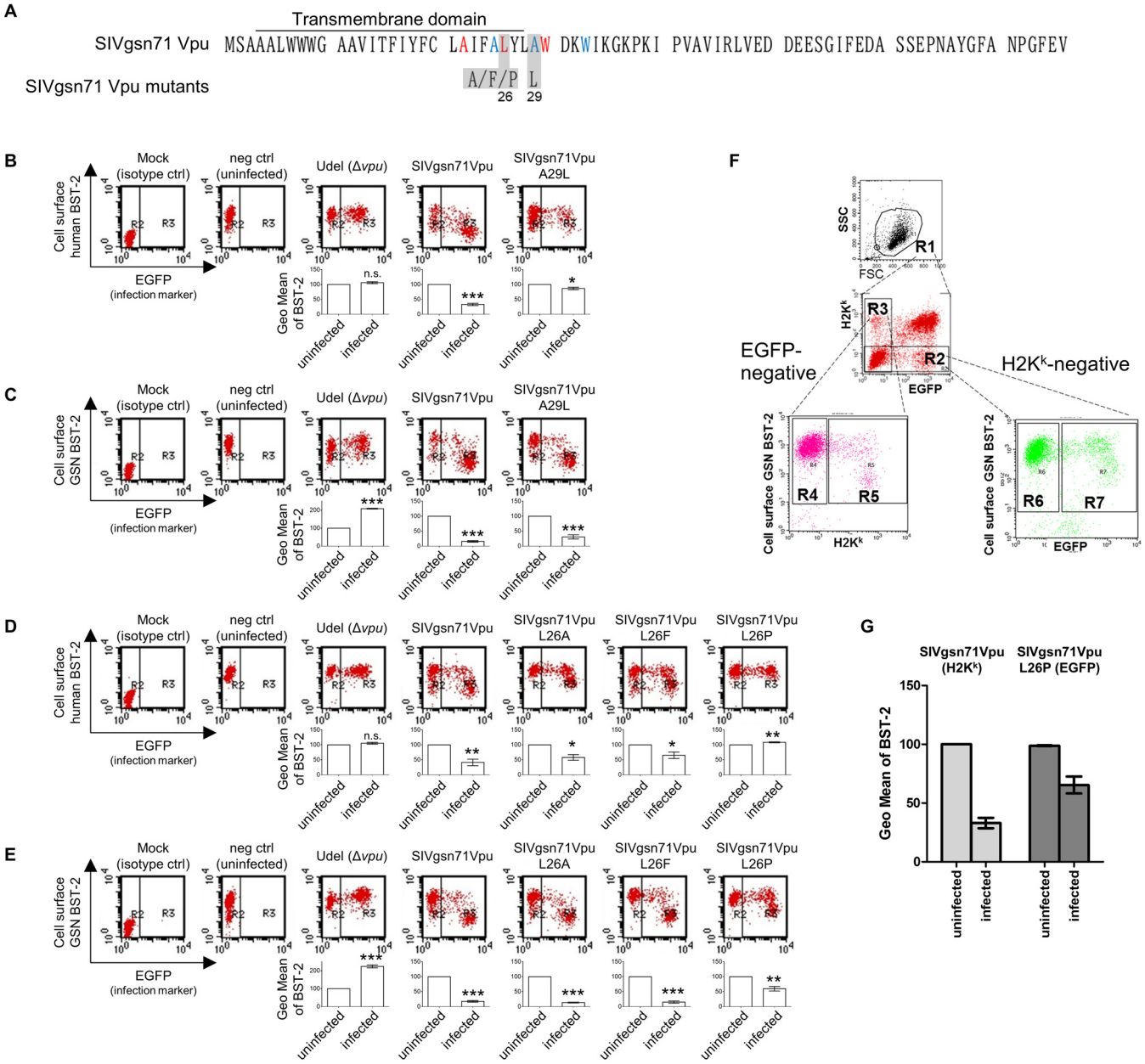

FIG 2 A^{29} of SIVgsn71 Vpu is important for downregulating human BST-2 expression. (A) Schematic representation of amino acid substitutions in the SIVgsn71 Vpu AxxxxxxxW motifs. $A^{22}L^{26}W^{30}$ and $A^{25}A^{29}W^{33}$ are indicated in red and blue, respectively. The mutated amino acids (L^{26} and A^{29}) are highlighted. (B to E) HeLa cells (B and D) and HeLa KO-GSN BST-2 cells (C and E) were infected with VSV-G-pseudotyped HIV-1 virus which carries a deletion (Udel), intact wild type (SIVgsn71 Vpu), and A^{29} or L^{26} mutants of the SIVgsn71 *vpu* gene, and the cell surface BST-2 expression was quantified with anti-BST-2 antibody. One representative dot plot is shown. Graphs are presented as described in Fig. 1D. (F and G) HeLa KO-GSN BST-2 cells were infected with VSV-G-pseudotyped HIV-1 which carries intact wild type (SIVgsn71 Vpu) and mouse *H2K*^{k} gene and VSV-G-pseudotyped HIV-1 virus which carries L26P mutant of SIVgsn71 *vpu* and *EGFP*. Cells were stained with anti-BST-2 and anti-H2K^k antibodies at 48 h after infection to detect cell surface BST-2 expression of uninfected cells (EGFP-, H2K^k-negative cells in R4), solely H2K^k-positive cells (EGFP-negative, H2K^k-positive cells in R5), uninfected cells (H2K^k-, EGFP-negative cells in R6), or solely EGFP-positive cells (H2K^k-negative, EGFP-positive cells in R7). The Geo Mean BST-2 fluorescence (*y* axis) in these cells is shown with the SEM from three independent experiments (first, second, third, and fourth bars, respectively). The Geo Mean value for BST-2 in EGFP-, H2K^k-negative cells was normalized as 100.

A29L mutant and prepared VSV-G-pseudotyped virus (Fig. 2A). HeLa cells and HeLa KO-GSN BST-2 cells were then infected with the virus expressing A29L mutant and cell surface expression of BST-2 was assessed by fluorescence-activated cell sorting (FACS) (Fig. 2B and C, respectively). Interestingly, SIVgsn71 Vpu A29L downregulated only GSN BST-2 but not human BST-2, which is consistent with the fact that the $A^{25}W^{33}$ motif is required for downregulation human but not GSN BST-2 by SIVgsn71 Vpu.

Since the $A^{22}W^{30}$ motif is crucial for SIVgsn71 Vpu to antagonize both human and GSN BST-2, we next assessed whether L^{26} plays a role in BST-2 downregulation. We constructed a pNL4-3EGFPΔ*env*Δ*nef* vector carrying the SIVgsn71 *vpu* gene capable of expressing SIVgsn71 Vpu with single amino acid substitutions L26A, L26F, or L26P (Fig. 2A). In control experiments, SIVgsn71 Vpu but not Udel downregulated both cell surface human and GSN BST-2 (Fig. 2D and E, respectively). Whereas L26A and L26F downregulated both human and GSN BST-2, L26P downregulated only GSN BST-2 but not human BST-2.

To assess how efficiently SIVgsn71 Vpu L26P downregulates GSN BST-2 compared to SIVgsn71 Vpu WT, we performed a persuasive experiment that enables us to analyze downregulating activity of two Vpus in a single cell culture. We generated HIV-1 mutant expressing SIVgsn71 Vpu and mouse $H2K^k$ as another infection marker (pNL4-3$H2K^k$Δ*env*Δ*nef* SIVgsn71 Vpu). HeLa KO-GSN BST-2 cells simultaneously exposed to this virus and VSV-G pseudotyped HIV-1 carrying *EGFP* and SIVgsn71 *vpu* L26P were stained with anti-BST2 and anti-$H2K^k$ antibodies. The middle panel in Fig. 2F shows the expression of EGFP and/or $H2K^k$ (R2 [$H2K^k$-negative] and R3 [EGFP-negative]); the lower left panel shows GSN BST-2 expression on cells infected solely with virus expressing SIVgsn71 Vpu ($H2K^k$-positive, EGFP-negative cells in R5); and the lower right panel shows GSN BST-2 expression on cells infected solely with virus expressing SIVgsn71 Vpu L26P mutant (EGFP-positive, $H2K^k$-negative cells in R7). Uninfected cells (cells negative in EGFP and $H2K^k$ expression) are in R4 and R6. As summarized in Fig. 2G, the level of cell surface expression of GSN BST-2 on cells infected with virus expressing SIVgsn71 Vpu L26P (fourth bar) is clearly lower than that on uninfected cells (first and third bars) but higher than that on cells infected with SIVgsn71 Vpu-expressing virus (second bar). These results indicate that A^{29} is essential for SIVgsn71 Vpu antagonism toward human but not GSN BST-2 and that L^{26} also plays an important role in antagonizing human BST-2 but is less important for its downregulation of GSN BST-2. Based on these findings, our results suggest that downregulation of human and GSN BST-2 by SIVgsn71 Vpu are mechanistically distinct.

Further evidence that antagonism of human and GSN BST-2 by SIVgsn71 Vpu is governed by distinct mechanisms. To identify additional amino acids in SIVgsn71 Vpu important for downregulating BST-2, we substituted all leucine (L) residues in the TM domain of SIVgsn71 Vpu for alanine (A), while all other amino acid residues in the TM domain were replaced by alanine in six consecutive clusters except for the $A^{22}L^{26}W^{30}$ and $A^{25}A^{29}W^{33}$ motifs, which we left unchanged (Fig. 3A). SIVgsn71 Vpu-TM_{RD}, whose TM domain was replaced with the corresponding randomized sequence from HIV-1 NL4-3 Vpu-TM_{RD} (48), was used as a negative control (17, 22, 29, 30, 46, 48). Figure 3B shows that HeLa cells infected with virus expressing SIVgsn71 Vpu mutant 2-5, 6-10, 11-15, or 16-20 (EGFP positive) expressed significantly lower levels of cell surface human BST-2 compared to that of uninfected cells (EGFP negative). In contrast, SIVgsn71 Vpu mutants 21-30 and 31-35 lost their downregulation ability, causing similar or even higher levels of cell surface human BST-2 expression in infected cells (Fig. 3B). In parallel, downregulation of cell surface GSN BST-2 was also evaluated (Fig. 3C). Similar to human BST-2, SIVgsn71 Vpu mutants 2-5, 6-10, 11-15, and 16-20 downregulated cell surface GSN BST-2 in infected cells, although some of them showed weaker activity than SIVgsn71 Vpu wild type. Importantly, SIVgsn71 Vpu mutant 31-35 strongly downregulated cell surface expression of GSN BST-2 (Fig. 3C), which is in contrast to its loss of downregulating activity toward human BST-2 (Fig. 3B). On the other hand, the surface expression of GSN BST-2 on cells infected with SIVgsn71 Vpu mutant 21-30 was higher than that on uninfected cells (Fig. 3C).

To assess whether the downregulation activity of mutant 21-30 against GSN BST-2 was completely abolished, we generated a *vpu*-deficient HIV-1 mutant expressing mouse $H2K^k$ (pNL4-3$H2K^k$Δ*env*Δ*nef*Δ*vpu*) and carried out experiments as were done in Fig. 2F and G with virus expressing enhanced green fluorescent protein (EGFP) and SIVgsn71 Vpu mutant 21-30. The reduced upregulation of GSN BST-2 in cells infected with the SIVgsn71Vpu21-30 (solely EGFP positive) virus compared to cells infected with

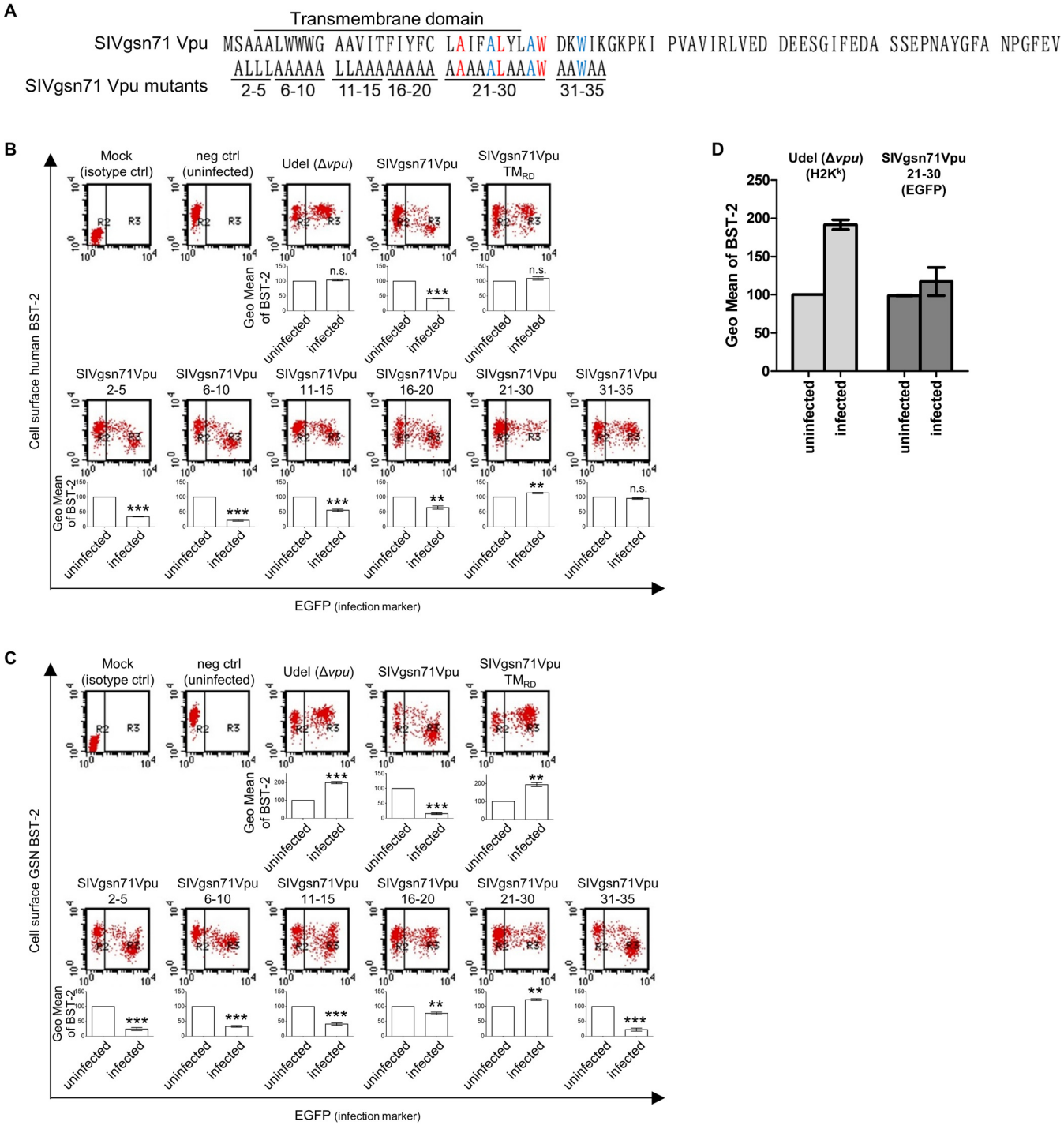

FIG 3 Identification of amino acid residues important for downregulation of human or GSN BST-2. (A) Schematic representation of amino acid substitutions in the SIVgsn71 Vpu N terminus. $A^{22}L^{26}W^{30}$ and $A^{25}A^{29}W^{33}$ are indicated in red and blue, respectively. (B and C) HeLa cells (B) and HeLa KO-GSN BST-2 cells (C) were infected with VSV-G-pseudotyped HIV-1 viruses which carry a deletion (Udel), intact wild type (SIVgsn71 Vpu), or the indicated mutants of SIVgsn71 *vpu* gene, and the cell surface BST-2 expression was quantified with anti-BST-2 antibody. One representative dot plot is shown. Graphs are presented as described in Fig. 1D. (D) HeLa KO-GSN BST-2 cells were infected with VSV-G-pseudotyped HIV-1 which carries deleted *vpu* (Udel) and mouse *H2K*^{k} gene and VSV-G-pseudotyped HIV-1 which carries mutant 21-30 of SIVgsn71 *vpu* and *EGFP*. Cells were stained, analyzed, and gated as described in Fig. 2F, and the graph is presented as described in Fig. 2G.

the Udel (solely H2K^k positive) virus suggests that this mutant has some residual activity against GSN BST-2 (Fig. 3D).

These results indicate that amino acids 2 to 20 of SIVgsn71 Vpu have a negligible role in the SIVgsn71 Vpu antagonism of both human and GSN BST-2 and that residues

21 to 30 are important for the downregulation of human and GSN BST-2. Finally, residues 31 to 35 of SIVgsn71 Vpu are only necessary to downregulate human BST-2.

Residues L^{21} and K^{32} of SIVgsn71 Vpu are essential for antagonizing human BST-2. Having demonstrated that residues 21 to 35 of SIVgsn71 Vpu are involved in human BST-2 antagonism and that residues 21 to 30 are involved in GSN BST-2 antagonism, we performed alanine scanning mutagenesis by generating sequential single amino acid mutants in the region between residues 21 to 35 (L21A, I23A, F24A, Y27A, L28A, D31A, K32A, I34A, and K35A in Fig. 4A) to identify amino acids essential for the SIVgsn71 Vpu function on BST-2. HeLa cells were infected with HIV-1 carrying these SIVgsn71 *vpu* mutants (Fig. 4B). Among the SIVgsn71 Vpu mutants, I23A, F24A, Y27A, L28A, D31A, and K35A downregulated cell surface human BST-2 in infected cells (Fig. 4B) to levels comparable to the positive control (SIVgsn71 Vpu). On the other hand, SIVgsn71 Vpu L21A and K32A did not downregulate cell surface human BST-2 and instead behaved like the negative controls (Udel and SIVgsn71 Vpu-TM_{RD}). Finally, the I34A mutant retained partial activity but caused only very modest downregulation of cell-surface human BST-2. Surprisingly, when the same Vpu mutants were tested for their effects on GSN BST-2 (Fig. 4C), we found that all of these mutants retained the ability to downregulate GSN BST-2 as efficiently as SIVgsn71 Vpu. Taken together, we conclude that residues $L^{21}$and K^{32}, and potentially residue I^{34} are important for antagonism of human BST-2, whereas none of these residues was necessary for targeting GSN BST-2. In summary, we found that amino acids in SIVgsn71 Vpu important for antagonizing human BST-2 are different from those required for antagonizing GSN BST-2, suggesting that distinct mechanisms mediate downregulation of human and GSN BST-2 by SIVgsn71 Vpu.

To assess whether the amino acids critical for downregulating human BST-2 are also important for the enhancement of virus release in the presence of human BST-2, we carried out TZM-bl assay to evaluate release of infectious virus as described previously (46). HeLa cells were transfected with proviral DNA pNL4-3EGFPΔ*env*Δ*nef* carrying SIVgsn71 *vpu* WT or each mutant (A29L, L21A, or K32A) gene together with a plasmid expressing VSV-G (pMISSION-VSV-G). As shown in Fig. 4D, release of infectious virus expressing SIVgsn71 Vpu WT was larger than those of the negative control (Udel virus) and other mutant viruses. Release of infectious virus expressing SIVgsn71 Vpu L21A was observed to be lower than that of Udel and other mutant viruses for unknown reason, but this mutant at least did not enhance release of infectious virus. Thus, these results suggest that L^{21}, A^{29}, and K^{32} are also important for SIVgsn71 Vpu to enhance virion release in the presence of human BST-2.

DISCUSSION

We previously reported that SIVgsn71 Vpu is able to antagonize not only BST-2 of its own natural host but can target human BST-2 (46). This feature is unique to SIVgsn71 Vpu and Vpu of other SIV isolates such as SIVgsn-99CM166 (SIVgsn166), SIVmon-99CMCML1 and SIVmus-01CM1085 can target only BST-2 of their own host but not human BST-2. In the same study, we identified two AxxxxxxxW motifs in SIVgsn71 Vpu but only one in Vpu from SIVmon and SIVmus. Whereas both AxxxxxxxW motifs are essential for antagonism of human BST-2 by SIVgsn71 Vpu, only one is needed for targeting GSN BST-2, suggesting that distinct mechanisms govern its antagonism toward GSN and human BST-2s (46). In the present study, we identified additional amino acids in SIVgsn71 Vpu important for antagonizing human BST-2, which were largely different from those required for antagonizing GSN BST-2. Four single amino acid mutants of SIVgsn71 Vpu (A22L, A25L, W30A, and W33A) all failed to bind and downregulate human BST-2 expression, while these same mutations did not completely abolish the ability to target GSN BST-2 (Fig. 1C to F). These results also indicate that the inability to target human BST-2 is not due to gross misfolding or mislocalization of the Vpu mutants but is a specific functional property. Indeed, despite the fact that the $A^{22}W^{30}$ motif is essential for GSN BST-2 antagonism, as evident from the loss of function of a double amino acid mutant, A22L and W30A mutants individually showed BiFC signals similar to wild-type Vpu, although they downregulate GSN BST-2 expression on the cell surface less

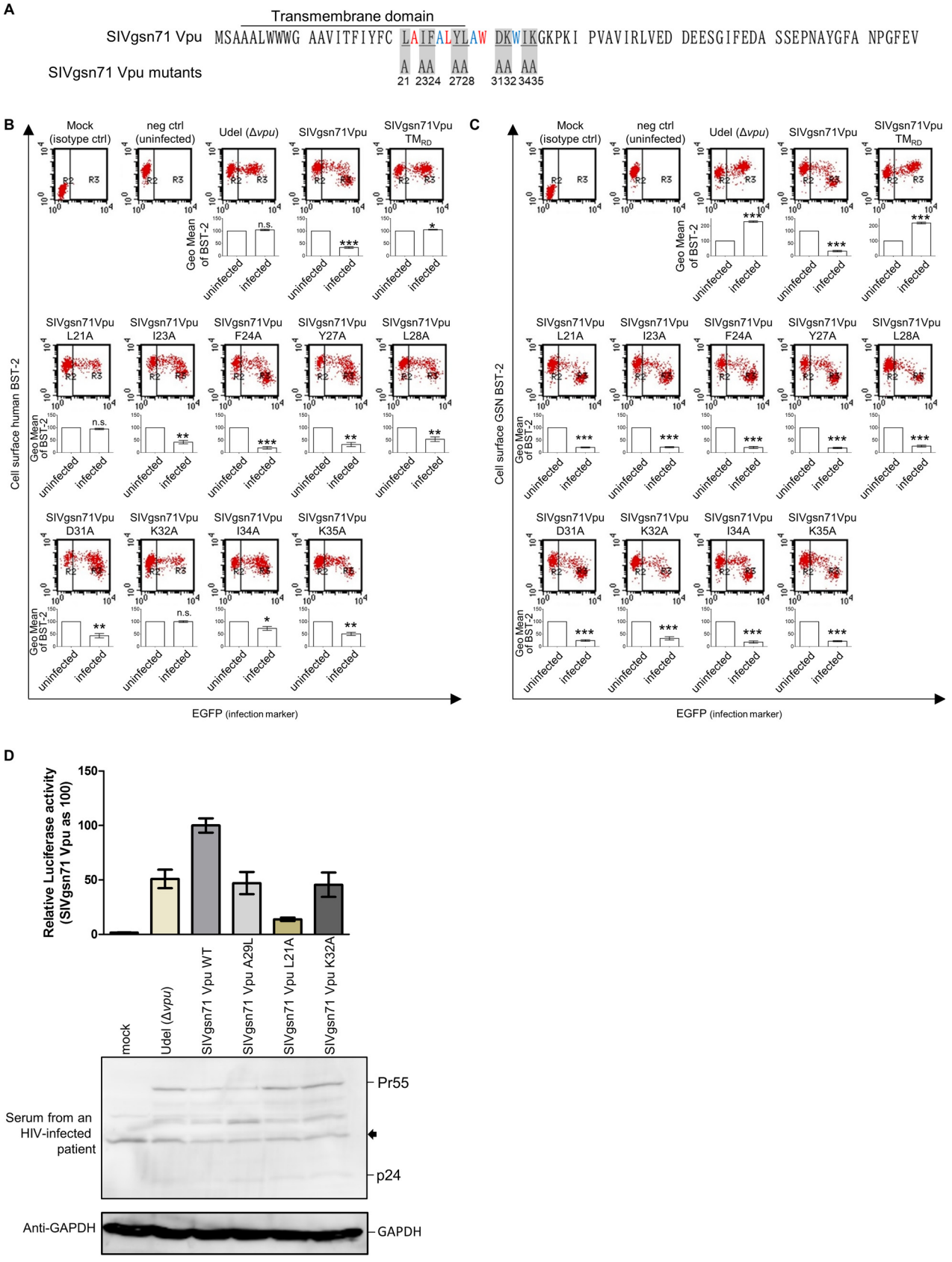

FIG 4 L^{21} and K^{32} are essential for downregulating human BST-2. (A) Schematic representation of single amino acid substitutions of SIVgsn71 Vpu. $A^{22}L^{26}W^{30}$ and $A^{25}A^{29}W^{33}$ are indicated in red and blue, respectively. (B and C) HeLa cells (B) and HeLa KO-GSN BST-2 cells (C) were infected with

(Continued on next page)

efficiently (Fig. 1E and F). These results led us to further mutational analyses to investigate roles of amino acids located inside the AW motifs.

Similar to residue A^{18} in the HIV-1 NL4-3 Vpu TM domain, which is located in the middle of its $A^{14}W^{22}$ motif and is important for antagonism of human BST-2 (28), we found that residue A^{29} in SIVgsn71 Vpu, which is located in the center of the $A^{25}W^{33}$ motif, was important to downregulate human BST-2 (Fig. 2B) but not GSN BST-2 (Fig. 2C). Since the $A^{25}W^{33}$ motif is necessary for human BST-2 antagonism but not for GSN BST-2 antagonism (46), it may be reasonable that A^{29} is not important for antagonizing GSN BST-2 (Fig. 2A and C). Along the same line, the amino acid residue L^{26} in the middle of the other AW motif ($A^{22}W^{30}$) of SIVgsn71 Vpu seems to be more important for antagonizing human BST-2 than GSN BST-2 (Fig. 2D and E), suggesting different modes of action depending on its targets. Notably, SIVgsn71 Vpu mutant 21-30 cannot downregulate human but has residual activity against GSN BST-2, and Vpu mutant 31-35 vitiated only human BST-2 downregulation (Fig. 3). This may be related to the fact that the $A^{25}W^{33}$ motif is necessary for human BST-2 antagonism but is not necessary for GSN BST-2 antagonism. Substitution mutations outside the AW motifs, i.e., L^{21} and K^{32} of SIVgsn71 Vpu, produced different effects on human and GSN BST-2 (Fig. 4B and C). In particular, the failure of the L21A mutant to downregulate only human BST-2 indicates that SIVgsn71 Vpu requires a broader region to downregulate human BST-2. Interestingly, instead of leucine at position 21 there is a valine residue in Vpu from SIVgsn-99CM166 (SIVgsn166), which can antagonize GSN BST-2 but not human BST-2 (38, 46). This difference might be the reason why Vpu from SIVgsn71 but not SIVgsn166 can antagonize human BST-2.

NMR experiments showed that the transmembrane domain (TMD) of HIV-1 NL4-3 Vpu assumes a hydrophobic alpha-helical structure in the membrane (49). It is interesting that mutation of leucine 26 to proline had a stronger impact on BST-2 antagonism than mutation to alanine or phenylalanine (Fig. 2D). Indeed, the L26P mutant of SIVgsn71 Vpu downregulated GSN BST-2 but not human BST-2. Because proline is known to act as a disruptor of secondary structures such as alpha helices and beta sheets, the amino acid substitution of proline for L^{26} may affect the TMD alpha-helical structure, which may affect interaction with human BST-2 but not, or to a lesser degree, GSN BST-2 (Fig. 2D and E).

Our data indicate that in addition to two AW motifs, L^{21}, A^{29}, and K^{32} in SIVgsn71 Vpu are critical not only to downregulate human BST-2 (Fig. 2B and Fig. 4B) but also to enhance virion release in the presence of human BST-2 (Fig. 4D). On the other hand, the ability of SIVgsn71 Vpu to downregulate GSN BST-2 was lost not by single amino acid substitutions (Fig. 2C and E and Fig. 4C) but by multiple mutations (Fig. 3C). The results shown in this study highlight a substantial difference in the mode of SIVgsn71 Vpu actions on human and GSN BST-2. In particular, the mechanism of antagonism by SIVgsn71 Vpu toward human BST-2 appears to be more complicated than toward GSN BST-2. The TMD domains of human and GSN BST-2 differ in 7 of the 27 residues, and there is an additional two-amino-acid deletion at the N-terminal end of the GSN BST-2 TM domain (Fig. 1B). These structural differences between human and GSN BST-2 TM domains could at least in part explain the need for different mechanisms of antagonism by SIVgsn71 Vpu. The diversity of primate *bst-2* genes might have resulted from distinct evolutionary pressures so that they have versatile phenotypes/functions.

FIG 4 Legend (Continued)
VSV-G-pseudotyped HIV-1 viruses which carry a deletion (Udel), intact wild type (SIVgsn71 Vpu), or single amino acid mutants of SIVgsn71 *vpu* gene, and the cell surface BST-2 expression was quantified with anti-BST-2 antibody. A representative dot plot is shown. Graphs are presented as described in Fig. 1D. (D) The production of infectious virus in the presence of human BST-2 was assessed by TZM-bl assay. Viruses were prepared by transfecting HeLa cells with HIV-1 *env*-deficient proviral DNA carrying the *vpu* gene from SIVgsn71 or its mutants together with a plasmid expressing VSV-G. TZM-bl cells were exposed with released virus and relative luciferase activity was determined (top). Luciferase activity caused by infection with virus expressing SIVgsn1 Vpu WT was set as 100. The results are shown as the means of triplicate samples ± the SEM. Whole-cell lysates prepared from producer cells were subjected to Western blotting (bottom) to monitor viral protein expression with serum from an HIV-infected patient. GAPDH was detected as loading control with the same membrane. The arrow represents nonspecific bands. HIV-1 proteins are identified on the right.

According to the Darwinian selection theory, genetic mutations in antiviral genes conferring a selective advantage should be maintained during evolution. The selective pressures could be ancient viral infections of enveloped viruses that were pathogenic and even life-threatening. Interestingly, human BST-2 lacks a DIWK motif in its cytoplasmic domain, which is conserved in nonhuman primate BST-2s (18, 24, 25). The lack of the DIWK motif is thought to play an important role as a barrier to SIV zoonosis. SIV Nef engages the clathrin adaptor AP-2 to downregulate primate BST-2 via its DIWK motif (50), and the lack of DIWK renders human BST-2 resistant to downregulation from the plasma membrane by SIV Nef. Thus, this may be one reason why group M HIV-1 strains switched from Nef to Vpu to antagonize human BST-2 (15, 18, 24, 25). As with the cytoplasmic domain, the TM domain of human BST-2 may have adapted its amino acid sequence under selective pressure. Indeed, human BST-2 is resistant to SIVmon and SIVmus Vpu even though they antagonize BST-2 of their own host (18, 46). The unexpected vulnerability of human BST-2 to SIVgsn71 Vpu, however, may also suggest compelling evolution under pressures from life-threating viruses other than SIVs.

In summary, we have found that amino acids L^{21}, A^{22}, A^{25}, A^{29}, W^{30}, K^{32}, and W^{33} of SIVgsn71 Vpu participate in antagonism of human BST-2 and that $A^{22}W^{30}$ and amino acids 21 to 30 of the SIVgsn71 Vpu are involved in GSN BST-2 antagonism. It is reasonable to assume that the human *bst-2* gene might have acquired a more resistant phenotype against viral proteins including, but not limited to, Vpu. Our results also suggest that the *bst-2* gene of primates has been subject to strong selective pressure, not necessarily limited to lentiviruses, during evolution. The results shown here potentially enable us to further investigate how similar and how different properties human and monkey BST-2s have and may help to understand the evolution of host genes as a result of host-pathogen interactions.

MATERIALS AND METHODS

Cells and transfection. HEK293T, HeLa, and HeLa KO-GSN BST-2 (46) cells were maintained at 37°C with 5% CO_2 in Dulbecco modified Eagle medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich Co., St. Louis, MO) and penicillin-streptomycin (Nacalai Tesque). HEK293T cells were transfected with polyethyleneimine (PolyScience, Niles, IL).

Plasmids. The plasmids expressing KGN-tagged SIVgsn-99CM71 Vpu, SIVgsn-99CM71 Vpu22/30, and SIVgsn-99CM71 Vpu25/33 and those expressing KGC-tagged human BST-2 and GSN BST-2 were described previously (46). The single-amino-acid mutants of the AW motifs in SIVgsn-99CM71 Vpu (A22L, A25L, W30A, and W33A) were constructed by overlap-extension PCR and inserted into phmKGN-MN vector (MBL International, Woburn, MA). The HIV-1 Vpu-deficient proviral vector pNL4-3EGFPΔ*env*Δ*nef*Δ*vpu* (Udel) and HIV-1 proviral vectors carrying *vpu* WT or *vpu* mutants of SIVgsn-99CM71 have been described previously (46, 51). The proviral vectors carrying SIVgsn-99CM71 *vpu* mutants (A22L, W30A, A25L, W33A, A29L, L26A, L26F, L26P, 2-5, 6-10, 11-15, 21-30, 31-35, L21A, I23A, F24A, Y27A, L28A, D31A, K32A, I34A, and K35A) were constructed by overlap-extension PCR using pNL4-3EGFPΔ*env*Δ*nef*-*SIVgsn71vpu* as the template. To generate pNL4-3 $H2K^k$Δ*env*Δ*nef*, the EGFP-coding region in pNL4-3 EGFPΔ*env*Δ*nef* was replaced with a PCR fragment of $H2K^k$-coding region amplified from CSII-CDF-GATEWAY-IRES-$H2K^k$ (52).

BiFC assay. The fluorescence protein (mKG) was split out into two fragments, the N terminus (mKGN) and C terminus (mKGC), which themselves are nonfluorescent and fused to Vpu and BST-2, respectively. The interaction of Vpu and BST-2 brings mKGN and mKGC fragments closer enough to form the active fluorescent protein (53). The fluorescent signal can be detected by flow cytometry in live cells and the geometric mean (Geo Mean) of BiFC signal represents the relative binding efficiency. HEK293T cells were transfected with 1 μg of pmKGC-huBST-2 or pmKGC-GSN BST-2 and 1 μg of pmKGN-SIV Vpu or its mutants, together with 2 μg of pmCherry as the transfection marker (17, 46). Cells were harvested at 48 h after transfection and subjected separately to flow cytometry and Western blotting. Flow cytometric analyses were performed after fixing of cells with 0.4% paraformaldehyde (Nacalai Tesque) in phosphate-buffered saline (PBS) by FACSCalibur flow cytometer (BD Bioscience, San Diego, CA) as previously reported (17, 22, 46, 54). The Geo Mean of bimolecular fluorescence complementation (BiFC) signals in mCherry-positive cells were analyzed with BD Cell Quest Pro software (BD Bioscience). Cells for Western blotting were resuspended with 150 μl of PBS and 150 μl of 2 $\times$ sample buffer (0.125 M Tris-HCl [pH 6.8], 10% [vol/vol] 2-mercaptoethanol, 4% [vol/vol] sodium dodecyl sulfate [SDS], 10% [vol/vol] sucrose, 0.01% [vol/vol] bromophenol blue). After incubation at 95°C for 10 min, 10 μl of supernatant was subjected to Western blotting for BST-2 using anti-KGC antibody (MBL International), Vpu using anti-KGN antibody (MBL International), and tubulin using anti-α-tubulin antibody (Sigma-Aldrich) and visualized by the LI-COR Odyssey imaging system (LI-COR Biosciences, Lincoln, NE) with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (American Qualex International, Inc., San Clemente, CA) and Western Lightning Plus-ECL (Perkin-Elmer, Waltham, MA).

BST-2 cell surface downregulation assay. HEK293T cells were transfected with 3 μg of proviral DNA pNL4-3EGFPΔ*env*Δ*nef* or pNL4-3 H2K^{k}Δ*env*Δ*nef* carrying SIVgsn-99CM71 *vpu* WT or *vpu* mutants and 1 μg of pMISSION-VSV-G (Sigma-Aldrich) to produce VSV-G pseudotyped HIV-1 vectors. The viral supernatants were harvested at 48 h posttransfection and filtered through 0.45-μm pore filters (Merck Millipore, Burlington, MA). HeLa and HeLa-KO GSN BST-2 cells were incubated with an optimized amount (to achieve a 40 to 50% EGFP-positive population) of viral supernatants. At 48 h after infection, the cells were harvested and stained for cell surface BST-2 using anti-BST-2 rabbit polyclonal antibodies (32) and donkey anti-rabbit IgG-conjugated Alexa Fluor 647 (Jackson Immuno Research Laboratories, Inc., West Grove, PA). For experiments using H2K^k-expressing virus, phycoerythrin-conjugated anti-H2K^k antibody (BioLegend, San Diego, CA) was used. After the cells were fixed with 0.4% paraformaldehyde (Nacalai Tesque) in PBS, the cell surface BST-2 expression (Geo Mean of the Alexa Fluor 647 signal) in EGFP-positive and EGFP-negative cells was analyzed on a FACSCalibur flow cytometer and analyzed using BD Cell Quest Pro software (BD Bioscience)

Quantification of virion release. Production of infectious virus was determined as described previously (46). Briefly, HeLa Cells were cotransfected with proviral DNA pNL4-3EGFPΔ*env*Δ*nef* carrying SIV *vpu* or its mutant and pMISSION-VSV-G using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA). The virus-containing supernatants were harvested 24 h after transfection, centrifuged at 8,000 rpm for 1 min and subjected to a TZM-bl assay. The TZM-bl assay was performed as described previously (17, 55). Protein expression in producer cells was evaluated by Western blotting as described previously (46). For Western blotting, viral proteins were detected with serum from an HIV-infected patient, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a loading control was detected with anti-GAPDH antibody (GeneTex, Irvine, CA). HRP-conjugated sheep anti-human IgG, HRP-conjugated rabbit anti-mouse IgG, and western Lightning Plus-ECL were used to detect the proteins with LI-COR Odyssey imaging system (LI-COR Biosciences).

Statistical Analysis. Statistical analyses were performed using GraphPad Prism 6. The data are presented as means, with error bars indicating the standard errors of the mean (SEM), from three independent experiments. Student *t* test was used for comparison between infected and uninfected cells in downregulation assays (Fig. 1D and F, 2B to E, 3B and C, and 4B and C). One-way analysis of variance (ANOVA) with the Dunnett's multiple-comparison test was used for BiFC assay results (Fig. 1C and E). Asterisk are used in the figures to indicate statistical significance (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; n.s., not significant [$P > 0.05$]).

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W.Y. and T.Y. designed the study. W.Y. and T.Y. performed the experiments. W.Y., K.S., S.Y., and T.Y. analyzed the data. W.Y., K.S., S.Y., and T.Y. contributed reagents and materials. W.Y., S.Y., and T.Y. wrote the paper. All authors reviewed the manuscript.

The authors have declared that no competing interests exist.

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